A FUNDAMENTAL STUDY OF EMULSIONS
FORMED IN A HEXANE-BASED LIPID EXTRACTION
FROM SLURRIES OF RUPTURED MICROALGAE

by

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Abstract

Microalgae are aquatic photosynthetic microorganisms that can convert and store solar energy in the form of energy-dense lipids. These algal lipids are highly desirable as they can be upgraded into biodiesel to be used directly as a liquid fuel in diesel vehicles. However, extracting the lipids from these oleaginous microbes has been a challenge. Conventional lipid extraction processes usually necessitate the use of energy-intensive techniques that can result in a net-negative energy balance. In addition, many published studies have been performed using laboratory techniques that are not applicable to large-scale processing.

One of the few promising lipid extraction techniques is wet biphasic solvent extraction. In this approach, cells in a wet concentrated biomass (15-25 % w/w solids concentration) are ruptured to release the intracellular lipids, typically by using a scalable and energy-efficient method such as high-pressure homogenisation. The released lipids are then extracted via a solvent extraction process using a non-polar (water immiscible) solvent. The use of a non-polar solvent, instead of a water-miscible solvent, is critical as it creates a biphasic solvent extraction environment that allows an easy solvent recovery route.

In the existing literature, emulsification of the solvent has occasionally been mentioned as a potential issue in the biphasic process. The formation of a stable emulsion can potentially inhibit the solvent recovery process and thus directly reduce the lipid recovery yield. However, there is a lack of systematic studies on the solvent recovery process and the reasons for stable emulsion formation have not been investigated.

Therefore, this thesis aims to further improve the current understanding of a biphasic solvent extraction process. In this thesis, the kinetics and the mechanism of the solvent recovery process in a hexane-based lipid extraction were characterised for the first time. The emulsified hexane droplets were highly stable, resisting phase separation during centrifugation up to 500 x g. Below a critical centrifugal force, the phase separation of emulsified hexane from the rupture-algae slurry was demonstrated to be limited by droplet-droplet coalescence, rather than creaming of the droplets. This finding has major implications on the algal biodiesel process, as specifying an industrial centrifuge for solvent recovery cannot be performed based on laboratory centrifuge tests due to the differences in equipment geometries that can affect the coalescence and the creaming behaviour. In addition, a fundamental understanding of the emulsion stabilising factors in the ruptured-algae slurry is required to improve the solvent recovery.

To identify the potential emulsifiers and surfactants in ruptured-algae slurries, a centrifugation-based method was used to separate the ruptured algal cell components into different biomass fractions, namely lipids, cell debris and serum. The interfacial properties and stability of the emulsions formed using these different components were characterised. Each biomass fraction was found to contribute to emulsion formation in different ways. The lipids were shown to have minimal emulsion-stabilising capacity with respect to coalescence, but were highly surface-active, allowing small hexane droplets to form and to
be stabilised by other emulsifiers. The serum was found to be protein-rich and thus able to form a viscoelastic proteinaceous interfacial film that stabilises emulsions against coalescence. The cell debris/particles were shown to stabilise emulsions by adsorbing strongly to the hexane-water interface via the hydrophobic sites on the cell walls. However, when these surface-active agents (lipid, debris, and serum) were present simultaneously in a solvent extraction process, they exhibited competitive adsorption behaviour at the hexane-water interface, thereby resulting in a less stable emulsion. Although the surfactants and emulsifiers in ruptured algal cells can be a barrier to complete solvent recovery, they were identified as having commercial promises for natural surfactants or emulsifiers that can be produced at scale.

Emulsion formation can inhibit solvent recovery from ruptured-algae slurry. Yet, the emulsion was theorised to be crucial for promoting lipid transfer into the solvent via additional solvent-water interfaces. To quantify the influence of emulsions on the lipid transfer rate, a lipid extraction protocol without involving emulsion formation was developed. The lipid transfer rate of this method (without emulsion) was compared against the conventional emulsion-based approach, and shown to be significantly slower. The interfacial area of the emulsions was demonstrated to be the most important factor accounting for the high lipid transfer rate and yield obtained in an emulsion-based approach, while the temperature, mixing rate and solids concentration could only increase the lipid transfer rate to a lesser extent. It was therefore concluded that emulsion formation is likely required for efficient biphasic lipid extraction from algae.

As emulsion formation is inevitable during lipid extraction, the ability of ruptured-algae slurry to form emulsions was used to advantage by using a lipase that benefits from emulsion formation. By performing a lipase-based in-situ transesterification, both the neutral lipids and the polar lipids, which were mostly unrecoverable using nonpolar hexane, were converted and recovered as FAME (biodiesel) in a single step. However due to the high moisture content of the slurry, a high methanol dosage was required to achieve high FAME conversion yields. This limitation may preclude the method from being applied for biodiesel production due to the energy loss in the form of unrecovered methanol. However, this approach can still be used for recovering polar lipids (which often contain most of the EPAs in an algae) without using a polar solvent that is difficult to recover.

This thesis is the first to systematically investigate the solvent-emulsions formed by ruptured-algae slurries. In this body of work, fundamental understandings of the emulsion formation and the demulsification process during solvent extraction, and the role of emulsions in lipid extraction were developed. These understandings are crucial for future process optimisation and for development of a feasible algal biofuel process. This work has also demonstrated that the potential of microalgae being a source of natural surfactants and emulsifiers which has been largely overlooked to date.
Declaration

This is to certify that:

i) The thesis comprises only my original work towards the PhD;

ii) Due acknowledgement has been made in the text to all other material used;

iii) The thesis is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Samuel Law

March 2018
Acknowledgement

Little did I know when I embarked on this journey 3.5 years ago, there is a long and challenging journey ahead. Fortunately, I have had great companies and mentors along the way which made all the difference. Thanks to them, the journey was enjoyable and fulfilling.

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Conference Presentations


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Nomenclature

% v/v  Volume percentage
% w/w  Weight percentage
DW    Dry weight
FAME  Fatty acid methyl esters
FFA   Free fatty acids
GL    Glycolipid
HPH   High-pressure homogenisation
*N. salina*  *Nannochloropsis salina*
NL    Neutral lipid
PL    Phospholipid
SL    Saponifiable lipids
TAG   Triacylglycerides or triacylglycerols
TL    Total lipids (including NL, GL and PL)
Chapter 1

1 Introduction

Microalgae (simply referred to as ‘algae’ in this thesis) are one of the few biofuel feedstocks that have the potential to be produced at a sufficient scale to have a significant impact on the global fossil fuel energy demand without negatively impacting food and water supply (Williams & Laurens, 2010). Compared to terrestrial plant-based biofuel feedstocks, microalgae have distinct competitive advantages such as higher biomass productivity, high lipid accumulation, and the ability to grow using brackish and salt water instead of fresh water (Brennan & Owende, 2010; Rodolfi et al., 2009). However, there are a few key issues that need to be addressed before algal biofuel can become an economically viable option.

Conventional algal processing typically involves a thermal drying step to completely remove the moisture from the algal biomass. Due to the high latent heat of water vaporisation, the thermal drying process is energy intensive and is therefore unfeasible (Chisti, 2013). Numerous life cycle analyses (M. J. Cooney et al., 2011; Lardon et al., 2009; Vasudevan et al., 2012) have concluded that any processing pathway that requires complete drying of the algal biomass (only achievable via thermal drying) will at best yield a low, or often negative, energy balance. As such, a dry algal processing route is unlikely to be feasible for algal biofuel production. Therefore, the lipids have to be extracted from a wet algal biomass. To minimise processing volumes and allow a positive energy balance in the system (Martin, 2016), the algae must first be concentrated to a paste or slurry, typically around 15-25 % w/w dry biomass, which is the highest biomass solids concentration achievable without thermal drying (Chisti, 2013; M. Cooney et al., 2009).

To date, extracting lipids efficiently from wet concentrated slurries of algal biomass remains a critical bottleneck in the algal biofuel process (Dong et al., 2016; Khoo et al., 2011). The majority of published studies have used polar (water miscible) solvents for algal lipid extraction, mainly due to the higher lipid yield when compared to using a nonpolar solvent (Ansari et al., 2017; Navarro López et al., 2016). However, polar solvents cannot be efficiently recovered from the wet slurry (Martin, 2016) without resorting to energy-intensive thermal heating processes. Others have developed novel lipid extraction methods that are seemingly promising at laboratory scale, but which are not applicable at large scale for various reasons (e.g. supercritical conditions, high temperature/pressure, high solvent:biomass ratio, use of novel/expensive solvents, and/or use of non-scalable techniques). There is a need to develop a wet lipid extraction process that is both energy feasible and scalable.

Using a water-immiscible (usually nonpolar) solvent provides a more energy efficient means of solvent recovery, as it can be physically recovered via a phase separation process. As the solvent does not mix with water and has a different bulk density, it can be recovered by centrifugation or possibly via simple
gravity settling. In addition to having a low water solubility, the selected solvent should have a low boiling point and heat of vapourisation, so that it can be removed from the lipid (through an evaporation/distillation process) with minimal energy. Hexane appears to be one of the only suitable solvents that is (i) cheap and abundant, (ii) has a low boiling point and (iii) a very low solubility in water. Further, hexane has been used as the extraction solvent in vegetable oil production and so the technologies to process hexane are readily available (Hernandez & Kamal-Eldin, 2013).

A process for extracting lipids from wet concentrated algal slurries using hexane has been developed with process scalability and feasibility as a priority (Halim et al., 2016; Olmstead et al., 2013). The process requires only mild conditions (low temperature and low pressure) and a low solvent-to-biomass ratio. Rupturing the algal cell walls is a prerequisite to wet lipid extraction processes that employ immiscible solvents such as hexane (Spiden et al., 2015). To address the need for cell rupture, a scalable technique (high-pressure homogenisation) was selected and an in-depth analysis had shown that this method is energy-efficient when used on a concentrated biomass slurry (Yap et al., 2015). Further, an analysis of the overall energy requirements of the cell rupture, lipid extraction, and biofuel production process was performed that concluded that the approach could be designed to be energetically-feasible (Martin, 2016).

In a biphasic solvent extraction process (which involves a water immiscible solvent), the solvent is initially mixed into the biomass slurry to form a solvent-in-water emulsion (Olmstead et al., 2013; Yap et al., 2014). As the lipids are more soluble in the solvent than the water, they preferentially dissolve into the solvent. The lipid-rich solvent can then be recovered from the biomass using centrifugation. Therefore, the recovery efficiency of this lipid-rich solvent will directly determine the lipid yield. However, the mechanisms of solvent separation that govern the recovery efficiency have not been investigated.

In the previous studies on hexane-based algal lipid extraction, intensive centrifugation (up to 8800 x g for 15 min) was required to separate the nonpolar solvent (Halim et al., 2016). This intensity of centrifugation would not be practical at large scale (Martin, 2016), however, the underlying reasons for the stability of these emulsions during centrifugation have not been explored. The possibility of undertaking lipid extraction without the formation of highly stable emulsions has also not been formally investigated. One of the major aims of this thesis is to understand the emulsion formation process and its role in the solvent extraction and recovery processes. Investigations to develop this understanding are the basis for chapters 3, 4 and 5 of the thesis.

After extraction, the algal lipid is typically converted into crude biodiesel through a transesterification process. Based on the insights gained in the prior thesis chapters, a lipase (biocatalyst) was explored in chapter 6 of this thesis as a way to improve the lipid extraction efficiency in a biphasic system. In the proposed process, the lipid extraction and the transesterification operations were combined into a single
step, thus achieving a more efficient process with a higher yield than the conventional wet hexane lipid extraction.

This thesis is divided into individual chapters with a specific focus in each chapter. Chapter 2 presents the current state-of-the-art knowledge of the algal lipid extraction processes and the background theory, and identifies the knowledge gaps that are targeted in the thesis. The experimental work performed for this thesis is described in Chapters 3 through 6. Each chapter includes an individual introduction before the presentation of the findings and conclusions. A list of references is provided at the end of each chapter. Appendices are also included at the end of the thesis that provide some experimental results that were important to the development of thesis but were not central to the primary scope of the investigations. The first three experimental chapters (chapter 3, 4 and 5) seek to develop mechanistic understandings of the biphasic solvent extraction process that were not available previously. In the last experimental chapter (chapter 6) a new strategy is developed to improve the biphasic wet lipid extraction using lipase. In the final chapter (Chapter 7), the findings and conclusions are brought together to form a united understanding of the solvent extraction process and directions for future studies are recommended.

References:


Chapter 2

2 Literature review

2.1 Overview – why microalgae?

Microalgae are a diverse group of aquatic eukaryotic microorganisms (as distinct from cyanobacteria) (Suganya et al., 2016). Microalgae usually range from several to a few hundred microns in cell diameter – thus the name micro-algae. Although small in size, they are highly abundant in fresh and marine waters and thereby play a significant role in the global carbon cycle and are accountable for producing about half the global atmospheric oxygen (Chapman, 2013; Field et al., 1998; Suganya et al., 2016).

Our current reliance on fossil fuels is not sustainable. Therefore, many alternative renewable energy sources have been investigated and considered (Ragauskas et al., 2006; Williams et al., 2009). Out of the many options for biomass production that can be used as a renewable feedstock to replace fossil fuels and chemicals, microalgae can be produced using the lowest amount of land and water resources (T. M. Mata et al., 2010). This advantage of microalgae is mainly attributed to the higher areal biomass productivity (two to tenfold) (Chisti, 2008; T. M. Mata et al., 2010; Sayre, 2010) and photosynthetic efficiency compared to most terrestrial plants (Melis, 2009; Weyer et al., 2010). In addition, marine (instead of fresh water) microalgae can be grown using brackish or seawater on a non-arable land (Brennan & Owende, 2010), thereby not competing for these resources with food production.

Microalgae are particularly promising as a feedstock for biofuel production due to their wide-spread ability to accumulate large quantities of lipids, in particular triacylglycerides (TAG). The TAG components can be readily converted into biodiesel (T. M. Mata et al., 2010). Other than being an excellent lipid source, microalgae can also produce protein and an array of valuable biomolecules (Table 2.1) with applications in the nutraceutical, food, and cosmetic industries (L. M. Laurens et al., 2017a; Spolaore et al., 2006). Therefore, the microalgae are not only interesting as a biofuel source but are also commercially relevant as a raw material for producing high-value products.
Table 2.1: Commercially valuable biomolecules that can be found in microalgae.

<table>
<thead>
<tr>
<th>Algal Components</th>
<th>Applications</th>
<th>Species</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipids</td>
<td>Nutraceutical supplements</td>
<td><strong>Crypthecodinium</strong>, <strong>Schizochytrium</strong></td>
<td>(Abril et al., 2003; Hammond et al., 2001; Jiang et al., 1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Nannochloropsis</strong>, <strong>Phaeodactylum</strong>, <strong>Nitzschia</strong></td>
<td>(Chini Zittelli et al., 1999; Molina Grima et al., 2003; Olmstead et al., 2013a; Wen &amp; Chen, 2003)</td>
</tr>
<tr>
<td></td>
<td>Infant formula</td>
<td><strong>Spirulina</strong>, <strong>Chlorella</strong>, <strong>Porphyridium</strong></td>
<td>(Hirano et al., 1990)</td>
</tr>
<tr>
<td>Pigments</td>
<td>Natural food color, anti-oxidant</td>
<td><strong>Haematococcus</strong></td>
<td>(Guerin et al., 2003)</td>
</tr>
<tr>
<td></td>
<td>Natural food color, Anti-oxidant</td>
<td><strong>Spirulina</strong>, <strong>Dunaliella</strong></td>
<td>(Dey &amp; Rathod, 2013; Guedes et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>Natural food color, anti-oxidant, anti-inflammatory</td>
<td><strong>Spirulina</strong></td>
<td>(Dasgupta, 2015; Romay et al., 1998)</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>Food Thickener</td>
<td><strong>Chlorella</strong>, <strong>Dunaliella</strong></td>
<td>(Goo et al., 2013; Yalcin et al., 1994)</td>
</tr>
<tr>
<td>Protein</td>
<td>Animal and fish feed</td>
<td><strong>Spirulina</strong></td>
<td>(Chew et al., 2017; Halim et al., 2016; Singh &amp; Gu, 2010)</td>
</tr>
</tbody>
</table>

2.2 The limit to dewatering and its impact on downstream processing

Despite the advantages of microalgae, there are still a number of technological obstacles that need to be overcome for biofuel production to become economically viable (Chisti, 2013; Hannon et al., 2010). Microalgae are aquatic microbes that grow in water, usually as a suspension culture. Owing to the limits of self-shading, these algal cultures are dilute, typically between 0.5 kg m⁻³ dry biomass (0.05 % w/w), when grown in an open raceway pond (Molina Grima et al., 2003), and 5 kg m⁻³ dry biomass (0.5 % w/w), when grown in a tubular photo-bioreactor under favourable conditions (abundant nutrients, light and carbon source) (Brennan & Owende, 2010; Chisti, 2013).

Due to the extremely low solids concentration (0.05-0.5 % w/w), a large volume of the microalgae culture has first to be removed in order to efficiently recover the algal products (K. Sharma et al., 2013). Processing of dilute algae is inefficient and will necessitate a high capital cost (Uduman et al., 2010). In addition, a high processing volume will increase the energy input requirement (e. g. heating, mixing, homogenising, or pumping) that can result in a negative energy balance (more energy being consumed in the process than the amount of usable energy generated) (Martin, 2016; Yap et al., 2015). Therefore,
the dilute algal suspension has to be concentrated through a dewatering process prior to further processing.

To date, the dewatering process has been a formidable challenge due to the small size of the microalgae cells (Gerardo et al., 2015). The choice of dewatering techniques is often a result of technical compromises (Gerardo et al., 2015; Uduman et al., 2010). Depending on the process requirement, the algal suspension can be concentrated via different techniques such as vacuum filtration, sedimentation, chemical and biochemical flocculation, air flotation and electrolytic coagulation (Al Hattab et al., 2015; J. Kim et al., 2013; Uduman et al., 2010; Vandamme et al., 2013). Centrifugation is often preferred (Brennan & Owende, 2010; Molina Grima et al., 2003) as it can be operated continuously with a high cell removal efficiency and without chemicals addition. However, a centrifugation process can be energetically demanding when applied to dilute suspensions. Thus, a step-wise approach with a pre-concentration step prior to centrifugation has been devised to reduce this energy load (Khoo et al., 2011; Soomro et al., 2016). A low-energy demand technique such as flocculation can be used to pre-concentrate the dilute algal culture 100-200 fold (K. Sharma et al., 2013) to obtain a slurry with a low viscosity (2-7 % w/w). This slurry can then be centrifuged to further concentrate to much higher solids, to what can be considered a thick slurry or paste (15-25 % w/w) (Molina Grima et al., 2003; Uduman et al., 2010). The energy required can be reduced by up to 70% via this strategy compared to a single-step centrifugation process (Soomro et al., 2016). Centrifugation removes a proportion of the extracellular water (as with other biomass systems such as sewage sludges (Skinner et al., 2015)), with the residual moisture contained intracellularly within the cell walls (L. Xu et al., 2011). As a result, a solids concentration of ca 30% solids appears to be the upper limit for mechanical dewatering on a time scale of relevance to industry.

For further drying, a thermal drying process has to be applied (Uduman et al., 2010). This can be achieved by solar drying, spray drying, drum drying, or lyophilisation (Molina Grima et al., 2003). However, this final step of complete moisture removal incurs a high energy cost. Due to the high latent heat of water vaporisation (2.3 MJ/kg), this thermal heating process can be very energy intensive (Chisti, 2013; M. J. Cooney et al., 2011). In practice, a thermal drying process is not fully efficient and can require an energy input higher than the heat of water vaporisation (3.3–3.9 MJ/kg for sewage sludge) (L. Xu et al., 2011).

The need to process wet, concentrated algal biomass to achieve a positive energy balance has been confirmed by multiple independent analyses:

1. Lardon found that wet extraction was the only route that resulted in a positive energy balance in both normal and high lipid accumulation scenarios (Lardon et al., 2009).
2. Lundquist argued that most drying methods (except for solar drying) would be unfeasible for biofuel production due to the excessive energy required for heat-drying the biomass (Lundquist et al., 2010).

3. A case study performed by Cooney et al. demonstrated that the biomass drying step is by far the largest energy load in the algal process (M. J. Cooney et al., 2011).

4. The analysis done by Vasudevan et al. concluded that a dry extraction scenario resulted in a net consumption of energy while a wet extraction process may still have a favourable energy balance (Vasudevan et al., 2012).

5. Quinn highlighted the importance of developing a wet lipid extraction process and that a biomass drying step prior to the lipid extraction will incur excessive energy consumption (Quinn et al., 2014).

6. Martin performed an in-depth analysis on a wet algal lipid extraction process and concluded that it is possible to obtain a positive energy balance via this approach (Martin, 2016).

In all the studies that have considered the energy balance, the unanimous conclusion was that a wet lipid extraction is the only option as further drying beyond the centrifugation process (15-25% solids concentration) will generally be unviable. Therefore, a practical algal biofuel process should be developed around working with a wet concentrated algal biomass (15-25% solids concentration) and any thermal drying process (including in the downstream) should be avoided. For that reason, the following review (and this thesis) will focus mainly on studies pertaining to processing wet concentrated algal slurry or paste (15-25 % w/w solids concentration).

2.3 Energy production from wet algal slurries

A wet algal slurry can be converted into useful energy forms via several methods, namely hydrothermal liquefaction, anaerobic digestion, fermentation, and lipid extraction and transesterification (Figure 2.1). The selection of a conversion process depends on several factors, such as (i) the composition of the algal biomass (e.g. lipid rich or carbohydrate rich), (ii) the desired form of energy (e.g. liquid fuel or gas), and (iii) economic considerations including the production of higher-value co-products (Tsukahara & Sawayama, 2005).
**Figure 2.1:** Diagram of the possible methods for converting a wet algal biomass (de Boer et al., 2012; Ghasemi et al., 2012).

Hydrothermal liquefaction is a thermochemical process performed at high temperatures below the supercritical temperature of water (300-370 °C) and elevated pressures (5-20 MPa) (A. Lee et al., 2016; López Barreiro et al., 2013). This process often requires addition of reactants such as hydrogen (Goyal et al., 2008), or acid or alkaline catalysts (Biller & Ross, 2011) to maximize the yield by minimising the formation of solid biochar (Dimitriadis & Bezergianni, 2017). Under these extreme conditions, the wet algal biomass is converted into a highly viscous bio-oil, not dissimilar to fossil crude oil, which can be upgraded into fuel. Recently, there has been growing interest in hydrothermal liquefaction as evident by the increasing numbers of publications (Dimitriadis & Bezergianni, 2017; López Barreiro et al., 2013). However, there are major challenges in scaling up the technology due to inefficient process heat recovery, high capital costs, reactor corrosion, and the production of toxic wastes (Dimitriadis & Bezergianni, 2017; A. Lee et al., 2016). Recent publications have shown that the composition of biomass feedstock can greatly affect the yield and quality of the bio-oil from the conversion process (Dimitriadis & Bezergianni, 2017; Kröger et al., 2018). Beyond this, most of the cell components (including any potential valuable co-products) are irreversibly destroyed in this process, thus making this conversion technology less likely to be used for developing value-added co-products.

In contrast to hydrothermal liquefaction, which is a thermochemical process, anaerobic digestion and fermentation are biochemical conversion processes. In anaerobic digestion, the wet algal biomass is first broken down by a series of biological processes (driven by bacteria) and then converted by methanogens to produce biogas (methane and carbon dioxide mixture) (Gonzalez-Fernandez et al., 2015; Sialve et al., 2009). For algal biomass with fermentable sugars (in the form of monomeric sugars such as glucose or mannose (L. M. Laurens et al., 2017a)), yeasts can be utilised to convert the sugar contents into bioethanol (Brexó & Sant’Ana, 2017). However, the common limitation of these processes are poor
yields. These have been attributed to the low biomass hydrolysis rate in the anaerobic digestion process (Córdova et al., 2017; Gonzalez-Fernandez et al., 2015), or the high amounts of unfermentable structural and storage carbohydrates, which requires a separate hydrolysis pretreatment step (de Farias Silva & Bertucco, 2016; T. Dong et al., 2016c; L. Laurens et al., 2015).

For microalgae biomass with high lipid contents, it is preferable to extract the lipids that can be readily converted into biodiesel, which can be used directly as a diesel replacement (T. M. Mata et al., 2010; Rodionova et al., 2017). The lipid-to-biodiesel conversion process is generally achieved by transesterification, which will be reviewed in detail in section 2.6.

It is becoming more evident that an algal fuel-only pathway will not be economically feasible due to the high production cost of algal biofuel and the low competing fossil fuel price (L. M. L. Laurens et al., 2017b; Vanthoor-Koopmans et al., 2013; L. Zhu, 2015). To improve the process viability, the cost of biofuel production has to be offset by the recovery of valuable co-products in the algal biomass (Chew et al., 2017; Ruiz et al., 2016; Suganya et al., 2016). This is made possible, at least in theory, in a biorefinery approach where a single algal biomass feedstock is used to generate multiple product streams (Table 2). This often involves a combination strategy of different energy conversion techniques and extraction methods to valorise all the components in the algae biomass.

A viable and sustainable algal biorefinery approach is still being developed (L. M. Laurens et al., 2017a). A recent development of biorefinery concepts include a so-called combined algal processing (CAP) that was developed by Dong et al. (T. Dong et al., 2016a). In this approach, the wet algal slurry is maximised for energy conversion by dilute acid hydrolysis at high temperatures and pressures. The hydrolysate with soluble carbohydrates is then fermented by yeasts to generate bioethanol and the residual lipids are recovered for transesterification.

Another alternative to the CAP process is a multi-stage extraction biorefinery. In this approach, the lipids and other valuable products are sequentially extracted (Halim et al., 2016; Pignolet et al., 2013; Suganya et al., 2016; L. Zhu, 2015) before being used as animal or aquaculture feed (Halim et al., 2016), or converted to an energy source via hydrothermal liquefaction (Garcia Alba et al., 2011) or fermentation (Nobre et al., 2013). To maximise the economic potential of the algal biomass in this approach, it is crucial that the upstream lipid extraction technique is mild and does not irreversibly damage the un-extracted components. For that reason, harsh conditions such as high temperature, high pressure, extreme pH and chemicals should be avoided (Vanthoor-Koopmans et al., 2013).
2.4 Lipid recovery via solvent extraction

2.4.1 Background theory

Solvent extraction is by far the most widely used approach to recover lipids from an oleaginous algal biomass (Chisti, 2013; Grima et al., 2013; Mercer & Armenta, 2011; Mubarak et al., 2015; Rawat et al., 2013). In a solvent-based lipid recovery process, an organic solvent is typically added to the oil-bearing biomass (aqueous phase) so that the lipid partitions into the solvent based on the difference in solubility (like dissolving like) (Grima et al., 2013; Halim et al., 2014). When the two phases are brought together, the solute (lipid) will distribute itself between the two phases until an equilibrium is achieved. The act of the solute moving (partitioning) to a phase in which it is more soluble is the underlying mechanism of a lipid extraction. The distribution ratio of the solute between the two phases, also known as the partition coefficient, depends on the solubility of the solute in each phase (Grima et al., 2013). The partition coefficient can be described mathematically (Tao Dong et al., 2016b) as follows:

\[
K = \frac{[A]_{\text{org}}}{[A]_{\text{aq}}}
\]

Where K is the partition coefficient, \(A_{\text{org}}\) and \(A_{\text{aq}}\) are the concentration of solute in organic and in aqueous phase respectively at a given temperature.

Compared to lipid extraction from plant oilseeds, lipid extraction from algal cells is more complicated due to the higher moisture content, the presence of the cell walls (M. Cooney et al., 2009) and the potential contaminants that could co-partition into the solvent (Foley et al., 2011). An ideal extraction solvent for algal lipids will have a high lipid solubility but a low solubility for other impurities in the algal cells (Grima et al., 2013). Therefore, matching the solvent solubility to the algal lipid is important for successful lipid extraction.

However, the term ‘lipid’ is broad, as it includes a highly diverse group of molecules that are common in their solubility in organic solvents, and there are many different types of lipids found in microalgae (Leblond & Dahmen, 2016). As will be discussed in the next section, only some of these lipids are suitable for biofuel applications, whereas others are problematic for biofuel (Foley et al., 2011) but potentially nutritionally valuable (Olmstead et al., 2013a). In addition, the algal lipid composition can vary greatly across different algal species (Martin et al., 2014) and growth conditions (K. Sharma et al., 2012). Therefore, the knowledge of the algal lipid composition and the desired product/application is crucial for selecting an appropriate solvent (Grima et al., 2013).

2.4.1.1 Algal lipid composition

Unlike other biomolecules (e.g. proteins or carbohydrate), lipids are a unique class of compounds that are grouped together based upon their hydrophobicity, rather than by a common structural feature (Belitz, 2009). Lipids can be classified by their polarity (Figure 2.2A) into neutral lipids (NL), glycolipids (PL) and phospholipids (PL)(Olmstead et al., 2013a). This classification is typically based
on column fractionation using solvents of increasing polarity (Olmstead et al., 2013a; Saoudi-Helis et al., 1994; C. Zhu et al., 1997). Different lipids serve different functions in cells. For example, the triacylglycerides recovered in the NL fraction are a form of energy reservoir (storage lipid) (K. Sharma et al., 2012). The polar lipids in the GL and PL are key structural components of the cell and organelle membranes (Martin et al., 2014; Sheffer et al., 1986).

For biodiesel conversion, a further distinction is made between saponifiable and non-saponifiable lipids (Figure 2.2B). Saponifiable lipid molecules include at least one acyl chain (fatty acid moiety) that is convertible to a fatty acid methyl ester (FAME) via a transesterification process. The converted FAME can be purified and used as fuel in diesel engines (T. M. Mata et al., 2010). Non-saponifiable lipids are all the remaining lipids that do not contain an acyl chain and therefore cannot be converted into biodiesel. Some examples of non-saponifiable lipids are wax esters, pigments (e.g. chlorophylls and beta carotene), sterols and hydrocarbons.

The conventional feedstock for biodiesel is TAG that comprises vegetable oils and tallow (da Cunha et al., 2009; Issariyakul & Dalai, 2014). Many studies have recommended that the triglycerides (TAG) contained within the NL fraction are also the most suitable algal lipid feedstock for biodiesel conversion (Tao Dong et al., 2016b; Foley et al., 2011; Rawat et al., 2013). This is because the presence of other lipids (e.g. phospholipids) can consume the catalysts during the conversion process and thus reduce the conversion efficiency. On the other hand, the free fatty acids (FFA) can cause soap formation complicating the downstream separation process (Freedman et al., 1984; J. Kim et al., 2013). In addition, EN 14214 and ASTM D6751 standards require that biodiesels must contain less than 10 ppm phosphorus (which is in the phospholipid) to avoid the poisoning of the catalytic converter in a vehicle (Hakan, 2001; Tyson & McCormick, 2006). Therefore, if phospholipid is present in the extracted lipid, a degumming step has to be performed prior to transesterification (L. Chen et al., 2012).

Therefore, an algal lipid extract with mostly NL (high TAG) and a low PL content is typically desired for biodiesel conversion (Dibenedetto et al., 2012; Foley et al., 2011).
2.4.1.2 Lipid accumulation in microalgae

The proportions and the composition of the lipid groups in microalgae can be manipulated to some extent during the cultivation process. It has been demonstrated that accumulation of the desired TAG can be achieved by exposing the culture to various stressful conditions. Most commonly, nitrogen/nitrate starvation has been suggested (Meng Chen et al., 2011b; Meng et al., 2015), but other stresses such as phosphate limitation (Feng et al., 2012), and brief exposure to UV-C radiation can also be used (K. Sharma & Schenk, 2015). These observations are consistent with the biological function of TAG as an energy reservoir for the microalgae. The onset of a stress triggers the accumulation of TAG, which can be used by the cell when the environment becomes favourable again.

This TAG induction strategy does come at a cost of lowered biomass productivity compared to non-stressed growth (Meng et al., 2015; Scott et al., 2010). However, sacrificing some biomass productivity for a high TAG content may be acceptable for biodiesel production. Previous analyses have identified that high TAG content in the biomass is one of the keys for achieving a favourable positive energy balance (Chisti, 2013; M. J. Cooney et al., 2011; Yap et al., 2015).

However, even after the TAG induction strategy, some quantity of polar lipids (GL and PL) will still be present in the algal cells due to their essential biological functions (Sheffer et al., 1986).

**Figure 2.2:** (A) Classification of the major lipid classes in algal lipids (Belitz, 2009; Graeve & Janssen, 2009; Martin et al., 2014; Olmstead et al., 2013a) (B) Examples of saponifiable lipids (Hartmann, 2016).
2.4.1.3 The polar lipid dilemma in a solvent extraction process

As the polar lipids (particularly phospholipids) can cause numerous complications in the downstream processes (Foley et al., 2011; Freedman et al., 1984; J. Kim et al., 2013), it is typically recommended to forgo the polar lipids and extract only the TAG-containing NL fraction for biodiesel conversion (Iyer, 2016). A nonpolar solvent such as hexane is used for this purpose as it is highly selective towards the NL fraction (Olmstead et al., 2013b). However, some of the saponifiable acyl molecules in the polar lipids (Figure 2.2) are not extracted. These polar lipids can form a significant fraction of the total saponifiable lipids in some microalgae (Foley et al., 2011) and therefore this approach of using nonpolar solvent will achieve a lower-than-theoretical biodiesel yield (Grima et al., 2013).

Dong et al. have developed a combined algal processing (CAP) technique that addressed this polar lipid dilemma (T. Dong et al., 2016a). In the CAP method, the macromolecules in the wet algal slurry are broken down into individual constituents via acid hydrolysis at high temperature (155 °C) prior to the solvent extraction. This approach allows the polar lipids to be recovered and converted to biodiesel and the hydrolysed carbohydrates to be fermented to ethanol, thus maximising biofuel production. However, in this method, other valuable molecules such as proteins are also denatured and hydrolysed in the process, thus potentially lessening their commercial value. Therefore, there is a need to develop a mild extraction technique that can extract the fatty acid moieties (acyl chain) of the polar lipid molecules without involving harsh conditions. One possible solution is the development a lipase-based in-situ transesterification which will be explained in the later section (2.6.5).

2.4.2 The requirement of cell rupture prior to wet solvent extraction

In previous studies, it was found that the high water content in an algal biomass (compared to a dried biomass) can significantly hamper the lipid extraction process (Ansari et al., 2017; Balasubramanian et al., 2013; Min Chen et al., 2011a; Islam et al., 2014; Wahlen et al., 2011). This was attributed in part to the reduced contact between the hydrophobic organic solvent and the biomass (Samarasinghe et al., 2012; Yoo et al., 2012). More importantly, the cell walls are a physical barrier that prevents solvent penetration into the cells, where the lipids are located (Mendes-Pinto et al., 2001; Yap et al., 2014).

The use of a polar or mixed solvent (e.g. chloroform and methanol mixture (Bligh & Dyer, 1959)) may allow solvent penetration through the cell walls. However, the lipid extraction process may still be slow and is limited by the diffusion rate across the cell wall (Mendoza et al., 2015; Yap et al., 2014). In addition, the polar solvents used cannot be recovered effectively from the wet algal biomass without the use of energy-intensive thermal heating (Martin, 2016).

Therefore, a cell rupture (interchangeable with the terms ‘cell disruption’ or ‘cell lysis’) step to break the cell wall is critical prior to a wet lipid extraction process to achieve a high lipid yield.
(Balasubramanian et al., 2013; J. Kim et al., 2013; Samarasinghe et al., 2012). A successful cell rupture process is also equally important to allow the recovery of other intracellular products such as carbohydrates and proteins (Günerken et al., 2015; Jubeau et al., 2013; Postma et al., 2017; Spiden et al., 2013).

2.4.2.1 Selection of a cell rupture technique

Most algal cell rupture techniques were developed based on existing techniques used on yeast and bacteria (Harrison, 1991). Examples of cell disruption techniques include high-pressure homogenisation (HPH) (Olmstead et al., 2013b; Samarasinghe et al., 2012), sonication (J.-Y. Park et al., 2015), bead beating (J.-Y. Lee et al., 2010), microwave-heating (J. Cheng et al., 2014; J.-Y. Lee et al., 2010), electroporation (Silve et al., 2018), heat treatment (Spiden et al., 2015), and osmotic shock (Yoo et al., 2012). In addition, chemicals such as detergent (Ulusoa et al., 2012), acid (T. Dong et al., 2016a), or alkali (Harun et al., 2011), or enzymes (Wu et al., 2017) have also been used for disrupting algal cells.

The effectiveness of a cell rupture technique is dependent on the algal species as the cell wall composition and strength varies greatly among the different species (S. Y. Lee et al., 2017; Spiden et al., 2013). More importantly for large-scale applications, other considerations such as scalability, process feasibility, and energy usage have to be taken into account. Unfortunately, most algal cell rupture methods were based on processes developed for pharmaceutical or food applications where energy consumption is not a high priority (S. Y. Lee et al., 2017). Many cell rupture techniques either (i) have a low rupture efficiency when used on a robust microalgae, (ii) are energy intensive, (iii) are non-scalable, and/or (iv) involve the addition of chemicals which are difficult to separate downstream.

Among the available options, high-pressure homogenisation (HPH) appears to be one of the few techniques that is viable for large-scale algal cell rupture. HPH operates by forcing the wet algal slurry through a small orifice using a large pressure differential across the valve (Yap et al., 2015). The algal cells are accelerated through the orifice and then impinged on an impact ring. The high shear environment as a result of the pressure differential (Clarke et al., 2010) and the impingement process (Kleinig et al., 1996) have been hypothesised to contribute to the cell rupture process.

A few studies have demonstrated the effectiveness of HPH at rupturing microalgae, including species that are commercially promising but physically robust such as *Nannochloropsis* sp. and *Haematococcus* sp. (Jiménez Callejón et al., 2014; Olaizola, 2000; Olmstead et al., 2013b; Ouyang et al., 2005; Spiden et al., 2013). In addition, HPH is readily scalable (for example it is widely used in the dairy industry) and has a high throughput capacity (between 5000 L h\(^{-1}\) – 50,000 L h\(^{-1}\) depending on the operating pressure). It is also capable of processing high-solids, viscous slurries (Samarasinghe et al., 2012; Yap et al., 2015), which is a requirement for an energy efficient wet algal process (Martin, 2016; Yap et al., 2015). The energy requirement of the process has been shown to be sufficiently low for biofuel
applications, provided a concentrated (up to 25 % w/w solids concentration), instead of dilute (<10 % w/w), algal slurry is used (Yap et al., 2015). These advantages position HPH as one of the promising techniques for large-scale algal cell rupture.

2.4.3 Differences between monophasic and biphasic solvent extraction

Once the algal cells are sufficiently ruptured, a solvent can be added to the slurry (Martin, 2016; Yap et al., 2015) (which is mostly water) to perform a solvent extraction. The process can be differentiated into a monophasic or a biphasic process based on the solvent miscibility/solubility in water (Spiden et al., 2015). In general, the more polar the solvent is, the more likely it will be soluble in water (Yalkowsky & Valvani, 1980). A solvent can be considered water-immiscible/insoluble when its solubility in water is less than 1 g.L⁻¹ (<0.1 w/v %) (Rogers & Stovall, 2000).

The fate of the solvent following extraction varies depending on its water solubility (Figure 2.3). For example, adding a polar solvent into a wet algal biomass will result in a fully miscible single liquid phase, thus making a monophasic extraction environment. On the other hand, mixing a water-immiscible solvent into a wet algal slurry will form two distinct liquid phases, thus resulting in a biphasic extraction system. The immiscible solvent in a biphasic system can be separated using centrifugation, whereas the miscible polar solvent can only be separated from the water using a distillation process.

In a biphasic extraction, a temporary dispersed droplet phase (also known as an emulsion) is formed (Lundquist et al., 2010) when the solvent and wet ruptured-algae slurry are mixed. Yap and co-workers (Yap et al., 2014) demonstrated that the dependence of cell rupture differed between biphasic (slurry and hexane) and monophasic solvent extractions (slurry, chloroform and methanol) (Figure 2.3). Without cell rupture, monophasic lipid extraction could be performed, however it was found to be diffusion limited. In contrast, lipids were only weakly extracted from unruptured cells using a biphasic system as the immiscible solvent could not penetrate the cells. After the cells were ruptured, the lipid recovery was increased for both extraction methods and the improvement was especially significant for the biphasic extraction (up to 100-fold increase in lipid yield).
Figure 2.3: Schematic diagram of the mechanistic difference between a monophasic and a biphasic extraction. Adapted from (Yap et al., 2014).

2.4.4 Selection criteria for the extraction solvent

As the choice of solvent not only affects the composition of the extracted lipids (Tao Dong et al., 2016b; Grima et al., 2013), it also determines the downstream process required for the solvent recovery (Halim et al., 2012). Therefore, it is important to select a solvent as part of process development.

In general, the selection criteria of an ideal solvent for the recovery of biodiesel feedstock lipids from microalgae can be summarised as follow (Grima et al., 2013; Halim et al., 2012; T. M. Mata et al., 2010; Mercer & Armenta, 2011; Ryckebosch et al., 2014; Tran et al., 2013):

i) High solubility/selectivity towards neutral lipids or TAG;

ii) Low solubility of polar lipids and other cell contaminants;

iii) Easily recoverable (water immiscible, low boiling point and heat of vaporisation, very different density to water);

iv) Cheap and abundantly available

v) Inert and non-toxic
Although many studies have reported on the use of polar solvents, they are infeasible for large-scale biofuel production due to the inability to be separated from the wet (high water) algal slurry without requiring energy-intensive distillation (Chisti, 2013; M. J. Cooney et al., 2011). Although lipid extractions performed using a polar solvent often have higher yields, this apparent increase in yield is likely due to the co-extraction of undesired fractions (Olmstead et al., 2013b; Ryckebosch et al., 2014) as the lipid yield is often evaluated gravimetrically without distinguishing (i) the saponifiable lipids from non-saponifiable matters, and (ii) the NL from the polar lipids (GL and PL) fraction (Ansari et al., 2017; Balasubramanian et al., 2013; Ryckebosch et al., 2014; Yao et al., 2012). A polar solvent such as ethanol can also potentially extract some contaminants such as sugars, amino acids and hydrophobic proteins in the biomass (T. M. Mata et al., 2010). In summary, nonpolar solvents are preferable to polar solvents due to their ease of recovery and selectivity to neutral lipids.

Recently, novel solvents have emerged such as ionic liquids (Y.-H. Kim et al., 2012), switchable amine solvents (Y. Du et al., 2017; Samorì et al., 2010) and supercritical solvents (C.-H. Cheng et al., 2011; Herrero et al., 2006). However, a comprehensive cost and feasibility analysis is not available for their use in algal biofuel applications. The preparation of ionic liquids is typically expensive (D. Fang et al., 2008; Zhao & Baker, 2013), thus making it unlikely for biofuel production. Switchable amine solvents promise an easy solvent recovery pathway, but they may have solvent stability issues (Supap et al., 2011; Wilson & Stewart, 2014). Supercritical extraction solvents have an excellent lipid extraction efficiency and provide an easy separation process. However, the extreme pressure and temperature required preclude their use for biofuel applications due to the very high capital and energy requirements (Chisti, 2013; Halim et al., 2012; Mercer & Armenta, 2011).

Among the nonpolar solvents, hexane (or n-hexane) appears to be a reasonable candidate for algal oil extraction. It is a highly nonpolar solvent that preferentially extracts the nonpolar NL fraction and has a low solubility in water for ease of recovery (Olmstead et al., 2013b). Hexane has also been used as the extraction solvent for plant oilseeds since the 1940s (Bredeson, 1983; Serrato, 1981). Therefore, the technology for using hexane are readily available. Although hexane is sometimes avoided due to its volatility and flammability, the history of its use demonstrate that these risks can be managed. Although it is not the perfect solvent, it is an acceptable compromise as technologies and regulations are in place with respect to the use and safety of hexane as an extraction solvent (Chappell, 2001; Wakelyn & Wan, 2004; Wan & Wakelyn, 1997).
2.4.5 Previous studies of solvent extraction on wet algal biomass

As discussed, the selection of an appropriate solvent is not a trivial task. In addition to the lipid yield and the extracted lipid quality, the practicality of the solvent in a scale operation has to be considered. Many published studies (not included in this review) have developed lipid extraction protocols involving a dilute cultures (<10 % w/w solids concentration) or a dried biomass powder, which has been shown to be non-viable for an algal biofuel process. Many others (Table 2.2) either used a polar solvent (isopropanol, ethanol, chloroform) or a polar solvent-containing mixture that cannot be efficiently recovered. Further, many of these studies have used extreme extraction conditions (high temperature, and/or high pressure), a high solvent ratio (solvent:wet biomass volume ratio >3:1), or techniques that are yet to be proven viable for large-scale applications such algal biofuel production (e.g. microwaves). Due to these shortcomings, many of these studies are not relevant to the advancement of a feasible algal biofuel technology. Hence, it is important to focus on the development of a biphasic lipid extraction process that is mild, energy efficient, and scalable.
Table 2.2: A comparison of the current wet lipid extraction methods grouped by the solvent miscibility in water (water miscible, partially miscible or immiscible). Non-conventional solvent technologies are also included. Only studies with wet biomass solids above 10% are included, and the optimal parameters from the studies are presented. Unfavourable or harsh extraction conditions are shaded grey.

<table>
<thead>
<tr>
<th>Solvent used</th>
<th>Extraction parameter</th>
<th>Solvent: biomass ratio (v/v or v/w)</th>
<th>Pretreatment</th>
<th>Solvent recovery method</th>
<th>Species</th>
<th>Solids concentration</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Water miscible solvents</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Chloroform / Ethanol</td>
<td>150 °C, 2h</td>
<td>2.7:1 (v/w)</td>
<td>N/A</td>
<td>Centrifugation, 3700 rpm for 10 min</td>
<td>Nannochloropsis</td>
<td>20%</td>
<td>(B. Kim et al., 2017)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>27 °C, 30 min</td>
<td>5:1 (v/w)</td>
<td>N/A</td>
<td>Centrifugation, N/A</td>
<td>Picochlorum</td>
<td>10%</td>
<td>(F. Yang et al., 2014)</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>80 °C, 1 h</td>
<td>2.3:1 (v/w)</td>
<td>N/A</td>
<td>Centrifugation, 3,000×10 min</td>
<td>Nannochloropsis</td>
<td>16%</td>
<td>(Yao et al., 2012)</td>
</tr>
<tr>
<td>Methanol</td>
<td>90 °C, 40 min</td>
<td>N/A</td>
<td>MW</td>
<td>Centrifugation</td>
<td>Nannochloropsis</td>
<td>80%</td>
<td>(Chee Loong &amp; Idris, 2017)</td>
</tr>
<tr>
<td>Supercritica 1 Ethanol</td>
<td>265 °C, 20 min</td>
<td>3.6:1 (v/w)</td>
<td>N/A</td>
<td>Centrifugation, 3200 rpm 10 min</td>
<td>Nannochloropsis</td>
<td>40%</td>
<td>(Reddy et al., 2014)</td>
</tr>
<tr>
<td><strong>Partially miscible or a mixture of solvents</strong></td>
<td></td>
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</tr>
<tr>
<td>Ethanol / Hexane</td>
<td>Temp N/A, 2 h</td>
<td>7.6:1 (v/w)</td>
<td>Pulsed electric field</td>
<td>Centrifugation, 10000×10 min</td>
<td>Auxenochlorella</td>
<td>10%</td>
<td>(Silve et al., 2018)</td>
</tr>
<tr>
<td>Isopropanol / Hexane</td>
<td>100 °C, 10 min</td>
<td>4:1 (v/w)</td>
<td>MW</td>
<td>Vacuum filtration</td>
<td>Scenedesmus</td>
<td>20%</td>
<td>(Ansari et al., 2017)</td>
</tr>
<tr>
<td>Methanol / Acetyl chloride</td>
<td>100 °C, 2.5 atm, 120 min</td>
<td>2.1:1 (v/w)</td>
<td>Sonication &amp; pressurisation</td>
<td>Gravity settling</td>
<td>P. tricornutum</td>
<td>18%</td>
<td>(Belarbi et al., 2000)</td>
</tr>
<tr>
<td>Methanol / Biodiesel</td>
<td>50 °C, 24 h</td>
<td>30.9:1 (v/w)</td>
<td>Mechanical pressing</td>
<td>Centrifugation, 7000 rpm 5 min</td>
<td>Chlorella</td>
<td>30%</td>
<td>(W.-C. Huang et al., 2017)</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>125°C, 2 h</td>
<td>2.7:1 (v/w)</td>
<td>N/A</td>
<td>Centrifugation, 3700 rpm 10 min</td>
<td>Nannochloropsis</td>
<td>20%</td>
<td>(J. Park et al., 2017)</td>
</tr>
<tr>
<td>Amine solvents</td>
<td>Room temp, 1-3 h</td>
<td>2:1 (v/w)</td>
<td>N/A</td>
<td>Centrifugation, N/A</td>
<td>Unknown</td>
<td>15%</td>
<td>(H.-Y. Yang et al., 2017)</td>
</tr>
<tr>
<td><strong>Water immiscible solvents</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>d-limonene, α-pinene, p-cymene</td>
<td>97 °C, 30 min</td>
<td>8.3:1 (v/w)</td>
<td>N/A</td>
<td>Distillation</td>
<td>Nannochloropsis</td>
<td>20%</td>
<td>(Dejoye Tanzi et al., 2013)</td>
</tr>
<tr>
<td>Hexane</td>
<td>35°C, 2.5 h</td>
<td>0.9:1 (v/w)</td>
<td>Incubation and HPH</td>
<td>Centrifugation, 784×15 min</td>
<td>Dunaliella</td>
<td>20-25%</td>
<td>(Olness et al., 2013b)</td>
</tr>
<tr>
<td>Hexane</td>
<td>22°C, 20h</td>
<td>1.4:1 (v/w)</td>
<td>HPH</td>
<td>Centrifugation, 7943×10 min</td>
<td>Nannochloropsis</td>
<td>14%</td>
<td>(Jiménez Callejón et al., 2014)</td>
</tr>
<tr>
<td>Hexane</td>
<td>Room temp, 5 min</td>
<td>4:1 (v/w)</td>
<td>MW</td>
<td>Centrifugation, N/A</td>
<td>Chlorella</td>
<td>23%</td>
<td>(J. Cheng et al., 2014)</td>
</tr>
<tr>
<td>Liquefied Dimethyl ether</td>
<td>20°C</td>
<td>79:1 (w/w)</td>
<td>N/A</td>
<td>Filtration with glass beads</td>
<td>B. braunii</td>
<td>26%</td>
<td>(Kanda et al., 2013)</td>
</tr>
</tbody>
</table>

Note: HPH = High pressure homogenisation; MW = Microwave treatment; N/A = details not available. Shaded = unfavorable criteria: temperature ≥80°C, solvent ratio >3:1.
2.4.6 Current knowledge gaps in biphasic lipid extraction from microalgae

Knowledge of the mechanisms and kinetics of biphasic lipid extraction from microalgae is very limited due to the unique complexities of this system (e.g. micron-sized algal cells with initially entrapped lipid droplets that are subsequently ruptured, a complex algal lipid profile, a high moisture environment, and the propensity for emulsion formation). Most of the research to date has only presented the final lipid yield of the extraction (Table 2.2) but not the kinetics of the extraction process. The studies that have considered lipid extraction kinetics were performed with a monophasic or a mixed solvent system (Halim et al., 2014; Ranjan et al., 2010). These are distinctly different from a biphasic system (Figure 2.3).

Jimenez et al. provided the bulk extraction kinetics of a biphasic hexane extraction process on a slurry of ruptured algae (Jiménez Callejón et al., 2014). However, details of the emulsion formation (emulsion droplet size), and the effects of other process variables such as temperature, mixing speed and solids concentration on extraction kinetics, were not considered.

The formation of emulsions during lipid extraction process has generally been reported to reduce lipid yield (M. Cooney et al., 2009; Fajardo et al., 2007; Grima et al., 2013; Hita Peña et al., 2015). However, based on the recent mechanistic understanding developed for a biphasic extraction system (Figure 2.3), it can be argued that the initial emulsion formation is likely necessary to provide the solvent interface area required for an efficient lipid transfer. More accurately, the reduced lipid yield was probably due to an inefficient emulsion destabilisation and therefore an inefficient recovery of the lipid-rich solvent. However, all the available lipid extraction studies (Table 2.2) have evaluated the extraction process solely on a lipid yield basis and none has considered the potential lipid loss via the unrecovered solvent (containing dissolved lipids). There is a need to evaluate the solvent recovery process in order to identify the limiting factors in a solvent extraction process. Thus, this will be reviewed in the next section.
2.5 Solvent recovery via phase separation

In a biphasic solvent extraction, the solvent (e.g. hexane) is used as the ‘lipid carrier’ (Figure 2.4). A fresh solvent (containing no solute) is initially introduced and emulsified into wet ruptured-algae slurry to allow lipid transfer. Once the lipid partitioning/extraction process has been completed, the solvent that now contains the dissolved algal lipid is recovered through a phase separation process (typically by centrifugation) (Jiménez Callejón et al., 2014; Olmstead et al., 2013b). This process splits the emulsion into separate immiscible phases based on their density difference (Martin, 2016). Phase separation can also potentially be obtained via other techniques such as gravity settling (Belarbi et al., 2000; Nadin & Semiat, 1995) and filtration (Ansari et al., 2017; Kanda et al., 2013). However, gravity settling is a slow process (Pragya et al., 2013) and filtration is likely only applicable for larger algal cells due to membrane clogging/fouling issues (Bhave et al., 2012; Zhang et al., 2013). Therefore, centrifugation is the most widely used technique for recovering a solvent (Table 2.2).

![Diagram of solvent recovery process](image)

**Figure 2.4:** The main processes in a biphasic solvent extraction using hexane solvent on a wet ruptured-algae slurry (Halim et al., 2016; Jiménez Callejón et al., 2014; Olmstead et al., 2013b).

Near-complete recovery of the solvent is important for a few reasons (Tao Dong et al., 2016b; Martin, 2016; Molina Grima et al., 2003; Wakelyn & Wan, 2004):

1. The quantity of the recovered lipid-rich solvent directly determines the yield of the lipid;

2. A complete removal of the solvent from the delipidated biomass is required to ensure its safety when utilised for other purposes (e.g. as a fish feed);

3. In an ideal process, the solvent will be recovered fully and recycled for use in the subsequent solvent extraction, thus eliminating the reliance on a constant supply of petroleum-based solvents (e.g. hexane).
Although the centrifugation is routinely used for phase separation, there is a lack of information on the centrifugation-based solvent recovery process from microalgae (Tao Dong et al., 2016b; Martin, 2016). More specifically, there is no detailed data available on the emulsions formed during biphasic solvent extraction, or studies that have considered the kinetics or the mechanisms of centrifugal solvent recovery. Given the possibility that stable emulsions inhibit the complete recovery of the solvent (Tao Dong et al., 2016b), it is important to understand the phase separation process to enable further process optimisation (Martin, 2016). As the information specific to solvent recovery from algae is not available, the existing knowledge from other related fields (e.g. food emulsions, petroleum crude oil separation and general surface chemistry studies) were reviewed to develop a basic understanding of solvent recovery processes.

### 2.5.1 General principles of centrifugation-assisted phase separation

In a biphasic lipid extraction process (Figure 2.4), initial emulsion formation is required to increase the interfacial area available for the lipid transfer to occur (Tao Dong et al., 2016b; Yap et al., 2014). Once the lipid partitioning has approached an equilibrium, the emulsion is then destabilised via centrifugation to recover the solvent from the emulsion. Therefore, a highly stable emulsion resistant to phase separation needs to be avoided for efficient solvent recovery (Lennie et al., 1990).

An emulsion is a mixture of two immiscible phases (typically oil and water) formed by dispersing one phase into the other, resulting in the creation of additional interfacial area (Leal-Calderon et al., 2007). Most emulsions are generally thermodynamically unfavourable due to the increase in interfacial area (Dickinson, 1994; Rousseau, 2000). Over time, the emulsions can usually be destabilised spontaneously via various mechanisms such as Ostwald ripening, flocculation, (partial) coalescence, creaming and phase inversion (Damodaran, 2005; Dickinson, 1994; Walstra, 1987). However, the rate of these destabilising mechanisms can be extremely slow for some emulsion, which can thereby be considered ‘kinetically stable’.

Centrifugation-assisted phase separation has been theorised (Krebs et al., 2012) to occur in a multi-stage process (Figure 2.5). Firstly, under the influence of the centrifugal force, the lighter oil droplets will rise (cream) to the top as a result of the density difference. A concentrated layer of oil droplets will therefore be formed at the top. When the oil droplets are forced together, they can coalesce (i.e. fuse together) to form larger droplets. When sufficient droplets have coalesced, a distinct oil layer can be observed on the surface (Damodaran, 2005; McClements, 2015). In the timescale of a centrifugation process, slower emulsion destabilisation processes such as Ostwald ripening (typically occurring over a period of days to months) are unlikely to be important (Taylor, 2003). Therefore, the two main mechanisms involved in a centrifugation process were proposed to be creaming and droplet coalescence. These will be discussed in detail.
2.5.2 Creaming

Due to the differences in the density of the dispersed oil/solvent phase (typically less dense) and the water phase (usually denser), there exists a buoyancy force under the influence of gravity or centrifugal force (Rousseau, 2000; Walstra, 1987). This buoyancy force allows the lighter phase to cream to the top while the heavier phase sediments to the bottom. Most emulsions are thermodynamically unstable (Dickinson, 1994) but they can still be kinetically stable due to a slow (e.g., considerably longer than the observation period) creaming rate. The only exceptions to this are thermodynamically stable microemulsions with extremely small droplets (<10 nm) (Lawrence & Rees, 2012; Solans et al., 2005). These are stable due to the dominance of Brownian forces over buoyancy/gravitational effects. The preparation of this microemulsion often involves special techniques (high surfactant concentration and high energy mixing) (McClements, 2012; Paul & Moulik, 1997) and is therefore not relevant to wet solvent extraction.

For thermodynamically unstable emulsions, the creaming velocity of the droplets can be mathematically described by the Stokes equation, provided the following conditions are met: (i) the droplets are mono-disperse in size, (ii) there are no interactions between the droplets, (iii) the droplets are rigid, smooth spheres, (iv) there are no wall effects, (v) there is a low Reynolds number, (vi) the droplet movement is driven solely by density difference (vii), and there is no hindered creaming (Badolato et al., 2008; Ward-Smith, 2012):

\[
\text{Equation 2.2 : } v = \frac{2d^2(\rho_p-\rho_l)}{9\mu}g
\]

Where \( v \) = creaming velocity of the droplet, \( d \) = diameter of the droplet, \( \rho_p \) = density of the continuous phase, \( \rho_l \) = density of the droplet, \( \mu \) = dynamic viscosity of the continuous phase, \( g \) = gravity acceleration.
Although the solvent droplets produced during algal lipid extraction may not meet all the conditions that were assumed (Equation 2.2), the equation is nonetheless a useful indicator of the major factors affecting the creaming process. For example, from the equation, the dispersed droplet diameter has the largest influence over the creaming velocity due to a squared dependency. The viscosity of the continuous phase (algal slurry) has an inverse relationship with the creaming velocity while the density difference has a positive relationship. Apart from the characteristics of the emulsion (e.g. density difference, viscosity, droplet size), a higher gravitational acceleration (in the form of centrifugal force) can also be applied on the emulsion to increase the creaming velocity, which subsequently contributes to a faster phase separation.

2.5.2.1 Droplet size

In a wet algal solvent extraction, the droplet size can be influenced by both the mixing intensity and the interfacial tension that arising due to the surfactants available in the system (Leal-Calderon et al., 2007; McClements, 2015; Walstra, 1987). A higher mixing intensity and a lower interfacial tension tend to produce smaller, often polydisperse, droplets (Badolato et al., 2008).

In a non-ideal emulsion, the dispersed droplets can also be brought closer by random movement and then interact to form droplet aggregates (Fredrick et al., 2010; Ivanov et al., 1999). Depending on the emulsion volume fraction, the droplet aggregation may increase the creaming velocity or it may hinder the creaming process due to a droplet-packing constraint (Damodaran, 2005; Dickinson, 2010a). In the former case, the new creaming velocity can be derived by accounting for the new effective diameter of the aggregates (Equation 2.2). In the latter case, the creaming velocity will have to be determined empirically by a modified Stokes equation (Richardson & Zaki, 1997).

2.5.2.2 Density of the droplet and continuous phase

The overall density of a typical algal slurry is close to the density of its growth media/water (e.g. ranging from 1.00-1.04 kg m\(^{-3}\) at 300K for a marine microalga) (Schneider et al., 2016). This value is unlikely to change significantly in an algal process as a highly concentrated algal slurry (15-25 % w/w) will still be mostly water (75-85%), and algal cells have a density fairly close to that of the medium. Therefore, to enhance the creaming velocity, selecting a solvent with a very different density to the algal slurry is critical to promoting a greater creaming velocity. For that reason, using a solvent such as the hexane that has a very different density to water (density = 0.66 kg m\(^{-3}\)) is desirable.

2.5.2.3 Viscosity of the continuous phase

For a microalgal suspension, the solids concentration (on a dry biomass basis) was found to be the largest factor influencing its viscosity (Cagney et al., 2017; Schneider et al., 2016; Wileman et al., 2012). Above a critical solids concentration (varying between 4-15 % w/w), the algal suspension transitions from a Newtonian fluid (water-like) to a complex pseudo-plastic fluid with shear thinning
characteristics. The behavior transition was hypothesised to be the result of the interaction between the closely-packed cell particles (Yap et al., 2016b). A previous attempt to model the viscosity of an algal slurry demonstrated that the viscosity increased exponentially as a function of the solids concentration (Schneider et al., 2016). Therefore, a solvent recovery process that involves a high-solids biomass is expected to have a highly viscous continuous phase during the creaming process.

2.5.2.4 Centrifugal force

Intuitively, a higher centrifugal force is desired as the creaming velocity will be enhanced and thus a better phase separation can be expected (Krebs et al., 2012). However, in practice, most industrial centrifuges have a limited rotational speed (Beveridge, 2000), and a high centrifugal force is negatively correlated to the throughput capacity. Therefore, knowing the minimal centrifugal force required to destabilise the emulsion and the expected separation performance is important for selecting an appropriate separator and for evaluating the cost and energy requirements of such a process (Navarro López et al., 2016b). In the solvent extraction studies performed to date on algae (Table 2.2), a wide range of centrifugal force (between 3000 x g-10000 x g) and time were used. However, little if any information was given about the separation efficiency and other important factors related to centrifugation (Equation 2.2). Thus the required specification of an industrial centrifuge unit during the scale up process cannot be determined from the available data (Martin, 2016).

2.5.3 Droplet coalescence

Coalescence refers to an irreversible process where two or more droplets merge into a bigger droplet (Dickinson, 1994), whereas creaming is the movement of droplets to the surface. Creaming and coalescence are distinct but often connected processes. Creaming can be accelerated by coalescence (larger droplets rise faster), and coalescence can result from creaming (as droplets are brought close together). In a solvent recovery process, the coalescence of the solvent droplets is important to achieve proper phase separation (Krebs et al., 2012).

To initiate droplet coalescence, two droplets first have to come into proximity for a long enough time (Walstra, 1987). However, the fate of the droplets upon contact can vary (Figure 2.6) depending on the emulsion characteristics, in particular the properties of surface-active molecules present at the oil/water interface (Badolato et al., 2008; Furtado et al., 2015; Ivanov et al., 1999). For example, droplets that are surfactant-free or have a weak or incomplete surfactant coverage can coalesce almost immediately upon contact (Fredrick et al., 2010). On the other hand, surfactant-rich emulsion droplets may be very stable against coalescence due to the adsorbed surfactant layer forming a steric or electrostatic barrier (McClements, 2015). In that case, the two droplets can either rebound from each other (repulsion) or become flocculated (attraction) by the intermolecular forces (Ivanov et al., 1999). To successfully
coalesce two stable droplets, the initial energy barrier required to rupture the droplet films has to be overcome (Rousseau, 2000).

![Diagram of droplet interaction](image)

**Figure 2.6**: The possible fates of two droplets upon coming into contact.

In general, emulsions are more stable when surface-active molecules (surfactants) fully cover the interface. These surface-active (often amphiphilic) molecules can be divided into three prevailing groups according to their size: low molecular weight surfactants (LMWS), high molecular weight surfactants (e.g. proteins), and solid particles (Tcholakova et al., 2008). The difference between these surface-active agents will be reviewed in the next section.

In literature, the terms emulsifier and surfactant are often used interchangeably for surface-active agents as they both can lead to emulsion formation (Uzoigwe et al., 2015). However, their modes of action can be distinguished - a surfactant acts mainly by lowering the interfacial tension by adsorbing to the interface, while an emulsifier can stabilise an emulsion without necessarily relying on a surface tension reduction (Dickinson, 2009; Mnif & Ghribi, 2015). In addition, a surface-active agent can act as a surfactant or an emulsifier, or both (Mnif & Ghribi, 2015; Uzoigwe et al., 2015). Therefore, in this review, the term ‘surface-active molecule/agent/component’ and ‘surfactant’ are used interchangeably to refer to agents that are capable of adsorbing to an oil/water interface to reduce the interfacial tension, whereas the term ‘emulsifier’ is used specifically to refer to its emulsion stabilising capacity.
2.5.3.1 Low molecular weight surfactant (LMWS)-based emulsions

The LMWS are perhaps the most widely known due to their widespread practical applications (A. Bos & van Vliet, 2001). Some examples of LMWS are the various soaps and synthetic detergents formulated for both domestic or industrial (petroleum processing) uses (Salager, 2002), or the monoglycerides and phospholipids (lecithin) that are widely used for creating food emulsions (Kralova & Sjöblom, 2009). The LMWS can be categorised into non-ionic or ionic surfactants and they can either be water-soluble or oil-soluble for different applications (A. Bos & van Vliet, 2001; Salager, 2002). The LMWS are often characterised based on their hydrophilic-lipophilic balance (HLB) to predict the type of emulsions that will be formed (e.g. oil-in-water, water-in-oil, or water-oil-water, or oil-water-oil) (Adams et al., 2007).

Due to their small size compared to other surface-active agents, LMWS have a high diffusivity (Damodaran, 2005) and mobility at the interface. This means they can form mobile (as opposed to rigid) interfacial films and rapidly come to equilibrium (Kralova & Sjöblom, 2009; Walstra, 1987). For example, in a complex surfactant system (e.g. LMWS mixed with colloidal solid particles), the LMWS will typically adsorb to the interface more rapidly compared to the slower-adsorbing colloidal particles. The presence of other surfactants alongside LMWS can result in complex behaviour, such as a synergistic emulsion stabilisation effect (Pichot et al., 2010). LMWS has also been found to readily compete with and displace adsorbed protein molecules from an interface (i.e. an antagonistic effect) (Walstra, 1987). In general, LMWS can lower the interfacial tension to a much greater extent than bigger surfactants (e.g. protein molecules or solid particles). However, the resulting emulsions are typically less stable against coalescence (A. Bos & van Vliet, 2001).

2.5.3.2 Protein-stabilised emulsions

High molecular weight emulsifiers include proteins and polysaccharides (hydrocolloids) (Kralova & Sjöblom, 2009). Hydrocolloids (e.g. xanthan gum or pectin) typically function as thickening agents or stabilisers by retarding the creaming process, and are therefore not strictly considered to be surfactants (Dickinson, 2009; Kralova & Sjöblom, 2009). As such, this section will focus on proteins and their role in stabilising emulsions.

In contrast to LMWS-stabilised emulsions, protein-stabilised emulsions are characterised by the formation of a gel-like adsorption film at the interface via noncovalent interactions (Dickinson, 2001). Proteins contain both hydrophilic and hydrophobic amino acids (Damodaran, 2005). In native globular proteins the hydrophobic residues (such as non-polar and sulfhydryl containing amino acids) tend to be present in hydrophobic cores folded within the tertiary structure. If protein molecules are exposed to a solvent/oil interface, this structure can unfold (Damodaran, 2005; McClements, 2004), exposing the hydrophobic residues which can associate with the hydrophobic phase, while the remaining parts of the protein can stay immersed in the aqueous phase. Such an arrangement of proteins at an interface can provide a steric stability against flocculation and coalescence (Damodaran, 2005; Y. Fang & Dalgleish,
In addition to providing steric stability (McClements, 2004; Narsimhan, 1992; Slavka Tcholakova et al., 2006), protein interfacial films can also protect the droplets from flocculation by modifying the electrical potential (Kulmyrzaev & Schubert, 2004; McClements, 2004; Narsimhan, 1992) and/or the Van Der Waals interactions between the droplets (Slavka Tcholakova et al., 2001).

Adsorbed protein molecules can spontaneously rearrange to form an ordered structure at the interface (Beverung et al., 1999; Norde, 2011). This structure can be observed as a viscoelastic film with mechanical strength (Dickinson, 2001; Lam & Nickerson, 2013). This film provides an additional protection against coalescence for droplets that have managed to overcome the steric and electrostatic barriers and been brought in close contact. Due to these traits, proteins are often a superior emulsifier for long-term emulsion stability in comparison to the LWMS (Damodaran, 2005). In protein-coalescence studies, it is often observed that a sufficient compression or a mechanical force larger than the disjoining pressure (i.e. the pressure required to ‘disjoin’ the two droplet films in contact, and is defined as force per unit area of the film (Narsimhan, 1992)) is required to coalesce protein-stabilised emulsions (Robins et al., 2002; Slavka Tcholakova et al., 2006).

2.5.3.3 Solids-stabilised emulsions

A solids-stabilised emulsion is also known as a particle-stabilised emulsion or a Pickering emulsion (Pickering, 1907). As its name implies, solids-stabilised emulsions are stabilised by small solid particles with a size ranging from nm to µm. Solids-stabilised emulsions have recently gained interest due to their newly discovered commercial applications (Aveyard et al., 2003; Berton-Carabin & Schroën, 2015) and their unique characteristics in comparison to conventional surfactant systems (LMWS and protein-based) (Arditty et al., 2003).

Some of the unique properties of a solids-stabilised emulsion were summarised and explained by (S Tcholakova et al., 2008):

(i) The process of Ostwald ripening (a common phenomenon that can destabilise a protein-stabilised and a surfactant-stabilised emulsions) can be arrested completely in a solids-stabilised emulsion. This behavior was hypothesised to be a result of the high desorption energy required to displace solid particles from an interface. As the droplets shrink (due to initial Ostwald ripening), this leads to particle jamming at the interface (instead of being desorbed from the interface), which eliminates the capillary pressure required for Ostwald ripening.

(ii) Long-term emulsion stabilisation (up to years) can be achieved with minimal or no surfactant.

(iii) The solid particles do not have to be amphiphilic to stabilise an emulsion.

(iv) A solids-stabilised emulsion is characterised by a high energy barrier for particle adsorption and a high desorption energy barrier post-adsorption.
2.5.3.4 Characteristics of emulsions formed by different surfactant groups

Emulsions are dynamic in that the surfactants can continually adsorb and desorb from the interface (Malhotra & Wasan, 1987; Yan & Masliyah, 1996). In many cases, the stability of an emulsion is primarily due to the characteristics of the interfacial film (Dickinson, 2010b, 2012). Stable emulsions often have full surfactant coverage at the interface that provides repulsive interactions between droplets. In contrast, unstable emulsions often arise if the surfactants are easily desorbed or there is only partial surfactant coverage of the interface.

The size of the adsorbed surfactants has been found to have a significant influence over the emulsion characteristics (S Tcholakova et al., 2008). These key differences are summarised in Table 2.3. In brief, a small LMWS is the most mobile and can both adsorb and desorb from the interface rapidly. In contrast, larger surfactants including the solid particles have a high energy barrier to both adsorption and desorption and thus adsorb and desorb less readily. Due to the high energy barrier to desorption, solid particles often appear to be irreversibly absorbed on an interface, and the resulting emulsions are highly stable against coalescence (B. P. Binks et al., 2010; Dickinson, 2010b; Giernmanska-Kahn et al., 2005; S Tcholakova et al., 2008).

Table 2.3: Qualitative comparison of emulsions formed by different types of surface-active materials (A. Bos & van Vliet, 2001; Berton-Carabin & Schroën, 2015; Dickinson, 2010b; S Tcholakova et al., 2008).

<table>
<thead>
<tr>
<th>Surfactant Molecule Size</th>
<th>LMWS</th>
<th>Protein</th>
<th>Particles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interfacial Film Thickness</td>
<td>Small (nm)</td>
<td>Moderate (nm)</td>
<td>Generally larger (nm to μm)</td>
</tr>
<tr>
<td>Adsorption energy</td>
<td>Low</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Desorption energy</td>
<td>Low</td>
<td>Moderate</td>
<td>High</td>
</tr>
<tr>
<td>Adsorption process</td>
<td>Reversible</td>
<td>Practically irreversible</td>
<td>Practically irreversible</td>
</tr>
<tr>
<td>Arrangement at interface</td>
<td>Tightly packed</td>
<td>Mesoscopically more porous than LMWS</td>
<td>Rigid disordered layer/network of particles</td>
</tr>
<tr>
<td>Characteristic of the interfacial film</td>
<td>Substantial surface lateral diffusion</td>
<td>Immobile, viscoelastic interfacial films with non-Newtonian behavior</td>
<td>Typically behaves as a rigid shell</td>
</tr>
</tbody>
</table>
2.5.4 Potential surfactants and emulsifiers in microalgae

As the type surface-active molecule will influence the emulsion stability, an understanding of the surfactants/emulsifiers present in a microalgal cell will be required to explain the emulsions in wet lipid extraction.

Like other microbes, the basic components of microalgal cells (e.g. proteins, cell walls, membrane lipids) can be surface-active. In addition, microalgae can produce a variety of surface-active agents to perform biological functions (e.g. to grow at water/air boundaries, to attach to solid surfaces and to improve the bioavailability of a hydrophobic substrate) (Holmberg, 2001; Mnif & Ghribi, 2015; Rosenberg & Ron, 1999; Sunde et al., 2017). As such, there are numerous molecules and cell components that may emulsify the organic solvent used in a solvent extraction process, resulting in difficulties in product separation downstream.

2.5.4.1 Extracellular polymeric substances (EPS)

As microalgae cells grow in suspension, it can be advantageous for them to perform cell-to-cell or cell-to-environment interactions (Barnes, 2003). This function can be performed by secreting so-called extracellular polymeric substances (EPS, distinguished here from the exopolysaccharide that is also commonly abbreviated as EPS but is only a subset of the extracellular polymeric substances) into their immediate environment. EPS includes polysaccharides, proteins, nucleic acids and lipids (R. Xiao & Zheng, 2016) and can be divided into soluble, loosely-bound and tightly-bound EPS depending on their degree of attachment to the cell surface (Basuvaraj et al., 2015; Sheng et al., 2010). Of relevance here, the EPS from some microalgae have been found to be potent emulsifiers, comparable with or even better than commercial surfactants such as Tween 20 and Triton X-100 (Bramhachari & Dubey, 2006; J. A. Mata et al., 2006; Mishra & Jha, 2009).

2.5.4.2 Protein

Protein can comprise up to 55-70% of the dry biomass in a microalgal cell (T. M. Mata et al., 2010). Protein can exist in many forms, either as a standalone entity (i.e. a pure protein molecule such as an enzyme or receptor protein) or as a complex molecule associated with lipids or carbohydrates (e.g. lipopeptide or glycopeptide) (Alberts, 2017). A majority of these proteins are contained intracellularly and therefore can only be accessed after cell rupture (Jubeau et al., 2013; Schwenzfeier et al., 2011). Previous studies have found that cytosolic protein extracted from microalgae can form stable emulsions (Schwenzfeier et al., 2013; Ursu et al., 2014). In wet solvent extractions, for which cell rupture is required, it is hypothesised that the release of these cytosolic proteins may lead to the formation of stable emulsions. However, the effect of the released intracellular proteins on the solvent recovery process has not yet been investigated.
2.5.4.3 Neutral and polar lipids

As reviewed in section 2.4.1.1, the neutral lipids (NL) include membrane structural lipids such as sterols and TAG which serves as a carbon and energy store for the cells, often existing as distinct oil droplets in the cytosol (Goold et al., 2015). To maintain the lipid stability in an aqueous environment, the highly nonpolar TAG is surrounded by a monolayer of polar lipids (N.-L. Huang et al., 2013). This polar lipid monolayer membrane is intercalated with hydrophobic proteins (also known as lipid droplet proteins) to prevent the droplets from coalescing (X. Wang et al., 2017a; Yoneda et al., 2016). Lipid droplet proteins in microalgae (Nannochloropsis) have been found to be analogous to the hydrophobic protein (oleosin) found in higher plants (Vieler et al., 2012). In a soybean oil extraction process, the presence of oleosin was found to reduce the free oil recovery, and to cause the formation of stable emulsions if it was co-extracted with the oil (Chabrand et al., 2008). It is possible that this lipid droplet protein can play a similar role in algal lipid extraction.

In addition, polar lipids (including glycolipids and phospholipids) found in the cell membrane and the membranes of the cellular organelles, may also contribute to a stable emulsion in a solvent extraction process. The ability of polar lipids (from non-microalgae sources) to form emulsions has been demonstrated in the production of food emulsions and in the bioremediation of marine oil spills (Holmberg, 2001; Kralova & Sjöblom, 2009; Maneerat, 2005). Therefore, it is hypothesised that algal polar lipids may play a role in stabilising emulsions during lipid extraction.

Another important but perhaps unexpected surface-active agent, are the algal cells, which can act as surface active particles (Maneerat, 2005; Mnif & Ghribi, 2015). At a sufficiently high solids concentrations (>1 % w/w for latex and hydrophobic starch particles), small solid particle have been found to stabilise emulsions through the formation of a Pickering emulsion (Frelichowska et al., 2010; Song et al., 2015). In addition, the smaller cell debris generated from the cell rupture process (Yap et al., 2014) may also increase the emulsification properties of the cell particles (B. Binks & Lumsdon, 2001). Therefore, both the high solids concentration of the algal cell particles and the presence of the cell debris are likely to affect the solvent recovery process.

2.5.5 Current knowledge gaps in the solvent recovery process

To achieve an efficient solvent recovery, destabilising the emulsified solvent droplets via coalescence is key (Figure 2.6). Therefore, understanding the stability and composition of emulsions formed during solvent extraction is critical for process optimisation. Slurries of ruptured algal cells are compositionally complex and contain various surface-active agents (section 2.5.4) that each could lead to a stable emulsion. However, it is unclear what happens when all of these emulsifiers/surfactants are simultaneously present during solvent extraction. Surface chemistry studies of emulsions (S Tcholakova et al., 2008) have mostly been performed with one or two types of surfactants/emulsifiers, but
information and understanding of systems as complex as ruptured wet algal biomass is currently lacking. It is not known if or how (e.g. synergistically or antagonistically) the various surface-active molecules in a wet ruptured-algae biomass can interact with each other at the solvent interface, or how this affect a wet solvent extraction and separation process. Information on the emulsion properties of the algal components is generally scarce, and where available, a purified component was studied in isolation (Kabalnov et al., 1995; Schwenzfeier et al., 2013; R. Xiao & Zheng, 2016). Therefore, there is a need to assess the ruptured-algae slurry as a whole from surface chemistry perspective.
2.6 Transesterification of algal lipids

Once the algal lipids have been recovered, the solvent can be removed via evaporation and the lipids can be converted into biodiesel via transesterification (Rawat et al., 2013; Robles-Medina et al., 2009). Transesterification is a multi-step chemical reaction (Figure 2.7) in which a mole of TAG (or other molecule containing acyl chains) is sequentially hydrolysed and trans-esterified into three moles of fatty acid methyl esters (FAME) (Fjerbaek et al., 2009; Kaieda et al., 1999; Robles-Medina et al., 2009). A transesterification reaction is not spontaneous and therefore requires a catalyst as well as a high temperature to lower the activation energy. The transesterification reaction is also reversible, with a hydrolysis reaction (FAME hydrolysis to produce FFA) as the opposing reaction. Thus, a high FAME yield is typically favoured by using an excess of methanol and a low water content to minimise the competing hydrolysis reaction.

![Figure 2.7: Generalised reactions during lipase transesterification. Adapted from (Adlercreutz, 2013).](image)

2.6.1 In-situ transesterification

In-situ transesterification, also known as direct transesterification, is a technique where both the lipid extraction and the transesterification reaction are performed simultaneously in a single step. Due to the integration of process steps, the capital and operating costs and the energy consumption can be reduced (J. Kim et al., 2013). As such, in-situ transesterification appears to be a promising strategy for improving the energy balance of a wet lipid extraction process.

To date, studies of in-situ transesterification have mostly been performed using homogenous acid or alkaline catalysts on dried algal powders (Pragya et al., 2013; Rawat et al., 2013). This is because the presence of free fatty acids and water (both which can be abundant in a wet ruptured-algae biomass) cause soap formation and catalyst loss during the process (Cao et al., 2013; Pragya et al., 2013; Sathish et al., 2014; Szczęsna Antczak et al., 2009). In addition, a chemical-catalysed transesterification reaction
usually requires a high reaction temperature (60-100°C). The high temperature is likely to degrade the heat sensitive products in the biomass and can have a high energy cost (J. Kim et al., 2013). Furthermore, neutralisation of the acid and alkaline catalyst can generate a large amount of wastewater downstream. Therefore, the in-situ transesterification of a wet ruptured-algae slurry is unlikely to be energetically feasible using a chemical catalyst.

2.6.2 Application of lipase as a biocatalyst

Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3.) are biocatalysts widely used in the cheese industry for lipid hydrolysis and in the pharmaceutical industry for synthesising esters (esterification) (Hasan et al., 2006). Recently, lipases have also been evaluated as a biocatalyst for biodiesel conversion, due to their ability to achieve high yields even in the presence of water and FFA. Compared to chemical-catalysed reactions, lipase requires much milder reaction conditions (20-50°C), avoiding the degradation of heat-sensitive algae components (Fjerbaek et al., 2009; Szczęsna Antczak et al., 2009). Furthermore, an enzymatic reaction is highly selective. Therefore, the unwanted side reactions that occur in an acid/alkaline-catalysed reaction can be avoided (J. Kim et al., 2013; Pragya et al., 2013; Szczęsna Antczak et al., 2009). For these reasons, lipases appear to be particularly promising for algal lipid conversion into biodiesel.

2.6.3 Interfacial activation of lipase

Lipases are an interesting subject for enzymology studies due to the heterogeneous reaction media involved (Hasan et al., 2006; Kanmani et al., 2015; Reis et al., 2009). A typical lipase-mediated reaction contains a minimum of two phases, where the lipase is often located in an aqueous phase while its substrate is typically located in an immiscible oil phase.

To deal with this phase disparity, lipases have evolved a unique structure and mechanism that allow them to access the substrate (Figure 2.8). In its inactivated form (when located in a fully aqueous phase), the hydrophobic catalytic site of the lipase, typically consisting of a catalytic triad (serine, histidine and aspartic amino acid), is shielded by a mobile lid structure (Carrasco-López et al., 2009; Derewenda et al., 1992). Upon coming into contact with an oil interface/droplet, the lipase can undergo a conformational change to open the lid, thus exposing the catalytic site for substrate binding (Rafael C Rodrigues & Fernandez-Lafuente, 2010a; Verger & De Haas, 1976). This mechanism is known as ‘interfacial activation’ and it allows the lipase to be adsorbed to the oil/water interface for catalysing the reactions. The consideration of this mechanism is especially important when designing a lipase-mediated reaction system (Reis et al., 2009) or when immobilising a lipase on a carrier (Hanefeld et al., 2009). In the former case, a lipase-mediated reaction media typically performs better in an emulsified
system. In the latter case, the lipase is usually immobilised on a hydrophobic carrier surface to ensure the lipase remains in the activated form (Adlercreutz, 2013).

![Figure 2.8](image.png)

**Figure 2.8**: A schematic of the interfacial activation of a lipase: (A) the lid is closed in a fully aqueous environment; (B) the lid is opened upon contacting a hydrophobic environment or interface (Adlercreutz, 2013; Hanefeld et al., 2009).

### 2.6.4 Factors affecting in-situ transesterification

In general, a biodiesel conversion yield above 90% can be attained using a lipase catalyst under optimal reaction conditions. The reaction time however, can vary between 9-96 hours, and the optimal temperature can be between 30-50 °C depending on the source of the lipase as well as the lipase formulation (Aransiola et al., 2014; Fjerbaek et al., 2009; Koh & Ghazi, 2011). Beyond this, there are other key factors that have to be considered in a lipase-catalysed transesterification (Szczęsna Antczak et al., 2009).

#### 2.6.4.1 Lipase specificity

Lipases can be grouped into 3 categories – i) non-specific, ii) sn-1,3 regio-specific, and iii) fatty-acid-specific lipases (Kapoor & Gupta, 2012; Macrae, 1983). A non-specific lipase shows no preference of the position in which it will attack the glycerol backbone of a TAG molecule. A sn -1,3 regio-specific lipase will preferentially cleave at the outer 1- and 3- positions on the glycerol backbone, generating a 2-monoglyceride. A fatty-acid-specific lipase will only react with a particular type of fatty acid on the TAG molecule. For the purpose of biodiesel conversion, the lipase should be non-discriminate as algal lipids contain a broad variety of fatty acids (Ma et al., 2016; Martin et al., 2014). In theory, a 1,3 regio-specific lipase will only convert a TAG molecule into two moles of FAME (instead of the potential three) as the acyl group on position 2- will not be hydrolysed. However, a spontaneous migration of the
2-acyl chain has been observed, and a near-complete FAME conversion can still be obtained by a 1,3 regio-specific lipase (W. Du et al., 2005; Kaieda et al., 1999; Rafael C. Rodrigues & Fernandez-Lafuente, 2010b). Therefore, either a non-specific lipase or a sn-1,3 regio-specific lipase may be used for catalysing a transesterification reaction.

2.6.4.2 The influence of water content

In general, lipases require a minimal amount of water to keep the enzyme surface-hydrated, which is needed to maintain its catalytic activity and structure (Timasheff, 1993; Yamane, 1987). Increasing the water content beyond this minimal level can enhance the enzymes flexibility and thus improve the lipase activity (Kaieda et al., 2001; Shah et al., 2004). However, when water is in excess, the opposing hydrolysis reaction can become more favourable (Figure 2.7), thus decreasing the FAME yield at the reaction equilibrium (Szczęsna Antczak et al., 2009). The optimal water content appears to vary depending on the choice of lipase and the reaction system used (Adlercreutz, 2013; Fjerbaek et al., 2009; Salis et al., 2005; Shah et al., 2004; Valivety et al., 1992). For example, the lipase isolated from a *Candida antarctica* (Novozyme 435) exhibited the highest FAME conversion when there was a minimal water content (<0.5 % w/w) (Shimada et al., 1999; Watanabe et al., 2005). In other studies, lipases isolated from *Rhizomucor miehei*, *Candida rugosa*, *Pseudomonas cepacia*, and *Pseudomonas fluorescens* showed an increasing transesterification conversion with increasing water content, up to 20 % w/w water (Al-Zuhair et al., 2006; Kaieda et al., 2001).

As a wet algal slurry is high in water content (75-85 % w/w moisture), it is desirable to select a lipase that can favour the FAME conversion in a high water environment.

2.6.4.3 Temperature

In a lipase-catalysed reaction, the reaction equilibrium (which dictates the maximal FAME yield) is not likely to be affected by the temperature range commonly required by the lipases (20-60 °C) (Fjerbaek et al., 2009). However, a high reaction temperature can increase the reaction rate of a lipase-catalysed transesterification reaction. This is because the rise in temperature can decrease the substrate viscosity, which then improves the mass transfer (Tran et al., 2013). However, increasing the temperature beyond the denaturation temperature of the lipase (typically >40 °C) can result in a decreased yield (Tran et al., 2012; M. Xiao et al., 2009; Y. Xu et al., 2003).

2.6.4.4 Solvent

In a lipase-based transesterification reaction, the purpose of solvent addition can be three-fold: (i) to partition the lipid; (ii) to decrease the substrate viscosity and thus to increase the diffusion rate; (iii) to reduce the inhibition effect of methanol (Fjerbaek et al., 2009; Royon et al., 2007; Soumanou & Bornscheuer, 2003; Tran et al., 2013). However, using a large volume ratio of solvent can also have a negative effect by diluting the substrate and thus decreasing the reaction kinetics (X. Li et al., 2007). In
the previous studies, organic solvents with different polarities (and thus different lipid partition coefficients) were used for converting algal lipids into FAME (X. Li et al., 2007; Tran et al., 2013). A higher FAME conversion was found to be inversely correlated to the polarity of the organic solvents, with n-hexane resulting in the highest FAME yield. This was attributed to three reasons: (i) the lipid is more soluble in a nonpolar solvent (Laane et al., 1987), (ii) a nonpolar solvent is better at trapping some water around the immobilised enzyme to create a micro-aqueous layer (Tran et al., 2013) and (iii) lipases can be deactivated in the presence of excessive concentrations of polar solvent (Kumar et al., 2016). Without the addition of solvent (i.e. solvent free media), no FAME conversion was observed (Tran et al., 2013).

Therefore, the addition of a nonpolar solvent (such as hexane) is critical in a transesterification reaction. This appears to align with the requirement of using a nonpolar solvent for algal lipid extraction, thus suggesting an in-situ transesterification may be applied during the hexane extraction process. This idea forms the basis of Chapter 6.

2.6.4.5 Acyl acceptor

In a transesterification reaction, the acyl chain from a substrate (e.g. TAG molecule) is transferred onto an acyl acceptor. A short-chain alcohol molecule such as methanol is the most commonly used acyl acceptor due to its low cost and abundance (Fjerbaek et al., 2009; Y. Sharma et al., 2008; Szczęsna Antczak et al., 2009). Stoichiometrically, only three moles of methanol are required to fully convert one mole of TAG (3:1 methanol:TAG molar ratio) into FAME. However, a molar ratio higher than 6:1 has often been required to achieve a maximal FAME yield (Noureddini et al., 2005; Szczęsna Antczak et al., 2009; Y. Xu et al., 2003). Yet, in a lipase-mediated reaction, an excess amount of methanol can denature the lipase and thus inhibit the FAME conversion process (Yong & Al-Duri, 1996). To mitigate this methanol inhibitory effect, methanol can be added in a stepwise manner to prevent the lipase inactivation (Noureddini et al., 2005; Shimada et al., 1999; Szczęsna Antczak et al., 2009). Alternatively, other acyl acceptors such as methyl acetate (Y. Xu et al., 2003) or tert-butanol (L. Wang et al., 2006) can be used to avoid lipase inhibition. However, the cost of these novel acyl acceptors is high and the reaction rate is slower (Fjerbaek et al., 2009; Tan et al., 2010; M. Xiao et al., 2009).

2.6.4.6 Lipase immobilisation

Despite its advantages, one major drawback of lipase catalysis is the high enzyme cost (Fjerbaek et al., 2009). To address the cost issue, the lipase can be immobilised on a carrier that allows the lipase to be recycled and reused. Several techniques can be used for lipase immobilisation, such as adsorption, cross-linkage, covalent bond, entrapment and encapsulation (Adlercreutz, 2013; Jegannathan et al., 2008; Tan et al., 2010). The adsorption method is the most widely used technique due to its simplicity and lower cost (Adlercreutz, 2013; Tan et al., 2010). However, as the enzymes are adsorbed to the carrier via weak forces such as hydrophobic interaction, enzyme leaching can be a significant issue that
results in activity loss in the subsequent reuse cycles (Jegannathan et al., 2008; Tan et al., 2010). Unfortunately, the other immobilisation methods are often expensive, and the immobilisation conditions can be harsh, thus decreasing the enzyme activity in the process (Tan et al., 2010).

2.6.4.6.1 To immobilise, or not

The added cost of enzyme immobilisation can be significant due to the expensive carrier material and the labour-intensive immobilisation process (Robles-Medina et al., 2009). In addition, some enzyme loss is expected as the immobilisation process is not fully efficient (typically 55-90% immobilisation efficiency reported) (Bhattacharyya et al., 2010; de Lathouder et al., 2008; Fernando Bautista et al., 2010; Godjevargova et al., 2006; Zaidan et al., 2012). For immobilised lipase to be a viable option, the benefit gained from the immobilisation process must outweigh the added cost. This is typically justified by a stable enzyme activity over a long lifespan spanning repeated uses.

While immobilised lipases have been shown to be stable for up to 200 reuse cycles when converting a refined vegetable oil (L. Li et al., 2006), applying the same method on a wet microalgal slurry had been a challenge. The existing studies have shown a rapid lipase activity loss (20-45% loss after only two cycles) when used in a wet algal slurry (Navarro López et al., 2016a; Tran et al., 2013). The enzyme inhibition was largely attributed to the presence of algal phospholipids that bind to the lipase/carrier (Amoah et al., 2016; W. Du et al., 2004; Y. Li et al., 2014; Navarro López et al., 2015). Further, when applied on high-solids reaction media (15-25% w/w solids), the adsorbed lipase could potentially be desorbed from the carrier surface due to the mixing action.

Compared to an immobilised lipase, a free soluble lipase can tolerate a higher level of phospholipids (Y. Li et al., 2013, 2014). In addition, a free lipase may also move freely to come in contact with lipids inside the ruptured cells to catalyse transesterification, whereas an immobilised lipase has limited mobility due to being fixed on an enzyme carrier. Therefore, a free (i.e. not immobilised) lipase with a lower preparation cost may be an alternative for performing in-situ transesterification on a wet algal biomass.

2.6.5 Potential application of free lipase to improve biphasic solvent extraction

As explained in section 2.4.1, a biphasic solvent extraction is required to enable energy efficient solvent recovery. However, water-immiscible solvents are unable to recover most of the polar lipids which may be convertible to biodiesel, as they are selective to TAG (Grima et al., 2013; Olmstead et al., 2013b). As the polar lipids (including GL and PL) can represent a high proportion (between 8-48%) of the overall saponifiable lipids (Iyer, 2016), these unrecovered polar lipids will be a significant untapped lipid source in a nonpolar solvent extraction process.
One strategy to address this is by hydrolysing the polar lipids (into individual fatty acid molecules) prior to the biphasic solvent extraction. This was performed by Dong et al. via an acid-mediated hydrolysis at high temperature (T. Dong et al., 2016a). While the polar lipid acyl chains were recoverable in this approach, the proteins and other potentially valuable biomass components were also indiscriminately hydrolysed in the process. This approach, which also releases fermentable sugars, appears suited to maximising fuel production from algae. However, it would be desirable to also have a process that can leave the proteins intact, while allowing recovery of all the saponifiable lipids.

This thesis investigates an alternative to the acid hydrolysis process, involving a free lipase in-situ transesterification. The lipase reaction is more specific (i.e. does not act on proteins or carbohydrates) and uses milder conditions (low temperature and low pressure). With this strategy, it is hypothesised that both the neutral and polar saponifiable lipids can be converted into FAME (Navarro López et al., 2016c) and that the converted FAME will then partition into the hexane phase. It is proposed that the emulsion formed during the biphasic solvent extraction will provide the interfaces necessary for activating the lipase (Brzozowski et al., 1991), and that the use of a nonpolar solvent can enhance the lipase efficiency (Tran et al., 2013). After the reaction, the undesired polar head groups (in particular the phosphate containing molecules) will be left in the aqueous phase due to their high water solubility, and only the acyl chains (water insoluble) will be recovered in the hexane as FAME. This seems especially promising when used on some microalgae that can accumulate valuable omega-3 fatty acids that are largely unrecoverable due to their association with the polar membrane lipids (Martin et al., 2014; Olmstead et al., 2013b).

### 2.6.6 Current gaps in lipase in-situ transesterification of wet algal biomass

To date, there have only been a few studies on lipase-based in-situ transesterification on wet algal slurries. Navarro et al. performed an in-situ transesterification on a wet ruptured-algae slurry using a water-miscible solvent (tert-butanol) (Navarro López et al., 2016a), which as explained, is difficult to recover. The in-situ transesterification protocol proposed by Tran et al. (Tran et al., 2013) involved a large amount of methanol (>10:1 v/v methanol:wet biomass ratio) for the cell disruption step and was performed using an energy-intensive technique (sonication), which is yet to be proven to be feasible (A. K. Lee et al., 2012). In the in-situ transesterification study performed by Wang et al. (Y. Wang et al., 2017b), a water-miscible solvent (tert-butanol), was again used as the extraction solvent, and a toxic and expensive solvent (chloroform) was used to recover the FAME from the tert-butanol phase.

Further, all the above-mentioned studies were performed exclusively using immobilised lipases, and rapid lipase activity loss was observed in all cases. Therefore, an immobilised lipase is likely not justifiable to use in a wet algal slurry with a high solids concentration and a high phospholipid content. A free lipase can tolerate a higher level of phospholipids compared to an immobilised lipase (Y. Li et
al., 2013) and it has a lower preparation cost (Y. Li et al., 2014). Moreover, the advancements in biotechnology have allowed the mass production of some lipases (via genetic engineering and overexpression) for the detergent market and thus they may be used as a cheap source of biocatalyst (Crooks et al., 1995). Therefore, it is worthwhile to investigate if a free-lipase can be used to recover the polar lipid acyl chains in a biphasic solvent extraction.

2.7 Design of the experimental framework to address the major knowledge gaps

As reviewed above, the majority of existing studies on wet algal solvent extraction have used techniques that are unlikely to be feasible for biofuel applications. Most have been performed using either (i) non-scalable or energy-intensive techniques, (ii) a high solvent:biomass ratio (>3:1 v/v), (iii) harsh conditions (high temperature and/or high pressure), and/or (iii) polar solvents which cannot be recovered without excessive energy. A more viable approach is to use a nonpolar solvent as a biphasic extraction system.

One consideration of this thesis is that the selected wet lipid extraction process should aim to be a scalable process in the long term. Therefore, the equipment and techniques selected for this study were mainly based on a previously developed algal process for which scalability and feasibility have been considered (Halim et al., 2016; Martin, 2016; Olmstead et al., 2013b; Yap et al., 2015). The process was performed using scalable techniques (e.g. incubation for cell weakening and high-pressure homogenisation for cell rupture), a water-immiscible nonpolar solvent (e.g. hexane), a low solvent:biomass ratio (≤ 1:1 v/v), low pressure and low temperature.

A marine microalga species, *Nannochloropsis salina*, was selected as the model of the study due to its commercial potential and its several advantages in handling:

i) *N. salina* is a marine species and so it can potentially be grown using unwanted brackish or sea water (Chisti, 2013; Sousa et al., 2014).

ii) The cultivation process is more resistant to biological contamination due to the high salinity of the growth media (Mendes & Vermelho, 2013).

iii) It is a promising species for biodiesel production due its ability to accumulate TAG and its high biomass productivity (Ma et al., 2016; Rodolfi et al., 2009).

iv) It is capable of producing omega-3 fatty acids, which have a high commercial value (Adarme-Vega et al., 2012).

v) It can be a feedstock for multiple coproducts such as β-carotene, chlorophyll, sugars, and proteins and is thus a suitable candidate for a biorefinery strategy (Leu & Boussiba, 2014).
vi) A wet lipid extraction process for this species has already been established (Halim et al., 2016).

Although *N. salina* was used as the model microalgae, it is expected that the understanding gained from this thesis can be translated to other wet oleaginous microbes (such as yeasts or other microalgal species) that are of similar cell size range (2-4 µm) (Yap et al., 2016a), have the ability to accumulate TAG, and have cell walls that need to be ruptured prior to lipid extraction.

The algal cultivation (Martin et al., 2014; K. Sharma et al., 2012; Yap et al., 2016a), the dewatering (Rwehumbiza et al., 2012), the incubation (Halim et al., 2016) and the cell rupture processes (Spiden et al., 2013; Yap et al., 2014) have been studied in-depth and are therefore not the focus of this thesis. Similarly, hexane was shown to be a suitable solvent for this process (Olmstead et al., 2013b) and it fits the selection criteria as reviewed in section 2.4.4. Thus, solvent selection is not within the scope of this study. From the literature review, it was found that a detailed understanding of the lipid extraction and the solvent recovery processes is not available for biphasic solvent extraction from microalgae. In particular, the mechanisms behind the formation of stable emulsions and the impact of this on solvent recovery efficiency have not been investigated.

**Figure 2.9**: The focus of the current thesis in relation to a previously established lipid extraction process (Halim et al., 2016; Olmstead et al., 2013b).

The aim of this thesis is therefore to address the major knowledge gaps identified in a hexane-based wet lipid extraction (Figure 2.9). This thesis focuses primarily on developing a mechanistic understanding of the lipid extraction and the solvent recovery processes when applied to concentrated
 slurries of ruptured *N. salina* cells (15-20% solids concentration). Based on the insights developed during the thesis, a modification to the existing biphasic lipid extraction process is then proposed as a way to improve polar lipid recovery.

The knowledge gaps are divided and presented sequentially in each of the following chapters (chapter 3-6). A background for each of these topic is presented in the introduction section of each chapter. As a whole, the body of work presented in this thesis aims to provide a comprehensive understanding of the biphasic solvent extraction process in a wet lipid extraction utilising a nonpolar solvent (e.g. hexane). These theoretical and mechanistic understandings are crucial for future process development and optimisation. The conclusions and understanding drawn from the experimental chapters are brought together in chapter 7 and recommendations for future work are presented.

Summary of the identified knowledge gaps to be addressed in this thesis:

**Gap 1: Chapter 3: Investigation of the phase separation of an algal slurry-hexane emulsion**

Wet lipid extraction has been conceptually demonstrated to be an energetically feasible process (Martin, 2016). A high lipid yield is attainable using a nonpolar solvent provided the algal cells are sufficiently ruptured (Halim et al., 2016). In this approach, the hexane solvent is used as a carrier for dissolving and recovering the algal lipids. Thus, the final yield of the lipid is directly linked to the recovery of the lipid-rich solvent. A majority of current studies assessed the process efficiency solely based on the lipid recovery while overlooking the solvent recovery (Table 2.2). More than one solvent contact is commonly employed to enhance the lipid recovery and this indicates the lipid-rich solvent may not be sufficiently recovered in the first place (Olmstead et al., 2013b). In addition, a relatively high centrifugal force and a long centrifugation time, which may not be feasible in a large-scale process, were used (Halim et al., 2016). Without knowing the underlying mechanisms and the kinetics of phase separation, it is not possible to specify an appropriate separator for a large-scale process.

This knowledge gap is addressed in chapter 3 by developing a method for monitoring the phase separation process of the algal-solvent mixture using an analytical centrifuge. This method was used to investigate the kinetics and yield of solvent recovery during centrifugation and the phase separation mechanisms. A detailed characterisation of the emulsions formed during the wet extraction process was also performed to enable a descriptive mechanism for a phase separation process, specific to a wet ruptured-algae slurry (15-20 % w/w), to be proposed and the limiting factor in the solvent recovery process to be identified.
**Gap 2: Chapter 4: Investigation of the emulsifying properties of a ruptured-algae slurry**

Like other microbes, microalgae produce an array of molecules that could be utilised as emulsifiers and surfactants (Rosenberg & Ron, 1999). Similar surface-active molecules such as the phospholipids and the proteins (from non-algal sources) have been found to be excellent surfactants and emulsifiers. When alone, these surface-active molecules can stabilise a solvent emulsion by adsorbing at the oil-water interface. However, it is unclear what happens in a wet solvent extraction process in which these emulsifying agents are present simultaneously. Understanding the interaction of these surface-active molecules with the solvent interface will be crucial to improving the solvent recovery process.

Chapter 4 investigates the emulsifying properties of the surface-active agents present in a ruptured algal cell. A centrifugation-based fractionation approach was developed to separate the emulsifying components into different fractions, namely cell debris, serum, and lipid. The behaviour of these biomass fractions in the presence of a nonpolar solvent interface was characterised and the first systematic study of the emulsifying properties of ruptured-algae performed. The mechanisms and the roles of the surfactants and emulsifiers found in the algal cell in the hexane extraction process were proposed based on the outcome of this study.

**Gap 3: Chapter 5: Understanding the role of emulsions in biphasic lipid extraction from algae**

In most published studies on wet biphasic lipid extraction, shear/mixing was used to form a homogenous phase (emulsion) to promote lipid extraction. It was theorised that the emulsion formation creates additional interfacial area that allows lipid transfer from the aqueous algal slurry to the solvent phase. Yet, these emulsions could make recovery of solvent downstream more difficult. Therefore, it would be ideal if sufficiently rapid lipid transfer could be attained without emulsion formation. However, the properties and role of the emulsions formed during biphasic lipid extraction has not been investigated systematically. Further, the effect of the various process variables such as temperature, solids concentration and mixing rate on the lipid extraction kinetics has not been investigated in detail for a wet biphasic system.

Chapter 5 aims to develop a mechanistic understanding of the lipid transfer process in a typical wet hexane extraction process that is facilitated by emulsion formation. To enable the rate limiting factors in lipid extraction to be identified, a method was developed to perform lipid extractions without the formation of emulsions, which was compared against an emulsion-based lipid extraction process.

**Gap 4: Chapter 6: Utilisation of emulsions and lipase to enhance biphasic lipid extraction yields**

Algal lipids can be converted to biodiesel via transesterification. However, *N. salina* contains a significant amount of polar lipids (e.g. phospholipids) that can reduce transesterification efficiency. The
negative effect of polar lipids can be avoided using a nonpolar solvent such as hexane that preferentially extracts the neutral lipids (e.g. TAG). However, this means that some of the saponifiable polar lipids are not recovered, thus resulting a lower-than-theoretical biodiesel yield. Additionally, much of the valuable omega-3 fatty acids such as eicosapentaenoic acid (EPA) remain largely unextracted as they are primarily associated with the polar lipids.

As chapter 5 will reveal, emulsion formation is likely inevitable during biphasic solvent extraction. Therefore, emulsions were instead explored as a means to improve lipid extraction by applying lipase, which in fact benefits from the high oil-water interfacial area resulting from emulsions. Chapter 6 investigates the use of a free lipase in an emulsified system to achieve a direct transesterification in a wet ruptured N. Salina slurry. The aim was to simultaneously convert and extract the saponifiable lipids (including polar lipids) into hexane as crude biodiesel (FAME), to increase the biodiesel yield and enhance EPA recovery.

2.8 References


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Chapter 3

3 Investigation of the phase separation of an algal slurry-hexane emulsion

The aim of this chapter is to develop understanding of how hexane is mechanically separated from slurries of ruptured *Nannochloropsis salina* during centrifugation. This work provides the foundational understanding for further development of the hexane-based lipid extraction process. The discussion is focussed on identifying the rate limiting factors and the development of a mechanistic understanding of the separation process. The implications for large-scale processes are then considered.

This chapter is based on the paper “Centrifugal recovery of solvent after biphasic wet extraction of lipids from a concentrated slurry of *Nannochloropsis* sp. biomass” by Law, S. Q., Chen, B., Scales, P. J., & Martin, G. J.O. (2017). Algal Research, 24, 299-308.
3.1 Introduction

Microalgae is a promising source of triacylglyceride (TAG) lipids that can be used as food or a feedstock for biodiesel. In addition to efficient growth of microalgae (Williams & Laurens, 2010), large-scale TAG production requires energy-efficient and cost-effective means of recovering these intracellular lipids (Cooney et al., 2011). This is more challenging than for conventional oil seeds where oil can be recovered by pressing and simple solvent extraction from the dry, oil-rich material. In contrast, microalgal lipids are enclosed within small, robust cells that are surrounded by water. An energy-efficient approach needs to recover the lipids from a wet, concentrated slurry of microalgal cells (e.g. 15-25% w/w solids) (Molina Grima et al., 2003; Vandamme et al., 2013).

In the recovery of lipids, polar solvents such as ethanol or isopropyl alcohol can penetrate the cells but these solvents are fully miscible with water and can only be recovered by energy-intensive (non-feasible) distillation (Ozer, 2014; Yap et al., 2014). Non-polar solvents such as hexane are required (Halim et al., 2016). Additionally, hexane is selective to neutral lipids including TAGs and is immiscible in water resulting in a biphasic extraction process (Jiménez Callejón et al., 2014; Olmstead et al., 2013b). This requires the cells to be ruptured for the solvent to contact and dissolve the TAG droplets (Yap et al., 2014). The TAG-rich hexane (or other non-polar solvent) can then be physically separated from the delipidated biomass and the solvent evaporated from the TAG (Jiménez Callejón et al., 2014; Olmstead et al., 2013b).

To date, the actual recovery of lipid-rich solvent after extraction has yet to be examined in detail (Tao Dong et al., 2016b; Martin, 2016). The emulsion that is formed upon mixing the solvent and the slurry of ruptured cells is yet to be characterised. The kinetics and mechanisms of the de-emulsification process have also not been investigated and the associated costs remain unknown. A detailed understanding of these issues is required to determine the feasibility of this approach and to understand what opportunities are available for improvement.

There appears to be no existing study that investigates the centrifugation process of solvent recovery from wet microalgal slurry (15-25% w/w). Past studies investigating the behaviour of concentrated microbial biomass during centrifugation have been limited to unruptured yeast and bacteria in the absence of solvent (Dorobantu et al., 2004; Lopes & Keshavarz-Moore, 2012; Tustian et al., 2007). Although there are studies that have used centrifugation to recover solvent after lipid extraction from microalgae, none of these have characterised the resulting emulsion or considered the kinetics or mechanisms of centrifugal solvent recovery in any detail (T. Dong et al., 2016a; Halim et al., 2016; Huo et al., 2015; Jiménez Callejón et al., 2014; Lee et al., 2013; Olmstead et al., 2013b). Given the role of emulsification in inhibiting complete solvent recovery (Heeres et al., 2014; Lennie et al., 1990) and preventing an effective solvent recycling process, it is important to understand the mechanism to allow
future optimisation. (Furtado et al., 2015). Complete recovery of solvent is also important as any lipid associated with the retained solvent in the emulsion will also remain unextracted.

In this chapter, the emulsification and separation behaviour of hexane in concentrated slurries of ruptured *Nannochloropsis salina*, a promising candidate for commercial applications, is investigated. Cells were weakened, then ruptured by high-pressure homogenisation (HPH), which has been shown to be an effective and scalable method for processing high solid biomass (Olmstead et al., 2013b; Yap et al., 2015). Hexane was chosen as the extraction solvent due to its very low solubility in water, low boiling point, established use in vegetable oil manufacturing and selectivity to neutral lipids suitable for biofuel production (Jiménez Callejón et al., 2014). The extraction method was based on the previous work of Olmstead et al. (Olmstead et al., 2013b), and key parameters of the resulting emulsion characterised. The kinetics of emulsion separation was investigated using a centrifuge with optics (LUMiFuge) that enabled the biphasic interface to be monitored in real time. This information was used to examine the mechanisms of phase separation. The implications of the results for large-scale centrifugal recovery of solvent and lipids from slurries of ruptured microalgae are discussed, and strategies for potential improvements suggested based on the identified mechanisms.

### 3.2 Materials and Methods

#### 3.2.1 Microalgal biomass

A strain of marine microalgae *N. salina* was grown in an outdoor facility in Karratha, Australia under nitrogen-replete conditions (Halim et al., 2016). The microalgae biomass was harvested via chitosan-assisted flocculation and further concentrated into a high concentration slurry (paste) via centrifugation. The concentrated slurry was stored at -20°C and kept refrigerated at 4°C upon thawing. All experiments were conducted within 3 weeks of thawing. The dry weight (% w/w) of the biomass slurry was determined to be 19.0 ± 0.1% w/w by oven drying at 60 °C until a consistent weight was obtained (Olmstead et al., 2013b). The total lipid content of the *N. salina* slurry was determined to be 23 ± 1% w/w by a modified Bligh & Dyer protocol (Olmstead et al., 2013b). The lipid extract comprised 62 ± 2% neutral lipids, 28 ± 2% glycolipids, and 10 ± 1% phospholipids as determined by solid phase extraction (SPE) (Olmstead et al., 2013a). The protein composition of the biomass was 50-53% w/w with a conversion factor of 6.25 as determined previously using the Kjeldahl nitrogen method (Halim et al., 2016). The remainder of the biomass includes the carbohydrates, ash content, and the flocculants that remain with the biomass (Chatsungnoen & Chisti, 2016). The density of the slurry was determined using a 5.0 ml density bottle (Blaubrand, BRAND GmbH, Wertheim, Germany) calibrated at room temperature (22 °C).
3.2.2 Mechanical cell rupture

The cell weakening and mechanical rupture methods of Olmstead et al. (Olmstead et al., 2013b) were modified to accommodate smaller experimental batches. Batches of thawed biomass (100 ± 20 g) were incubated at 37 °C in a rotator-mixer (LabQuake, Barnstead International, Iowa, USA) and subsequently subjected to a single-pass through a high pressure homogeniser (Panda 2K NS1001L, GEA Niro Soavi, Parma, Italy) at 1050 ± 100 bar. The extent of cell rupture was approximately 50% as determined by cell-counting using a Neubauer-improved haemocytometer (Yap et al., 2014). All the experiments, including cell rupture, lipid extraction, and centrifugation analysis, were performed in at least duplicate.

3.2.3 Lipid extraction using hexane

Lipids were extracted from the homogenised biomass by contacting with hexane. 4.0 ± 0.1 g of homogenised biomass and 1.3 ± 0.05 g of n-hexane (95% AR, Ajax Finechem, Victoria, Australia) were added into air-tight 14 mL screw-cap glass vials (20 mm cylindrical diameter x 72 mm height). The vials were hand-shaken 5-10 times until the mixtures formed a single phase. The contents were then mixed by rotating the vials at 8 rpm (Labquake, Barnstead International, Iowa, USA) for 2 hours at 37°C. The hexane-biomass mixtures (emulsions) were cooled to 25 °C in a water bath before loading into LUMiFuge centrifuge tubes.

3.2.4 LUMiFuge centrifugation analysis

The kinetics of phase separation was investigated using a centrifuge instrument with an integrated photo-analyser (LUMiFuge®, L.U.M. GmbH, Berlin, Germany). Aliquots (1.3 ± 0.1 g) of the emulsion mixtures were loaded into the LUMiFuge tubes (10 mm optical path length, part no. 110-135XX, L.U.M. GmbH, Berlin, Germany). Centrifugation was conducted between 36 x g to 2330 x g (radius = 130 mm) for a pre-determined time. The LUMiFuge instrument and the samples were allowed to equilibrate to the target temperature separately before initiating centrifugation. Duplicate samples were analysed for each of the repeated experiments. The representative data is the result of quadruplicate experiments.

During centrifugation, the emulsions (mixtures of hexane, ruptured biomass and salt water) were separated into distinct layers of hexane, emulsion, aqueous and biomass layers (Halim et al., 2016). The positions of the hexane-emulsion and emulsion-water interfaces were tracked via transmission of near-infrared light along the length of the centrifuge tube during the centrifugation (Figure 3.1). Based on the interface heights and the cross-sectional area of the LUMifuge tube (72.1 mm²), the percent recovery of hexane and water were calculated based on the mass and composition of the sample according to Equation 3.1 and Equation 3.2. The hexane interface heights obtained by the LUMiFuge light transmission profile were verified to be in good agreement with physical measurements using a Vernier caliper. The sample masses were recorded throughout the experiment to verify that the loss of hexane by evaporation was minimal (< 1% w/w). The hexane layers separated from LUMifuge centrifugation
were subsequently collected and dried under nitrogen to obtain the gravimetric measurements of the extracted dry total lipids.

**Equation 3.1:** \( \text{Hexane recovery (\%)} = \frac{\text{Upper interface height} \times \text{area of centrifuge tube}}{\text{Sample mass} \times \text{hexane:biomass ratio} \times \text{hexane density}} \times 100\% \)

**Equation 3.2:** \( \text{Water recovery (\%)} = \frac{\text{Lower interface height} \times \text{area of centrifuge tube}}{\text{Sample mass} \times \text{water content of sample} \times \text{water density}} \times 100\% \)

**Figure 3.1:** Typical LUMifuge data with an illustration of the centrifuge tube at the top. The positions of the interface between the layers can be determined based on differences in the light transmission % through the centrifuge tube (indicated by the dash lines). As centrifugation progresses, the colour of the traces changes from red to green.

### 3.2.5 Microscopic imaging of hexane-biomass emulsions

To determine the size distribution of hexane droplets in the emulsified hexane-biomass mixtures, samples were visualised using a bright-field light microscope with a digital camera attachment (BX51, Olympus, Tokyo, Japan) at 100x and 400x total magnification. The emulsion mixtures were imaged
before and after centrifugation. A drop of the emulsion mixtures was placed onto a microscope slide and a cover slip was applied evenly on top. Multiple images were taken to ensure a representative sampling of the droplet population ($n_{\text{droplets}} > 1500$).

Images were processed using an open-source software (ImageJ). In the software, Gaussian blurring was used to minimise the graininess resulting from the algal solid particles. An area size threshold of $<0.25 \mu m^2$ was applied to avoid noise being counted by the software. The area of each droplet was measured by the software and, by assuming that the droplets were spherical, the diameter determined. A volume-based particle size distribution was then derived, again assuming spherical droplets. The results obtained from ImageJ were verified by manually checking against randomly selected raw images.

### 3.2.6 Rheological analysis

The viscosity of the ruptured biomass was measured using a stress-controlled rheometer (AR-G2, TA Instruments). A vane-in-cup setup, the most suitable method for high viscosity biomaterials such as microalgal slurries, was used to minimise wall-slip effects (Yap et al., 2016). The cup had a diameter of 30 mm and a height of 80 mm. The vane had a diameter of 10 mm and a height of 20 mm. The shear rate, shear stress and viscosity profiles were calculated from the torque and displacement data as measured directly by the instrument based on the vane-in-cup geometry (Fisher et al., 2007; Yap et al., 2016). The viscosity profiles of samples were determined using a stepped flow profile. As the rheology of microalgal slurry is sensitive to previous shear history (Yap et al., 2016), any intense mixing action was avoided during sample handling.

### 3.2.7 Experimental reproducibility

Physical characterisation (i.e. dry weight, density and lipid fraction measurements) of the microalgal biomass was performed on triplicate samples. Rheological measurements were performed in duplicate. Droplet size measurements were made from duplicate samples of an emulsified slurry. All LUMiFuge centrifugation runs were performed in duplicate on duplicated experiments (i.e. in which homogenisation and solvent extraction was replicated). For clarity, representative separation kinetic data were selected and presented. The variability of the LUMiFuge separation results was presented as the standard deviation of the percentage yield of hexane and water separated at the end of centrifugation.

### 3.3 Results and Discussion

#### 3.3.1 A typical wet lipid hexane extraction

The phase partitioning of a typical hexane-based wet lipid extraction process (Halim et al., 2016) is shown in Figure 3.2. When the hexane is added and mixed into the slurry of ruptured biomass, a homogenous single-phase emulsion is formed. After centrifugation, instead of three phase layers that can be expected after destabilising the emulsion (a cell solids pellet at the bottom, an aqueous
supernatant middle layer, and a light hexane layer on the top), a fourth layer (an emulsion layer) is still present between the aqueous supernatant and the light hexane layer. A density measurement revealed that this fourth emulsion layer (0.973 g cm\(^{-3}\)) must include lipid-rich hexane which has a much lighter density (0.679 g cm\(^{-3}\)). The resulting density of this emulsion layer is between that of the cell debris, aqueous supernatant and hexane, depending on the quantity of hexane that is retained. In the context of a hexane-based wet lipid extraction process, this fourth emulsion layer has to be eliminated to completely recover the solvent and allow full solvent recycling.

**Figure 3.2:** A typical hexane extraction process on a slurry of ruptured *N. salina* (19% w/w total solids concentration). The density measurements were carried out with a density bottle and the average of triplicate measurements are represented. The standard deviation of the measurements was within ±0.002 g mL\(^{-1}\). Note that the proportion of each layer was not drawn to scale.

### 3.3.2 Emulsion characteristics related to creaming

In this study hexane droplets (the dispersed phase) are emulsified in a viscous slurry of ruptured biomass (the continuous phase). Under centrifugal force these droplets ‘rise’ (move inwards in relation to the centrifugal force) through the slurry. A typical sedimentation or creaming velocity of particles under gravity or centrifugal force can be described by Stokes’ equation given the following assumptions: uniform distribution of particles, no interaction between particles, rigid spherical particles, no wall effects, low Reynolds number, and movement driven solely by density difference (Ward-Smith, 2012). For systems with a high concentration of particles (or in this case droplets), a modified Stokes’ equation (Equation 2.2) with a Richardson Zaki hindered factor (Richardson & Zaki, 1997) (Equation 3) is typically used to account for the increased drag resulting from particles in close proximity to each other:

\[
\text{Equation 3.3 : } v = \frac{2d^2 (\rho_p - \rho_l)}{9 \mu} g (1 - \varnothing_f)^n
\]

Where \(v\) = creaming velocity of droplet, \(d\) = diameter of droplet, \(\rho_p\) = density of droplet, \(\rho_l\) = density of continuous phase, \(\mu\) = viscosity of continuous phase, \(g\) = gravity acceleration, \(\varnothing_f\) = volume fraction of droplet, and \(n\) = hindered factor constant.
3.3.2.1 Viscosity and density

From Equation 3.3, the density of both the continuous and dispersed phase, and the viscosity of the continuous phase are key physical parameters affecting droplet creaming. The densities of the bulk phase and hexane were measured to be $1.042 \pm 0.001 \text{ g mL}^{-1}$ and $0.660 \pm 0.001 \text{ g mL}^{-1}$ respectively at room temperature (ca 22°C).

As shown previously (Wileman et al., 2012; Yap et al., 2016; Zhang et al., 2013), concentrated microalgae slurries exhibit shear-thinning non-Newtonian fluid behaviour. Additionally, the viscosity of the slurry can be affected by prior shear history (Yap et al., 2016). In these previous studies, slurries of whole cells were investigated, whereas in the current study approximately 50% of the cells in the slurries were ruptured by homogenisation. The shear-dependence of the slurry viscosity is important to understanding how variations in shearing during centrifugation might affect the creaming rate. Slurries containing ruptured cells were found to be highly shear-thinning (Figure 3.3), with the viscosity decreasing by more than two orders of magnitude across the shear rates tested (0.05 – 100 s$^{-1}$).

![Figure 3.3](image)

**Figure 3.3:** The viscosity of 19% w/w solids slurries of ca 50% ruptured *N. salina* cells as a function of shear rate at 25 °C. The different symbols represent duplicate measurements.

3.3.2.2 Emulsified hexane droplet size distribution

In theory, the rate of creaming has a squared dependency on the droplet diameter (Equation 3) and is therefore a critical parameter. Image analysis was used to obtained size distributions of the hexane droplets observed microscopically in the emulsions formed in both unhomogenised and homogenised microalgal slurry (Figure 3.4). Direct visualisation via microscopy is a common method (Robins, 2000) for quantifying emulsion droplet sizes. The emulsion mixtures were visualised after 2 hours of mixing (as described above) to produce an emulsion representative of a biphasic extraction process. This mixing regime was sufficient to allow lipids released from the ruptured cells to be recovered into the solvent (Olmstead et al., 2013b). This was verified by thin layer chromatography (data not shown) and is evidenced by the green hue of the hexane droplets in the homogenised samples (Figure 3.4C & 3.3D).
Figure 3.4: Hexane droplet size distribution on a volume-percent basis for non-ruptured sample when mixed with hexane vigorously (A) and ruptured sample mixed with hexane for 2 hours according to the standard procedure (B). On the right are the representative microscopic images at 400x total magnification for non-ruptured sample (C) and ruptured sample (D). Droplet size measurements were made on duplicate samples of an emulsified slurry. For clarity the histograms are presented with data pooled from both measurements, with standard deviations provided for the percentiles.

The volume-weighted median diameter of the hexane droplets in the homogenised slurry containing ruptured cells was 21 µm +/- 4 µm, compared to 127 µm +/- 48 µm in the unhomogenised slurry containing whole algal cells. The sample containing whole cells could emulsify a limited portion of the hexane after being mixed vigorously. Although relatively large, the presences of emulsified droplets (Figure 3.4) demonstrates the ability of the whole cells to stabilise the water-solvent interface, presumably via particle stabilisation referred to as Pickering emulsion (Leal-Calderon & Schmitt, 2008; Pickering, 1907). In addition, the hexane droplets in these samples remained colourless even after vigorous mixing confirming the need to rupture the cells to enhance the extraction of intracellular lipids. The smaller size of the droplets in the ruptured slurries could be explained by the release of surface-active cell components (e.g. proteins and phospholipids) (Heeres et al., 2014; Lennie et al., 1990) or by the increased interfacial stabilisation of the cell debris (e.g. organelles and cell wall fragments) compared to the whole cells. While cell rupture is crucial for effective solvent extraction of microalgal
lipids (Olmstead et al., 2013b; Yap et al., 2014), this shows that the rupture process also enhances the emulsifying properties of the slurry.

3.3.3 Kinetics of separation of hexane and water during centrifugation

The kinetics of hexane and water separation from emulsified slurries containing ruptured cells was investigated by tracking interfaces in a LUMifuge instrument. To study the effect of centrifugal force, an initial test was performed in which the centrifugation force was periodically increased from a low initial value of 36 x g up to 2330 x g, a force typical of an industrial centrifuge (Figure 3.5). The test was performed over an extended period to allow the material to approach a compressive equilibrium (Curvers et al., 2009) before stepping up to the next rotation speed.

In this test, a water layer (which formed below the bulk emulsion) evolved progressively even at the lowest force (36 g). In comparison, the hexane layer (which formed above the bulk emulsion) developed to a minimal extent from 36 to 292 x g, before increasing at a greatly accelerated rate at 584 x g. The kinetic profile of the water layer formation during this test is typical for the dewatering of a compressible material, in which the dewatering rate decelerates as a function of increasing cake solids fraction (Curvers et al., 2009). It is interesting that water separates from the solids in the direction of the gravitational force despite being less dense. This suggests that the hexane and solids must be associated strongly enough for the hexane to provide buoyancy for the biomass.

Figure 3.5: Separation of hexane and water as a function of time during centrifugation with stepped increases in force from 36 x g to 2330 x g. The theoretical solids content of the continuous phase (on a hexane-free basis) is also presented based on the separated water. Data are shown from a duplicated experiment.
The hexane separation rate increased almost seven-fold when the centrifugation force was doubled from 292 x g to 584 x g, considerably more than the two-fold increase that would be expected based on hindered Stokes’ separation (Equation 3.3). This suggests that there may be additional factors beyond buoyancy and drag forces that limit the rate of hexane separation, and that there is a force threshold above which the mechanisms limiting the rate of hexane separation are altered.

Further separation experiments were performed at forces close to the previously observed threshold of 584 x g (Figure 3.6A). A run at 2330 x g was also included to represent a centrifugation force closer to that of an industrial centrifuge. At forces below the apparent threshold (500 x g and 550 x g), after an initial delay of ca 5 min, hexane separation occurred almost linearly with time during the ca 30 min of testing. Above the apparent threshold (600 x g and 2330 x g), hexane separation occurred in three reasonably distinct stages: i) an initial lag (0-2 min); ii), rapid recovery of about 65-80% of the hexane (2-5 min) and iii) a slower and progressive recovery of the remaining hexane (beyond 5 min).

Consistent with the previous test (Figure 3.5), the dewatering rate did not vary greatly between 500 x g to 600 x g (Figure 3.6B). The presence of a clear water phase was only detected after ca 10 min at which point the change in the average solids content of the biomass-emulsion phase could be derived. The viscosity of a biomass slurry is dependent on the solids content (Schneider & Gerber, 2014; Wileman et al., 2012). However, as a similar dewatering rate was observed regardless of centrifugation force, changes in the viscosity of continuous phase cannot account for the vast difference in hexane separation rate between 500 x g and 600 x g.

**Figure 3.6:** Hexane (A) and water separation (B) during centrifugation at different forces close to the threshold. For clarity, data presented from one representative run from duplicates measurement of duplicate experiments. The standard deviations of the experiment are in the range of 2-15% w/w (for the final hexane recovery value) and 1-2% w/w (for final separated water value).
3.3.4 Mechanisms of hexane separation

Understanding the mechanisms of phase separation, in particular what is rate limiting, is required to improve wet extraction from microalgae. Broadly, phase separation of emulsions occurs via two major mechanisms: 1) migration of droplets to the surface (creaming), and 2) transfer of dispersed droplets into a separate immiscible phase via coalescence. It was not possible to examine the processes of creaming and coalescence separately, as the instrument only provides information of the rate of formation of the separated phase (i.e. a product of both creaming and coalescence). It was also not possible to compare these kinetics with current available theoretical models. To predict the creaming kinetics of an emulsion with a concentrated suspension of polydisperse droplets, advanced numerical simulations are required (Basson et al., 2009; Xue & Sun, 2003). Beyond this, current models cannot account for the following complications present in this situation: i) droplet flocculation, ii) coalescence and phase separation, and iii) complex rheology of the continuous phase (in this case a shear-thinning material with changing properties in time and depth due to dewatering).

Nevertheless, consideration of the available kinetic data can help elucidate the key mechanisms. In particular, what is responsible for the apparent force-threshold is worth considering. Comparing the separation kinetics at 500 x g and 600 x g (Figure 3.6), a small increase in force resulted in a dramatically faster separation. According to hindered settling behaviour (Equation 3.3), the rate of droplet migration (creaming) would be expected to be in proportion to the increase in force (i.e. only about 20% faster at 600 x g compared to 500 x g). None of the factors accounted for in the hindered Stokes model can explain this behaviour, indicating that the force threshold is not related to droplet migration and must instead be related to a different process, for instance coalescence/phase separation.

The idea that droplet migration is not rate-limiting is further supported by considering the extent of separation achieved at the different forces after, for instance, 5 min of centrifugation. At 600 x g more than 60 % of the total emulsified hexane had been recovered in a distinct upper phase, whereas at 500 x g, less than 5% had been recovered. This large difference can be explained by the accumulation of droplets near the surface that were yet to coalesce and phase separate. The accumulation of concentrated un-coalesced droplets in the upper regions of the emulsion at 500 x g were confirmed by microscopic examination of the emulsion mixture immediately prior to the formation of a distinct hexane layer (Figure 3.7A).
3.3.4.1 Collective migration of hexane droplets during centrifugation

Although a high proportion of the droplets migrate rapidly to the surface (i.e. at least 60% v/v of the droplets in 5 min at 600 x g), some droplets are left behind (Figure 3.7C). The persistence of both small
(5-10µm) and large droplets (up to ca 25 µm) in the upper and lower regions of the emulsion suggest that the droplets do not migrate individually or at a rate strictly proportional to their size. Such a phenomenon has been observed before in a study investigating the creaming of polydisperse droplets (Kumar et al., 2000). It was found that at a volume fraction of ca 0.24, all droplets migrated at a velocity expected of the largest size droplets regardless of their size. This was attributed to a collective migration of the droplets, in which the smaller droplets are swept along in the wake of the larger droplets. This effect is likely to be coupled with and intensified by droplet flocculation resulting from colloidal interactions (Dickinson, 2010), given the high volume fraction of the droplets (Robins et al., 2002). It is likely that a similar collective migration of hexane droplets occurred in the current study, in which the hexane droplets size are polydisperse and has a high initial volume fraction of 0.33. Such collective creaming occurs much more rapidly than the migration of individual droplets experiencing hindered creaming, providing a possible explanation for why droplet migration was not limiting despite the highly viscous continuous phase. The droplets observed to remain in the lower regions of the emulsion may therefore have been decoupled from the collective creaming process.

3.3.4.2 Droplet-droplet coalescence cascade

It is clear that coalescence is an important factor. Coalescence requires the breakage of two droplets films, therefore requiring a sufficient force driving the droplets together that overcomes this barrier (George A van Aken & Zoet, 2000). The formation of a nascent second phase (i.e. a distinct hexane layer above the emulsion and shall be referred to as homophase) requires an initial droplet-droplet coalescence. Beyond this, growth of the homophase layer occurs via coalescence of additional droplets into the homophase (droplet-homophase coalescence) (Krebs et al., 2013).

During centrifugation the droplets cream and accumulate near the surface (Figure 3.7). As the droplets at the surface cannot migrate any further the droplets rising from below are forced into those above, allowing droplet-droplet coalescence and thus phase separation to commence. The subsequent growth of a homophase layer occurs via additional droplets coalescing into the homophase.

At this point, if the droplet-homophase coalescence rate is limiting (as suggested by the evidence), it is possible that droplet-droplet coalescence in the bulk emulsion phase can occur via compression of the droplets beneath the homophase. This droplet-droplet coalescence likely requires both a critical force to rupture the inter-droplet film (Tcholakova et al., 2002) and the presence of a minimum volume fraction of droplets (George A. van Aken, 2002). A step-change increase in the rate of such droplet-droplet coalescence as a function of centrifugation force has recently been observed (Krebs et al., 2013). Further, initiation of droplet-droplet coalescence has been shown to be a trigger for a cascade of coalescence events in a viscous fluid system (Martula et al., 2000; George A. van Aken, 2002). These mechanisms provide a tangible explanation for the greatly accelerated phase separation observed above the force threshold at 600 x g (Figure 3.6A).
It was also observed that there was much greater variability in the repeated experiments performed near the threshold (i.e. at 550 x g and 600 x g), indicating some potential instability in this region. The yield of hexane after 30 min was 34 ± 3% at 500 g, 36 ± 15% at 550 g, 70 ± 11% at 600 g and 87 ± 2% at 2330 g. The high variability near the force threshold is consistent with coalescence. Large variations have been seen in other centrifugation-coalescence studies and are likely due to the unstable nature of coalescence phenomenon (Krebs et al., 2013; Krebs et al., 2012) especially near the critical coalescence force.

At 2330 x g the rate of separation was even higher than at 600 x g (Figure 3.6A). The initial phase of rapid separation occurred earlier and faster than at 600 x g, and recovered more hexane (ca 80 vol%) before slowing down. The increase in the extent of hexane recovered in this initial phase could be due to an increase in the proportion of droplets included in either or both the collective migration or the cascade of droplet-droplet coalescence events.

### 3.3.5 Proposed mechanism of hexane separation in high solids ruptured microalgal biomass

Based on this discussion, a mechanistic description of centrifugal separation of hexane from high solids (ca 20 % w/w) ruptured microalgal biomass can be proposed (summarised graphically in Figure 3.8). During the initial stage of centrifugation, collective creaming concentrates droplets at the surface that coalesce to form a nascent hexane layer.

At forces below a threshold value, the compressive pressure is insufficient to maintain continued droplet-droplet coalescence, and the development of the hexane layer relies primarily on droplet-homophase coalescence. This is a relatively slow process as only the droplets at the actual surface can coalesce. The area for droplet-homophase coalescence should remain constant, which is consistent with the approximately linear progression of the observed hexane recovery (Figure 3.6A). At forces above the threshold, the compressive pressure from centrifugation is sufficient to rupture inter-droplet films and sustain continued droplet-droplet coalescence within the upper region of emulsion. This triggers a cascade of coalescence events resulting in a rapid evolution of the droplets into the homophase.

As the concentration of droplets decreases, the rate of droplet-droplet coalescence decreases and phase separation transitions to droplet-homophase coalescence, which is approximately linear with respect to time (Figure 3.6A). The droplets that are not separated during the initial coalescence are removed from the emulsion at a much slower rate. As centrifugation continues, the solids content of the emulsion phase increases, which could further restrict the migration and separation of these droplets. The separation of these residual droplets presents a key challenge to achieving a complete recovery of the solvent.
3.3.6 Lipid recovery

The aim of the wet solvent extraction process is to recover the lipids from the microalgal cells, particularly the neutral lipids including the TAGs. In this study, hexane droplets containing dissolved algal lipids were recovered from the emulsion via centrifugation in the LUMifuge instrument and were then dried under a stream of nitrogen gas to gravimetrically determine the amount of lipid recovered. The total lipid concentrations in the hexane samples recovered from the LUMifuge centrifugation at 600 x g and 2330 x g were determined to be 7.6 ± 0.8 mg and 7.9 ± 0.5 mg (per 100 mg of recovered
hexane). The similar lipid-in-hexane concentrations of the samples obtained at 600 g and 2330 g show that lipid recovery is increased at higher centrifugation speed due to the additional hexane recovered, thus supporting the need for an efficient solvent recovery for high lipid extraction yield.

In this experiment, an overall recovery yield of 47 ± 3% of total extractable lipids (based on the Bligh & Dyer extraction method), or 10.8 ± 0.7 mg recovered lipid per 100 mg of N. salina dry biomass, was achieved for centrifugation at 2330 x g following a single hexane contact. There was insufficient material to further perform accurate SPE measurements on the dried lipid samples, however, similar information obtained using the same extraction process has been previously published (Halim et al., 2016; Olmstead et al., 2013b). Assuming a similar composition of neutral (90%), glyco-(6%) and phospho-(4%) lipid fractions in the recovered hexane (Halim et al., 2016; Olmstead et al., 2013b), a recovery of about 68% of the total available neutral lipids and 10% and 19% of the GL and PL fractions respectively (Figure 3.9) can be derived.

Although the actual conditions of the experiment such as cell rupture % and biomass composition may vary the actual lipids composition in the hexane phase, hexane has been shown to be a non-polar solvent preferentially extracting the neutral lipids when used in a total lipid extraction process (Halim et al., 2016; Olmstead et al., 2013b). The recovery yield of neutral lipids was less than the overall hexane recovery of 87 ± 2%, indicating incomplete partitioning of the available neutral lipids into the hexane. It has previously been shown that for immiscible extraction solvents such as hexane, neutral lipids can only be recovered from ruptured cells (Yap et al., 2014). Therefore, a higher percentage of cell rupture is expected to increase the amount of neutral lipids extractable by hexane during the solvent contact (up to 100% of the neutral lipid contained within the algal cells). However, based on the 50% cell rupture observed in this paste, and the recovery yield of hexane of 87%, only 43.5% of the neutral lipids would have been expected to be recoverable, suggesting somewhat better than expected recovery (ca 68% was recovered instead in this study). This may be due to an underestimated cell rupture (Figure A2). The incomplete recovery of solvent, and therefore incomplete recovery of the associated lipids, explains why additional lipid yields were obtained on a second hexane contact in the previous study by Olmstead et al. (2013b).

In this study, about 32% of the total neutral lipids (contained in the algal cells) remained unrecovered after the first hexane extraction. Of these remaining neutral lipids, 31.9% (or 10.2% of the total neutral lipid content) was not recovered due to being associated with the unrecovered hexane. The remaining 68.1% (or 21.8% of total neutral lipids) were unextracted likely due to being contained in the unruptured cells or not being able to partition into the hexane phase for some reason (such as insufficient mixing time or intensity). To improve the recovery of neutral lipids associated with unrecovered hexane, a second hexane contact can be performed. To recover the lipids contained within the intact cells, a higher homogenisation pressure or a more intense cell-rupture technique is likely required to achieve more extensive cell rupture.
3.3.7 Implications for predicting scale-up performance and throughput analysis

These experiments give an understanding of the mechanisms that limit both the rate and the yield of hexane recovery from concentrated slurries of ruptured microalgae by centrifugation. This understanding has implications for scale up. Specification of industrial centrifuges for a given flow rate of material (Q) is typically based on the so-called sigma factor (Σ), which is equivalent to the surface area required for separation under gravity (Equation 3.4) (Fujita et al., 2000). Specification in this manner thereby requires knowledge of the settling (or creaming) velocity of the limiting particle (or droplet) under gravity (νg).

\[
Q = \nu_g \times \Sigma
\]

However, as the rate of separation was limited by coalescence rather than droplet migration, it is not possible to determine the limiting creaming velocity for this system. While the kinetics gives an indication of the rate of separation, this is dependent on the rate of coalescence resulting from compressive forces that are dependent on the geometry and configuration of the centrifuge. In addition, industrial centrifuges such as disk stacks and decanters have very different geometry and flow shear forces that would impact on the viscosity of the shear-thinning continuous phase (Axelsson, 2002). In addition, the high shear forces might encourage phase separation via flow-induced coalescence,
provided there is a sufficient concentration of droplets volume (George A. van Aken, 2002). Thus, selecting the right centrifuge design which optimises these conditions for coalescence would be a key consideration for selection and specification of a large-scale centrifuge. However, it is not possible to accurately predict the kinetics of coalescence in a large-scale centrifuge, meaning that pilot- or even full-scale trials would be needed to determine the required capacity and speed of a given centrifuge.

The separation kinetics observed in these experiments can nonetheless be considered to be slow (e.g. 80% recovery in 2 min at 2330 x g). The identification of coalescence as the rate limiting step point to several possible improvements that could be explored. The rate of coalescence, and therefore hexane separation, could be increased by destabilising the interfacial film. This could achieved by lowering the viscosity of the continuous phase (e.g. by dilution, pre-shearing, or increased temperature), or by targeting the surface-active components stabilising the interface. This requires further investigation, as the effect of differences in biomass composition was not included in the current study. To achieve a near-complete solvent recovery, targeting the smaller droplet population (<20um) decoupled from the collective migration would be crucial.

### 3.4 Conclusion

Hexane was shown to be strongly emulsified into small droplets (< 50 µm) when mixed with concentrated slurries of ruptured microalgae. The recovery of hexane by centrifugation was found to be limited primarily by coalescence rather than droplet creaming. The unexpectedly rapid creaming movement of droplets was attributed to collective migration of the flocculated droplets. A step-change increase in the rate of hexane separation was found to occur at a critical force of approximately 550 x g. This was attributed to the induction and propagation of droplet-droplet coalescence above a critical compressive force. As coalescence is the rate limiting mechanism, these results and others using laboratory scale batch centrifuges, cannot be directly used to specify large-scale centrifuge capacity. Rather the results show the importance of targeting approaches that can improve the recovery kinetics by increasing the rate of droplet coalescence, for instance by destabilising the interfacial film. Therefore, an understanding of the emulsion stabilising components in the rupture-algae slurry is required.
3.5 References


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Chapter 4

4 Investigation of the emulsifying properties of a ruptured-algae slurry

This chapter follows from the discovery in the previous chapter that the emulsions are unexpectedly stable and that a layer of recalcitrant emulsion is always present after centrifugation. The aim of this chapter is to investigate the interfacial properties of the components in the biomass and their roles in emulsion stabilisation. The fundamental understanding of the emulsification process is essential to developing demulsification strategies to obtain a full solvent recovery. A novel fractionation approach was developed to identify the specific roles of the major components of the biomass in contributing to the recalcitrance of the emulsions. Based on the insights obtained, a full mechanistic understanding of the emulsification and demulsification (phase separation) process in a wet lipid extraction was developed.

This chapter is based on the paper submitted for journal publication “Emulsifying properties of ruptured microalgae cells: Barriers to lipid extraction or promising biosurfactants?” by Law, S. Q., Srinivas, M., Scales, P. J., & Martin, G. J. O. (2018).
4.1 Introduction

To extract algal lipid in an energy efficient manner, a water-immiscible solvent with a low boiling point (e.g. hexane) is required that can be physically recovered from wet concentrated algal slurry by centrifugation (Martin, 2016). The use of water-immiscible solvents results in a biphasic (emulsified) extraction system (Yap et al., 2014). In chapter 3, an analytical centrifuge was used to investigate the kinetics of hexane recovery from a hexane-emulsified slurry of ruptured cells. The emulsion exhibited remarkable stability against coalescence even under a centrifugal force of up to 500 x g (Figure 3.6A). Further, some hexane was shown to be unrecoverable from the wet algal slurry even after prolonged centrifugation at over 2000 x g (Halim et al., 2016), a force sufficient to induce coalescence of the droplets at the surface as demonstrated in chapter 3. This is currently a major challenge, as near-complete recycle of the solvent is required for sustainable biofuel production (T. Dong et al., 2016a).

Although emulsions formed by wet algal biomass have been reported in the past (Cooney et al., 2009; Fajardo et al., 2007; Grima et al., 2013; Hita Peña et al., 2015; Navarro López et al., 2016), no systematic investigation has been performed to understand the fundamental contributing factors. The emulsifying properties of individual components of the ruptured algae have not been studied and yet this understanding will be required to develop targeted demulsification strategies to make lipid recovery from microalgal biomass feasible.

An emulsion is a mixture of two immiscible phases formed by dispersing one phase into the other, resulting in the creation of additional interfacial area (Leal-Calderon et al., 2007). For an emulsion to be stable, a third component (typically an amphiphilic molecule that is surface-active) is required to reduce the interfacial tension and stabilise the interface. These surface-active molecules can be termed surfactants or emulsifiers (Rosenberg & Ron, 1999). Although the terms emulsifier and surfactant are sometimes used interchangeably, it is important to distinguish them here on the basis of differences in their modes of action (Dickinson, 2009; Uzoigwe et al., 2015). Whereas a surfactant acts mainly by lowering the interfacial tension, allowing small dispersions to form, an emulsifier can stabilise an emulsion via various mechanisms (Dickinson, 2009) but does not necessarily rely on the reduction of the interfacial tension (Uzoigwe et al., 2015). In this way, a surfactant is related to short-term emulsion stability, while an emulsifier is more relevant to long-term stability. In addition, a surface-active agent can act as a surfactant, an emulsifier, or both (Mnif & Ghribi, 2015a; Uzoigwe et al., 2015).

Like other microbes (Mnif & Ghribi, 2015a; Rosenberg & Ron, 1999; Sunde et al., 2017), microalgae produce an array of biomolecules, both intracellularly and extracellularly, that could behave as emulsifiers or surfactants (Paniagua-Michel et al., 2014). One of the more commonly studied group of surface-active molecules of microalgae is the so-called extracellular polymeric substances (EPS, as distinct from the exopolysaccharide which is also commonly abbreviated as EPS but is only a subset of extracellular polymeric substances) (Mnif & Ghribi, 2015a). EPS includes polysaccharides, proteins,
nucleic acids and lipids (Xiao & Zheng, 2016), and can be divided into soluble, loosely-bound and tightly-bound EPS based on their degree of attachment to the cell surfaces (Basuvaraj et al., 2015; Sheng et al., 2010).

Inside the cells there are cytosolic proteins, membrane lipids and other molecules such as DNA (Alboresi et al., 2016; Coustets et al., 2015). Proteins are known to be excellent emulsifiers due to their amphiphilic nature (Lam & Nickerson, 2013), and protein extracts from microalgae have been demonstrated to be no exception (Medina et al., 2015; Schwenzefer et al., 2013). The cell membrane and membranes of the cellular organelles are lipid bilayers comprised mostly of neutral and polar lipids (e.g. phospholipids and glycolipids (Cortés-Sánchez et al., 2013)), which are naturally surface-active (Mnif & Ghribi, 2015a; Pacwa-Płociniczak et al., 2011). For biofuel applications, microalgae can be induced to accumulate large amounts of TAG, for instance by deprivation of nitrogen (Sharma et al., 2012). Inside the cells the TAG is stored as lipid droplets that are surrounded by a layer of polar lipids, intercalated with structural hydrophobic proteins, such as oleosin (Vieler et al., 2012). These polar lipids and hydrophobic proteins can potentially continue to stabilise the lipid droplets after cell rupture.

In addition, the ruptured algal cell wall debris (Yap et al., 2014) may adhere to the hydrophobic surface of the solvent droplets (Mnif & Ghribi, 2015a), resulting in a highly stable Pickering emulsion. The need to process at a high-solids concentration (i.e. 15-25% w/w) means that the slurry is highly viscous (Yap et al., 2016). This can stabilise an emulsion kinetically by retarding the creaming and coalescence process.

There is clearly an abundance of potential surfactants and emulsifiers in a slurry of ruptured algae that can stabilise an emulsion, however it is not currently known what roles they play in stabilising the emulsions and which are the most important (e.g. to perhaps selectively target for removal or degradation).

To help develop this understanding, a systematic study was performed in which the complex wet ruptured-algae slurry was fractionated into four major components: the cell debris, the delipidated debris, the serum, and the lipid. A commercially-relevant marine microalga (N. salina) was chosen as the model for the study due to its ability to accumulate TAG and omega-3 fatty acids. The N. salina cells were concentrated and ruptured using a previously developed method based on scalable processes (Halim et al., 2016). Pendant drop tensiometry and interfacial shear rheology were used to study the interfacial properties of emulsions produced using the various biomass fractions and a nonpolar solvent (e.g. hexane or hexadecane). Finally, the stability of the emulsions was evaluated using an optical-centrifugation technique (Law et al., 2017). The results from this systematic study were used to identify the role of each biomass fraction in forming and stabilising emulsions. A full mechanistic understanding of the emulsification and demulsification processes in the wet lipid extraction is then presented based on the insights from the study.
4.2 Materials and Methods

4.2.1 Microalgae cultivation and harvest

A strain of *N. salina* was cultured and maintained as described previously (Halim et al., 2016). To generate the biomass, cultures were grown in a series of 15 L carboys with a modified f-medium (Olmstead et al., 2013a). The cultures were cultivated indoors at 20 °C with a light:dark cycle of 14:10 hours. Gas exchange was delivered via an aquarium air pump (Stellar 380D, Aqua One, China) at a total flow rate of 190 L/h, which was split into 4 air stones. The biomass cultures were harvested after a 14-day growth period by centrifugation using a disc stack centrifuge (Separator OTC 2-02-137, GEA Westfalia, Italy). The pastes were stored frozen at -20 °C and were used within 2 months of harvesting. The total solids concentration of the pastes was determined based on the dry weight of the biomass after oven drying at 60 °C (Yap et al., 2015).

4.2.2 Cell rupture via high pressure homogenisation

To perform the subsequent experiments, the *N. salina* paste (ca. 30% w/w total solids) was diluted to 11-15% w/w solids using 3% artificial sea water (Coral Pro Salt, Red Sea Fish Pharm LTD, Israel). The slurry was pre-treated by incubating at 37 °C for 24 hours to weaken the cell walls prior to a rupture by high-pressure homogenisation (Halim et al., 2016; Yap et al., 2015). The incubated slurry was passed once through a high-pressure homogeniser (Panda 2K NS1001L, GEA Niro Soavi, Italy) at 1400 ± 100 bar. A typical cell rupture efficiency of this process was around 55%-75% as determined by counting the residual intact whole cells post-homogenisation under the microscope as previously described (Yap et al., 2015).

4.2.3 Fractionation of the ruptured-algae slurry

After cell rupture, the algal slurry was separated into different fractions using centrifugation (Figure 4.1). The overall slurry of ruptured algal cells is designated here as the *ruptured total*. Next, a portion of the *ruptured total* was fractioned by centrifugation (Allegra X-30R, fixed-angle rotor F0685, Beckman Coulter, Victoria, Australia). The supernatant obtained from centrifuging the *ruptured total* was filtered with a 0.22μm PVDF syringe-filter (low protein binding Durapore, Millex GC, Cork, Ireland) to exclude any insoluble particulate. This filtered supernatant is designated as the *serum*. The remaining pellet was washed multiple times (5-7 times) by removing the supernatant post-centrifugation and resuspending with 3% artificial sea water, before repeating the centrifugation. To maintain the starting biomass solids concentration, the final resulting pellet was resuspended to the original volume. This washed pellet is designated as *cell debris*. All centrifugation was carried out at 3000 x g for 10 minutes (Allegra X-30R, fixed-angle rotor F0685, Beckman Coulter, Victoria, Australia) and the 3% artificial sea water was used as both the diluent and the resuspension fluid. Between each centrifugation interval, a mixing step was performed by hand mixing followed by rotating of the tube on a rotary tube.
mixer (RSM6, Ratek Instruments, Victoria, Australia) for 1 hour at room temperature to ensure a homogenous resuspension.

On a separate portion of the ruptured total, n-hexane (AR grade, Ajax Finechem, Victoria, Australia) was added at a 1:1 volume ratio to the wet algal slurry (i.e. 10 mL of hexane to 10 mL of wet algal slurry) to extracted the lipids. The hexane and wet slurry were mixed (RSM6, Ratek Instruments, Victoria, Australia) for 2 hours at room temperature before a centrifugation at 9000 x g for 10 minutes. The hexane layer obtained is referred to as the lipid in this study. For subsequent experiments requiring the lipid to be dissolved in hexadecane, the lipid-rich hexane was dried under nitrogen (Olmstead et al., 2013a) and then resuspended in n-hexadecane (99% ReagentPlus, Sigma Aldrich, MO, USA). After decanting the hexane layer, the remaining pellet was repeatedly mixed with 10 mL of fresh hexane and centrifuged, to perform multiple hexane extractions (up to 6 cycles) to remove the hydrophobic components. The end of the hexane extraction was indicated by a colourless phase-separated hexane layer. The remaining wet solids from this process was then washed multiple times with 3% artificial seawater to remove the supernatant components. The resulting pellet (excluding the emulsion layer) was retrieved carefully and is designated as the delipidated debris.

The total nitrogen in the fractionated serum was measured by combustion in a CNS elemental analyser (TruMac CNS, Leco Corp., MI, USA) and a nitrogen-to-protein conversion factor of 5.86 was used based on a previously determined conversion factor for protein extracts from Nannochloropsis oculata (Safi et al., 2013). The lipid was fractionated into neutral lipids, glycolipids and phospholipids using solid phase extraction (SPE) as described previously (Olmstead et al., 2013a).

![Figure 4.1](image-url)

**Figure 4.1**: The separation scheme to fractionate the slurry of ruptured *N. salina* cells from a single batch into different biomass fractions following three different routes (color-coded red, blue and orange).

In order to normalise the concentration of the biomass fractions for subsequent experiments, the term ‘biomass concentration’ will be used to represent the concentration of the isolated component as present in the ruptured total. For instance, the serum and cell debris isolated from an 11% w/w total solids
concentration of the ruptured total will be described as an 11% w/w biomass concentration. The biomass concentration of the delipidated debris was obtained by accounting for the dry weight of the removed hexane-extracted materials as well as the dry weight of the serum that was removed.

4.2.4 Interfacial tension measurement

A pendant drop tensiometer (OCA20, Dataphysics, Germany) was used to determine the interfacial tension between the aqueous and organic phases. In preliminary tests the viscosity of the ruptured total at 11% solids concentration appeared to affect the pendant drop formation due to its non-Newtonian fluid and yield stress behaviour at high solids concentration (Wileman et al., 2012). As the validity of the assumptions underlying the Young-Laplace equation could not be confirmed (e.g. that the drop contour is solely affected by gravity and the density difference between the phases) (Berry et al., 2015), the subsequent interfacial tension measurements of all components were performed at an equivalent of 5.5% w/w biomass.

In all measurements, n-hexane was used as the organic bulk phase in a 10 mm x 10 mm solvent-resistant cuvette. A PTFE cuvette stopper with a centre hole was used to minimise the solvent evaporation while allowing the needle to pass through. A drop of the aqueous phase was formed from the top using a gas-tight glass syringe with a stainless steel Luer lock needle (21 gauge). The tensiometer was checked and calibrated using clean distilled water as the drop phase with air as the bulk phase. All measurements were performed with the droplet volume close to the critical detachment volume to ensure a more accurate measurement (Berry et al., 2015). Images of the droplets were captured using an integrated camera unit and the interfacial tension was calculated by the software using the Young-Laplace equation.

The densities of the solutions were measured on a calibrated 5 cm$^3$ pycnometer (Blaubrand, Germany).

4.2.5 Interfacial shear rheology

The interfacial shear rheology was performed in an interfacial rheological cell with a biconical geometry using an electro-driven rheometer (MCR 702, Anton Paar, Austria). The bicone had a radius of 68.3 mm with a 5° angle. The cell had an inner diameter of 79.8 mm and a height of 55.2 mm. The experimental method and derivation of the complex interfacial viscosity were based on previously presented principles and calculations (Erni et al., 2003; Rühs et al., 2013). In brief, the bicone was placed at the interface (Figure 4.2) to detect the presence of an interfacial film by applying an extremely small oscillatory shear strain (0.1%) at low frequency (1 rad s$^{-1}$). The measured torque was converted to a complex interfacial viscosity value by accounting for the geometry and the viscosity of the two bulk phases (Rühs et al., 2013). For brevity, only the complex interfacial viscosities are presented.

To perform the experiments, 50 mL of the diluted aqueous samples (biomass fractions described in Section 4.2.3) were added into the interfacial rheological cell. The bicone was positioned exactly at the interface using a protocol in the supplied software (Interfacial Gap Sensing app, Anton Paar) by sensing the change in force as the bicone was lowered into the interface. The accuracy of the bicone placement
at the interface was visually confirmed. Then 15 mL of n-hexadecane (with or without lipid) was gently added to the top along the stem of the bicone so as not to disturb the interface. All experiments were conducted at 23°C. The kinetics of the film formation were tracked through the interfacial elastic modulus. For experiments taking longer than 6 hours, 0.1 % w/w sodium azide was added to the aqueous phase to prevent any possible bacterial activity. A strain amplitude sweep (0.1%-1000% strain) was also performed at the end of the interfacial rheology measurements to check the final breakage strength of the film formed.

![Diagram](image)

**Figure 4.2:** Illustration of the bicone interfacial setup used to obtain the complex interfacial film viscosity.

### 4.2.6 Emulsion stability tests

To determine the emulsion stability at high solids concentrations (>10% w/w), 3 mL of each biomass fraction (as defined in Section 4.2.3) and 3 mL of n-hexane were added to an airtight and solvent-resistant conical centrifuge tube (15 mL polypropylene tube, Tarsons, Kolkata, India). The contents were mixed at 24 rpm for 2 hours at room temperature on a rotator tube mixer. An analytical centrifugation instrument with an integrated photo-analyser (LUMiFuge®, L.U.M. GmbH, Berlin, Germany) was used to accelerate and monitor the phase separation process as described previously in chapter 3. 1.3 g of the emulsified mixtures were loaded into the supplied LUMiFuge tubes (synthetic polyamide cells, L.U.M. GmbH, Germany) for centrifugation tests. As the separated hexane layer is transparent to the near infrared light used by the instrument, and the emulsion layer is light-opaque, the height of the emulsion layer was tracked to monitor the phase separation process.

In the centrifugation tests, a small centrifugal force (30 x g) was applied initially to allow the hexane droplets to cream. After 12 minutes, the centrifugal force was incrementally doubled every 5 minutes until the maximum centrifugal force of the instrument (2330 x g) was reached. The volumes of the phase-separated hexane layers were determined based on the heights of the initial and final emulsion layers. The percentage of hexane recovered as a phase separated layer above the emulsion was presented as a function of both the centrifugation time and the centrifugal force.
4.2.7 Microscopic examination of emulsions

Selected emulsions from the emulsion stability testing experiments (section 4.2.6) were examined by placing a drop of the emulsion phase between a microscope slide and a cover slip and inspecting by bright field microscopy (BX51, Olympus, Tokyo, Japan). For samples with coarse emulsions, the samples were also observed without cover slip to ensure the application of cover slip did not artificially change the droplet size of the emulsion.

4.2.8 Experimental reproducibility and analysis

All the experiments were performed in at least duplicate. The interfacial tension measurements were performed in duplicate on duplicated samples. The interfacial rheology experiments were performed once for each duplicated biomass fraction sample and the ruptured total from two different batches were measured twice each (i.e. four measurements in total). The emulsion stability tests were performed on duplicated samples. The trend was observed to be consistent between the duplicates and a representative set of the data was presented for clarity.

4.3 Results and Discussion

The effect of various components of the ruptured N. salina cells on stabilising emulsions was investigated individually by studying the fractions separated by centrifugation (Figure 4.1). This method avoided harsh treatments (e.g. chemicals, heat or extreme pH) (Sheng et al., 2010; Ursu et al., 2014), allowing the emulsifying properties of the fractions to be preserved (Xiao & Zheng, 2016). The different fractions included the total slurry of ruptured cells (ruptured total), the serum, cell debris, lipid, and delipidated debris. The mass fraction of each component in the ruptured total (on a salt-free basis) was ca 22% w/w serum, 18% w/w lipid, and 60% w/w delipidated debris. The cell debris was essentially the ruptured total minus the serum, consisting of ruptured and intact cells and some lipids (as confirmed in extraction tests). The serum consisted of soluble intracellular components, in particular protein, that comprised 52-57% w/w of the soluble material (based on total nitrogen measurement and a conversion factor of 5.86 (Safi et al., 2013)). The lipid included hydrophobic molecules that partitioned into the nonpolar n-hexane during the first solvent contact (Olmstead et al., 2013b), and consisted mainly of neutral lipids (91-93% w/w) and a small amount of polar lipid (5-7% w/w glycolipid and 1-2% w/w phospholipid). The delipidated debris included ruptured and intact cells with the nonpolar fraction removed via repeated hexane extraction.

4.3.1 Interfacial tension

Interfacial tension is an indirect measurement of the work required to increase the interfacial area (A. Bos & van Vliet, 2001; Leal-Calderon et al., 2007). A lower interfacial tension will allow smaller
droplets (i.e. tighter emulsions) to be formed, which generally leads to more kinetically-stable dispersions. The interfacial tension was measured using the pendant drop technique, with n-hexane as the bulk phase and seawater as the drop phase. The biomass fractions were then introduced individually into the appropriate phases (hexane or seawater) and the resulting interfacial tension measured as a function of time (Figure 4.3).

**Figure 4.3:** Interfacial tension as a function of various biomass fractions dissolved either in the hexane (bulk) or salt water (droplet) phase (3% artificial sea salt). All the biomass fractions were normalised to 5.5% biomass concentration, except for the lipid which was diluted to an equivalent of 0.4% biomass concentration as appropriate pendant drop images could not be obtained above that concentration. The term ‘biomass concentration’ was used to represent the concentration of the isolated components as present in the ruptured total. For example, the serum isolated from ruptured total of 5.5% w/w solids concentration is designated as having 5.5% biomass concentration.

To compare the relative surfactant strength of the biomass fractions the pseudo-stable interfacial tension at 1000s will first be considered, before discussing the kinetics. In the absence of any surfactants, the seawater-hexane interface had an expectedly high interfacial tension of 46.5±0.3 mN/m after 1000 sec (Figure 4.3), close to that of a pure water-hexane system (50.8 mN/m) (Zeppieri et al., 2001). The addition of the individual biomass fractions reduced the interfacial tension to varying degrees. To assess the impact of cell rupture on the emulsifying properties of the algae, both unruptured (intact cells) and ruptured cells were compared. At 1000 sec, the sample with unruptured cells had an interfacial tension of 26.4 mN/m, much higher than the ruptured total at 12.0 mN/m, showing that the emulsifying capabilities of the wet biomass were enhanced by the release of the intracellular contents that act as biosurfactants.
The interfacial tension resulting from the presence of the individual cell debris and serum fractions were 16.0 mN/m and 18.6 mN/m at 1000s, respectively. In both cases this was higher than the ruptured total in which both the serum and the cell debris were combined. This shows that both the cell debris and serum contained surface-active components, and that they were more effective in combination than individually. Even though the delipidated debris had the lipids and serum components removed, it was still able to lower the interfacial tension significantly (17.1 mN/m at 1000s). This suggests that the cell (wall) fragments can adsorb at the interface and reduce the interfacial tension.

The lipid (added to the hexane phase) was able to reduce the interfacial tension the most (7 mN/m), resulting in an even lower interfacial tension than the ruptured total. This was unexpected considering the ruptured total also contained the lipids. In addition, the result was based on the lipid at 0.4% biomass concentration instead of 5.5%, as an acceptable pendant drop image could not be obtained above that concentration. This shows that the lipids are stronger surfactants than any of the other biomass components. The apparent ineffectiveness of the lipid molecules in the aqueous ruptured total could be due to displacement at the interface by less effective surfactants, or the inability of the lipids to reach the interface due to being trapped within the cell debris.

The kinetics of interfacial tension decline, or the so called dynamic interfacial tension, can provide information relating to the adsorption process of the surfactants at the interface (Beverung et al., 1999). When the dynamic interfacial tension of a dilute surfactant system is plotted on a log-linear relationship, three distinct adsorption regimes (I-III) can often be observed (Beverung et al., 1999; Ferrari et al., 1997; Ferri et al., 2010; Gilcreest et al., 2006; Kutuzov et al., 2007; Okado et al., 2012; Tripp et al., 1995). Regime I, also known as the induction phase, is identified by an initial (often transient) lag period where the interfacial tension remains relatively unchanged. This lag phase represents a period of low surfactant coverage, before surfactants from the bulk reach the interface (Tripp et al., 1995). After the induction phase, the monolayer saturation phase (regime II) can begin and is characterised by a rapid decline in the interfacial tension. This regime is largely controlled by the diffusion process of the surfactants to the interface from the bulk phase (Beverung et al., 1999; Zhang et al., 2018). Once the interface is close to saturated, the adsorption process transitions to regime III (multilayer adsorption), which can be observed as a slow decline in the interfacial tension over an extended period. This is attributed to surfactant adsorption on the sublayer of the interface, forming a multiple adsorption layer, and/or the formation of a gel-like network (e.g. for a typical protein system) at the interface as a result of surfactant aggregation or a conformational change of the adsorbed layer (Beverung et al., 1999; Zhang et al., 2018).

At the high concentration (5.5% w/w biomass) used in the previous experiments (Figure 4.3), the surfactants were highly abundant and could therefore rapidly adsorb at the interface (e.g. interfacial tensions were close to stable after about 100 s). No apparent lag phase or saturation phase were observed. Thus, to better investigate the adsorption kinetics (Beverung et al., 1999), the samples of ruptured total, cell debris and serum were diluted to reduce the rate of adsorption/diffusion (Figure 4.4).
As expected, the interfacial tension (after 1000 s) generally decreased with decreasing concentration of the surface-active molecules (Figure 4.4A), with the *ruptured total* having lower interfacial tensions than the *cell debris* and the *serum* at all the tested biomass concentrations.

**Figure 4.4:** (A) The effect of biomass concentration on the interfacial tension at 1000 sec. (B) Comparison of the dynamic interfacial tension for (B) *ruptured total* (C) *serum* and (D) *cell debris* at different biomass concentrations.

At 0.1% biomass concentration (Figure 4.4C), the *serum*, which was rich in protein, displayed an adsorption regime typical of a dilute protein system (Beverung et al., 1999), where all the induction (<10s), saturation (10-500s) and multilayer adsorption (>500s) phases can be observed on a log-linear plot. The *serum* at 0.01% biomass concentration exhibited an induction phase (<100s) and a saturation phase (>100s) but the multilayer adsorption phase was not apparent within the period of measurements.

In comparison to the *serum*, the *cell debris* at lower biomass concentrations (0.01% - 0.1%) generally had a shorter lag period and a less steep saturation phase (Figure 4.4D). The lack of a steep saturation phase can be explained by the diffusion limitation of the surfactants as a function of their size. In this study, the cell particles in the *cell debris* are at least a magnitude larger (*N. salina* cells have a diameter of 2-4 µm) than the soluble molecules in the serum (typically in the nanometre range for soluble proteins).
(Erickson, 2009)). Therefore, the cell particles have a limited diffusivity in the bulk phase and the transport of the cell particles/debris to the interface will rely on gravity and any shear forces applied during lipid extraction.

In the ruptured total samples (Figure 4.4B), the interfacial tension reduction was more rapid than either of the serum or the cell debris samples at similar biomass concentrations, with no lag evident within the limits of the time measurements. Similar to the previous results (Figure 4.3), the ruptured total provided a synergistic effect with respect to its combination of serum and cell debris components, reducing the interfacial tension more than either of the fractions individually. At 0.01% biomass concentration, the serum and the cell debris had interfacial tensions of 42.3 mN/m and 40.8 mN/m respectively after 1000 s, whereas the ruptured total (which has both the serum and the cell debris components) had a significantly lower interfacial tension at 22.7 mN/m. This effect cannot be accounted solely by the higher amount of surfactants in the ruptured total, as the ruptured total at 0.01% biomass concentration still had a lower interfacial tension (20.4 mN/m) than either the serum at 0.1% biomass concentration (ca 0.022% w/w in an absolute salt-free solids concentration) with 22.9 mN/m, or the cell debris at 0.1% biomass concentration (ca 0.078% w/w) with 23.9 mN/m. This synergistic effect is likely because both the cell debris and the serum were able to complement each other at the interface due to their difference in size.

4.3.2 Interfacial viscosity

Although a lower interfacial tension is often used to predict the emulsifying capacity, it does not always correlate to the emulsion stability (Aderangi & Wasam, 1995; Kang et al., 2011). Rather, the formation of a rigid pseudo-plastic interfacial film or a high interfacial viscosity are better predictors of a stable emulsion (Aderangi & Wasam, 1995). The presence of such an interfacial film can be detected by interfacial rheometry, a technique that has been successfully applied to protein- (A. Bos & van Vliet, 2001; Murray & Dickinson, 1996), particle- (Madivala et al., 2009) and asphaltene-stabilised (Fan et al., 2010; Rane et al., 2013) emulsions.

In an interfacial shear rheological measurement, it is assumed that the interfacial flow is decoupled from the bulk phases (water or oil phase) and thus the measured effect only originates from the interfacial film region. This is typically confirmed by a large Boussineq number (>1) which is primarily a function of the bulk phase viscosity (Rühs et al., 2013). A more detailed explanation has been provided previously (Erni et al., 2003). In our study, the interfacial shear measurements were affected by the bulk phase viscosity at solids concentration above 8% w/w. This was indicated by an artificially high ‘interfacial’ viscosity (Figure 5A). Another limitation of the current system was the sedimentation of the particulate matter (i.e. the cell debris) over time (Figure 4.5A). Thus, all comparison studies were
performed at 4% w/w biomass concentration (Figure 4.5B) and the measurement period limited to <7 hours.

![Figure 4.5](image)

**Figure 4.5**: (A) The progression of the interfacial viscosity when the measurement was performed with ruptured total (8% biomass concentration) and hexadecane. The high initial torque (which was recorded as the interfacial viscosity) was due to the resistant of the bulk phase (i.e. the algal slurry) rather than the interfacial film. As the cell particles sediment, the measured torque dropped to close to that of the seawater-hexadecane interface. (B) The interfacial viscosities as formed by different biomass fractions at 4% w/w biomass concentration. The seawater-hexadecane (blue-X) was used as a control, with measurements close to or lower than the control (<0.25 mPa· s· m) indicating an absence of a continuous interfacial film.

Under the conditions of this study the serum, containing intracellular protein, was the only biomass fraction that was able to form a rigid viscoelastic interfacial film, as indicated by the positive complex interfacial viscosity (Figure 4.5B). The interfacial film was detectable almost instantaneously upon sample loading and continued to strengthen as it was allowed to age. Although a positive interfacial viscosity was also detected for the lipid samples (Figure 4.5B), this was likely due to lipid crystallisation and deposition at the interface, rather than a true interfacial film (Tavernier et al., 2016). This was evidenced by both microscopic and visual inspections (not shown) that revealed the presence of a crystalline material at the interface. During the measurements, interfacial viscosity would spontaneously decrease after reaching a peak. This behaviour was consistently observed in repeated measurements with the lipid samples, although the peak interfacial viscosities varied. Attempts to perform a strain amplitude sweep irreversibly damaged the microstructure (as evident by a significant reduction in the interfacial viscosity) and the interfacial viscosity did not recover even after several hours (data not shown). In a wet lipid extraction process it is unlikely that this lipid crystals film would form, as shear is continuously applied.

Interestingly, the ruptured total did not generate a measurable interfacial viscosity despite containing the serum components. Although no rigid film was detected by the rheometer, some of the cell particles
were observed to physically attach to the hexadecane interface. This suggests that the presence of cell-particles at the interface can interrupt the formation of a strong protein film, despite the apparently synergistic effect of the *cell debris* and *serum* components in lowering the interfacial tension (Figure 4.4). This is conceptually similar to other studies that have shown that the addition of low molecular weight surfactants into protein-stabilised dispersions can destabilise the emulsions by interrupting the protein film layers (Dickinson, 2001; Pelipenko et al., 2012).

During a wet lipid extraction process, the neutral lipids from the ruptured-algae slurry are expected to partition into the organic solvent phase. To test if these lipid molecules could interfere with a protein-based interfacial film, the *lipid*, which was primarily neutral lipids, was added to a serum-hexadecane system. It was observed that the interfacial viscosity decreased significantly after the addition of the *lipid* into the hexadecane phase (Figure 4.6A). An amplitude strain sweep of the serum-hexadecane interface without the lipid addition showed a highly viscoelastic interfacial film (Figure 4.6B), similar to a protein-stabilised interface (Erni et al., 2003). After the lipid addition, the interfacial film was significantly weaker and less elastic, with the G’ and G” cross-over point occurring at a much lower strain. The interfacial shear rheological measurements demonstrate that the simultaneous presence of multiple surfactants and emulsifiers (*cell debris*, *lipid* and *serum*) can result in weaker interfacial films than those formed with protein (*serum*) alone.

![Figure 4.6](image)

**Figure 4.6:** (A) Complex interfacial viscosity of a *serum*-hexadecane interface with or without the *lipid*. (B) A strain amplitude sweep was performed for both of the interfaces at the end of the measurements presented in (A) (*t* = 24 h for the *serum* and *t* = 32 h for the *serum-lipid*). The cross over points of G’ and G” in the strain amplitude sweeps are indicated, showing that the *serum*-hexadecane film was much stronger in the absence of *lipid*. 126
4.3.3 Emulsion stability

When hexane is mixed into the slurry of ruptured algae cells, a homogenous oil-in-water emulsion is formed, and the neutral lipids are extracted into the hexane droplets. After centrifugation, four distinct layers are formed: a cell solids pellet at the bottom, an aqueous supernatant layer, an emulsion layer, and a hexane-lipid top layer (Halim et al., 2016). The emulsion layer is recalcitrant to centrifugation, but the solvent must be recovered from this layer to allow full solvent recycling. The emulsion stability is therefore critical to a wet lipid extraction process.

As both the prior techniques (interfacial tension and interfacial shear rheology) required diluted samples and did not directly measure the emulsion stability, direct tests were performed to evaluate the emulsion stability at high biomass concentrations (section 4.2.6). Briefly, equal volumes of the biomass fractions (at 14 % w/w biomass concentration) and n-hexane were mixed for 2 hours and the resulting emulsions subjected to centrifugation. In preliminary emulsion stability tests, all the biomass fractions except the lipid (i.e. ruptured total, serum, cell debris, and delipidated debris) were able to fully emulsify the hexane. The lipid samples were able to produce dispersed hexane droplets that were observable during mixing, however they phase separated within a few minutes in the absence of mixing. This confirmed that while the lipid can act as a surfactant and lower the interfacial tension (Figure 4.3), it is not an effective emulsifier. This is consistent with interfacial rheology measurements that showed breakdown of the interfacial protein film with the addition of lipids (Figure 4.6). Thus, the lipid was eliminated from subsequent emulsion stability tests.

A more quantitative analysis of emulsion stability was performed using a LUMiFuge, an analytical centrifugation unit with an integrated optical component. In chapter 3, it was determined that the phase separation of ruptured-algae stabilised emulsions was limited by the coalescence of the hexane droplets (as opposed to the creaming rate). Therefore, the phase separation of the emulsified hexane will not occur unless a critical centrifugal force is exceeded that is sufficient to destabilise the emulsions through the coalescence of droplets.

In these emulsion stability tests, a low centrifugal force (30 x g) was applied initially to allow the creaming of the hexane droplets. After 12 minutes of centrifugation at 30 x g, the centrifugal force was roughly doubled every 5 minutes, reaching a final force of 2,330 x g. The critical centrifugal force at which a sudden increase in the hexane layer was observed was 50 x g for the delipidated debris, 200 x g for the ruptured total, 500 x g for the cell debris, and 1000 x g for the serum (Figure 4.7). The relatively high stability of the serum-stabilised emulsion is consistent with the presence of a highly viscoelastic proteinaceous interfacial film as measured in the interfacial shear rheology (Figure 4.6).
Figure 4.7: The phase separation of hexane from emulsions as monitored by a LUMifuge analytical centrifuge. (A) Hexane separation from emulsions formed by different biomass fractions at a biomass concentration of 14% w/w. (B) Hexane separation as a function of solids concentration for the ruptured total. The x-axes indicate the centrifugation time and the centrifugal force applied at different times. The y-axis is the percentage of the separated hexane on a height basis (based on the initial added hexane volume). The centrifugal forces at which major coalescence events occurred are noted in the figures.

The cell debris emulsion was more stable than the ruptured total emulsion at low centrifugation forces, not exhibiting any hexane separation prior to a catastrophic coalescence event at the critical centrifugal force (500 x g). Even though the ruptured total contained serum in its composition, the emulsion was not as strong as the emulsion produced by the serum alone. These results again suggest that the presence of other surface-active molecules can disrupt the protein film, resulting in a less stable emulsion. Other than proteins, the carbohydrate content (e.g. complex polysaccharides in which carbohydrate moieties are coupled to surface-active proteins or nonpolar molecules) of the algal cells (Schwenzfeier et al., 2013) may have also contributed to the emulsion stability via electrostatic or steric repulsion, not dissimilar to protein-carbohydrate emulsifiers used in food hydrocolloids (Dickinson, 2009).

The emulsion stability of the biomass fractions also did not appear to correlate with their interfacial tension (Figure 4.3). In addition to providing the critical centrifugal force, the emulsion stability tests reveal the amount of the hexane retained in the emulsion. The least stable emulsion, the delipidated debris, exhibited near-complete hexane recovery (97.7%) at the end of the centrifugation tests. Despite having different kinetics, about 81% of the hexane was recovered from both the cell debris and ruptured total emulsions. Only about 60% of the hexane could be recovered from the emulsions produced with the serum indicating that even higher forces would be required.

In a wet lipid extraction process, being able to process a high solids concentration is required (Yap et al., 2015) to achieve a high process efficiency and throughput. To investigate the effect of solids
concentration on the emulsion stability, tests were performed on emulsions produced with ruptured total at 14% w/w, 10% and 5% biomass concentration. The results (Figure 4.7B) show that as the solids concentration decreased (from 14% to 5%), the critical centrifugal force required to coalesce the droplets also decreased (from 200 x g to 75 x g) and the amount of hexane recovered increased. This could be explained by the reduced viscosity and amount of surface-active agents at decreased solids concentrations.

4.3.3.1 Qualitative analysis of the emulsion droplets

All else being equal, an emulsion with smaller droplets will be more stable with respect to creaming (Leal-Calderon et al., 2007). To evaluate the droplet sizes in emulsions formed with the different biomass fractions (at 14% w/w biomass concentration), samples of the emulsions from the stability tests (in Figure 4.7A) were visualised under a microscope (Figure 4.8). The droplet sizes were only assessed qualitatively, as the dispersed hexane droplets were tightly packed and flocculated, preventing an accurate quantitative analysis. Representative images of the emulsions formed by each biomass fraction (Figure 4.8A-D) suggest that the droplet sizes correlate to the interfacial tension measurements. In particular, the emulsions containing the lipid (i.e. the cell debris and the ruptured total), which contributed to the lowest interfacial tension, had smaller droplets (<200 µm), than the delipidated debris and the serum samples which did not contain the lipids (up to 500 µm). In contrast, the droplet size does not appear to relate to the results of interfacial rheology or stability experiments, suggesting that the droplet size has relatively little influence on coalescence.

As both the ruptured total and the cell debris appeared to be stabilised by particles (Figure 4.8C and 8D), further investigations were made to test if the emulsions could be destabilised by removing the non-associated or weakly bound particles from the emulsified droplets. To test this hypothesis, emulsions were diluted (9-fold) with seawater, mixed, and allowed to settle overnight. Unexpectedly, an emulsion layer was still present the next day without any visible hexane layer on top. By inspecting the emulsions under the microscope, it appeared that the hexane droplets in the cell debris emulsion were largely covered by apparently whole cell particles (Figure 4.8F), while the ruptured total droplets had some areas without any visible cell particles attached (Figure 4.8E). The exposed areas must have been stabilised by other surfactant molecules (e.g. proteins and lipids that are not visible under the microscope). These observations provide further evidence that the cell particles can stabilise the interfaces of the emulsion droplets but can also be displaced by the other surface-active molecules.
Figure 4.8: The images (8A-D) are representative of the hexane droplets formed by (A) the serum, (B) the delipidated debris, (C) the ruptured total, and (D) the cell debris. The qualitative differences between the hexane droplets from the emulsified (E) ruptured total and (F) cell debris were assessed after being diluted with seawater and allowed to settle overnight.

After washing, the cell particles were still adhered to the surface of the hexane droplets (Figure 4.8F). This is unexpected given that algal cells are hydrophilic, being natively well-dispersed in water. In addition, even though the delipidated debris and the cell debris should have both stabilised the emulsion via a Pickering mechanism (i.e. solids-stabilised), the removal of the nonpolar components via hexane
extraction seems to have reduced both its emulsifying ability and hexane retention (Figure 4.8B). One explanation is that the adherence of the cell particles to the hexane droplet was caused by the surface-bound EPS on the cells. Microbes such as microalgae often excrete EPS in order to interact with each other or to adhere to a surface (Shen et al., 2016; Xiao & Zheng, 2016). Some of these EPS can contain hydrophobic moieties (proteins or lipids) that are typically hidden in a hydrophobic core (Xiao & Zheng, 2016), but can be exposed upon contact with a nonpolar solvent (Damodaran, 2005; McClements, 2004).

Prior studies (Chevalier & Bolzinger, 2013; Rayner et al., 2014; Tavernier et al., 2016) have demonstrated that solid particles, especially those with dual wettability (amphiphilic), have a much lower free energy (more stable) when adsorbed at an interface. As such, after the initial adsorption, the detachment of the solid particles from the hexane-water interface would be practically irreversible due to the high desorption energy requirement. Consistent with this, it was observed that if a low centrifugal force (less than 600 x g) is used for separating the hexane from the emulsions, only three of the four layers will be formed (namely the light hexane layer, the emulsion layer and the aqueous supernatant), with the bottom wet solids pellet (containing solid particles detached from the hexane droplets) being absent. In this case, the low centrifugal force may not be sufficient to overcome the desorption energy barrier and release solid particles from the hexane droplet interface.

These results suggest that the hydrophobic components on the cell surfaces (likely the surface bound EPS) are responsible for the retention of some of the solvent after centrifugation (Figure A4). Thus, these surface-hydrophobic components could be targeted to increase solvent recovery. Another strategy could be to add a low molecular weight surfactant to occupy both the water-oil interface and the hydrophobic cell surfaces, to encourage the release of the cell particles from the solvent interface (Figure A5).

### 4.3.4 Mechanisms of emulsification in a nonpolar solvent-based wet algal lipid extraction

Based on the current study, a mechanistic understanding of the emulsification and demulsification processes during wet lipid extraction from microalgae can be consolidated (Figure 4.9). After the cells in the wet biomass have been ruptured and the intracellular contents released, a nonpolar solvent (e.g. hexane) is added to the ruptured-algae slurry to extract the neutral lipids (Tao Dong et al., 2016b; Yap et al., 2014). The application of shear during the extraction process facilitates the transfer of the lipids into the solvent phase. The presence of the lipid in the hexane phase decreases the interfacial tension significantly, allowing small hexane droplets to be formed by the shear. In the aqueous phase, the protein in the serum is able to stabilise the emulsified droplet by forming a viscoelastic interfacial film while the cell debris adsorbs to the interface via hydrophobic sites on the cell surface. The presence of multiple surface-active molecules means that there is competitive interfacial adsorption between the
lipids, cell particles and proteins. Importantly, the strong protein film resulting from the serum can be weakened by the presence of lipids or cell particles.

Based on these findings, the recovery of the solvent from the emulsified biomass can now be considered as a two-fold problem. Firstly, a critical centrifugal force is needed to destabilise the bulk emulsion by overcoming the interfacial layer, enabling droplet coalescence. This allows up to 80-90% hexane to be recovered as a separate phase. However, to recover the hexane remaining in the recalcitrant emulsion layer, the solids particles have to be detached from the solvent interface. However, as there is a high energy barrier to detachment, a more effective strategy than centrifugation will be required.

![Diagram](image)

**Figure 4.9:** A summary of the emulsifying roles of the surface-active components present in a slurry of ruptured algal cells. Note that the components are not drawn to scale.

### 4.3.5 Microalgae as a source of biosurfactants and bioemulsifiers

This study has demonstrated that ruptured microalgae contain a variety of surface-active agents with different characteristics. The fractionation approach taken in this study is a first step towards understanding the emulsifying properties of these surface-active agents. In the future it may be interesting to isolate individual surface-active molecules for in-depth characterisation. In this regard, microalgae may be considered as a source of biosurfactants and bioemulsifiers, which may have more immediate commercially promise than biofuel production. Industries such as the food, cosmetic and pharmaceutical industries are looking to substitute synthetic surfactants with naturally derived
alternatives (Dickinson, 2009; Mnif & Ghribi, 2015b; Paniagua-Michel et al., 2014). Microalgae could be a particularly attractive source that is non-toxic and highly productive (William & Laurens, 2010). In addition, the surface-active fractions can be readily produced at scale and appear to have interesting and distinct properties. For example, the lipid is an excellent surfactant that has almost no stabilising effect. On the other hand, the serum is highly effective as a long-term emulsifier but is only a moderately effective surfactant producing relatively coarse emulsions. These distinct and varied properties may allow them to be tailored for specific applications.

4.4 Conclusion

A systematic investigation of the emulsifying properties of different components of ruptured microalgal cells was performed for the first time. Effective surface activity was exhibited by the cell debris, the lipid and the serum. The interfacial tension and the interfacial shear rheology demonstrated that each of the biomass fractions contributed to the stability of emulsions in different ways. The lipid acts primarily as a surfactant that lowers the interfacial tension, but does not contribute to emulsion stability. Conversely, the protein-rich serum is only moderately effective as a surfactant, but it is able to strongly stabilise the emulsion through the formation of a strong film layer. The cell debris solids can also help to stabilise the emulsion to a lesser extent by adsorbing at the interface. When multiple components are present there is competitive interfacial adsorption. As such, lipids and cell debris can interrupt the protein film, so that emulsions produced with all the biomass fractions were somewhat easier to break than those produced using solely the protein-rich serum. To ensure a full solvent recovery in a wet lipid extraction process, the detachment of the adsorbed cell particles from the solvent interface appears to be needed. The insights from this study suggest it would be worthwhile investigating the use of individual surface-active agents or combinations of the biomass fractions as bioemulsifiers or biosurfactants for commercial applications.
4.5 References


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Chapter 5

5 Understanding the role of emulsions in biphasic lipid extraction from algae

Emulsions are usually formed as part of a biphasic solvent extraction process. This is because the emulsion provides additional interfacial area that enhances the lipid extraction rate. However, as shown in chapter 4, the emulsion formation impairs downstream solvent recovery. Therefore it would be preferable if effective lipid extraction could be achieved without emulsifying the solvent. This chapter investigated a method to extract lipids using hexane without emulsion formation. Using this approach, the slurry-hexane interfacial area could be reliably quantified and thus the effect of process variables such as temperature, slurry concentration and mixing speed on the lipid extraction kinetics could be studied independent of the effect of the interfacial area. The lipid transfer rate in this method was then compared against a conventional emulsion-based extraction process to gain a deeper understanding of the rate limiting factors in a biphasic lipid extraction process.

The data presented in this chapter are the result of a joint effort between the author and Erwin Adrian Macario, a research student who was under the author’s guidance as part of his Master’s course. The experimental data was included in the thesis with consent from Erwin. The author of this thesis contributed significantly to the conception of the experiments, performing the preliminary experiments, finalising the experimental design, and assisting actively throughout the execution of the experiments. The final data analysis and interpretation presented here were performed by the author.
5.1 Introduction

The kinetics of monophasic lipid extraction from wet algal slurries has been investigated (Halim et al., 2014; Ranjan et al., 2010). However, the same information is not available for biphasic lipid extraction from slurries of ruptured algae. Understanding of the kinetics of lipid extraction (the process of lipid transferring into the solvent phase) is important for process equipment selection and optimisation (Halim et al., 2014).

The lipid transfer process in a biphasic extraction is different from a monophasic extraction due to the immiscibility of the solvent (Yap et al., 2014). While a monophasic extraction is mostly driven by the solute-solvent concentration gradient, this driving force is not available in a biphasic extraction as the immiscible solvent cannot penetrate through the algae cell walls (Yap et al., 2014). In a typical biphasic extraction, hexane is mixed into a wet ruptured slurry to form a homogenous mixture (emulsion) (Jiménez Callejón et al., 2014; Lundquist et al., 2010; Olmstead et al., 2013b). The emulsion creates additional slurry-solvent interfacial area that facilitate the lipid transfer process (Dong et al., 2016; Yap et al., 2014). The increase in the interfacial area depends largely on the emulsion droplet size and the emulsion volume fraction (Jurado et al., 2007). However, the emulsion properties in this extraction process (particularly the droplet size distribution) was not documented in previous studies (Jiménez Callejón et al., 2014; Olmstead et al., 2013b), and therefore the interfacial area available during lipid extraction process has not been characterised. As such, there is no knowledge of the mass transfer coefficients.

It is known that cell rupture is critical to biphasic lipid extraction (Halim et al., 2016; Jiménez Callejón et al., 2014; Yap et al., 2014). However, the influence of other variables including the slurry solids concentration, the extraction temperature, and the stirring/mixing rate have not yet been studied. Previous studies of biphasic extraction were performed with these process parameters fixed (Jiménez Callejón et al., 2014; Olmstead et al., 2013b). In addition, changing these process variables may also change the emulsion droplet size (Jiang et al., 2016; Weiss & Muschiolik, 2007) and its associated interfacial area. Therefore, it is desirable to study the lipid extraction kinetics with the effect of interfacial area isolated from the other variables.

In this study, two methods of lipid extraction from a slurry of ruptured algae were performed and compared. The first lipid extraction method was a constant interfacial area (CIA) extraction with a defined slurry-hexane interfacial area. The second method was a conventional emulsion-based lipid extraction process using hexane. The interfacial areas resulting from these two approaches were quantified, allowing a normalised comparison to be made. The information from this study was then used to identify the rate-limiting processes during biphasic lipid extraction.
5.2 Materials and Methods

5.2.1 Generation of ruptured-algae slurry

Marine microalgae *N. salina* grown outdoors under a nitrogen-replete regime was harvested and stored at -20°C until use (Halim et al., 2016). The dry weight of the algal slurry was determined to be 19-20% w/w by oven drying at 60 °C (Yap et al., 2015). The frozen slurry was thawed before rupturing the cells according to a previously established protocol (Halim et al., 2016; Olmstead et al., 2013b). Briefly, the *N. salina* slurry was incubated overnight at 38 °C to weaken the cell walls. The next day, the incubated slurry was passed through a high-pressure homogeniser at 1200 ± 100 bar (Panda 2K NS1001L, GEA Niro Soavi, Parma, Italy) for cell rupture. To minimise the effects of variation in the extent of cell rupture that would otherwise affect the lipid extraction results, a large batch (ca. 300g of 19 % w/w slurry) of slurry was processed at once and was stored at 4 °C for use within a month.

5.2.2 Determination of the total lipid content and the hexane-extractable lipids

The total lipid content in the algal slurries was determined to be 22 ± 2% w/w of the dry biomass (e.g. 0.22g total lipid per 1g dry biomass) using a modified Bligh & Dyer protocol (Olmstead et al., 2013a). The overall lipid was 61 ± 4 % w/w neutral lipid, 27 ± 4% w/w glycolipid and 9 ± 1 % w/w phospholipid based on a solid phase extraction (SPE) fractionation (Olmstead et al., 2013a).

To quantify the amount of lipids released by the cell rupture process (Halim et al., 2016; Jiménez Callejón et al., 2014), a theoretical maximum lipid yield (on a hexane-extractable lipid basis) was determined by performing three consecutive hexane contacts on the ruptured-algae slurry. Briefly, 4 mL of ruptured-algae slurry (from section 5.2.1) was mixed with 8 mL n-hexane (AR grade, Ajax Finechem, Victoria, Australia) – at a 1:2 v/v wet biomass:hexane ratio. The slurry and hexane were mixed (RSM6, Ratek Instruments, Victoria, Australia) for 2 hours prior to centrifugation at 9000 x g for 15 minutes (Allegra X-30R, fixed-angle rotor F0685, Beckman Coulter, Victoria, Australia). The separated hexane layer was decanted and collected separately. The pellet and the emulsion layer were mixed with 8 mL of fresh hexane for 2 hours prior to another centrifugation. This hexane extraction process was performed a total of three times. The lipid-rich hexane layers from all three extractions were pooled and dried at 60 °C under a stream of nitrogen (Olmstead et al., 2013a). The lipid extract obtained from this protocol was defined as the ‘hexane-extractable lipid’ (0.12 ± 0.1 ghexane extractable lipid/g dry biomass, which was 54% w/w of the total available lipid). This hexane-extractable lipid yield was used as the basis of comparison for quantifying lipid recoveries in the subsequent experiments.

5.2.3 Constant Interfacial Area (CIA) lipid extraction

To remove the slurry-solvent interfacial area as a variable in the lipid extraction process, an extraction protocol with a constant interfacial area (CIA) was used (Figure 5.1). 15 mL of the diluted ruptured-algae slurries (diluted to 4-11% w/w solids concentration) was carefully added to a 100 mL air-tight
Duran Schott bottle (to prevent hexane evaporation). After that, 40 mL of hexane was carefully layered on top of the ruptured-algae slurry without inducing mixing or emulsification. The weight of the entire sample was monitored to ensure there was no significant hexane evaporation (typically < 2 % w/w loss). A Teflon-coated magnetic stirrer bar was placed gently in the ruptured-algae slurry and the Schott bottle placed on a magnetic stirrer plate (LTF Labortechnik GmbH & Co. KG, Wasserburg, Germany) to provide a uniform mixing in the slurry phase. The stirring rate was calibrated to the setting on the stirrer prior to the experiment, and was varied between 200-500 rpm. The interfacial area of the slurry-hexane interface was determined based on the inner diameter of the Schott bottle, which was determined based on measuring its outer diameter and wall thickness, and assuming the bottle is a perfect cylinder.

For temperature-controlled experiments, the Schott bottle was placed in a water bath above a magnetic stirrer. After a predetermined extraction time (24 hours or 96 hours), 10 mL of the hexane phase was taken and dried under nitrogen. The total extracted lipid in the CIA experiments was calculated based on the sampled lipid dry weight and the total hexane volume used. The final lipid yield (recovery %) was obtained by comparing against the hexane-extractable lipid (section 5.2.2). Post lipid-extraction, the algal slurries were examined under the microscope to confirm there was no unintended emulsification.

**Figure 5.1:** The experimental setup used to provide a constant interfacial area (CIA) during hexane extraction.

### 5.2.4 Conventional lipid extraction via emulsification

The results from the CIA experiments were compared against a typical lipid extraction process involving hexane-emulsion. Briefly, 2 mL of the ruptured-algae slurry from section 5.2.1 was mixed with 1 mL of hexane (Olmstead et al., 2013b). To determine the lipid extraction kinetics using this method, the algae-hexane slurry was mixed via vortexing for 1, 10, or 20 min, and then centrifuged at 3,273 x g for 25 min (Allegra X-12, swing bucket rotor SX4750, Beckman Coulter, Victoria, Australia). For kinetic modelling, the final lipid-in-hexane concentration at equilibrium was required. Thus, another sample was vortexed for 20-minute and then mixed further via rotational (RSM6, Ratek
Instruments, Victoria, Australia) at room temperature for 72 hours. The collected hexane layers from
the centrifugation process were dried at 60 °C under a stream of nitrogen (Olmstead et al., 2013a). The
weight of the lipid-rich hexane (pre-drying) and the weight of the dried lipid were recorded to obtain
the lipid-in-hexane concentration required for the kinetic modelling.

5.2.5 Determination of the droplet size distribution in the emulsified extraction

To quantify the total interfacial area of the algae-hexane emulsion (from section 5.2.4), samples were
loaded onto a microscope slide for an image analysis. Images of the emulsions were taken using a
bright-field light microscope with a digital camera attachment (BX51, Olympus, Tokyo, Japan). The
droplet diameters were assessed manually by drawing lines through all droplets using AutoCAD
software and a droplet size distribution was obtained. At least 500 individual hexane droplets were
counted for each of the mixing times (1 min and 10 min) to ensure a representative sample. The total
droplet surface area and volume were then calculated assuming that the droplets were spherical and that
the droplet cross-sections represented the maximum diameter.

5.2.6 Experimental reproducibility and statistical analysis

The dry weight and the lipid content analyses were performed in duplicate. The CIA extraction studies
(effect of temperature, mixing, and solids concentration) were repeated at least once to confirm
reproducibility. The emulsion-based lipid extraction was performed once. The results were presented
as the average ± standard deviation where available.

5.3 Results and Discussion

5.3.1 Constant Interfacial Area (CIA) extraction

In general, it was observed that the lipid extraction kinetics in the CIA experiments were slow, taking
almost 24 hours to extract a sufficient amount of lipid for a reliable gravimetric measurement (>10 mg).
This is in contrast with the emulsion-based processes where a majority of the accessible lipids (after
cell rupture) could be extracted within an hour (Jiménez Callejón et al., 2014; Olmstead et al., 2013b).
This result is consistent with the idea that the emulsification process in a typical hexane extraction
generates a large interfacial area that facilitates lipid transfer. It also demonstrates that the interfacial
area in a biphasic extraction can be the rate-limiting factor in lipid extraction. However, accurately
quantifying the interfacial area in a emulsification process can be a challenge as the droplet size can
change dynamically during the mixing process (Tcholakova et al., 2008). Therefore, to accurate
determine the mass transfer coefficient, there is a need to perform the lipid extraction without inducing
emulsion formation. Hence, the CIA setup was used. The interfacial area for the CIA extraction was
estimated to be about 3100 ± 100 mm² (or 3.1 x 10⁻⁴ m²) based on the inner diameter of the vessel. The
lipid extraction process was performed over a 24-hour period, and the lipid yield was obtained by
recovering and drying the hexane phase. The average lipid transfer rate was determined using Equation 5.1. Importantly, only a small fraction of the available lipids were extracted (typically <10% w/w, which will be discussed later) over the 24 period, meaning the average rate is reasonably representative of a maximum initial extraction rate.

**Equation 5.1:** Lipid Transfer Rate \((g \text{ m}^{-2} \text{hr}^{-1}) = \frac{\text{Recovered Dried Lipid in Hexane Phase (g)}}{\text{Interfacial Area (m}^2\text{)} \times \text{Extraction period (hr)}}\)

### 5.3.2 The influence of mixing in different phases

During preliminary experiments performed in the set-up of the CIA extraction method, the difference between providing mixing in the aqueous phase or the hexane phase was investigated. Mixing of the aqueous phase was achieved by placing a magnetic stirrer in the bottom slurry phase (Figure 5.1). Mixing of the upper hexane phase was achieved by shaking the entire Schott bottle with its content on a rotary shaker platform (the highly fluid hexane was mixed much more thoroughly than the viscous slurry). The result showed that the lipid transfer rate in the rotary shaker platform was significantly slower than the magnetic stirrer setup (Figure 5.2). This suggests that the mixing of the ruptured-algae slurry, instead of the hexane phase, is likely to be the limiting factor in the CIA lipid transfer process. In addition, even without active mixing of the hexane phase, the diffusion of the extracted lipid was rapid, as evidenced by a homogenously green hexane layer. Therefore, all further experiments were conducted with the magnetic stirrer setup shown in Figure 5.1.

![Figure 5.2](image)

**Figure 5.2:** The influence of the mixing in different phases on the lipid transfer rate. Mixing of the hexane phase was achieved by placing the bottle on a rotary shaker platform at 200 rpm, while mixing of the aqueous phase was achieved by a magnetic stirrer bar located in the slurry phase.

### 5.3.3 Effects of stirring speed, temperature and slurry concentration on lipid transfer rate

Mixing is a crucial parameter in a solvent extraction as it promotes the diffusion or the movement of the solute (Halim et al., 2014; Sivaramakrishnan & Incharoensakdi, 2017). However, comparing the mixing rate across different studies can be challenging due to the different fluid dynamics that are
determined by the equipment geometries, mixer type, slurry rheology etc. (Bridgeman, 2012). Nonetheless, a general trend can still be observed. In a low-mixing regime, an increase in the stirring speed can improve the lipid extraction efficiency (Sivaramakrishnan & Incharoensakdi, 2017). At higher mixing speeds, further increases will not improve the extraction rate (Kadi & Fellag, 2001), and will only increase the mixing energy input (Bridgeman, 2012).

In this study, an increase in the stirring rate from 200 rpm to 300 rpm improved the lipid extraction rate slightly (from 0.19 g m$^{-2}$ hr$^{-1}$ to 0.34 g m$^{-2}$ hr$^{-1}$) (Figure 5.3A). Although a further increase in the stirring rate (to 500 rpm) also appeared to improve the lipid transfer rate (1.14 g m$^{-2}$ hr$^{-1}$), this was likely due to the formation of an emulsion as confirmed by microscopic observation. Therefore, subsequent experiments were performed below 500 rpm to prevent emulsion formation.

The role of the extraction temperature can be two-fold in a lipid extraction process. An increase in the temperature can generally increase the preferential solubility of the solutes in the solvent (also known as the partition coefficient) (Grima et al., 2013), thereby increasing the final lipid yield (Sivaramakrishnan & Incharoensakdi, 2017). A higher temperature can also improve the mass transfer coefficient (Sivaramakrishnan & Muthukumar, 2014) by decreasing the viscosity of the algal slurry (Schneider et al., 2016). In the CIA extraction, the lipid transfer rate at 40 °C was more than two times higher (1.6 g m$^{-2}$ hr$^{-1}$) than at room temperature (0.7 g m$^{-2}$ hr$^{-1}$) (Figure 5.3B). While this shows that an above-ambient temperature can improve lipid extraction kinetics, the elevated temperature would require a heat source. Fortunately, waste heat generated from high-pressure homogenisation and hexane distillation could be used to provide this energy (Navarro López et al., 2016).

The solids concentration in the ruptured-algae slurry can affect the lipid extraction process in two ways. By increasing the solids concentration, faster extraction can be expected due to the increased lipid volume fraction. This was confirmed experimentally (Figure 5.3C), with the lipid transfer rate generally found to increase with an increase in solids concentration. However, a very highly concentrated algal slurry can actually negatively influence lipid transfer due to an exponential increase in viscosity (Schneider et al., 2016). Also, at very high solids concentrations algal slurries can become highly viscous, shear-thinning fluids (Yap et al., 2016). The shear-thinning behaviour of the slurry was evident in this study, with the magnetic stirrer was not able to mix the slurry in experiments performed at a solids concentration ≥15% w/w. Instead, there was only localised mixing around the magnetic stirrer rather than uniform mixing throughout the slurry (not shown).
Figure 5.3: The lipid transfer rate during CIA extraction as a function of (A) the stirring speed (rpm), (B) the temperature and (C) the slurry solids concentration. The stirring rate experiments (A) were performed with an 8 ± 0.5% w/w slurry at room temperature. The temperature experiments (B) were performed with a slurry of 9 ± 0.5% w/w solids with a stirrer speed of ca 400 rpm. The solids concentration experiments (C) were performed at room temperature and a stirrer speed of ca 400 rpm.

5.3.4 Kinetics and yield of CIA extractions

To investigate the lipid extraction kinetics and recovery, CIA extractions were performed for 24 hours and 96 hours. The quantities of the recovered lipids in the hexane phase were compared against the maximum hexane-extractable lipids (based on repeated emulsified hexane extractions) to estimate the lipid recovery yield. An increase in the extraction time from 24 hours to 96 hours increased the lipid recovery from 4.7 ± 1.4% to 8.1 ± 3.4% (Figure 5.4A). However, this was still a much lower yield than was typically achieved in emulsion-based extractions (Jiménez Callejón et al., 2014; Olmstead et al., 2013b).

Interestingly, the lipid transfer rate at 24 h (0.4 g m⁻² hr⁻¹) was slightly higher than the lipid transfer rate at 96 h (0.2 g m⁻² hr⁻¹) despite still being far from the extraction equilibrium (100% of hexane extractable lipid) (Figure 5.4B). The two-fold reduction in lipid transfer rate cannot be accounted for simply by the reduction in the overall lipid volume fraction in the aqueous phase. Rather, it could be due to localised depletion of lipids near the interface or the recovery of more easily recoverable lipids. For example, loose, large lipid droplets would be more likely to be able to rapidly migrate to the hexane layer than
droplets entrapped in the cell debris. Another possible reason for the reduced lipid transfer rate could be due to the solid particles adsorbing to the interface more strongly over time and therefore limiting the effective interfacial area available for lipid transfer. Determining the exact mechanisms would require further investigation, however these (Figure 5.4) show that it would take much longer than 96 h to achieve a lipid yield comparable to an emulsion-based extraction.

![Figure 5.4](image)

**Figure 5.4**: (A) Lipid recovery kinetics on a hexane-extractable lipid basis and (B) lipid transfer rate as a function of extraction time. The experiments were performed on a 7 ± 1% w/w solid slurry at room temperature, with a stirring rate of *ca* 400 rpm. The 24 h data point was the average of 4 experiments and the 48 h was the average of 3 experiments.

### 5.3.5 Emulsion-based lipid extractions

#### 5.3.5.1 Effect of mixing on emulsion surface area

To compare CIA and emulsion-based extractions, lipids from the ruptured-algae slurry were also extracted via a mixing process that formed a homogenous emulsion. The additional interfacial area created in the emulsification process was characterised by obtaining the hexane droplet size distribution via microscopy after 1 min and 10 min of mixing (Figure 5.5A and 5B). The emulsions formed after 20 min of mixing appeared to be similar to those after 10 min, so only the size distribution from 10 min samples were quantified and presented.

After 1 min of mixing, *ca* 50% (on a number basis) of the droplets had a diameter less than 21-25 µm. After 10 min mixing, more than 50% of the droplets had diameters less than 11-15 µm. This droplet size distribution was comparable to what was observed in the previous work presented in chapter 3.

As a known volume of hexane was added to the wet ruptured-algae slurry, the hexane volume can be converted to an interfacial area based on the droplet size distribution. In brief, a volume-to-area
conversion factor was obtained via the ratio of the total droplet surface area to the total droplet volume specific to the droplet size distribution. In this experiment, the conversion factors for 1 min and 10 min mixing were $1.92 \times 10^5 \text{ m}^{-1}$ and $3.2 \times 10^5 \text{ m}^{-1}$, respectively. Applying these, the total interfacial area per 1 m$^3$ of emulsified hexane calculated to be $1.92 \times 10^5 \text{ m}^2$ and $3.2 \times 10^5 \text{ m}^2$ after 1 min and 10 min of mixing, respectively.

Figure 5.5: Droplet size distributions on a number basis obtained from microscopy image analysis of emulsions formed after 1 min (A) and 10 min (B) mixing. (C) Cumulative number distributions comparing the emulsions from both mixing times, replotted from the data presented in (A) and (B).

5.3.5.2 Lipid transfer rate and extraction yield normalised by interfacial area

As expected, the lipid recovery in the emulsification approach (Figure 5.6A) was significantly faster than the CIA extractions (Figure 5.4A), with more than 40% lipid recovery (on hexane-extractable lipid basis) after only one minute of mixing and about 80% lipid recovery after 20 minutes.

For a simplified estimation of the lipid transfer rate, the emulsion formation process was assumed to be instantaneous, so that the final interfacial area (measured after either 1 min or 10 min) could be considered to be the interfacial area available for the entire extraction period. Based on this approach, the lipid transfer rate in the emulsion-based extraction process was calculated to be $5.2 \text{ g.m}^{-2}\text{.hr}^{-1}$ over 1 minute, $0.5 \text{ g.m}^{-2}\text{.hr}^{-1}$ over 10 minutes, and $0.1 \text{ g.m}^{-2}\text{.hr}^{-1}$ over 20 minutes. Interestingly, when
normalised based on the interfacial area, the lipid transfer rates over 10 min and 20 min were of a similar order of magnitude to those of the CIA extractions. This suggests that the rapid kinetics of emulsion-based extractions can largely be attributed to the increased interfacial area.

However, over one minute, the lipid transfer rate was 5.2 g.m\(^{-2}\).hr\(^{-1}\), close to 10 times faster than the lipid transfer rates observed in the CIA extractions (Figure 5.3 and Figure 5.4). This could be due to the more intense mixing regime, which was the only main difference between the two experiments other than the interfacial area, that may for instance have been able to liberate lipid droplets from the cell debris allowing them to partition into the solvent. Over 10 minutes and 20 minutes, the lipid transfer rate was considerably reduced to 0.5 g.m\(^{-2}\).hr\(^{-1}\) and 0.1 g.m\(^{-2}\).hr\(^{-1}\) respectively. This was likely due to the decreased lipid volume fraction left in the aqueous phase, as more than 40% lipid had already been extracted in the first minute.

The results of this chapter appear to be in agreement with Yap et al.’s proposed mechanism (Yap et al., 2014). Based on their observations, the lipid transfer in a biphasic system occurs through a ‘washing’ step that allows direct physical contact of the solvent and the oil droplets in the aqueous phase. In an emulsion-based extraction, the likelihood of the hexane-lipid contact (also known as the collision frequency in other emulsion studies (McClements, 2015)) will largely depend on the emulsion droplet size and volume fraction (often as a function of solvent ratio and the concentration of the surfactants/emulsifiers (McClements, 2015; Shtyka & Sek, 2016; Urbina-Villalba, 2004)), the lipid volume fraction, as well as the shear forces from mixing (McClements, 2015). Therefore, when an intense mixing was applied in the emulsion-based extraction, the hexane was more likely to come in contact with the oil droplets. As the lipid fraction decreases due to partitioning into the hexane phase, the probability of hexane-lipid contact decreases, resulting in a decreased lipid extraction kinetics (Figure 5.6B).

There is another explanation why an intense mixing can be beneficial to the lipid transfer process. As has been shown in Chapter 4, many of the cellular components (such as cell wall fragments and proteins) are surface-active and can adsorb to the aqueous-solvent interface. This could retard the rate of lipid transferring from the aqueous phase into the hexane phase (Dong et al., 2016; Lewis, 1954). While the actual lipid molecules are much smaller in size (than cell solids and proteins) and can theoretically diffuse across the adsorbed-surfactant layer, the available interfacial area for lipid transfer may be limited as a result of the adsorbed-surfactants. In addition, the layer of adsorbed cell components could provide a steric barrier preventing the contact of the lipid bodies with the hexane droplets. An intense mixing could potentially facilitate the lipid extraction process by disturbing this adsorbed-surfactant layer. This may explain the higher initial lipid transfer rate (5.2 g.m\(^{-2}\).hr\(^{-1}\)) observed in the emulsion-based approach compared to the CIA extraction (0.4 g.m\(^{-2}\).hr\(^{-1}\)) even after the interfacial area has been accounted for. To confirm this hypothesis, further investigation using a model system (instead of ruptured-algae) to study the lipid transfer process will be required.
5.3.6 Kinetic analysis of emulsion-based lipid extraction

To model the lipid extraction process in the emulsion-based approach, the lipid concentration as a function of mixing time was obtained from the experiment. The experimental data were fitted to the linearised integrated rate equations commonly used for modelling dissolution/leeching processes (Cho et al., 2012; Rakotondramasy-Rabesiaka et al., 2007), chemical reactions, and adsorption systems (Ho, 2006). The linearised equations used are as following:

\[
\begin{align*}
\text{Equation 5.2:} & \quad C_0 - C = k_0 t + C_0 \quad \text{(Zero Order)} \\
\text{Equation 5.3:} & \quad \ln(C_0 - C) = -k_1 t + \ln(C_0) \quad \text{(First Order)} \\
\text{Equation 5.4:} & \quad \frac{1}{C} \cdot \frac{dC}{dt} = k_2 t + \frac{1}{C_0} \quad \text{(Second Order)}
\end{align*}
\]

where \( C_0 \) is the maximum achievable lipid concentration in the extraction process, also known as extraction capacity (Jiménez Callejón et al., 2014). In this study, the \( C_0 \) was obtained by gentle mixing of the slurry-hexane emulsion for a further 72 hours after the initial 20 min of vigorous mixing. \( C \) is the lipid concentration in the hexane phase at an arbitrary time (t). \( k_n \) is the corresponding rate constant for the zero, first and second order models (n = 0, 1 and 2). Value of the rate constants \( k_n \) were determined by the method of least square. Plots of the integrated rate equations are shown in Figure 5.7.

Overall, the experimental data followed the second order model best, with an \( R^2 \) of 0.98 and a rate constant \( (k_2) \) of 0.0061 min\(^{-1}\). A slightly larger deviation from the second-order model fit was observed particularly for the data point at 1 min mixing. This is probably in part due to the limitation of the current experimental design where lipids continue to be extracted after the mixing had been stopped (i.e. during sample handling and centrifugation). This effect is especially significant for the data point at 1 min where the concentration driving force is the highest. The real lipid concentration (\( C \) term in
equation 5.2, 5.3, and 5.4) for 1 min is expected to be lower than what was measured and therefore should result in an even better fit with the second-order model.

The rate constant $k_2$ obtained in this study (0.0061 min$^{-1}$) was close to the rate constant observed by Cho et al. (0.0053 min$^{-1}$) for an extraction from ruptured Scenedesmus sp., albeit using a monophasic solvent (chloroform/methanol) instead of hexane (Cho et al., 2012). The model fitting result was however in contrast with the study performed by Jimenez et al. (Jiménez Callejón et al., 2014). In their study, they found that the lipid was extracted slowly and continuously (using hexane) for approximately 20 hours, following a first-order model. This was likely due to the insufficient mixing regime used in their study, as their slurry-hexane mixture was agitated on an orbital shaker at 200 rpm (similar to the rotary shaker setup used in the preliminary experiment in this study - Figure 5.2), which was found unable to mix the slurry effectively at high solids concentration (14% w/w). This comparison suggests that the extraction kinetics of a biphasic system (with finite interfacial area) can approach a monophasic system provided it is a well-mixed system.

**Figure 5.7**: Fitting of the experimental data to (A) zero order (B) first order and (C) second order integrated rate equations. The linearised integrated rate equations used to fit the data and the regression coefficients are displayed in the figures.
5.3.7 The role of interfacial area in a conventional emulsion-based lipid extraction

As lipids are not soluble in water, there is no lipid concentration gradient to drive mass transfer in a biphasic extraction process (Yap et al., 2014). Instead, the lipid transfer in a biphasic system can only occur via a physical contact of the lipid with the solvent interface through collisions, the likelihood and intensity of which are determined by various factors such as the mixing intensity, the lipid volume fraction, and the solvent interfacial area (as a function emulsion volume fraction and droplet size) (McClements, 2015). This idea is consistent with the findings of this chapter, which showed that the slurry-hexane interfacial area was the single most important factor for increasing lipid transfer rate in an emulsion-based extraction. Other factors such as the temperature and the solids concentration also affected the extraction kinetics, likely by influencing the collision frequency as discussed earlier. The findings in this chapter represent an extension to the mechanic model proposed by Yap et al. (Yap et al., 2014). They suggested that a biphasic extraction on a wet ruptured-algae slurry occurred through a physical ‘washing’ of lipids from the cell debris, however the role of interfacial area and the hexane-lipid collision frequency were not considered.

These findings establish the importance of creating an emulsion via shear to improve lipid extraction in a biphasic system. Avoiding emulsion formation or creating only a coarse emulsion using reduced shear or an insufficient mixing intensity (Jiménez Callejón et al., 2014; Olmstead et al., 2013b) will come at the cost of slower extraction kinetics. The collision between the hexane and the lipid droplets is key to the lipid transfer process in a biphasic system.
5.4 Conclusion

In this study, the influence of temperature, mixing rate, and solids concentration on biphasic lipid extraction kinetics was studied independently of the slurry-solvent interfacial area. The efficiency of biphasic lipid extraction was mainly attributed to the formation of an emulsion that increases the interfacial area. This interfacial area is required to enhance the collision frequency between the lipid droplets and the hexane, which is required for lipid transfer into the solvent. Similarly, a well-mixed system is also required to intensify hexane-lipid collisions for an efficient lipid extraction kinetics. Therefore, emulsion formation is likely inevitable in a biphasic lipid extraction.

**Figure 5.8**: The proposed rate-determining factors in biphasic extraction from ruptured algae. Note that the figure was not drawn to scale.
5.5 References


Chapter 6

6 Utilisation of emulsions and lipase to enhance biphasic lipid extraction yields

In the previous chapter, it was found that emulsion formation is inevitable in a biphasic solvent extraction as it is required for efficient lipid transfer. As the nonpolar solvent (e.g. hexane) in a typical biphasic extraction preferentially extracts the nonpolar neutral lipids over the polar lipids, much of the saponifiable polar lipid will not get extracted when hexane is used as an extraction solvent. In a typical biofuel pathway, this practice is acceptable as the presence of phospholipids in the polar lipids had been shown to reduce the biodiesel conversion yield and cause complications downstream. In this chapter, a free (e.g. non-immobilised) lipase biocatalyst was explored as a way to enhance the recovery of the acyl chains in the polar lipids. In this method, the saponifiable lipids (including both NL and polar lipids) were simultaneously extracted and converted into FAME in a single step, thus eliminating an extra transesterification step in the downstream. The acyl chains of both the NL and the polar lipids were recovered as FAME in the hexane phase. Similar to a conventional hexane extraction, minimal amounts of polar lipid were found in the hexane phase. A higher biodiesel yield and an enhanced EPA recovery were made possible by transesterifying the polar lipids during the extraction process. The practical aspects of this method in a biodiesel process were considered and possible improvements recommended.

This chapter is based on the paper submitted for journal publication “Recovery of microalgal saponifiable polar lipids using a nonpolar solvent via lipase-assisted extraction” by Law, S. Q., Halim, R., Scales, P. J., & Martin, G. J. (2018).
6.1 Introduction

Sustainable production of microalgal biofuel is seen by many as a long-term goal. Microalgae contain triacylglyceride (TAG) and other saponifiable lipids (SL) (Olmstead et al., 2013a) that can be converted into fatty acid methyl esters (FAME) to be used as a fuel in diesel engines (Mata et al., 2010). For biodiesel production, the selective extraction of TAG using nonpolar solvents is generally preferred (Tao Dong et al., 2016b; Foley et al., 2011; Olmstead et al., 2013b), because the phospholipids must be removed to avoid detrimentally affecting the biodiesel quality (Atadashi et al., 2010). However, the unrecovered polar lipids (both glycolipids and phospholipids) are an untapped biodiesel feedstock, especially for microalgae with a high polar lipid content (Iyer, 2016).

One potential strategy to enhance the recovery of polar lipids is to hydrolyse the lipids into free fatty acids that can partition into a nonpolar solvent (T. Dong et al., 2016a). However, acid hydrolysis requires a high temperature (155°C) and pressure, and degrades the protein in the biomass. An alternative to acid hydrolysis is to convert the lipids into FAME (i.e. biodiesel) prior to the solvent extraction step. Conversion of SL into FAME is generally performed separately in a transesterification reaction involving an acyl acceptor (methanol) and a catalyst, typically an alkali. In an effort to improve process efficiency, the two unit operations of lipid extraction and transesterification can be combined into a single step, also known as in-situ transesterification (Pragya et al., 2013). In-situ transesterification was originally performed using acid or alkaline catalysts. However, a high reaction temperature is required (Carrero et al., 2015; Im et al., 2014; Park et al., 2015), which is not energetically favorable for biofuel applications and is detrimental to the sensitive co-products (Vanthoor-Koopmans et al., 2013) such as protein and omega-3 fatty acids that are present during in situ transesterification.

More recently, lipase has been proposed as a promising biocatalyst for transesterification of algal oils (Park et al., 2015; Tan et al., 2010; Tran et al., 2013). A high FAME conversion can be achieved in the presence of water (Guan et al., 2010) under mild reaction conditions (typically 40°C) (Tran et al., 2013). To reduce costs, the lipase is often immobilised to allow reuse of the enzyme (Tan et al., 2010). However, immobilised lipases have been prone to inactivation when used in wet algal slurries (Tan et al., 2010; Tran et al., 2013; Tran et al., 2012; Wang et al., 2017), likely due to the inhibition by the polar lipids in the microalgae (Amoah et al., 2016; Navarro López et al., 2015). As lipase immobilisation adds a significant cost (Zhao et al., 2015), the instability of the immobilised lipase is unacceptable. In addition, large quantities of polar solvents (tert-butanol), which are difficult to recover, were used in these studies (Navarro López et al., 2016; Wang et al., 2017).

In this work, the use of a free (i.e. soluble) lipase was evaluated for the first time as a potential biocatalyst in a biphasic wet algal in-situ transesterification. A fungal lipase, Rhizomucor miehei lipase (RML), was chosen as it is a well-studied lipase (Rodrigues & Fernandez-Lafuente, 2010) that has been demonstrated to be an effective biocatalyst for conventional lipase-catalysed transesterification.
reactions in high water system (up to 20 % water) (Al-Zuhair et al., 2007; Rodrigues & Fernandez-Lafuente, 2010; Soumanou & Bornscheuer, 2003). Compared to an immobilised lipase, soluble lipase can tolerate a higher level of phospholipids (Li et al., 2014) that are present in a wet algal slurry. In this chapter, a modified protocol for in-situ transesterification is proposed, based on a previously developed wet hexane extraction process (Olmstead et al., 2013b). The process is based on scalable techniques and requires a low amount of solvent as well as low temperatures (40°C) and pressures. However, as hexane is nonpolar, it was not able to recover the polar lipids that include most of the valuable omega-3 fatty acids such as EPA (Olmstead et al., 2013b). The possibility of recovering both TAG and polar SL in the nonpolar solvent (hexane) is investigated in this chapter. To assess whether polar lipids as well as TAG could be converted, experiments were performed on N. salina grown under low and high nitrate conditions that had very different polar lipid-to-TAG ratio. This method provides a new route to the recovery of EPA located on polar saponifiable lipids while avoiding the need for drying the biomass.

6.2 Materials and Methods

6.2.1 Microalgae cultivation

A previously maintained N. salina (Halim et al., 2016) was inoculated into multiple 15 L-carboys and grown indoors at 20 °C with a light:dark cycle of 14:10 hours. Each bioreactor was aerated with an aquarium air pump (Stellar 380D, Aqua One, China) at a total flow rate of 190 L/h, which was split into 4 air stones. A modified f medium (Olmstead et al., 2013a) and f/2 medium with half the nitrate concentration were used for high-nitrogen (N-replete) and low-nitrogen (N-deplete) cultures respectively. At the end of a 14-day growth cycle, both N-replete and N-deplete cultures were harvested and concentrated into pastes using a disc stack centrifuge (Separator OTC 2-02-137, GEA Westfalia, Italy). The resulting pastes were 28-32% w/w solids, as established by oven drying at 60 °C (Olmstead et al., 2013b). The pastes were stored frozen at -20 °C and were used within 2 months of harvesting.

6.2.2 Determination of the lipid composition in the biomass

In this study, six batches of algal paste (three N-replete and three N-deplete as described in section 6.2.1) were used to perform the experiments. Thus, the lipid content of each batch was determined separately for normalisation in the subsequent comparison study. The total lipid content of the N-replete and N-deplete biomass was quantified gravimetrically using a modified Bligh & Dyer extraction protocol as previously described (Olmstead et al., 2013a). The extracted lipids were dried and stored at -20 °C until analysis. The TAG in the lipid extracts was measured using an HPLC-ELSD as described in section 6.2.7. The SL in the samples was first derivatised to FAME by chemical transesterification (section 6.2.5) before GC-FID analysis (section 6.2.6).
6.2.3 Cell rupture

Prior to enzymatic transesterification, the cells were ruptured. The harvested biomass (ca 100 g) was first diluted to a slurry of about 17-21% w/w with 3% artificial sea water. The slurry was then incubated at 38 °C for 24 hours to weaken the cell walls prior to cell disruption (Halim et al., 2016). To rupture the cells, the incubated slurry was passed once through a high-pressure homogeniser (Panda 2K NS1001L, GEA Niro Soavi, Italy) at 1400 ± 150 bar. Due to some variation in the homogenisation pressure and cell properties, some minor variation in cell rupture can be expected between the batches. Therefore, the extent of cell rupture in each batch was also determined separately by counting the intact whole cells post-homogenisation (Yap et al., 2015).

6.2.4 Enzymatic in-situ transesterification

Preliminary experiments (table A1 and Figure A6, A7, A8 and A9) were performed to select a suitable lipase and suitable reaction parameters (e.g. lipase dosage and methanol). The final enzymatic in-situ transesterification reactions were performed by mixing 3.0 g of ruptured slurry (at 17-21% w/w solids) and 0.99 g of hexane (95% AR, Ajax Finechem, Victoria, Australia) with varying amounts (0.015-0.690 g) of methanol (99.8% AR, Chem-Supply, SA, Australia) and either 0.018 g or 0.006 g of RML lipase (20,000 U/g, Novozyme, Denmark). Control samples were run alongside the reactions. These also contained 3.0 g of ruptured slurry and 0.99 g of hexane, but without any RML lipase or methanol. The reaction mixtures were rotated at 8 rpm (Labquake, Barnstead International, Iowa, USA) while being incubated at 40 °C (Unimax 1010 & Incubator 1000, Heidolph Instruments, Germany). Parallel reactions were stopped sequentially at 4 h, 8 h, 16 h, 24 h or 48 h at which point the slurry mixtures were cooled to room temperature in a water bath and centrifuged at 3,273 x g for 25 min (Allegra X-12, swing bucket rotor SX4750, Beckman Coulter, Victoria, Australia). The hexane fractions were recovered carefully and immediately dried under a stream of nitrogen and stored at -20 °C until subsequent analysis.

To emulate a 2-stage hexane extraction process used to improve the lipid yield (Halim et al., 2016), another 0.99 g hexane was added to resuspend the pelleted slurries. For the kinetic experiments, the mixtures (with hexane removed) were first heated at 85 °C for 30 min to deactivate the lipase prior to the second hexane extraction. The slurries containing fresh hexane were mixed for 2 hours at room temperature (ca 20 °C) before another centrifugation. After the second hexane fraction was collected, the leftover biomass was subjected to a 4-stage chloroform/methanol-based total lipid extraction protocol (Olmstead et al., 2013b) to extract the residual lipids. To verify the extraction efficiency, a C15:0 FAME standard (>99%, Nu-Chek Prep, USA) was dissolved in hexane and added to selected samples at the beginning of the in-situ transesterification reaction.

To analyse the lipase conversion efficiency, the dried lipids from the two hexane extractions and the residual total lipid were pooled together and analysed by GC-FID (as described in Section 6.2.6). To
perform a mass balance analysis and to check the lipid distribution during the extraction process, each extract was analysed separately using GC-FID.

6.2.5 Two-step chemical transesterification

Chemical transesterification of the lipid samples was performed by first dissolving the dried lipids in a 1:2 v/v chloroform:methanol mixture (HPLC grade, Ajax Finechem, Victoria, Australia). Excess acid catalyst (0.15 mL of 10 w/w% sulphuric acid in methanol) was added to 1 mL of dissolved lipid and agitated on an orbital shaker (300 rpm) at 55 °C for 3 hours. After the acid-transesterification, excess potassium methoxide (0.5 mL) was added to neutralise the acid and to complete the reaction. The alkaline reaction was performed at 55 °C for 2 hours. C17:0 TAG internal standard (>99% glyceryl triheptadecanoate, Sigma, USA) was added to the lipid samples prior to chemical transesterification to measure the extent of FAME conversion. In preliminary experiments (results not shown), it was confirmed that this chemical transesterification method was able to convert all the available SLs (including glyco- and phospholipids) to FAME.

6.2.6 FAME determination by GC-FID

To determine the FAME conversion by lipase, the two dried hexane fractions and the residual lipid extract from section 6.2.4 were combined and dissolved in a 1:2 v/v chloroform/methanol solution for GC-FID analysis. The measured FAME was used as the numerator in equation 1, and the denominator is the total SLs available in the biomass as determined in section 6.2.2. Overall, the FAME conversion percentage by lipase is defined by Equation 6.1:

\[
\text{Conversion} \% = \frac{\text{FAME converted by RML(mg)}}{\text{Total SLs in the biomass as FAME(mg)}} \times 100\%
\]

All the samples were filtered via 0.45 µm N66 syringe filters prior to analysis. To determine the FAME concentration in the transesterified samples, gas chromatography (GC 7890B, Agilent Technologies, USA) with flame ionisation detector (GC-FID) was used with a DB-23 column of 30 m x 0.320 mm (Agilent Technologies, USA). The front inlet injector was set to 250 °C with a split ratio of 200:1 and an injection volume of 6 μL. The GC was programmed to start and hold at 50 °C for 1 minute, then ramp up to 180°C at 20 °C/min and end with a final ramp to 210 °C at 2 °C/min.

6.2.7 Lipid class analysis by HPLC-ELSD

To analyse the lipids by their lipid class, the samples were analysed directly without any derivatisation using a normal-phase column (Chromolith Performance Si 100-4.6mm, Merck Milipore, USA) on a HPLC system (Agilent 1290, Agilent Technologies, USA) with an evaporative light scattering detector (ELSD). The mobile phases (all HPLC grade, Chem-Supply, SA, Australia) and gradient programme were adapted from Graeve and Janssen (2009) with slight modification to account for the differences in the hardware configuration. To prevent possible degradation of the PEEK coating on the Chromolith column (Graeve & Janssen, 2009), no chlorinated solvents were used. Instead, the lipid samples were
dissolved in toluene/methanol 2:1 v/v (dry lipid concentration range of 15-30 mg/mL) and analysed within the day with an injection volume of 2 μL.

For TAG determination, an 8-point-to-point standard calibration curve was generated using C16:0 TAG (2 - 35 mg/mL) as the ELSD response is non-linear (Jones et al., 2012; Kobayashi et al., 2013; McLaren et al., 2011). As it was not possible to get individual peaks for the FFA and polar lipids, these lipid classes are only compared qualitatively in this study.

6.2.8 Experimental reproducibility and analysis

All experiments were performed in duplicate. Each lipid sample was analysed once by HPLC-ELSD and GC-FID. The variation of these measurements was verified to be less than 2% (typically <1%) in repeated reinjections of the same lipid samples. Thus, data represent the average and standard deviation of single measurements of samples from duplicated experiments.

6.3 Results and Discussion

6.3.1 Principles behind the biphasic in-situ transesterification process

Hexane was chosen as it is a non-polar organic solvent that is highly immiscible with water enabling physical recovery of the solvent after lipid extraction (Olmstead et al., 2013b). When mixed with an aqueous algal slurry (e.g. 80% moisture content) in the presence of surface-active components, an emulsion of hexane-in-water will be formed (Law et al., 2017). As microalgal cell walls are impermeable to non-polar hexane, which is present as large emulsion droplets, the cells have to be ruptured to achieve a meaningful level of lipid extraction (Yap et al., 2014). Moreover, neutral lipids (including TAG) are preferentially dissolved into the hexane phase as compared to the glycolipids and phospholipids (Olmstead et al., 2013b).

RML lipase is a water-soluble enzyme and is located in the aqueous phase during the reaction (Reis et al., 2009). For transesterification to occur, an acyl acceptor (i.e. methanol) must be added. Based on the water-methanol-hexane ternary phase diagram (Liu et al., 2002), >99 mole % of the methanol will partition into the aqueous phase. The reaction is therefore biphasic, with hexane and dissolved algal lipid in an organic phase and the lipase, methanol and algal slurry (intact cells, cell debris, growth media, and intracellular components) in the aqueous phase. In its inactive form, RML has a single-helical lid structure that covers the catalytic site. Upon adsorption at a solvent-water interface, the lipase is activated through the displacement of the lid structure, allowing substrate binding (Brzozowski et al., 1991; Derewenda et al., 1992). Previously it was demonstrated that lipase-based direct-transesterification did not work well in a solvent-free system (Tran et al., 2013), which strongly suggests that RML requires a solvent-water interface to catalyse the transesterification reaction.
Figure 6.1: Proposed working model of in-situ transesterification of algal lipids using free RML lipase. The progression of the reaction and the locations of the different components are illustrated. Step 1: the lipids from the ruptured cells are extracted into the hexane droplets. Step 2: as the lipase comes in contact with a hexane-droplet it is activated at the interface and the substrates (TAG and methanol) are brought together at the active site. Step 3: the transesterification reaction proceeds to produce FAME that is released into the hexane phase and glycerol that is released into the aqueous phase. Centrifugation can then be used to recover the FAME-rich hexane. Note that the reaction components are not drawn to scale.

Based on this hypothesis, a working model of a free-lipase in-situ transesterification can be proposed (Figure 6.1). First, when hexane is added into the slurry of ruptured cells an emulsion is formed. The non-polar algal lipids, including TAG, dissolve into the hexane droplets due to their high solubility in hexane compared to water. As the lipase comes in contact with the water-hexane interface it is activated, allowing the substrates (TAG and methanol) to come together to form an enzyme-substrate complex. With excess methanol in the aqueous phase the reaction is directed towards transesterification instead of hydrolysis (Adlercreutz, 2013), forming FAMEs and glycerol that partition into the hexane and aqueous phases respectively. Following the transesterification reaction, the emulsion mixture can be centrifuged to separate the FAME-rich hexane from the aqueous slurry.

6.3.2 Kinetics of lipase transesterification

Enzymes are typically costly, and as lipase is soluble it cannot be reused. It is therefore desirable to rapidly reach a high yield of FAME using only a minimal amount of lipase. In theory, the amount of
catalyst should only affect the kinetics and length of the reaction, and not the final equilibrium yield, which is instead determined by the amounts of water and methanol (Adlercreutz, 2013). The amount of water depends on the solids concentration of the biomass (ca 80% water in a 20% solids biomass), while the required amount of methanol is typically determined experimentally. A suitable methanol dosage range was identified in preliminary experiments (Figure A8). The effect of methanol dosage was systematically investigated later.

To determine the impact of lipase loading on the reaction kinetics, two different lipase loadings in a similar range to previous studies (Navarro López et al., 2016; Tran et al., 2013) were compared for an N-deplete biomass. As expected, a higher lipase loading resulted in a higher reaction rate (Figure 6.2A). Also, in accordance with the ca 3-fold increase in enzyme concentration, the reaction was approximately 3-times faster for the higher loading. For instance, the yield after 8 hours (72.6%) with 2500 U.gSL⁻¹ (enzyme unit per gram of total SL) of lipase was similar to that obtained after 24 hours (72.3%) with 850 U.gSL⁻¹.

An apparent reaction equilibrium was attained after 24 h with a lipase loading of 2500 U.gSL⁻¹, reaching a final FAME conversion yield of 85.5%. Although the FAME yield was less than 100%, this yield was determined on the basis of all the SL in the biomass, which would include the total lipids remaining in the unruptured cells. A cell count performed on the biomass indicated that approximately 74% of the cells were visibly disrupted. The conversion yield (85.5%) was actually greater, suggesting the method could access lipids in apparently whole cells. This anomaly is likely due to the presence of partially ruptured cells (Yap et al., 2014), which may be counted as whole cells, as well as the methanol acting as a monophasic extraction system to some extent (Ryckebosch et al., 2014). It was confirmed in a separate experiment that some lipids could be extracted from unruptured cells by methanol:hexane mixtures at the ratio used in this method. However, the quantity of lipid extracted was minor, with less than 10% of the saponifiable lipid in the unruptured cells extracted after 24 hours of mixing. Therefore, the majority of the converted FAME originated from the lipids released by high-pressure homogenisation.

Although the reaction with 850 U.gSL⁻¹ of lipase did not reach equilibrium within 48 h, the final equilibrium FAME yield was not very different to that obtained with 2500 U.gSL⁻¹ of lipase (Figure 6.2A). Through fitting of the experimental data, the in-situ lipase transesterification reactions in this study appeared to follow pseudo-second-order kinetics. The high initial reaction rate indicates that the initial dissolution of lipid into hexane occurred rapidly relative to enzymatic conversion into FAME. This is consistent with hexane-based extraction processes on ruptured wet biomass, in which most of the accessible neutral lipids partitioned into the hexane within 0.5-2 h (Jiménez Callejón et al., 2014; Olmstead et al., 2013b).
Figure 6.2: In-situ transesterification reaction kinetics as a function of (A) lipase loading, (B) solids concentration, and (C) biomass/lipid composition (ND = nitrogen depleted biomass containing 25.4% saponifiable lipid and 74% ruptured cells; NR = nitrogen replete biomass containing 11.7% saponifiable lipid and 67% ruptured cells). FAME conversion is presented on the basis of the transesterified FAME relative to the total saponifiable lipids in the original biomass. Reactions were performed with 0.99 g hexane, 0.345 g methanol, and 3 g of homogenised biomass with a solids concentration and enzyme loading (U.g\textsubscript{SL}^{-1}) as specified in the legends. The data represented by squares in (B) and diamonds in (C) are replotted from (A). It is worth noting that the amount of lipase used in all experiments was constant (either 0.018 g lipase as the standard dosage or 0.006 g for the low lipase dosage), however, the final enzyme loading varied due to difference in the lipid content of the biomass.

To obtain a favourable energy balance for microalgal biofuel production, it is desirable to operate with as high a solids concentration as possible within the limits of physical dewatering, typically in the range of 15-25\% solids (Yap et al., 2015). To investigate the effect of solids concentration on the transesterification reaction, the kinetics were compared between biomass at 14.0\% (obtained via post-rupture dilution) and 18.3\% w/w solids, with the concentrations of lipase (0.006 g lipase in 3 g wet
biomass) and methanol, and the hexane ratio held constant (Figure 6.2B). The results indicate that the difference in solids concentration did not influence the progression of the reaction. Higher solids concentrations (>21% w/w) were not tested in this study. This is because at close to 25% w/w solids concentration, the algal slurries were highly viscous and difficult to pass through the high-pressure homogeniser without a pressurised hopper (Navarro López et al., 2016; Yap et al., 2015). In addition, the high viscosity hinder mixing of the algal-lipase mixture during the reaction.

6.3.3 Effect of algal lipid composition on transesterification

The lipid content in microalgae can be influenced greatly by the growth conditions (Sharma et al., 2012). In particular, if algae such as N. salina are deprived of a nitrogen source for extended periods, they accumulate TAG, which is the preferred feedstock for conventional transesterification (Tao Dong et al., 2016b). One possible advantage of lipase transesterification is the ability to also convert polar SLs (e.g. phospho- and glycolipids) to FAME. In addition to increasing the overall FAME yield (compared to a separate lipid extraction and transesterification approach), this leaves the undesired polar moieties (e.g. phosphorus-containing phosphate groups) (Atadashi et al., 2010) in the aqueous phase, which would otherwise have to be removed (Chen et al., 2012). This has the potential to enable nitrogen replete biomass (containing a high polar lipids:TAG ratio) to be used, which can be grown at higher overall productivities than nitrogen starved cultures (Meng et al., 2015).

A comparison of N-deplete and N-replete biomass was performed to investigate the effect of lipid composition on the in-situ transesterification reaction and to determine if FAME could be produced from the polar lipids. Table 6.1 shows the lipid profiles of the N. salina in this study when cultivated under different nitrogen conditions. Comparing the N-replete and N-deplete cultures, the amount of non-saponifiable lipids, and non-TAG SLs were reasonably constant. The total lipid per gram of dry biomass was increased by 47% as a result of the increased TAG content, which was four times higher than in the N-replete biomass. Overall there was a two-fold difference in the total SLs. The proportion of C20:5 (n-3) fatty acid (EPA) in the N-deplete SLs was half that of the N-replete, however, the net quantity of EPA per gram biomass was similar (0.036 g/g dry biomass under N-replete versus 0.037 g/g dry biomass under N-deplete), as the EPA does not accumulate significantly in the TAG (Martin et al., 2014; Meng et al., 2015). This suggests that forom EPA production perspective, an N-replete growth condition is preferred due to the higher biomass productivity.
Table 6.1: Summary of lipid profile of *N. salina* when subjected to N-deplete and N-replete growth conditions.

<table>
<thead>
<tr>
<th></th>
<th>N-replete</th>
<th>N-deplete</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Lipid (g/g dry biomass)</td>
<td>0.283 ± 0.010 g/g</td>
<td>0.416 ± 0.008 g/g</td>
</tr>
<tr>
<td>Non-saponifiable lipid</td>
<td>0.166 ±0.02</td>
<td>0.162 ±0.02</td>
</tr>
<tr>
<td>Saponifiable lipid (g/g dry biomass)</td>
<td>0.117 ± 0.008 g/g</td>
<td>0.254 ±0.005 g/g</td>
</tr>
<tr>
<td>TAG (g/g dry biomass)</td>
<td>0.043 ± 0.002 g/g</td>
<td>0.174 ± 0.002 g/g</td>
</tr>
<tr>
<td>Non-TAG SL (g/g dry biomass)</td>
<td>0.074 ±0.01 g/g</td>
<td>0.080 ± 0.01 g/g</td>
</tr>
</tbody>
</table>

\[^1\]As determined gravimetrically by 4 stages of Bligh & Dyer total lipid extraction  
\[^2\]By subtraction of saponifiable lipid (SL) from the total lipid  
\[^3\]As determined by GC-FID (section 6.2.9 and 6.2.10)  
\[^4\]As determined by HPLC-ELSD (section 6.2.11)  
\[^5\]Determined by subtraction of TAG from SL.

Despite the different lipid composition of the N-replete and N-deplete biomass (low TAG versus high TAG), lipase:SLs ratio (2500U.gSL\(^{-1}\) versus 4300U.gSL\(^{-1}\)), and solids concentration, both reactions followed similar kinetics (Figure 6.2C). This suggests that the lipase was likely not affected by the higher polar lipid content. While both reactions reached equilibrium at a similar time (i.e. within 24 h), the final FAME yield was only 70% for the N-replete biomass compared to 85% for the N-deplete biomass. The lower final FAME yield for the N-replete biomass is likely due to the difference in the extent of cell rupture. According to the cell counts, approximately 67% of the N-replete cells were ruptured, compared to 74% of the cells in N-deplete cultures. This is supported by a subsequent experiment (Figure 6.6C) in which a higher FAME yield (89% conversion at 24h) was possible in N-replete biomass with more extensive cell rupture (79%).

Nonetheless, 70% FAME conversion is still greater than the 37% percent of TAG in the SLs (Table 6.1), indicating that the polar lipids were also converted to FAME. To confirm this, the composition of total lipids (including the lipids in the unruptured cells) before and after in-situ lipase transesterification were compared by HPLC (Figure 6.3A). The results clearly indicate the near disappearance of both TAG and polar SLs (using non-saponifiable sterol as a basis of comparison) and the emergence of FAME.
Figure 6.3: Comparison of HPLC chromatograms of (A) the total lipid classes of the N-replete biomass from before (red) and after (blue) in-situ transesterification. The post-lipase transesterification data correspond to the 48 h time point of the NR curve presented in Figure 3C. The sterol peak, which was not affected by the transesterification reaction, was labelled to serve as a comparison basis. Chromatogram (B) shows the lipid classes extracted by hexane (red) and the residual lipids (blue) extracted by subsequent chloroform-methanol extractions on ruptured N-replete biomass. No lipase or methanol were added. The detector response is different for the different lipid classes meaning that the peak areas are not proportional to the conversion. The inset figures are the same data presented on a different scale.
While the polar lipids do not dissolve in hexane, as surface-active molecules they can be present at the hexane-water interface (Tao Dong et al., 2016b). Their near complete conversion could be facilitated by the fact that the lipase is also situated at the interface. There is another possible reason for the high degree of polar SL conversion despite their low solubility in hexane. Based on the HPLC analysis of the control samples without lipase and methanol addition (Figure 6.3B), the extended reaction time (>24h at 38 °C) resulted in a considerable amount of free fatty acids being released from polar SL. As FFAs are highly soluble in hexane, this provides an alternative pathway for lipase conversion of polar SL (Figure 6.4).

**Figure 6.4:** Schematic representation of proposed mechanisms for lipase transesterification of polar SLs. Pathway 1: Polar SLs are transesterified at the water-hexane interface. Pathway 2: Polar SLs are first hydrolysed into FFA that partition into hexane droplets before being converted into FAME.
6.3.4 FAME recovery after in-situ transesterification

To investigate the overall SL recovery after in-situ transesterification, complete SL mass balances were performed over the process on both N-deplete and N-replete biomass (Figure 6.5). The extracted lipids from all extraction stages (hexane extractions and the residual lipid extraction) were analysed separately. To determine the level of unreacted SL, the FAME generated from the lipase in-situ transesterification reactions were analysed directly (numbers 1 and 3 in Figure 6.5) before being subjected to additional chemical transesterifications for further analysis (numbers 2 and 4). To determine the residual lipids in the biomass, the leftover biomass was subjected to a chloroform/methanol-based extractions that can indiscriminately extract all of the lipids (numbers 5 and 6) (Bligh & Dyer, 1959). Finally, control experiments without methanol and lipase were conducted in parallel, in which similar solvent extractions (sequential hexane and chloroform-methanol extractions) were also performed.

During the in-situ transesterification (Figure 6.5), C15:0 FAME standard was also added to the reaction mixture to track the FAME recovery yield. It was found that between 82-87% of the FAME standard was recovered in the hexane fractions (13-18% remaining unextracted) across all the experiments including the control. This is lower than expected as >96% w/w FAME was found to partition in the hexane phase with the solvent ratio used in this study (data not shown). This result can be explained by the FAME-containing hexane emulsion that is retained in the biomass after centrifugation, as has been demonstrated in chapter 3 and in Figure A10. To obtain a greater recovery, either more hexane or a demulsification strategy to break the hexane-biomass emulsion will be required.

**Figure 6.5:** Flow diagram of the saponifiable lipid mass balance performed around the lipase-catalysed in-situ transesterification and solvent recovery process. The numbers in circles correspond to the data presented in Figure 6.6.
In the N-deplete experiments (Figure 6.6A and Figure 6.6B), there was no significant difference in the hexane-recoverable FAME/SL (ca 70% recovery) between the in-situ transesterification and the control (without lipase or methanol addition). For the N-replete biomass (Figure 6.6C and Figure 6.6D) only a slightly higher percentage of FAME/SL was recovered in the in-situ transesterification biomass than in the control (80% and 73% respectively). This difference was unexpectedly small as the initial N-replete biomass should contain a high proportion of polar lipids (Table 6.1) that are not usually recovered in biphasic hexane extractions (Halim et al., 2016; Olmstead et al., 2013b). This result can be explained by the release of the acyl chains from some of the polar lipids through lipid hydrolysis during the prolonged hexane extractions (24 h at 40 °C). The hexane extracts from both the lipase in-situ transesterification and control samples were analysed by HPLC. A near-absence of polar lipids was confirmed (Figure 6.3B), indicating that the hexane did not extract the unconverted polar lipids, as expected. In addition, the mass of TAG per dry biomass in the control samples remained relatively unchanged before and after the prolonged hexane extraction (Figure 6.3B). Thus, the increase in recoverable SL as FFA in the control experiments likely came from the polar SL which were previously not recoverable by hexane. This supports the possible second pathway for lipase transesterification of polar lipids via FFA generation (Figure 6.4).

By comparing the proportion of FAME within the hexane fractions and the unextracted SLs (Figure 6.6A and Figure 6.6C), a higher percentage of FAME conversion was found in the hexane fractions (87.1% for ND and 92.5% for NR biomass) than in the unextracted residual SL (70.2% for ND and 74.5% for NR biomass). This observation again supports the suggestion that some of the SL remained inaccessible to the lipase and therefore unconverted. This SL was presumably located in completely unruptured cells and were not extractable by hexane.

To determine if the RML lipase exhibits any substrate preference (e.g. different fatty acid carbon chain length and saturation degree) during the transesterification process, the fatty acid profiles of the FAME from different transesterification methods (chemical versus lipase in-situ transesterification) were compared (Figure 6.7). The crude FAME from the lipase in-situ transesterification were also subjected to an additional chemical transesterification step to convert the remaining unconverted SL (Figure 6.5). As expected, the final quantity and proportion of each fatty acid (from combining the FAME of both the hexane fractions and the residual lipids) after both lipase in-situ transesterification and chemical transesterification were similar to the fatty acid profile of the total FAME extracted by Bligh and Dyer protocol, where the total lipids were extracted first and then chemically transesterified. In general, the fatty acids composition of the lipase-converted lipids correlates closely to the fatty acids converted by chemical transesterification (extracted by Bligh and Dyer protocol). The slight differences of the FAME quantity observed between the chemical transesterification and the lipase in-situ transesterification lipids were due to the incomplete conversion due to inaccessible lipids in intact cells and high water
This suggests that the lipase in-situ transesterification process is likely non-discriminate and is not affected by the type of the SL.

**Figure 6.6:** The mass balance, on a percentage basis, of total saponifiable lipids for: (A) in-situ transesterification of N-deplete biomass; (B) hexane-only extraction of N-deplete biomass as a control; (C) in-situ transesterification of N-deplete biomass; and (D) hexane-only extraction of N-deplete biomass as a control. The numbers in the brackets refer to the source of the lipid samples as indicated in Figure 6. All reactions were performed with 3 g ruptured biomass (18.0% solids concentration for N-deplete and 18.6% for N-replete), 0.99 g hexane, 0.345 g methanol, and 0.018 g lipase for 24 h at 38 °C.
Figure 6.7: The fatty acid profiles of the FAME from lipase in-situ transesterification experiments from Figure 6.6 for both the (A) N-replete and (B) N-deplete biomasses were compared against the FAME obtained by 4-stage Bligh and Dyer extraction followed by chemical transesterification. The amount of each fatty acid was normalised on the basis of 1 gram dry biomass for direct comparison. The lipase-converted FAME were the FAME converted by RML lipase during in-situ transesterification (combined samples of number 1, 3 and 5 in Figure 6.5). The lipase and chemical transesterified FAME were obtained by chemically transesterifying the crude FAME that was in-situ transesterified (combined samples of number 2, 4 and 6 in Figure 6.5).

6.3.5 Effect of methanol dosage on in-situ transesterification

Transesterification reactions require an excess amount of methanol to favour FAME formation over hydrolysis (Adlercreutz, 2013). However, a high methanol concentration can risk deactivating the lipase. To determine the effect of methanol dose rate on the FAME yield, an additional series of experiments was performed at different methanol:TAG equivalent mole ratios (Figure 6.7). To allow comparisons to existing literature, the data are presented on a SL basis as TAG-equivalents (instead of the total lipids which would include non-saponifiable lipids), by equating to 3 moles of saponifiable acyl chains that could be present on TAG or other SL. In this study (Figure 6.7), the FAME conversion continued to increase when the methanol:TAG equivalent mole ratio was increased from 6:1 to 120:1, with no significant inhibition effect up to 145:1. This ratio is much higher than the typically reported inhibitory ratio of 9:1 for RML lipase (Guan et al., 2010). This is presumably due to the dilution of methanol by the water content in the system. This is also in agreement with a previous study using RML in the presence of water (Huang et al., 2012), where the inhibition effect was only observed at methanol concentrations above 30 v/v % (corresponding to >120:1 mole ratio in this study).
Figure 6.8: Total FAME conversion as a function of methanol:TAG-equivalent ratio. Each mole of TAG-equivalent equates to 3 moles of saponifiable acyl chains. Experiments were performed on 3g of N-replete and N-deplete biomass of 17.1% (67% cell rupture) and 17.3% (69% cell rupture) solids respectively, with 0.018 g lipase for 24 h of in-situ transesterification reaction.

The optimal methanol dosage (defined as the minimum methanol:water ratio needed for maximal conversion) found in the current study (72:1) was significantly lower than reported previous in-situ transesterification studies on wet algal slurries (Table 6.2). The higher methanol dosages required in some previous studies could be have been due to the methanol concentration effectively being diluted in the reaction by the water-miscible solvent that was used (tert-butanol). Similarly, the use of a more dilute biomass slurry (ca 9-14%) (Tran et al., 2013) meant that a higher methanol:TAG-equivalent ratio was required.
Table 6.2: Comparison of in-situ transesterification conversion of wet biomass using enzymatic approach. As the results were reported on either a SL- and on a TL- basis (the proportion of SL:TL was not reported), the comparison was done on a SL-basis and is noted otherwise where applicable.

<table>
<thead>
<tr>
<th>Feedstock</th>
<th>(Navarro López et al., 2016)</th>
<th>(Tran et al., 2013)</th>
<th>(Wang et al., 2017)</th>
<th>Current work</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td>Nannochloropsis gradihana</td>
<td>Chlorella ESP-31</td>
<td>Nannochloropsis IMET-1</td>
<td>Nannochloropsis salina</td>
</tr>
<tr>
<td>Starting biomass</td>
<td>25% w/w</td>
<td>9-14% w/w</td>
<td>39.4% w/w</td>
<td>18-20.9% w/w</td>
</tr>
<tr>
<td>Solids content</td>
<td>12.1% SL</td>
<td>29.82% SL¹</td>
<td>ND-25.4% SL</td>
<td></td>
</tr>
<tr>
<td>SL(%SL/dry biomass)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzyme type</td>
<td>Novozyme 435 (immobilised)</td>
<td>Burkholderia lipase (immobilised)</td>
<td>Novozyme 435 (immobilised)</td>
<td>R. miehei Lipase (soluble)</td>
</tr>
<tr>
<td>Loading²</td>
<td>2875 U g⁻¹ SL, or 1150 U g⁻¹ TL</td>
<td>6014 U g⁻¹ SL</td>
<td>550 U g⁻¹ TL³</td>
<td>2500 U g⁻¹ SL, or 1500 U g⁻¹ TL</td>
</tr>
<tr>
<td>Conversion</td>
<td>99.5%</td>
<td>90-94%</td>
<td>81.5%</td>
<td>90%</td>
</tr>
<tr>
<td>Reaction time</td>
<td>56h</td>
<td>48h</td>
<td>16h</td>
<td>24h</td>
</tr>
<tr>
<td>Reaction conditions</td>
<td>HPH</td>
<td>Resuspension in methanol, sonication, and drying</td>
<td>None</td>
<td>Incubation at 38°C and HPH</td>
</tr>
<tr>
<td>Co-solvent</td>
<td>Tert-butanol</td>
<td>Hexane</td>
<td>Tert-butanol</td>
<td>Hexane</td>
</tr>
<tr>
<td>MeOH:TAG molar ratio (SL or TL basis)²</td>
<td>230:1 (SL)</td>
<td>128.9:1(SL)</td>
<td>120:1 (TL)³</td>
<td>72:1(SL) 44:1 (TL)</td>
</tr>
</tbody>
</table>

Notes:
1. The result for 29.82% SL biomass (out of the 14-60% SL assessed in the study) was selected for comparison
2. Unit conversion was made assuming methanol = 0.792 g/mL, algal saponifiable TAG = 800 g/mol, methanol = 32.04 g/mol, Novozyme 435 = 5000 U/g (Wang et al., 2017)
3. The fraction of SL in the TL was not reported and thus the value was compared on a TL basis

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6.3.6 Practical considerations

As methanol is highly miscible in water, any unreacted methanol can only be recovered via an energy-intensive distillation process, which would not be feasible (Martin, 2016). It must therefore be accepted that the unreacted methanol (or other alcohol) will be lost in this process. In addition to the cost of supplying excess methanol, any unrecovered methanol represents a parasitic energy load on the process (Martin, 2016) as it is a relatively energy-dense material (19.7 MJ/kg). A simplified analysis was done to determine how much excess methanol can be used in the in-situ transesterification reaction before the embodied energy exceeds the energy contained in the recovered lipids. This is represented as an energy return on investment (ROI) and was calculated according to the following equation:

\[
\text{Equation 6.2 : Energy ROI} = \frac{\text{Parasitic energy load (MJ)}}{\text{Energy content of biodiesel produced (MJ)}}
\]

\[
= \frac{\text{Methanol dosage (kg)} \times \text{Methanol energy content (MJ)}}{\text{Wet algae (kg)} \times \text{Solids content (\%)} \times \text{SL (\%)} \times \text{Conversion (\%)} \times \text{Recovery (\%)} \times \text{Biodiesel energy content (MJ)}}
\]

The energy ROI was also performed for a dry extraction approach where dried algae is used for lipid extraction and transesterification. For dry extraction, the parasitic energy included the energy contained in the methanol that is converted to biodiesel and the energy required to thermally evaporate the water in a 20% solids biomass slurry. Excess methanol was assumed to be fully recovered after dry extraction and the energy used to recover any excess methanol was considered negligible.

Table 6.3 shows that based on the reaction parameters in this study the in-situ lipase transesterification can be energetically feasible but only at methanol:TAG-equivalent ratios <20:1. The FAME conversion will be reduced at this ratio (Figure 6.7). At 6:1 methanol:TAG-equivalent mole ratio for the N-deplete biomass (25% SLs), the energy losses via the unrecovered methanol are ca 14% of the energy content in the biodiesel generated (Table 6.3), however the FAME conversion yield at this methanol dosage was only 45% (Figure 6.7). It may be possible to reduce the required methanol dosage by developing or using a lipase that shows a greater affinity for FAME production over fatty acid production (Amoah et al., 2016) in a water rich system, or by replacing methanol with a water-insoluble acyl acceptor such as ethyl acetate (Kim et al., 2007) that could be recovered in the hexane.

Even after considering the loss of methanol, in-situ transesterification with a water-immiscible solvent could still have a lower parasitic energy load than extraction of EPA or polar lipids from thermally dried biomass (Table 6.3) or via monophasic wet extraction processes that use unrecoverable water-immiscible solvents (Table 6.2). For the sole purpose of biodiesel production this method is not likely to be competitive with two-stage hexane-transesterification (Martin, 2016), however it can be offered as a means of recovering EPA. In this study, the method was able to recover most of the EPA in the hexane (69-72% of total EPA recovered from ND biomass and 77-80% from NR biomass, for the experiments presented in Figure 6.6), which would otherwise be largely unrecovered by conventional hexane extraction processes (Olmstead et al., 2013b).
Table 6.3: Simplified comparison of the parasitic energy loss from the methanol used during lipase in-situ transesterification/wet hexane extraction to the energy required to thermally dry biomass prior to conventional extraction and transesterification. Data presented are the energy ROI ratios on the basis of the energy contained in biodiesel generated from the recovered FAME/lipids. Indices higher than one are shaded grey and indicate net loss of energy. For the in-situ transesterification process the parasitic energy is in the form of unrecovered methanol. For dry extraction the parasitic energy considers only the energy contained in the methanol that is converted to biodiesel and the energy required to thermally evaporate the water in 20% solids biomass. Excess methanol was assumed to be fully recovered after dry extraction and the energy used to recover any excess methanol was negligible. The influence of methanol ratio, SL content (25% for N-deplete or 12.5% for N-replete biomass) and lipid recovery yield are presented.

<table>
<thead>
<tr>
<th>Conversion Method</th>
<th>SL content of dry biomass</th>
<th>FAME conversion</th>
<th>Energy ROI&lt;sup&gt;3,4,5&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry extraction</td>
<td>25%</td>
<td>~100%</td>
<td>1.14</td>
</tr>
<tr>
<td></td>
<td>12%</td>
<td></td>
<td>2.30</td>
</tr>
<tr>
<td>Lipase</td>
<td>25%</td>
<td>45%</td>
<td>0.14</td>
</tr>
<tr>
<td>6:1 MeOH:SL</td>
<td>12%</td>
<td></td>
<td>0.29</td>
</tr>
<tr>
<td>Lipase</td>
<td>25%</td>
<td>65%</td>
<td>1.07</td>
</tr>
<tr>
<td>20:1 MeOH:SL</td>
<td>12%</td>
<td></td>
<td>2.22</td>
</tr>
<tr>
<td>Lipase</td>
<td>25%</td>
<td>90%</td>
<td>1.54</td>
</tr>
<tr>
<td>72:1 MeOH:SL</td>
<td>12%</td>
<td></td>
<td>3.21</td>
</tr>
</tbody>
</table>

Assumptions:
1. Dry extraction or lipase process with different MeOH:SL ratios from 20 w/w% solids algae slurries
2. Lipase FAME conversion based on the data in the current study assuming ca 80% cell rupture
3. Lipid recovery efficiency was assumed to be 90% for lipase in-situ transesterification
4. Methanol energy density = 19.7 MJ kg<sup>-1</sup>; algal biodiesel energy density = 37.5 MJ kg<sup>-1</sup>
5. Unused methanol was assumed to be recovered fully in the dry extraction process
6. Heats of vaporisation: water = 2.254 MJ kg<sup>-1</sup>; methanol = 1.1 MJ kg<sup>-1</sup>

Another improvement to this process will be to further minimise the cost of the enzyme such that it is outweighed either by the value of the extracted EPA or the reduction in energy costs from avoiding thermal drying of the biomass (which is otherwise required for EPA recovery). Enzyme immobilisation may offer a means of reusing the lipase. However, to date immobilised lipase has exhibited slow kinetics.
and a rapid loss of activity (to around 70-80\% after only 3 cycles) when used on wet algae slurries (Navarro López et al., 2016; Tran et al., 2012).

By using the current biphasic system (Figure 6.1 and Figure 6.4), a reverse-micelle enzyme (Moya-Ramírez et al., 2016) that partitions in the hexane phase could potentially be explored as an alternative to the free lipase used here. Locating the lipase in the hexane phase would mean the enzyme could be recovered along with the solvent. As such, the combined biphasic transesterification and extraction process could allow reuse of the enzyme without requiring immobilisation.

6.4 Conclusion

A free-lipase-based in-situ transesterification was demonstrated on ca 20\% w/w ruptured N. salina biomass using hexane as a solvent. Up to ca 90 wt\% of the SL were converted to FAME after 24 h of reaction. Approximately 75\% of the FAME could be recovered in the hexane after centrifugation. In addition to TAG, it was shown that polar lipids were also converted to FAME, allowing enhanced recovery of EPA. Two possible mechanisms for the conversion and recovery of the FAME from polar lipids were identified. Although the dilution effect from high water content (75-80\% water) in the biomass avoided the methanol inhibition of the lipase, the high water content also necessitates a high methanol:TAG ratio to promote maximal FAME conversion. If the yields could be improved or the loss of methanol reduced or avoided this process could be more energy efficient than extracting EPA from thermally dried algae biomass.

6.5 References


Chapter 7

7 Conclusions and recommendations

In this final chapter, the insights gained from the experimental chapters are brought together to develop a unified understanding of the role of emulsions in a hexane-based lipid extraction process from a slurry (15-20% w/w solids concentration) of ruptured microalgae cells. This thesis helps to bridge existing knowledge across algal biofuel technology, surface chemistry and biochemical process engineering. The ruptured-microalgae slurry was an interesting material to investigate in surface chemistry studies. Its potential as a supply of biosurfactants and bioemulsifiers (chapter 4) is especially promising as higher-value products for the food, cosmetic, chemical and pharmaceutical industries. Emulsion formation was found to be integral for successful lipid recovery (chapter 5) and yet, at the same time, was found to be a barrier to solvent recovery (chapter 3). However, the ability of ruptured algae to produce stable emulsions was used to advantage to enhance the effectiveness of lipase in-situ transesterification (chapter 6). Identification of the mechanisms of emulsification by ruptured algae (chapter 4) will be useful in guiding future microalgae research studies and process development. Based on the insights gained in the thesis, recommendations for future work are suggested at the end of the chapter.

7.1 Key conclusions

The key conclusions of the thesis are summarised as follows:

1. A stable slurry-solvent emulsion can be a barrier to complete solvent recovery and therefore future solvent extraction studies should consider solvent removal efficiency as one of the evaluation parameters.

Solvent recovery yield has been an overlooked aspect in most previous lipid extraction studies. Chapter 3 demonstrated that a significant quantity of solvent can remain unrecovered in the emulsified slurry even after centrifugation. In addition to the solvent loss, the lipid dissolved within the unrecovered solvent will also remain unrecovered. The emulsions formed in the process were surprisingly stable. This was shown to be due to the ability of the algae cell components to prevent coalescence of the solvent droplets. Considering the significance of the unrecoverable solvent and lipid, future solvent extraction studies on wet algal slurry (e.g. Figure A3) should consider the solvent removal efficiency, alongside with the lipid recovery, as part of the research outcomes.
2. Microalgae contain highly effective emulsifiers and surfactants that can form stable emulsions.

Having shown that the ruptured microalgal cells can form stable emulsions, Chapter 4 identified the roles of major components in contributing to emulsion stability. The slurries of ruptured algal cells were separated into three main fractions, namely the cell particles, the lipids and the proteins-rich serum. The lipids did not form a stable emulsion on their own, but did allow the dispersion of small hexane droplets that could be stabilised by other emulsifiers. Both the serum and the cell particles were able to form stable emulsions on their own. The serum, lipids and cell particles exhibited competitive interfacial adsorption in the presence of each other. The presence of lipid reduced the stability of protein or cell-stabilised emulsions, suggesting the possibility of adding surfactants with similar properties to enhance solvent recovery. The cell particles were proposed to be the main reason for the recalcitrant emulsion layer observed after centrifugation and are therefore the key target to improving solvent recovery. Importantly, this experimental chapter also highlighted the potential of microalgae as a source of diverse biosurfactants and bioemulsifiers that can be produced using scalable processes.

3. The interfacial area provided by emulsions is required to achieve a high lipid extraction rate.

Although emulsion formation is undesirable for solvent recovery, it was shown to be needed for efficient lipid extraction (Chapter 5). The key rate limiting factor during biphasic lipid extraction kinetics was determined to be the slurry-solvent interfacial area. Both a high interfacial area and a well-mixed system are required to promote the direct contact/collision between the lipid droplets and hexane droplets that allow lipid transfer into the solvent. Therefore, it is likely that emulsion formation will remain an essential aspect of a biphasic extraction process.

4. In-situ transesterification can enhance the recovery of acyl groups from polar lipids in an emulsified system.

In chapter 6, a free lipase requiring interfacial-activation (provided by the emulsification process) was used to perform an in-situ transesterification. This process concurrently extracts and converts the extracted algal lipid into FAME in a single unit operation from a wet ruptured-algae slurry. Up to 90% FAME conversion could be achieved within a similar or shorter reaction time compared to existing studies. Although a high dosage of methanol was required in this approach compared to a conventional transesterification, the methanol dosage was still lower compared to existing in-situ transesterification studies using immobilised lipases. The method proposed in this chapter can be utilised as an alternative to recover the high-value acyl chains from polar lipids such as omega-3 fatty acid while leaving behind
the phosphorus moiety. A mass balance and energy balance on the process were performed and future improvement was identified based on the analysis.

7.2 Recommendation for future studies

Based on the outcomes and insights gained from the thesis, recommendation for future investigation are suggested:

- **The impact of upstream processes (algal species, growth regime, media composition, dewatering process, rupture process) on slurry viscosity**

  In a centrifugation process (chapter 3 and chapter 4), the slurry viscosity is one of the main parameters determining the kinetics of a phase separation process (Equation 2.2). A less viscous slurry tends to result in an emulsion that can be demulsified more easily due to a higher creaming rate. During the project, it was observed that there were slight variations in the slurry viscosity from different batches at a similar solids concentration. However, a detailed investigation into this behaviour was beyond the scope of the thesis. Currently, there is no study in the literature that has investigated the slurry viscosity as a function of the upstream processes. Understanding the factors that influence the slurry viscosity could be a key to improving solvent recovery and lipid extraction kinetics.

- **Optimisation of surfactant formulation to improve demulsification**

  In this thesis, the adsorption of algal cells and debris particles onto the solvent interface was found to be the main reason for the residual unrecoverable solvent. To displace the particles from the interface, a surface-active LMWS may be used to promote competitive adsorption at the interface. In preliminary experiments (Figure A5), surfactants were used in an attempt to improve solvent recovery. However, the improvement was not as significant as expected, possibly due to the unsuitable choice of surfactant. Based on the prior experiences in petroleum crude oil industry (Al-Sabagh et al., 2011; Angle, 2001; Wu et al., 2003), demulsification is often a complex process, requiring the selection of an appropriate surfactant for the system, finding the optimal dosage range, the optimal mixing regime etc. Therefore, going forward, a systematic surfactant selection process needs to be performed based on the understanding developed in this thesis. This will enable an effective demulsification protocol to be developed that can improve solvent recovery.

- **Isolation and identification of the surfactants in ruptured algal cells for practical applications**

  In the thesis, the biomass fractions exhibited diverse emulsion-forming and stabilising properties. To gain a full understanding of these biosurfactants and bioemulsifiers, the fractions need to be isolated further to identify the active components in the fraction. This will allow a
more accurate feasibility study to be performed and for specific application-surfactant combinations to be targeted.

- **The effect of growth conditions on the algal surfactant profile**
  The lipid profile of microalgae cells has been shown to be affected greatly by growth conditions (Sharma et al., 2012). Similarly, the cell also responds to the environmental condition by expressing different biomolecules profiles, including the protein composition (Lourenço et al., 2004), the cell wall/membrane thickness and composition (Boussiba, 2000), the composition of EPS (Sheng et al., 2010) etc. Therefore, it can be expected that changes in the growth phase will affect the surfactant activity of the ruptured cells in the lipid extraction and solvent recovery processes.

- **Lipid extraction via membrane-emulsification**
  The quantity of the interfacial area was found to be the rate-limiting factor in biphasic solvent extraction. In the conventional wet hexane extraction method, this interfacial area is created by emulsification using shear. However, this typically produces an emulsion with a polydisperse droplet size distribution, and includes undesirably small droplets which are difficult to recover. Recent advances in membrane emulsification have made a promising technology for producing highly monodisperse emulsion droplets (Charcosset et al., 2004). By manipulating the resulting emulsion droplet size, it may be possible to create larger emulsion droplets that can be separated more easily and yet meeting the minimum required lipid transfer rate for solvent extraction.

- **Investigation of alternative acyl acceptors as a means for recovery**
  In wet algal in-situ transesterification, free lipase was found to be a viable alternative to immobilised lipases that inadvertently lose their activity, likely due to exposure to the polar lipids in the aqueous phase (Navarro López et al., 2016). However, as has been shown in the energy ROI analysis (Chapter 6), the unrecoverable methanol remains a limitation to the process. To take advantage of the emulsification and the separation processes, water-immiscible acyl acceptors that are soluble in the organic phase can be investigated as a means for recovery (Carvalho & Cabral, 2000).

In conclusion, emulsion formation is an integral part of hexane-based solvent extraction. Understanding the fundamental mechanisms of emulsion formation is critical to optimising the solvent extraction process. This thesis has provided a mechanistic understanding of the emulsion formation and the demulsification process in a biphasic solvent extraction from a wet slurry of ruptured microalgae. The thesis has also demonstrated the importance of solvent recovery, which has been largely neglected to date. The process-limiting factors in both lipid extraction and solvent recovery were identified. These findings provide the required groundwork for future studies. In particular, future research efforts can be
directed towards utilising the microalgae as a source of surfactants and emulsifiers as well as to further developments to solvent recovery that can lead towards a sustainable algal biofuels production process.

7.3 References


8 Appendices

A significant amount of preliminary experimentation was performed to better understand the hexane-extraction process carried out in the thesis. While some of the insights gained from the preliminary experiments were beyond the primary scope of the thesis, the understanding developed from these experiments was sufficiently important to include as part of this thesis. They are presented here as a series of self-contained experiments that were designed to answer short research questions that arose during the work of the thesis. Each section includes a brief introductory paragraph, details of the test methods/approaches, a description of the results, and a summary of the key findings.

8.1 The effect of algal paste storage temperature and the incubation process on the lipid profile

In most experiments, it was not logistically possible to complete the experiments within days of harvest. Therefore, an appropriate long-term storage condition for preserving the algal paste was required. In addition, the incubation step (38 °C for 24 h) was found crucial to weaken the cell walls for a high level of cell rupture (Halim et al., 2016). Therefore, the effect of this incubation process on the algal lipid profile needed to be verified.

To test the effect of storage temperature and the incubation process on the algal lipid profile, a batch of frozen N-replete *N. salina* slurry (19% w/w) was obtained (Halim et al., 2016) and then subjected to a 2-month storage period under four different conditions. The slurries were then subjected to a total lipid extraction (modified Bligh & Dyer protocol) without cell rupture (Olmstead et al., 2013). The four tested conditions were:

(i) Stored at -20°C (freezer) for 2 months with no incubation
(ii) Stored at -20°C (freezer) for 2 months and subjected to 24h incubation at 40°C
(iii) Stored at 4°C for 2 months with no incubation
(iv) Stored at 4°C for 2 months and subjected to 24h incubation at 40°C

The extracted total lipids were analysed by HPLC-ELSD as described in chapter 6 (section 6.2.7).

The results (Figure A1.A) demonstrated that the algal lipids in the intact cells can undergo noticeable lipid hydrolysis over a 2-month period when stored at 4°C. This was indicated by an increase in the FFA level in samples stored at 4°C. However, the level of TAG remained consistent, suggesting the lipid hydrolysis occurred mostly among the polar lipids which was confirmed by the HPLC chromatograms (Figure A1.B). On the other hand, there was no significance difference in the level of
both TAG and FFA before and after the incubation process. Therefore, the lipid profile was unlikely to be affected by the incubation process.

**Highlights:**

(i) Freezing was a better alternative for long-term storage of harvested algal paste.

(ii) Lipid hydrolysis occurred mostly among the polar lipids, rather than TAG which had remained consistent during the test period.

(iii) The incubation process did not affect the level of TAG and FFA.

**Figure A1:** (A) The level of TAG and FFA per 100mg of dry biomass under different test conditions as analysed by HPLC-ELSD. (B) The chromatograms of the algal lipids after being subjected to two-months of storage. The incubated sample did not vary from the non-incubated samples and was therefore not presented. The results in (A) were the average ± standard deviation of duplicate samples.

### 8.2 Alternative methods to quantify cell rupture

In wet lipid extraction using a nonpolar solvent (e.g. hexane), the extent of cell rupture is the key factor to determining the final lipid yield (Halim et al., 2016). Therefore, an accurate method to quantify cell rupture is crucial to predict the lipid yield enabled by the cell rupture process. Previously, whole cell counting was found to be the most reliable method for quantifying cell rupture (Spiden et al., 2013). However, this method has several shortcomings that may limit its usability. For example, partially ruptured cells may be counted as whole cells (Yap et al., 2014), which thereby underestimates the rupture percentage. Cell clumping (particularly for algal cells harvested via flocculation-assisted dewatering process) will likely result in an underestimated cell count. The cell counting procedure can also be time-consuming for processing large numbers of samples. Therefore, alternative methods were investigated in this experiment.
In this experiment, *N. salina* slurry harvested via centrifugation (chapter 6, section 6.2.1) was weakened by incubation and ruptured using high pressure homogenisation (HPH) as previously described (Halim et al., 2016). Two different approaches were used to characterise the ruptured cells. First, the particle size distribution of the *N. salina* cells were analysed by a laser particle analyser (Malvern Mastersizer 3000) before and after the cell rupture process.

In the second approach, the extent of cell rupture (after HPH) were assessed via cell counting (Spiden et al., 2013) and a hexane extraction method. In the cell counting method, a haemocytometer chamber was used to count the cells using a light microscopy with a camera attachment (Law et al., 2017). The rupture % was determined by cell counting as follows:

\[
Rupture \% \text{ (whole cell basis)} = (1 - \frac{\text{Number of cells after HPH}}{\text{Number of cells before HPH}}) \times 100\%
\]

On the same batch of ruptured cells, the total lipid and hexane-extractable lipid yield were determined based on the procedures as described in chapter 5 (section 5.2.2). Briefly, the total lipid content was measured using a modified Bligh and Dyer total lipid extraction protocol (Olmstead et al., 2013). The hexane-extractable lipid yield was measured by performing repeated hexane extraction on the ruptured cells (4-5 hexane contacts). Both the total lipid and hexane-extracted lipid were chemically transesterified and analysed by GC-FID to quantify the FAME (as described in 6.2.6). The rupture % based on hexane-extractable lipid was determined by:

\[
Rupture \% \text{ (hexane-extractable lipid basis)} = \frac{\text{Hexane extractable lipid (mg FAME per g dry biomass)}}{\text{Total Lipid (mg FAME per g dry biomass)}} \times 100\%
\]

The particle size analysis (Figure A2.A) showed that the cell particles clumped significantly after the HPH process, thus contributing to larger particles and a broader particle size distribution. The particle clumping may be due to the release of the charged or hydrophobic components. Therefore, particle size analysis is not a suitable method to characterise cell rupture.

The rupture % established by the two methods (Figure A2.B) showed a significant difference (67% rupture on whole-cell counting basis, and 80% rupture on hexane-extractable lipid basis). This suggests that cell counting may underestimate the cell rupture in some cases. For lipid extraction studies, hexane-extractable lipid yield appeared to be a suitable alternative for quantifying cell rupture.

**Highlights:**

(i) Laser particle analysis is not suitable for characterising cell rupture of *N. salina* due to particle clumping.

(ii) Cell rupture can be evaluated based on a hexane-extractable lipid basis and can be offered as an alternative to the standard cell counting method. This is also likely a more relevant metric for lipid extraction studies.
Figure A2: (A) The particle size distribution of *N. salina* cell particles before and after cell rupture by HPH. (B) The extent of rupture as established by two different methods (hexane-extractable lipid basis and whole-cell counting basis) on the same homogenised slurry. The results in (B) are the average ± standard deviation of duplicate samples.

### 8.3 Evaluation of alternative solvents based on solvent recovery efficiency

In chapter 3, some hexane was found retained in the emulsion layer even after a prolonged centrifugation. The retention of solvent may preclude the delipidated biomass from being used for other purposes such as fish feed (Spolaore et al., 2006) unless the emulsion layer is thermally heated to remove the residual hexane. In addition, the hexane is a volatile solvent which requires expensive gastight equipment. Therefore, two alternative solvents (namely refined soybean oil and methyl oleate) were investigated as a potential hexane substitute. Refined soybean oil is food safe and consists mainly of TAG. Thus it can be used directly as a transesterification feedstock without a solvent removal step. Methyl oleate is a form of FAME/biodiesel and therefore does not need to be removed from the lipid extract (Park et al., 2014).

The refined soybean oil was obtained from a local grocery store, and was determined to be mostly TAG as evaluated previously by thin layer chromatography (TLC). The methyl oleate was a technical grade reagent (70% purity) sourced from Sigma Aldrich Australia. In this experiment, the *N. salina* slurry (19 % w/w dry biomass and had a total lipid content of 22%) was incubated and ruptured as previously described (Halim et al., 2016). 2 mL of solvent (either hexane, refined soybean oil or methyl oleate) was added into 4 mL of ruptured-algae slurry. The solvent and algal slurry were mixed for 0.5 h before centrifugation using an analytical centrifuge (LUMifuge) (Law et al., 2017). The solvent recovery was calculated based on the height % as described in chapter 4 (section 4.2.6). During centrifugation, a single speed (either at 582 x g or 2330 x g) was applied for 16 minutes.
All the solvents used were able to form a stable emulsion (single homogenous phase) during lipid extraction. Compared to hexane (Figure A3.A), both the methyl oleate and soybean oil appeared to be more stable against coalescence at 582 x g, with no phase separation observed. At the maximum centrifugation speed of the instrument (2330 x g), most of the emulsions were successfully separated (Figure A3.B) - with 84.9% final solvent recovery for hexane, 81.3% for soybean oil and 90.4% for methyl oleate. In this experiment (assuming algal lipid density = 0.91 g mL\(^{-1}\)), 1 unit volume of solvent was used to recover 0.1 unit volume of lipid. Therefore, a loss of 10% volume solvent means that there was no net energy gained in the process, assuming the solvent and the algal lipid have a similar energy density (ca 38 MJ/kg). Hence, both the soybean oil and the methyl oleate are unlikely to be energetically feasible for a biodiesel process if evaluated solely from the perspective of solvent recovery.

**Figure A3:** (A) Phase separation of the slurry-solvent emulsion at 582 x g, which was the centrifugal force required to coalescence the hexane emulsion found in chapter 3. (B) Phase separation of the slurry-solvent emulsion at maximum instrument speed (2330 x g) for 16 min (C) The final solvent recovery yield was replotted from B. The values presented are the average of duplicate experiments.
**Highlights:**

(i) A small amount of solvent (either soybean oil, methyl oleate or hexane) was retained in the emulsion layer after 16 min of centrifugation at 2330 x g.

(ii) The emulsions formed by soybean oil and methyl oleate were more stable against coalescence as compared to hexane, with no solvent phase separation at 582 x g.

(iii) The methyl oleate achieved a higher solvent recovery (90%) as compared to hexane (85%) and soybean oil (81%). However, the unrecovered soybean oil and methyl oleate means that it is not feasible for biofuel production due to the energy loss in the form unrecovered solvent.

### 8.4 Emulsifying properties of intact *N. salina* cells

In Chapter 4, it was found that the intact (non-homogenised) cells were able to reduce the hexane-water interfacial tension. Therefore, the emulsifying properties of intact *N. salina* slurry was further investigated in this section.

About 100 mL of *N. salina* slurry (19% w/w) was incubated at 38 °C for 24 h (Halim et al., 2016). The next day, the incubated slurry was split into two portions. A portion of the sample was allocated for use as an ‘intact’ sample, while the other portion was subjected to cell rupture using HPH at 1200±100 bar to be used as a ‘ruptured’ sample. In a 15 mL centrifuge tube, 2 mL hexane was added into 4 mL algal slurry (either intact or ruptured). The hexane and algal slurry was hand mixed rigorously for about 15 sec and then was mixed further on a rotary mixer for 15 min and overnight (20 h) at room temperature. At the end of the mixing, the slurry-hexane emulsion was centrifuged at 9000 x g for 15 min. The lipid-rich hexane was recovered and then weighted. The yield of hexane recovered was calculated based on the weight of the initially added hexane and the weight of the hexane recovered after centrifugation.

This experiment revealed that while the intact cells had less emulsifying capacity (e.g. less hexane was incorporated into the slurry phase) than the ruptured cells, the intact cells can still contribute to hexane retention in the slurry phase (Figure A4.A). This appeared to be consistent with the hypothesis proposed in chapter 4, where the hexane retention was likely caused by the adsorption of the hydrophobic/amphiphilic cell surfaces onto the hexane droplets. Therefore, the hexane recovery did not appear to differ greatly between the ruptured and the intact samples (Figure A4.B), as the particle adsorption process does not depend on cell rupture. A longer mixing time allowed more cells to adsorb to the hexane droplets and therefore causing a higher level of unrecovered hexane. This was evident by a thicker emulsion layer in the intact sample (Figure A4.C & A4.D) and a reduction in hexane recovery (Figure A4.B) following overnight mixing.
**Figure A4:** (A) The emulsions resulting after 15 sec of rigorous hand mixing. (B) The percentage of the recovered hexane as a function of mixing time was plotted. The images of the separated layers after centrifugation for a mixing for (C) 15 min and (D) overnight.

**Highlights:**

(i) Intact cells had less emulsifying capacity than ruptured cells.

(ii) Intact cells can still contribute to hexane retention in the slurry phase. This was likely caused by the hydrophobic/amphiphilic cell surfaces.
8.5 The influence of the surfactant profile on the phase separation process

In chapter 5, it was found that a ruptured algal cell contains a variety of biosurfactants and bioemulsifiers. To improve the solvent recovery process, targeting these surfactants and emulsifiers in the ruptured cells is key. This section documents the attempts undertaken to understand the influence of surfactants in the solvent recovery process.

In this experiment, *N. salina* slurry (14% w/w) was incubated and ruptured using HPH at 1200±200 bar as described in Chapter 6. Different approaches were used to modify the surfactant profile, namely adjustment of the pH, enzyme treatment and surfactant addition. In the pH tests, the homogenised slurry was adjusted from pH 6.1 to the desired pH (pH 4 or 8) using concentrated 1M hydrochloric acid and 1M sodium hydroxide. For the enzymatic treatments, 3 mL of homogenised slurry was added with 20 µL trypsin (Sigma Aldrich, USA), or 20 µL Lecitase Ultra (Novozyme, Denmark). The reaction was performed for 16 h at 40 °C. A negative control was included alongside the treated sample mixed at 40 °C. In the surfactant test, a low molecular weight surfactant commonly used for food emulsion was used, namely the lactic acid ester of monoglyceride (Lactem) (Gaupp & Adams, 2004). The Lactem was dissolved in hexane at 5 mg mL⁻¹ prior to being added into the homogenised slurry. The emulsion mixtures were mixed on a rotator for 2 h prior to centrifugation using LUMifuge as described in chapter 4 (section 4.2.6).

pH modification is a commonly used techniques for destabilising emulsions (Strassner, 1968). However, in this experiment (Figure A5.A), changing the pH in either direction (pH 4 or 8) appeared to result in a more stable emulsion. This was likely due to the protein denaturation in the serum (observed as a white-solid precipitate when the pH of an isolated serum was adjusted in a similar fashion). The denatured proteins likely became less surface active and thus allowed other surfactants in the slurry to dominate the interface to form a more uniform interfacial film.

Similarly, treating the homogenised slurry with a protease (trypsin) and phospholipase (Lecitase) also resulted in a more stable emulsion than the control sample with no treatment (Figure A5.B). From the enzymatic treatment experiment, the emulsion mixture after the phospholipase-treatment was significantly more stable. This was in part due to the increase in viscosity (as observed visually) after the enzymatic digestion. The reason for the increased viscosity was however unclear.

By adding surfactants to the homogenised slurry (Figure A5.C), the emulsion stability appeared to decrease slightly, with more hexane separated at the lower centrifugal force. However, the amount of finally recovered hexane did not vary much from the control samples. This was likely because the surfactant was not strong enough to displace the cell particles from the solvent interface. More work is needed to identify a suitable surfactant.
Figure A5: The effect of (A) pH modification, (B) enzymatic treatment (C) surfactant addition, on the resulted slurry-hexane emulsion stability

Highlights:

(i) Changing the pH resulted in protein denaturation that led to a more stable emulsion.

(ii) Hydrolysing the proteins and phospholipids resulted in a more stable emulsion.

(iii) The phospholipase-treated slurry-hexane emulsions were highly viscous after the enzymatic treatment

(iv) Adding a surfactant slightly improved the coalescence at low centrifugal force, however, the amount of hexane retained in the emulsion layer was not affected.
8.6 Lipase selection for in-situ transesterification of wet ruptured *N. salina*

This section describes the preliminary work performed for chapter 6 and before the development of HPLC-ELSD method.

In-situ transesterification is a strategy where the lipid extraction and the biodiesel (FAME) conversion were performed in a single step. Different lipases may have a different optimal conditions and catalyst properties (Fjerbaek et al., 2009). Therefore, selecting a suitable lipase is an important first step.

The lipases were purchased commercially from Australian suppliers. *Rhizomucor miehei* lipase (RML) and *Candida rugosa* lipase (CRL) were short-listed due to their demonstrated ability in catalysing transesterification reaction in a water-rich system (Crooks et al., 1995; Kuo et al., 2015) and their relatively low cost per enzyme unit. The Lecitase Ultra is a phospholipase that was provided as a sample from Novozyme. The Lipozyme was included in the experiment as it is the *Rhizomucor miehei* lipase immobilised on methacrylate polymer carriers (for a free lipase versus immobilised lipase comparison). The Novozyme 435 was included in the selection process as it is one of the most widely used immobilised lipase for biodiesel conversion (Du et al., 2004; Hernández-Martín & Otero, 2008).

**Table A1** Some information pertaining to the lipases tested in the thesis. Note that the enzyme per unit cost were only an estimation based on the purchase cost (year 2016) from Sigma Aldrich Australia and may not reflect the actual cost in an industrial process.

<table>
<thead>
<tr>
<th>Catalysts</th>
<th>Enzyme Unit</th>
<th>Cost (AUD)</th>
<th>Supplier</th>
<th>Note/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rhizomucor miehei</em> lipase (RML)</td>
<td>&gt;20,000 U/g</td>
<td>0.12</td>
<td>Sigma Aldrich, USA</td>
<td>Soluble lipase isolated from <em>Rhizomucor miehei</em></td>
</tr>
<tr>
<td><em>Candida rugosa</em> lipase (CRL)</td>
<td>&gt;700 U/mg</td>
<td>0.02</td>
<td>Sigma Aldrich, USA</td>
<td>Soluble lipase isolated from <em>Candida rugosa</em> (in powder form) and was prepared with distilled water/PBS</td>
</tr>
<tr>
<td>Lecitase Ultra</td>
<td>&gt;10,000 U/g</td>
<td>0.21</td>
<td>Novozyme, Denmark</td>
<td>Soluble phospholipase isolated from <em>Thermomyces lanuginosus</em></td>
</tr>
<tr>
<td>Lipozyme</td>
<td>&gt;300 U/g</td>
<td>45.30</td>
<td>Sigma Aldrich, USA</td>
<td>Immobilised form of RML</td>
</tr>
<tr>
<td>Novozyme 435</td>
<td>&gt;5000 U/g</td>
<td>8.10</td>
<td>Sigma Aldrich, USA</td>
<td>Immobilised lipase from <em>Candida antarctica</em></td>
</tr>
</tbody>
</table>

For the catalyst-selection experiments, a standardised condition was used: 4 mL ruptured-algae slurry, 2.0 mL hexane, 0.05 mL methanol (corresponding to the optimal 7:1 methanol: total lipid mole ratio reported by (Guan et al., 2010)) and 50 µL chemical catalysts (either acid or alkaline of 1% w/w) or 10 µL biocatalyst/lipase (or enzyme unit equivalent to 10µL RML lipase). The reactions were performed at 40 °C (65 °C for chemical catalyst) for 20 h. All experiments were performed in a 14 mL glass vial with an airtight cap on a rotary device (Labquake, Barnstead International, Iowa, USA) in a
temperature-controlled incubator (Unimax 1010 & Incubator 1000, Heidolph Instruments, Germany). A heater block (Techne Sample Concentrator) was used for the chemical catalyst samples requiring above 40 °C and was shaken periodically.

To evaluate the FAME conversion, a modified thin layer chromatography (TLC) method was verified for separating and identifying TAG, FAME and FFA. The mobile phase used was hexane/diethyl ether/acetic acid (Kaluzny et al., 1985) in a modified volume ratio of 80:20:1. After the transesterification reactions, 1 mL of reaction mixture was taken and centrifuged at 9400 x g (Eppendorf 5424, Australia) to recover the hexane layer which contained the converted lipids. The hexane layers were diluted 100-fold and 0.5 µL of the diluted hexane solution was spotted on a silica plate (HPTLC Silica gel 60 F254, Merck, Germany). The spotted silica plate was developed in a glass chamber saturated with mobile phase. The developed silica plate was then stained with 13% w/w phosphomolybdic acid in ethanol and heated with a heat gun for sample visualisation. A typical separation profile of the algal lipids is shown below. The major lipid spots were identified via co-spotting of lipid standards.

![Typical separation profile of algal lipids](image)

As there were slight variations in the spot intensity due to the staining procedure (but were consistent within a set of experiment), a negative control (with no methanol and catalyst) was included in each set of experiment as a comparison.

In the preliminary experiments, chemical-catalysed in-situ transesterification was performed to compare against a lipase-based in-situ transesterification (Figure A6.A). It was found that no FAME was formed with either the acid or the alkaline catalyst. This was likely due to the high water content in the system, which requires a much higher catalyst concentration (Im et al., 2014). The RML appeared to be a more effective catalyst compared to the CRL as indicated by the presence of an intense FAME spot (Figure A6.B). Interestingly, the immobilised form of RML (Lipozyme) yielded less FAME than its soluble form (RML). This was likely due to an inefficient mixing as the ruptured-algae slurry was highly viscous. Lecitase Ultra is a microbial phospholipase commonly used for degumming vegetable oil (Yang et al., 2006). Surprisingly, the Lecitase was able to catalyse the transesterification reaction despite being widely used a phospholipase (Figure A6.C). Conversely, the RML also exhibited a ‘phospholipase’ activity as evident by the decrease in the polar lipid spot. It is worth noting that in all of the experiments the TAG spot intensity did not decrease as much as expected despite the apparent formation of FAME. This was likely due to the coelution with other molecules (such as pigments). The results also suggest that the FAME conversion coming from non-TAG lipids can be significant when
using an N-replete algal culture. Therefore, an in-situ transesterification comparison using N-deplete (high TAG) and N-replete (low-TAG) \( N. \text{salina} \) biomass was made subsequently in chapter 6.

From these tests, the RML appeared to be more effective at transesterification (with a more intense FAME spot) and has a lower cost per enzyme unit compared to the other lipases. Therefore, RML was selected for further studies in the thesis.

**Figure A6:** Comparison experiments of in-situ transesterification (with wet ruptured-algae slurries as a reaction feedstock) between (A) chemical catalysts (1% w/w sulfuric acid and sodium hydroxide) versus lipase (RML) catalyst (ii) different lipases (CRL, RML, and immobilised RML), and (iii) lipase (RML) versus phospholipase (Lecitase)

To evaluate the performance of the immobilised lipases, Novozyme 435 was compared against the Lipozyme. In this reaction, a higher methanol dosage (0.2 mL) and an algal slurry with a lower solids concentration (17% w/w) were used to increase the reaction yield. The Lipozyme was able to produce a more intense FAME spot with a lower FFA ratio (Figure A7.A). This is consistent with previous studies where the Novozyme 435 required a low water content to achieve a high level of FAME conversion (Lu et al., 2009). To assess the reusability of the immobilised enzyme, the enzyme beads (Lipozyme) were recovered from the first reaction and used again in a second in-situ transesterification. A significant reduction in enzyme activity was observed as evident by the lower FAME: TAG ratio (Figure A7.B).
**Figure A7:** (A) Comparison of in-situ transesterification between different immobilised lipases (Lipozyme and Novozyme). (B) FAME conversion of Lipozyme before and after reuse.

**Highlights:**

(i) RML was more effective compared to CRL and Lecitase as a soluble transesterification catalyst in wet ruptured-algae slurry.

(ii) RML was more efficient in soluble form than in the immobilised form (Lipozyme).

(iii) RML was able to convert the polar lipids, similar to a phospholipase (Lecitase).

(iv) Lipozyme (immobilised RML) was more effective in a water-rich system (higher FAME yield) compared to Novozyme 435.

(v) A significant reduction in enzyme activity was observed in Lipozyme after one reuse.

### 8.7 The effect of various reaction parameters on transesterification

This section is a continuation to the previous experiment (section 8.6) performed to understand the effect of the various reaction parameters on FAME conversion (transesterification). The main parameters tested included types of acyl acceptor, pH, methanol dosage, lipase dosage, reaction time, and water content.

In this experiment, the in-situ transesterification reactions were performed with standardised parameters of 4 mL wet ruptured *N. salina* slurry (19% w/w), 2 mL hexane, 0.4 mL methanol and 10 µL RML lipase. The reactions were performed at 40 °C for 20 h. Only one variable was changed in each experiment to allow a direct comparison.
Figure A8 shows the overall effect of changing each reaction variable. A higher FAME conversion was desired and was indicated by a more intense FAME spot (with generally less intense TAG and FFA spots). Methanol appeared to be a better acyl acceptor than either ethanol or ethyl acetate (Kim et al., 2007). Only a minimal amount of FAME was formed when ethanol was used as an acyl acceptor. The use of ethyl acetate as an acyl acceptor appeared to favor FFA formation (lipid hydrolysis) rather than FAME conversion (transesterification). Increasing the dosage of ethyl acetate decreases the FAME spot intensity due to a dilution effect (ethyl acetate is miscible with hexane and is minimally soluble in water). Adjusting the pH from 4.9 (unadjusted) to 7.2 (within the cited optimal pH 7-9 for RML) did not appear to improve the FAME conversion. Methanol inhibition of lipase was only seen at 5 mL dosage, which was 10 times higher than the optimal dosage found in chapter 6 (section 6.3.5). Increasing the lipase dosage from 1 µL to 80 µL did not appear to affect the FAME intensity. This may be due to the small difference in FAME yield being was less than the detection limit of the TLC. The FAME conversion increased with increasing reaction time as expected, up to the 20 h tested.

In the methanol dosage experiment (Figure A8.D), the RML lipase appeared to be mostly unaffected by methanol up to 5 mL methanol dosage, which corresponded to ca 650:1 methanol: total lipid mole ratio. This was much higher compared to the typical inhibitory methanol ratio (9:1 methanol: lipid mole ratio) reported for RML (Guan et al., 2010). It was theorised that the high water content (ca 80% water) in the algal slurry attenuated the methanol inhibition effect. To test this hypothesis, additional transesterification reactions were performed on a model oil system with refined soybean oil consisting of mostly TAG (instead of wet ruptured-algae slurry). The standard reaction conditions used were 0.2 mL soybean oil (similar to the oil content in 4 g N. salina slurry), 0.04 mL methanol, 0.032 mL distilled water, 10 µL lipase and 2 mL hexane. The amount of water and methanol was varied to observe the relationship and its effect on the FAME conversion.
**Figure A8:** The effect of various variables on FAME conversion in wet ruptured-algae slurries. (A) The effect of acyl acceptor (methanol versus ethanol). (B) The effect of ethyl acetate as acyl acceptor at different dosages. (C) The effect of pH (D) The effect of methanol dosage (E) The effect of lipase dosage (F) The effect of reaction time. Note: the TLC separation in (A) was performed with a different mobile phase (toluene/ethyl acetate/diethyl ether/acetic acid 80:10:10:1) (Touchstone, 1995) as an unsuccessful attempt to resolve TAG from the co-eluted molecule. The order of TAG and FAME was reversed when using this mobile phase.

Figure A9 showed the differences in FAME conversion when the amounts of water and methanol were varied. By increasing the water content, the FAME conversion for RML increased along with more FFA formation (lipid hydrolysis). The Novozyme 435 reacted differently to the increase in water content and had the highest FAME conversion with minimal water content (0.2%).

When the methanol dosage was increased from 0.04 mL to 0.2 mL methanol (in a 2% water content system), a significant decrease in FAME conversion was observed. This was in contrast with the previous results (in a wet algal slurry) where no lipase inhibition was observed up to 5 mL. This revealed that the high water content in the algal slurry can attenuate methanol inhibition on the lipase.
Figure A9: (A) The effect of water content on soybean oil transesterification for different lipases (RML and Novozyme) (B) The effect of methanol dosage in a transesterification reaction (using soybean oil).

**Highlights:**

(i) Methanol was the most suitable acyl acceptor for lipase-based in-situ transesterification.

(ii) RML lipase appeared to perform similarly between pH 4.9-pH 7.2 and thus pH adjustment of the algal slurry is likely not required.

(iii) RML was fairly resistant to methanol inhibition in a water-rich system (e.g. wet algal slurry)

(iv) Novozyme achieved the highest FAME conversion at minimal water content (0.2%) while RML had higher FAME conversion in a high-water system (up to the 20% water content tested).

### 8.8 The effect of in-situ transesterification on solvent recovery

As has been demonstrated in appendix 8.5, modification of the surfactant profile in the homogenised slurry may impact the hexane recovery process. In an in-situ transesterification reaction, the polar lipids, which are surface active, were shown to be converted into FAME. Therefore, it is expected that the solvent removal efficiency may be affected by this process.

To test this hypothesis, lipase-catalysed in-situ transesterification was performed according to the protocol described in chapter 6 (section 6.2.4). Control samples without methanol and lipase were prepared alongside the in-situ transesterification samples. After 24 h of reaction time at 40 °C (on both the control and the in-situ transesterification samples), the reaction mixtures were centrifuged in the LUMifuge at the maximum setting (2330 x g) to access the solvent recovery yield. To verify the solvent
recovery yield, the samples were also concurrently centrifuged in a typical centrifuge instrument at 3,273 x g for 25 min (Allegra X-12, swing bucket rotor SX4750, Beckman Coulter, Victoria, Australia). A second hexane contact was also performed as described in chapter 6. The separated hexane layers from each centrifugation step were recovered and weighed to obtain the recovery yield.

The LUMifuge centrifugation revealed that the solvent separation was slower in the transesterified sample than in the control sample (Figure A10.A). In addition, the final recovered hexane yield was also lower (ca 75% hexane recovery for the control sample and 70% for the transesterified sample). These results were close to the centrifugation results obtained via recovering the hexane physically (Figure A10.B), with 73% w/w hexane recovered for control samples and 69% w/w for transesterified samples. Surprisingly, the hexane added in the second solvent contact was fully recovered (close to 99%). This suggests that most of the amphiphilic cell particles had been saturated by hexane in the first contact and thus no additional solvent was retained. Therefore, a second hexane contact can be used to recover the lipids associated with the initially unrecovered hexane without losing more solvent in the process.

Figure A10: (A) The hexane separation kinetics and yield as measured on a LUMifuge. (B) The solvent recovery in both centrifugation steps (from two sequential hexane contacts) as evaluated by physically recovering and measuring the hexane layer gravimetrically.

Highlights:

(i) In-situ transesterification resulted in a slightly lower solvent recovery.
(ii) The hexane added in the second solvent contact step was fully recovered.
(iii) A second hexane contact can be used to improve the lipid/FAME recovery without losing more solvent.
8.9 References


