Downstream processing of lipids and lipases from thraustochytrids

by

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I am the author of the thesis entitled:

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marine environment for producing next generation biofuels. SMBB conference, Mexico 2014

4. **Avinesh Byreddy**, Munish Puri, Colin Barrow, Production and extraction of lipases from the *Schizochytrium* sp. S31. AISRF conference, March 12-14, 2014

**Sequences deposited in GenBank database**

1. DT1  *Ulkenia* sp.  KF682136
2. DT2  *Ulkenia* sp.  KF682137
Table of contents

Acknowledgements......................................................................................................i
List of publications .....................................................................................................ii
Sequences deposited in GenBank database.............................................................iv
Table of contents.........................................................................................................v
List of figures .............................................................................................................ix
List of tables..............................................................................................................xii
List of abbreviations................................................................................................xiv
Abstract ....................................................................................................................xvi

Chapter 1: Introduction and Literature review ..................................................1
  1.1. Introduction....................................................................................................2
  1.2. Omega-3 FAs.............................................................................................3
    1.2.1. Health benefits of omega-3 FAs .........................................................4
    1.2.2. Dietary sources of omega-3 FAs .........................................................5
    1.2.3. Techniques used for the concentration of omega-3 FAs .................7
    1.2.4. Microalgae as an alternative source for omega-3 fatty acids ..........9
  1.3. Thraustochytrids .........................................................................................10
    1.3.1. Potential of Thraustochytrids .............................................................12
    1.3.2. Strain selection and sustainable lipid production.............................12
    1.3.3. Estimation of lipid content and fatty acid profile ............................13
  1.4. Extraction of lipids from dry cell biomass .................................................15
    1.4.1. Selection of suitable extraction solvents ...........................................15
    1.4.2. Lipid extraction from wet biomass .....................................................19
    1.4.3. Microalgae cell disruption for lipid extraction .................................20
    1.4.3. Effect of cell disruption methods on lipid extraction .....................21
  1.5. Lipases ........................................................................................................25
    1.5.1. Marine lipases ...................................................................................25
    1.5.2. Isolation of lipase producing microbes and screening methods ........26
    1.5.3. Extraction of intracellular lipases .....................................................28
    1.5.2. Lipase based methods for concentration of omega-3 fatty acids .......28
  1.6. Research aim and objectives.......................................................................33
Chapter 7: Phospholipase A1 as a catalyst to enrich omega-3 fatty acids in anchovy and algal oil

7.1. Introduction

7.2. Material and Methods

7.2.1. Chemicals

7.2.2. Hydrolysis of anchovy oil

7.2.3. Thraustochytrid oil hydrolysis by phospholipase A1

7.2.4. IATROSCAN analysis

7.2.5. Methylation and GC analysis

7.2.6. Analysis of positional distribution of fatty acid by NMR

7.3. Results and discussion

7.3.1. Lipase activity of Phospholipase A1

7.3.2. Hydrolysis of Anchovy oil by PLA1

7.3.3. 13C NMR studies of the anchovy oil hydrolysis by PLA1 and BSL

7.3.4. Thraustochytrid oil hydrolysis by phospholipase A1

7.4. Conclusion

Chapter 8. Summary and future aspects
List of figures

Chapter 1:
Figure. 1.1 Chemical structures of omega-3 FAs, ALA; C18:3n-3 (cis-9,cis-12-cis-15-octadecatrienoic acid, EPA; C20:5n-3 (Δ5,8,11,14,17-eicosapentaenoic acid)and DHA; C22:6n-3 (Δ4,7,10,13,16,19-docosahexaenoic acid) .................................................................3
Figure. 1.2. Microscopic images of thraustochytrid cells; (A) Schizochytrium S31, (B) Schizochytrium DT3 and (C) Thraustochytrium AMCQS5-5 .........................................................11
Figure. 1.3. Classification of cell disruption methods ..........................................................................................................................................................................................21

Chapter 2:
Figure. 2.1. Schematic diagram of SPV method ........................................................................38
Figure. 2.2. Standard curve representing relationship between Schizochytrium oil concentration and increase in the absorbance ..............................................................................................................40
Figure. 2.3. Comparison of various lipids and other compounds with sulfo-phospho-vanillin reaction ........................................................................................................................................41
Figure. 2.4. Profile of colorimetric detection of thraustochytrid whole cell lipids. ..............................43
Figure. 2.5. Change of lipid colour after reaction with sulfo-phospho-vanillin reagent .........................................................................................................................................................44

Chapter 3:
Figure. 3.1. Lipid extraction percentage from dry biomass using various solvents from Schizochytrium sp. S31 ............................................................................................................................56
Figure. 3.2. Effect of different cell disruption methods for lipid extraction from thraustochytrids .................................................................................................................................................57
Figure. 3.3. Effect of cell disruption on thraustochytrids cells. Thraustochytrid cells before (A) and after (B) cell disruption (scale bar 20 μm) ...............................................................................................58
Figure. 3.4a. Effect of different cell disruption methods on fatty acid profiles of Schizochytrium sp. S31 .................................................................................................................................................61
Figure. 3.4b. Effect different cell disruption methods on SFA, MUFAs and PUFAs of Schizochytrium sp. S31 ...............................................................................................................................62
Figure. 3.5a. Effect of different cell disruption methods on fatty acid profiles of Thraustochytrium sp. AMCQS5-5 .............................................................................................................................................62
Figure. 3.5b. Effect of different cell disruption methods on SFA, MUFAs and PUFAs of Thraustochytrium sp. AMCQS5-5 .............................................................................................................................................63
Chapter 4:
Figure. 4.1. Cluster analysis of thraustochytrids based on various enzyme activities. ............................................................75
Figure. 4.2. Microscopic observation of thraustochytrid after cell disruption. ....77
Figure. 4.3. Effect of bead vortexing on thraustochytrid cell disruption and lipase activity. ..................78
Figure. 4.4. Effect of grinding on thraustochytrid cell disruption and lipase activity. .................................................................79
Figure. 4.5. Effect of sonication on thraustochytrid cell disruption and lipase activity. .................................................................81
Figure. 4.6. Effect of homogenisation on thraustochytrid cell disruption and lipase activity. .................................................................82

Chapter 5:
Figure. 5.1. Effect of Tween 80 concentration on lipase production by Schizochytrium S31 at shake flask, pH 6.0 and incubation temperature 25 °C........89
Figure. 5.2. Fermentation profile for the production of lipase by Schizochytrium S31 in a stirred tank reactor.................................................................92
Figure. 5.3. Biomass, lipid and DHA production reached a maximum after five days of incubation.................................................................91
Figure. 5.4. Fatty acid composition of Schizochytrium S31.................................92
Figure. 5.5. Cell disruption optimisation of Schizochytrium S31 a) as a function of sonication power, b) time duration.................................................................95
Figure. 5.6. Substrate specificity of lipase from Schizochytrium S31..................96

Chapter 6:
Figure. 6.1. Schematic diagram of experimental process followed from thraustochytrid cell disruption to enzymatic omega-3 fatty acids concentration.....104
Figure. 6.2. Thraustochytrid cell disruption (40 X magnification) employing bead mill.................................................................104
Figure. 6.3. Optimisation of lipid extraction by bead mill as function of biomass concentration. .................................................................105
Figure 6.4. Effect of bead mill agitator speed (rpm) on lipid yield from thraustochytrid.................................................................................................................................106

Figure 6.5. Fatty acid composition of lipid extracted from thraustochytrids S31 and DT3........................................................................................................................................108

Figure 6.6. Capillary chromatography (iatrascan) profile of thraustochytrid oil .................................................................................................................................109

Figure 6.7. Optimisation of enzyme concentration..................................................................................................................................................................................110

Figure 6.8. Lipid class analysis of thraustochytrid oil after hydrolysis with Candida rugosa lipase at different time intervals........................................................................110

Figure 6.9. AG and FFA are separated hydrolysed oil and analysed by gas chromatography................................................................................................................111

Chapter 7:

Figure 7.1 Phospholipase A1 activity towards \( p \)-NP esters with different acyl chains. ..................................................................................................................................119

Figure 7.2. Time course of hydrolysis of anchovy fish oil by PLA1 and BSL A ... 120

Figure 7.3. Time course of hydrolysis of anchovy oil and fatty acid distribution in the hydrolysate. ...........................................................................................................122

Figure 7.4. Percent hydrolysis of various fatty acids (from Fig.7.3) at 32% hydrolysis of anchovy oil by PLA1 (light) and BSL (dark).................................124

Figure 7.5. 13C NMR spectra of the unhydrolysed oil fraction before (blue) and after (red) 30% hydrolysis of anchovy oil by PLA1 (A) and BSL (B) ........................125

Figure 7.6. Percent accumulation of different fatty acid classes in TG fraction after 30% hydrolysis of anchovy oil by PLA1 (dark) and BSL (light). .........................126

Figure 7.7. Lipase hydrolysis of structured triglyceride 1, 3-dioeyl-2-myristic triglyceride......................................................................................................................127

Figure 7.8. Lipid class analysis of thraustochytrid oil after hydrolysis with phospholipase at different time intervals.................................................................129

Figure 7.9. Fatty acid profiles of hydrolysed and unhydrolysed DT3 oil.............130
List of tables

Chapter 1:
Table 1.1. Contents of EPA and DHA in some fish species. .............................................6
Table 1.2. Dietary recommendation of omega-3 FAs by various scientific bodies. ....7
Table 1.3. Recent studies investigating the use of organic solvent in the extraction of microalgal lipids. ......................................................................................................................16
Table 1.4. Extraction of lipids improved by cell disruption methods. .........................24
Table 1.5. Main patents relating production of omega-3 fatty acids processing using lipases from the last 10 years. .................................................................29

Chapter 2:
Table 2.1. Biomass and lipid production in glycerol medium by thraustochytrids....39
Table 2.2. Comparison of thraustochytrid lipid content by SPV, gravimetric and GC methods. ......................................................................................................................45

Chapter 3:
Table 3.1. Biomass, lipid contents and productivity of thraustochytrids. ....................54
Table 3.2. Comparison of cell disruption methods employed to extract total lipids from different microalgae........................................................................................................58
Table 3.3. Advantages and disadvantages of the investigated cell disruption methods. .................................................................................................................................60
Table 3.4. Biodiesel properties of the given thraustochytrids strains. .........................64
Table 3.5. Energy consumption comparison. ................................................................66

Chapter 4:
Table 4.1. Lipase activity of thraustochytrids new isolates and ATCC cultures.
Lipase activity was determined by p-NPP assay using whole cell as catalyst. ....76
Table 4.2. Disruption of thraustochytrid cells for lipase extraction...........................83

Chapter 5:
Table 5.1. Effect of sonication on protein release and lipase activity from
Schizochytrium S31. ....................................................................................................93
Chapter 6:
Table 6.1. Energy consumption analysis for different mass concentrations and lipid yields. ........................................................................................................................................................................106

Chapter 7:
Table 7.1. Positional distribution of various fatty acids in anchovy oil. .................123
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<td>ω</td>
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<td>DHA</td>
<td>Docosahexaenoic acid</td>
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<td>GLA</td>
<td>Gamma-linolenic acid</td>
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<td>DGLA</td>
<td>Di-homo gamma-linolenic acid</td>
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<td>SFAs</td>
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<td>Polyunsaturated fatty acids</td>
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<td>FAs</td>
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TFA - Total fatty acids
TAGs - Triacylglycerols
DAGs - Diacylglycerol
MAGs - Monoacylglycerol
PLA1 - Phospholipase A1
BSL - Bacillus subtilis lipase
DU - Degree of unsaturation
SV - Saponification value
IV - Iodine value
CN - Cetane number
LCDF - Long chain saturated factor
CFPP - Cold filter plugging point
CP - Cloud point
APE - Allylic position equivalents
BAPE - Bis-Allylic position equivalents
OS - Oxidation stability
HHV - Higher heating value
KV - Kinematic Viscosity
MJ - Mega joules
BHT - Butylated hydroxytoluene
GYP - Glucose, Yeast extract, Peptone
sp. - Species
GC - Gas Chromatography
FID - Flame Ionisation Detector
DCW - Dry cell weight
v/v - Volume by volume
w/v - Weight by volume
w/w - weight by weight
SD - Standard deviation
OD - Optical density
FAMEs - Fatty acid methyl esters
ATCC - American Type Culture Collection
Abstract

Marine microalgae are a renewable alternative source to fish oil for the sustainable production of omega-3 fatty acids. The potential use of marine microalgae for nutraceutical and biofuel production is of global interest owing to increasing concerns with sustainable production and environment impact. Thraustochytrid are common marine heterotrophs, capable of producing large amounts of lipid, particularly omega-3 fatty acids. These organisms are also potential sources of bioactive proteins, enzymes, pigments and exopolysaccharides. The development of rapid lipid quantification method for the isolation of new strains from diverse marine habitats is important for future cost-effective production of these bioactive metabolites. Cell disruption methods for improving lipid and lipase extraction has the potential use in economising large scale algal processing for biofuel production. Further, development of enzymatic methods for concentrating omega-3 fatty acids from fish and algal oils is desirable because of their beneficial health effects.

Isolation and screening of suitable microalgae with high lipid productivity are important for achieving sustainable commercial production of omega-3 oils and biofuel. Current methods of lipid quantification are time consuming and costly. Quantification of microalgal lipids using the sulpho-phospho-vanillin (SPV) reaction is a rapid colorimetric method. This method was successfully tested on in-house thraustochytrid strains. Conventional gravimetric analysis confirmed the accuracy of the colorimetric method with $R^2 = 0.99$. The total lipid contents of thraustochytrids were determined gravimetrically was DT3 33.3%, S31 25.8%, AMCQS5-5 22.8% and PRA 296 18.0%, respectively. Carbohydrates, proteins and glycerol did not interfere with the SPV reaction.

Lipid extraction is an integral part of biodiesel production, as it facilitates the release of fatty acids from algal cells. We evaluated the extraction efficiency of various solvents and solvent combinations for lipid extraction from Schizochytrium sp. S31 and Thraustochytrium sp. AMCQS5-5. The maximum lipid extraction yield was 22% using a chloroform:methanol ratio of 2:1. The highest lipid extraction yields were obtained using osmotic shock method with 48.7% lipid yield from Schizochytrium sp. S31 and 29.1% from Thraustochytrium sp. AMCQS5-5. Saturated and monounsaturated fatty acid contents were more than 60% in Schizochytrium sp. S31 which suggests their suitability for biodiesel production.
Marine microorganisms are a potential source of enzymes with structural stability, high activity at low temperature and unique substrate selectivity. We screened for a range of enzymatic activities from the seven thraustochytrid strains. The relationship between isolate(s) and enzyme activity was investigated using cluster analysis. The two strains AMCQ-4b27 (34 IU/g) and AMCQS1-9 (36 IU/g) exhibited the highest lipase activity of the strains investigated. Four different cell disruption methods, bead vortexing, grinding, sonication and homogenization, were evaluated for their impact on lipase extraction yields. Sonication was found to be the best method for enhancing lipase extraction yields from *Thraustochytrium* sp AMCQ-4b27 (0.903 IU/μg) and AMCQS1-9 (1.44 IU/μg).

The effect of Tween 80 as a carbon source was investigated with regard to biomass, lipase and lipid productivity in *Schizochytrium* sp. S31. Tween 80 (1%) and 120 h of incubation was the optimum period for biomass, lipid and lipase productivity in a stirred tank reactor. The yields obtained were 0.9 g L⁻¹ of biomass, 300 mg g⁻¹ of lipid and 39 U g⁻¹ of lipase activity. Sonication was optimised in terms of time and acoustic power to maximise the yield of extracted lipase. The extracted lipase from *Schizochytrium* S31 had unusual fatty acid selectivity in that it preferentially hydrolysed DHA over EPA.

A method for lipid extraction from marine thraustochytrids using a bead mill and enzymatic concentration of omega-3 fatty acids from the thraustochytrid oil was investigated. The optimised lipid extraction conditions were, bead size 0.4-0.6 μm, 4500 rpm, 4 min of processing time at 5% biomass concentration. The maximum lipid yield achieved at optimum conditions were 40.5% for *Schizochytrium* sp. S31 (ATCC) and 49.4% for *Schizochytrium* sp. DT3 (in-house isolate). DT3 oil contained 39.8% DHA as a percentage of lipid, a higher DHA percentage than S31. Partial hydrolysis of DT3 oil using *Candida rugosa* lipase was performed to enrich omega-3 polyunsaturated fatty acids (PUFAs) in the glyceride portion. Total omega-3 fatty acid content was increased to 88.7%.

For enrichment of omega-3 fatty acids in fish and algal oils, we have explored the possibility of using phospholipase A1 (PLA1), a *sn*-1 position specific phospholipid hydrolysing enzyme that also demonstrates lipase activity. PLA1 from *Thermomyces* species was initially tested for its substrate specificity with various *p*...
nitrophenyl esters. In this study, lipase from *Bacillus susbtilis* (BSL) was taken as a control. The pH stat based hydrolysis followed by gas chromatographic characterisation of the hydrolysate of *Anchovy* fish oil indicated a selective retention of omega-3 fatty acids in the triglyceride fraction by PLA1, whereas such discrimination was not observed with BSL. Selective accumulation of omega-3 fatty acids was also confirmed by $^{13}$C NMR spectra. Position-wise analysis of fatty acids in the unhydrolysed fractions suggests that PLA1 preferably retains docosahexaenoic acid. Phospholipase A1 was used to catalyse the hydrolysis of thraustochytrid DT3 oil to enrich DHA in glyceride portion, with short chain fatty acids being preferentially cleaved from the triacylglyceride. This results show that phospholipase A1 can be used to concentrate DHA from triacylglycerides.
Chapter 1: Introduction and Literature review
1.1. Introduction

Omega-3 fatty acids (FAs) are essential FAs, meaning that the human body cannot synthesise them, so they must be obtained from the diet. Omega-3 FAs are well known for their health benefits (Swanson et al., 2012). For example, high consumption of omega-3 fatty acids in their diet decrease the risks of sudden death particularly in those who already have had a heart attack (Blondeau, 2015; Hoen et al., 2013). DHA is added to the infant formulas, as it is necessary for eye and brain development. These fatty acids may also decrease the risks of as depression, schizophrenia, Alzheimer’s and Parkinson’s diseases (Nichols et al., 2014; Tai et al., 2013). Multiple health benefits associated with omega-3 FAs have increased the demand for these ingredients in nutraceuticals and functional food industries. It has been forecast that the consumption of omega-3 FAs in 2014 was 134.7 thousand metric tons valued at US$ 2.5 billion. By 2020, it is been projected that the demand further will increase significantly to 241 thousand metric tons with a value of US$ 4.96 billion (Market watch, 2014). Most of the health benefits associated with omega-3 fatty acids are attributed to the long-chain FAs eicosapentaenoic acid (EPA, C20:5-3) and docosahexaenoic acid (DHA, C22:6-3). The most widely and naturally available sources of EPA and DHA are fish oils. Concentration of EPA and DHA from fish oils is normally achieved via chemical methods, which are not environmentally friendly, also result in partial degradation of long chain FAs and production of toxic waste. This has led researchers to seek an alternative, environmentally friendly method (Namal Senanayake, 2013; Shahidi & Wanasundara, 1998). Enzymatic concentration of EPA and DHA from fish oil using selective lipases is ecofriendly and causes less damage to chemically sensitive long chain FAs (Shahidi & Wanasundara, 1998).

Production of EPA and DHA from fish oils has resulted in increased pressure on fish species and number. Moreover, it has been recognised that mass fishing is not a sustainable approach for EPA and DHA production (Lenihan-Geels et al., 2013). In addition, these fish oils can be contaminated with heavy metal (methyl mercury) that are hazardous to human health, particular to pregnant and lactating women’s (Lin et al., 2014; Mahaffey et al., 2011). A viable alternative source of omega-3 FAs are heterotrophic microalgae (Adarme-Vega et al., 2012). Commercial scale production has been performed by using *Cryptochodinium cohnii* and *Schizochytrium* sp. as a source for DHA (Infant formulas) and EPA (Food and beverages) (Kuratko et al.,
Chapter 1

These algae can be grown in suitably sterile conditions and oil extracted in a manner that is suitable for use in infant formula and dietary supplements. Biomass harvesting and lipid extraction are expensive steps in the production of oil from algal biomass (Kim et al., 2013). Effective cell disruption methods and efficient solvent extraction methods are critical for high lipid yield and cost effective production (Ryckebosch et al., 2014).

1.2. Omega-3 FAs

The term ‘Omega-3’ refers that the first double bond between the third and fourth carbon atoms from methyl end, typically in cis configuration, hence called omega-3 FAs. In cis configuration the hydrogen atoms are attached to double bonds at the same side (Figure 1.1). Natural PUFAs consist of an even number of carbon atoms with various unsaturation points. The most common omega-3 FAs are DHA C22:6, EPA C20:5 and α-linolenic acid (ALA) C18:3 (Poudyal et al., 2011; Ratnayake & Galli, 2009). ALA is a precursor molecule in the biosynthesis of omega-3 FAs.

![Chemical structures of omega-3 FAs](Figure 1.1 Chemical structures of omega-3 FAs, ALA; C18:3n-3 (cis-9,cis-12,cis-15-octadecatrienoic acid, EPA; C20:5n-3 (Δ5,8,11,14,17-eicosapentaenoic acid) and DHA; C22:6n-3 (Δ4,7,10,13,16,19-docosahexaenoic acid). Modified from (Akanbi et al., 2014b).)

Generally omega-3 FAs are stored in adipose tissue in triacylglyceride (TAG) form and present in all cellular membrane in phospholipid and related forms. Cell membrane phospholipid composition determines the physiological characteristics of the cell membrane, including changes in response to the external environment and
functional activities of membrane-bound proteins (Murphy, 1990). The proportion of EPA and DHA to all other FAs present in cell membranes differs and impacts the function of cells, particularly storage and transport (Browning et al., 2012). DHA is predominantly present in specific regions such as brain and eye. So for example, DHA constitutes 18% of FA in adult human brain grey matter (Innis, 2007; Skinner et al., 1993). With demonstrated physiological roles and established clinical benefits, the link between human health and EPA and DHA is relatively well established.

1.2.1. Health benefits of omega-3 FAs

In the beginning of the 20th century dietary fats were recognised as a good source of energy and fat soluble vitamins. This view later changed following various studies, including a study conducted on rats reported that ‘dietary fatty acids were required to prevent deficiency disease’ (Burr & Burr, 1929). The health benefits of omega-3 FAs have been evaluated by a number of clinical studies. There is evidence for the efficacy of omega-3 FAs in the prevention of sudden death from cardiovascular disease and in ameliorating rheumatologic conditions (Barrow, 2010; Bhangle & Kolasinski, 2011; Cicero et al., 2015). An early study on fat consumption and health with Greenland Inuit indicated the cardiovascular benefits of omega-3 FAs. Further studies produced more evidence for the cardiovascular benefits associated with omega-3 FAs, including the reduction of the risk of arrhythmias (Asif, 2014), decreasing platelet aggregation (Gao et al., 2013), lowering plasma triglycerides (Qi et al., 2008), decreasing blood pressure (Cabo et al., 2012).

The consumption of dietary omega-3 FAs by ulcerative colitis and Crohn’s disease patients has been shown to produce beneficial weight gain and significant improvement in disease activity (Papadia et al., 2010). Omega-3 FAs have been recently shown to have anti-cancer activity, particularly against colorectal cancer (Cockbain et al., 2012). Many research investigations have been conducted to understand the positive effects of omega-3 FAs on growth, learning and behavioural problems of young children. Supplementation of omega-3 FAs during pregnancy resulted in an increase in birth size (Makrides et al., 2011). Brain and eye development in infants is particularly influenced by DHA, which has led to the addition of DHA to infant formulae. Learning capacity of school going children is increased by taking DHA enriched food supplements (Birch et al., 2007; Nunes et al., 2014; Richardson & Montgomery, 2005). A number of studies have provided evidence for the anti-
inflammatory effects of omega-3 FAs. Omega-3 FAs reduce the production of pro-inflammatory molecules (eicosanoids) and increase the production of anti-inflammatory molecules such as resolvins and protectins (Calder, 2012). The combined effect of these multiple health benefits has been a dramatic increase in the market demand for omega-3 FAs.

1.2.2. Dietary sources of omega-3 FAs

The main traditional sources of omega-3 FAs are marine fish. The marine ecosystem is a rich source of omega-3 FAs, which are naturally produced by microalgae and accumulated in fish and higher animal via the food chain (Lenihan-Geels et al., 2013). Some other common sources include crustaceans, such as lobsters, krill and molluscs, such as oysters and squid. Some land animals and plant oils also contain omega-3 FAs in small quantities. Plant oils, such as canola, walnut oil, olive oil and flaxseed oils are rich source of the omega-3 fatty acid ALA, a precursor for the biosynthesis of EPA and DHA. To meet current requirements for men and women, the recommended consumption of olive oil and flaxseed oil varies from 158–229 ml/d (olive oil) to 2.2–3.2 ml/d (flaxseed oil) (USDA, 2006). The recommended intake of omega-3 FAs from seeds and nuts to meet ALA recommended consumption for women and men varies, from 12.2–17.6 g/d of English walnuts, 4.8–7.0 g/d of flaxseeds, 612–890 g/d of pumpkin seeds (USDA, 2006). Many studies indicate that the conversion of ALA to long chain FAs EPA and DHA is very low (Burdge & Calder, 2005), because of this, diet rich in preformed EPA and DHA are required to achieve the associated health benefits.

Marine derived fish oils are an excellent source for EPA and DHA. The percentages of EPA and DHA in total fish oils vary depending on the fish species. Farmed fish have more fat than wild fish, but the level of omega-3 in that fat varies depending on fish feed use. Farm fish often has lower omega-3 levels than wild fish even, although in one study farm-raised Atlantic salmon has 2.128 g SFA/85 g and 1.825 g EPA+DHA/85 g; whereas wild Atlantic salmon has 1.068 g SFA/85 g and 1.564 g EPA+DHA/85 g, respectively (Gebauer et al., 2006). Some of popular wild caught fish and their EPA and DHA contents listed in table 1.1.
Table 1.1. Contents of EPA and DHA in some fish species.

<table>
<thead>
<tr>
<th>Common name</th>
<th>Scientific name</th>
<th>EPA</th>
<th>DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mackerel</td>
<td><em>Scomber scombrus</em></td>
<td>1.10</td>
<td>2.56</td>
</tr>
<tr>
<td>Red mullet</td>
<td><em>Mullus surmuletus</em></td>
<td>0.91</td>
<td>1.66</td>
</tr>
<tr>
<td>Sardine</td>
<td><em>Sardina pilchardus</em></td>
<td>0.62</td>
<td>1.12</td>
</tr>
<tr>
<td>Salmon</td>
<td><em>Salmo salar</em></td>
<td>0.50</td>
<td>1.00</td>
</tr>
<tr>
<td>Ton</td>
<td><em>Thunnus thynnus</em></td>
<td>0.24</td>
<td>0.98</td>
</tr>
<tr>
<td>Fresh anchovy</td>
<td><em>Engraulis encrasicolus</em></td>
<td>0.14</td>
<td>0.80</td>
</tr>
<tr>
<td>Sea bream</td>
<td><em>Pagellus bogaraveo</em></td>
<td>0.12</td>
<td>0.61</td>
</tr>
<tr>
<td>Cod</td>
<td><em>Gadus morhua</em></td>
<td>0.23</td>
<td>0.47</td>
</tr>
<tr>
<td>Hake</td>
<td><em>Merluccius merluccius</em></td>
<td>0.10</td>
<td>0.54</td>
</tr>
<tr>
<td>Conger eel</td>
<td><em>Conger conger</em></td>
<td>0.15</td>
<td>0.43</td>
</tr>
<tr>
<td>Swordfish</td>
<td><em>Luvarus imperialis</em></td>
<td>0.15</td>
<td>0.30</td>
</tr>
<tr>
<td>Dogfish</td>
<td><em>Galeorhinus geleus</em></td>
<td>0.04</td>
<td>0</td>
</tr>
</tbody>
</table>

This table has been reproduced and modified from Rubio et al., (2010)

Dietary recommendation for omega-3 FAs advised by various scientific bodies are given in Table 1.1. The Dietary Guidelines for Americans 2005 report states, “Evidence suggests consuming approximately two servings of fish per week (approximately 227 g; 8 ounces total) may reduce the risk of mortality from CHD (Coronary heart disease) and that consuming EPA and DHA may reduce the risk of mortality from cardiovascular diseases in people who have already experienced a cardiac event”. Another important challenge is meeting the recommended consumption of omega-3 FAs in vegetarians and people who do not enjoy the taste of fish. Alternative ways of getting omega-3 FAs for vegetarian is consuming fish oil supplements and plant oils with significant omega-3 fatty acid content (Gebauer et al., 2006; Maurer et al., 2012).
Chapter 1

Table 1.2. Dietary recommendation of omega-3 FAs by various scientific bodies.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Professional body (year)</th>
<th>Recommendation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>UK Scientific Advisory Committee on Nutrition (2004)</td>
<td>Minimum of 2 portions of fish/week that is equivalent to 450 mg EPA + DHA</td>
</tr>
<tr>
<td>2</td>
<td>World Health Organization (2003)</td>
<td>Up to two fish meals/week (400–1000 mg EPA+DHA)</td>
</tr>
<tr>
<td>3</td>
<td>US National Academies of Science, Institute of Medicine (2002)</td>
<td>1.4 g α-linolenic acid/day (140 mg EPA+DHA)</td>
</tr>
<tr>
<td>4</td>
<td>American Heart Association (2002)</td>
<td>1g/day omega-3 FA for secondary prevention of CHD</td>
</tr>
</tbody>
</table>

This table has been reproduced from Yashodra et al., (2009)

1.2.3. Techniques used for the concentration of omega-3 FAs

Fish is a proven natural source of omega-3 FAs, but its consumption may not meet recommended levels. A prior concentration and purification step is valuable, therefore, to convert fish oils into a suitable chemical form which can be easily metabolised in the human body, with the added benefit of improved oxidative stability. Some studies suggest that the acylglyceride form of omega-3 FAs are better absorbed than as ethyl esters and free fatty acid forms. In addition to that, some studies reported that their oxidative stability has been increased in acylglyceride form (Dyerberg et al., 2010). However, eating large amount of fish is also associated with the risk of heavy metal – including mercury absorption, which may discourage people from eating fish. (Lin et al., 2014). To achieve highly purified omega-3 fatty acids concentrate, physical, chemical and enzymatic methods may be employed. The common methods employed for the concentration and purification of omega-3 fatty acids from marine oils include urea adduction, chromatographic methods, low-temperature fractional crystallization,
supercritical fluid extraction, distillation, and enzymatic and integrated methods
(Namal Senanayake, 2013).

Urea adduction is simple and efficient technique for the concentration of omega-3 fatty acids from marine fish oils. The principle behind this method is to hydrolyse fish oil, using ethanolic KOH or NaOH, then mixing free FAs with urea solution to form a complex. Saturated and monounsaturated fatty acids form complex structures with urea and PUFAs include omega-3 FAs remain in the liquid portion, which is highly enriched with omega-3 fatty acids. These two portions can be separated by filtration and concentrated omega-3 FAs portion can be collected (Shahidi and Senanayake, 2006). The major disadvantage of this method is that it produces large amounts of urea waste, the disposal of which is expensive (Zuta et al., 2003).

Another important method for the concentration of omega-3 fatty acids from fish oils is chromatographic techniques. This method involves saponification, followed by treatment with urea solution. The non-complexed free fatty acids are converted into fatty acid methyl esters and separated by chromatography (Namal Senanayake, 2013). Guil-Guerrero and Belarbi used silver nitrate-impregnated silica gel column to purify EPA and DHA from cod liver oil. Multiple washings with mobile phases led to the recovery of DHA and EPA, 64% and 29.6%, with resulting purities of 100% and 90.6%, respectively (Guil-Guerrero & Belarbi, 2001).

Low-temperature fractional crystallisation is a simple method that depends on differences in melting points of different fatty acids. The melting point depends on the degree of unsaturation of each fatty acid. In general, the more saturate the fatty acids the higher the melting point. The omega-3 FAs EPA and DHA melting points were -54 and -44.5 °C, compared with 18:1 (Oleic acid) and 18:0 (Steric acid) of 13.4 and 69.6 °C, respectively (Namal Senanayake, 2013). As temperature decreases for a mixture of saturated and unsaturated fatty acids, saturated fatty acids first crystalise and the liquid portion enriched with unsaturated fatty acids (Shahidi and Senanayake, 2006).

Fractional distillation is a straight forward and widely accepted method for the concentration of omega-3 FAs from a mixture under ultralow pressure. Based on their boiling point and molecular weight, fatty acids are separated at reduced pressure and elevated temperature of about 250 °C. This method allows highly selective separation to concentrate selective fatty acids, but its high operating temperatures results in
increased operating costs and damage to the oxidatively sensitive omega-3 FAs (Breivik, 2007).

Large scale concentration of omega-3 FAs by physical or chemical methods is associated with some drawbacks in terms of low yield, use of large volumes of chemical, production of large volumes of waste effluents, high operation costs and damage to the resulting fatty acids (Senanayake, 2000). Enzymatic methods such as the use of lipase in concentrating omega-3 FAs is an alternative green method. Lipase work under mild operating conditions, which make them suitable for the concentration of omega-3 FAs from fish oils. Lipases have been used for many years in lipid modification and food processing. Lipase based concentration involves either cleaving short chain fatty acids from glyceride or selectively hydrolysing EPA and DHA from glyceride. Thraustochytrid represent a group of marine microalgae and a source from which to isolate lipases with novel activities. A novel lipase with selectivity towards EPA and DHA would be useful and no such lipase has been characterised from any source. Thraustochytrid are a potential source of omega-3 specific lipases. A detailed review is given in (Section 1.3.6) on lipase mediated concentration of omega-3 fatty acids.

1.2.4. Microalgae as an alternative source for omega-3 fatty acids

The current commercial source of omega-3 fatty acids for human consumption is marine fish. Although most fish oil comes from anchovy, which is considered a sustainable source, there are still concerns regarding overfishing, particularly for larger fish such as tuna. There are also concerns with the presence of contaminants such as mercury and polychlorinated biphenyls (PCBs), which are harmful to consumers (Seabert et al., 2014). Furthermore, the fish oils are not suitable for vegetarians due to their fish origins. New alternative sources must be found to supply the growing global omega-3 market (Ratledge, 2013). Various alternative sources such as bacteria, fungi, plants and microalgae have been explored for commercial production of single cell oils (SCOs) (Adarme-Vega et al., 2012; Delarue & Guriec, 2014).

Marine microalgae are the primary producer of EPA and DHA in the marine ecosystem. These naturally grow under a variety of culture conditions including autotrophic, mixotrophic and heterotrophic (Buono et al., 2014). Use of microalgae as a source of omega-3 FAs production has several advantages over traditional sources. They can be grown in a controlled environment with readily available substrates in the
fermentation process. They do not compete for land and fresh water and also eliminate the risk of chemical pollutant contamination (Ryckeboesch et al., 2012a). There are a number of algal species with higher EPA and DHA contents. Thraustochytrids species produce elevated quantities of omega-3 FAs with potential commercial application in infant formulas, food, cosmetic and pharmaceutical products (Sijtsma & de Swaaf, 2004).

1.3. Thraustochytrids

Thraustochytrids are marine heterotrophic fungi like microorganisms, widely distributed in a variety of habitats such as mangrove detritus, rocky shores, coral reefs, salt marshes, sandy sediment, coastal waters, and deep sea (Liu et al., 2014a; Raghukumar & Damare, 2011). The lipid content of thraustochytrids ranges from 50–77% on a dry weight basis with more than 90% as triacylglycerides. Thraustochytrids have recently emerged as a potential alternative source for the production of omega-3 fatty acids, particularly for DHA (Lee Chang et al., 2014). For example, Lee chang et al, 2013, reported that the DHA percentage of total fatty acid content was more than 40% (w/w) in *Aurantiochytrium* sp. TC20 (Lee Chang et al., 2013). Another study conducted by Ryu et al. (2013), observed that the DHA content of *Aurantiochytrium* sp. KRS101 was 38% (w/w) of total fatty acids (Ryu et al., 2013). These high DHA levels indicate that thraustochytrids are potential sources for commercial DHA production. Some of the important properties of thraustochytrids which could influence successfully commercial scale production of omega-3 fatty acids include high growth rates, a high proportion of DHA/EPA as percentage of total lipids and unaffected growth at low salinity conditions (William et al., 2005).

Thraustochytrids are fungi like microorganisms, but they are classified under the Labyrinthulomycetes, because ultrastructurally, they are closely related to heterokont algae (Honda et al., 1999). This phylum contains 8 genera: *Thraustochytrium, Schizochytrium, Ulkenia, Labyrinthuloides, Labyrinthula, Japonochytrium, Aplanochytrium* and *Althornia*. Thraustochytrium and Schizochytrium have a rapid growth rate and capable of producing large amount of DHA (Azevedo & Corral, 1997; Wong et al., 2008). Microscopic image of thraustochytrids used in this study are presented in Fig. 1.2.
Many strains available today for commercial production of EPA and DHA are the result of intensive procedures such as collection, isolation and screening. Extensive screening and characterisation of thraustochytrid from a wide variety of samples led to identification of thraustochytrids that can accumulate about 50% of total lipid, with more than 25% of DHA (Raghukumar, 2008). Thraustochytrids are typically abundant in marine sediments where large amounts of decaying organic matter accumulate, resulting in enzymatic degradation (Lucia & Fernando, 2002). Many studies have focused on the isolation and characterisation of thraustochytrids from various marine habitats such as oceanic and coastal marine environments (Burja et al., 2006; Yang et
Chapter 1

al., 2010) as well as low temperature environments (Zhou et al., 2010) and the colonial tunicate *Botryllus schlosseri* (Rabinowitz et al., 2006). There are two important techniques used for the isolation of thraustochytrids from marine samples, direct plating and pollen baiting. Direct plating techniques involve the spreading of samples on agar plates under sterilised conditions (Bremer, 2000). Pollen baiting is the most common method for isolation of thraustochytrids by which, sterilised pollen grains are spread in marine samples with anti-bacterial and fungal agents and incubated for 1-2 weeks. Thraustochytrids cell growth can be observed on the surface of the pollen grain, after which follows isolation of pure cultures on selective agar medium (Wilkens & Maas, 2012).

1.3.1. Potential of Thraustochytrids

Currently the most common microalgae used for commercial production of omega-3 fatty acids are marine derived, particularly from family members of *Thraustochytriaceae* and *Crypthecodiniaceae*. Thraustochytrids are known to produce several commercially interesting biotechnological compounds such as omega-3 fatty acids, carotenoids, sterols, steroids and surfactants and also enzymes and exopolysaccharides (Fan & Chen, 2007; Gupta et al., 2012a). The company DSM commercially produces DHA rich oil Life’s DHA™ using a *Schizochytrium* sp that accumulate about 40-45% of DHA and 2% of EPA, for the food, beverage and supplement industries. DSM uses a different strain of *Schizochytrium*, which produce DHA and EPA rich oils in 2:1 ratio, to compete with anchovy fish oil that generally has a higher EPA than DHA level. The trade name of this oil is Life’s Omega™, and it contains 24% DHA and 12% EPA (Winwood, 2013).

Carotenoids are commonly used as a food colouring agent, particularly astaxanthin that is used in aquaculture for salmon coloration. Carotenoids produced either synthetically or derived from microbial production. Production of carotenoids from microbial fermentation is an attractive area of research interest, as carotenoids have been found to have protective activity against cancer, aging, ulcers, heart attack, and coronary artery disease (Rao & Rao, 2007).

1.3.2. Strain selection and sustainable lipid production

Screening of high lipid producing microalgae is a key step for toward the success of large scale cultivation and biodiesel production from microalgae. These
microalgae are specific to particular environments and certain climate conditions (Brennan & Owende, 2010; Williams & Laurens, 2010). These organisms occur in various environments such as freshwater, lacustrine, brackish, marine, maturation ponds and hyper-saline conditions (Mutanda et al., 2011). During the selection of suitable microalgae for lipid production, organisms which have high biomass productivity and lipid accumulation are preferred for mass cultivation than high lipid storage with low productivity (Amaro et al., 2011; Mata et al., 2010). In addition, there are other factors which have to be considered such as lipid profile and the capacity to grow under specific conditions. Some microalgae produce fatty acids, making them suitable for biodiesel production, whereas others, such as polyunsaturated fatty acids, are not suitable because their unsaturation bonds are more prone to oxidative degradation (Chisti, 2007). Biodiesel production depends on lipid profiles, which can be regulated by the nutritional supply, processing and growth conditions (Amaro et al., 2011). In some microalgal species, lipid accumulation is maximum during the stationary phase. This can be induced by limiting one of the growth controlling nutrients such as nitrogen, phosphorous and silicon (Guschina & Harwood, 2006). Microalgae accumulate more lipid when subjected to stress conditions and by limiting one of the growth nutrients, which enhances the production of lipid in microalgae (Brennan & Owende, 2010; Pruvost et al., 2011).

In addition to bioprospecting for high lipid producing microalgae, finding a cheaper raw materials for lipid production can reduce production cost. Researchers have used molasses, crude glycerol and agricultural waste as a carbon source for lipid production from microorganisms (Karata & Dönmez, 2010).

1.3.3. Estimation of lipid content and fatty acid profile

Conventional lipid quantification methods involve microalgal biomass being exposed to extraction solvents that extract lipids from the microalgal cellular matrix. The whole mixture is centrifuged to separate the lipid layer from water and cell debris, and the solvent evaporated. The microalgal crude oil thereby obtained is measured gravimetrically and calculated as a percentage of dry biomass. This method only gives the amount of lipid present in microalgae cells and not the lipid profile. To analyse the lipid profile, obtained crude oil is subjected to transesterification process to convert microalgal lipids to fatty acid methyl esters (FAMEs). The lipid composition and quantities can be determined using gas chromatography (Bendikiene et al., 2011; Vyas
et al., 2010). This method provides accurate quantity of lipid weight, but requires many chemicals and multi-step process. In particular, this method is not favourable in screening microalgae strains for high lipid production.

### 1.3.3.1. Rapid quantification of lipids

Microalgae strain selection depends on biomass and lipid productivity since these are the key factors involved in economical biodiesel production. These factors are critical to achieve, because the conditions favour high biomass may not favour lipid accumulation, and vice versa (Huo et al., 2011). The biomass and lipid production, particularly triacylglycerols, can be modulated by varying the culture conditions such as nutrient limitation. In addition metabolic engineering can be applied as a relatively new strategy for enhancing lipid accumulation in different microbial species (Liang & Jiang, 2013). Therefore, a rapid and reliable lipid quantification technique is required in identifying potential lipid producing strain from environmental samples and screening large numbers of mutants. Traditional methods for lipid quantification from microalgae are labour intensive, require solvents and treatment steps (Wawrik & Harriman, 2010).

Staining of intracellular lipid using fluorescent lipophilic dyes, such as Nile red for lipid quantification, popularly used for the dyes affinity towards neutral lipids (Han et al., 2011; Rumin et al., 2015). Lipid content has been successfully quantified in certain microalgae using Nile red dye, but unsuccessful with other microalgae because of their thick cell wall that prevent the penetration of the dye. At elevated temperature, Chen et al (2009) assessed the effectiveness of different solvents as a stain carrier and found DMSO as good stain carrier for the species *Chlorella vulgaris*. Using this optimised method, they successfully quantified lipids in microalgae using 96 well plates (Chen et al., 2009). In a different study by Chen et al (2011), both conventional and improved Nile red methods were ineffective in quantifying the lipid content in green algae such as *Pseudochlorococcum* sp. and *Scenedesmus dimorphus*. This problem was solved by treating the samples with microwaves for 50-60 seconds prior to lipid quantification (Chen et al., 2011). Several studies have successfully quantified the lipid content in various microalgae using Nile red methods (Bertozzini et al., 2011; Gerde et al., 2012; Kou et al., 2013; Satpati & Pal, 2014).

Colorimetric quantification of lipids is achieved by sulfo-phospho-vanillin methods (SPV). The SPV method was initially introduced by Chabrol and Charonnat
Chapter 1

(1973) to quantify the total serum lipids in humans (B. Chabrol, 1937; Vatassery et al., 1981). This method was further improved by Frings and Dunn (1970) and is still widely used for serum lipid quantification.

1.4. Extraction of lipids from dry cell biomass

Marine microalgae are single-celled microorganisms with potential industrial applications: as a source of aquaculture feedstock and production of valuable bioactive compounds such as lipids, carotenoids and enzymes (Amin, 2009; Cao et al., 2013; Mercer & Armenta, 2011). Lipid extraction is the most costly and critical step in biodiesel production. Generally, organic solvent extraction method is most commonly used as it is well established (Bligh & Dyer, 1959; Folch et al., 1957). However, effective extraction of total lipids from microalgae is not possible due to the rigid cell wall. In case of slow extraction due to the presence of strong cell wall, it is advised to perform an extraction dynamic study in order to improve extraction efficiency (Cho et al., 2012a). The most commonly used organic solvents are benzene, hexane, acetone and chloroform and these have shown to be effective for lipid extraction from microalgae by disrupting their cell walls (Ramluckan et al., 2014). Selection of the solvent for lipid extraction should take into account its ability to enter into the algal cells efficiently and match the polarity of the desired compounds (for instance, hexane for non-polar lipids). Physical contact with solvent and lipid material can be achieved by mechanically disrupting the microalgal cells and adding the solvent later (Li et al., 2014b). The effect of cell disruption in lipid extraction from microalgae has been reported and shown to significant improve lipid extraction yields (dos Santos et al., 2015; Grimi et al., 2014; Halim et al., 2013; Lee et al., 2010b; Yap et al., 2014).

1.4.1. Selection of suitable extraction solvents

In order to optimise lipid extraction yields solvent selection is important. Chloroform / methanol (1: 2 v/v) is the most commonly used and reliable organic solvent mixture for total lipid extraction from microalgae (Bligh & Dyer, 1959). Using this organic solvent system, residual endogenous water in the microalgal cells acts as a ternary component that enables the complete extraction of both neutral and polar lipids. This method does not require the complete drying of microalgal biomass. Once the cell debris is removed, more chloroform and water are added to induce biphasic partitioning. The lower organic phase (chloroform) contains most of the lipids (both
neutral and polar) while the upper aqueous phase (water with methanol) constitutes most of the non-lipids (proteins and carbohydrates) (Medina et al., 1998). The chloroform and methanol lipid extraction process is fast and quantitative. However, the use of chloroform associated with health, security, and regulatory problems, and as such, its usage is undesirable. Folch et al, originally developed chloroform and methanol lipid extraction procedure to extract lipids from brain tissue (Folch et al., 1951). The lipid extraction yield depends on the solvent mixture used in the extraction process. As a result, its efficiency in lipid extraction from microalgal biomass need to be further investigated.

Jeon et al, examined 15 different solvents and analysed their efficiency in lipid extraction from *Chlorella vulgaris* biomass. Lipid extraction yields increased to 25% with methanol:dichloromethane as a solvent mixture compared with other solvents used (Jeon et al., 2013). Mandal et al, also studied the effect of different solvents and their combinations in lipid recovery from *Scenedesmus obliquus*. Around 13% of lipid extracted using chloroform and methanol (2:1) solvent (Mandal et al., 2013). Ramluckan et al, investigated the efficiency of 13 solvents and their combinations with a range of polarities and solubilities. The greatest lipid extraction efficiency (11.76) was obtained upon using 1:1 mixture of chloroform:ethanol (Ramluckan et al., 2014). Table 1.3 summarises the recent studies investigating the effect of various solvents on lipid extraction.

Table 1.3. Recent studies investigating the use of organic solvent in the extraction of microalgal lipids.
<table>
<thead>
<tr>
<th>Marine Microorganisms</th>
<th>Dried biomass (g)</th>
<th>Organic solvents v/v</th>
<th>Reaction conditions</th>
<th>Wt% of total extracted lipids</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Schizochytrium</em> S31</td>
<td>0.05</td>
<td>Chloroform/ethanol (2:1)</td>
<td>NA</td>
<td>22</td>
<td>(Byreddy et al., 2015)</td>
</tr>
<tr>
<td><em>Chlorella</em> sp.</td>
<td>1</td>
<td>Chloroform/ethanol (1:1)</td>
<td>3 h/NA/boiling point</td>
<td>11.76</td>
<td>(Ramlucken et al., 2014)</td>
</tr>
<tr>
<td><em>Scenedesmus obliquus</em></td>
<td>1</td>
<td>Chloroform/ethanol (2:1)</td>
<td>NA/NA/room temperature</td>
<td>12.9</td>
<td>(Mandal et al., 2013)</td>
</tr>
<tr>
<td><em>Nannochloropsis gaditana</em></td>
<td>0.05</td>
<td>N,N-dimethylcyclohexylamine</td>
<td>24 h/stirring/room temperature</td>
<td>29.2</td>
<td>57.9</td>
</tr>
<tr>
<td><em>Tetraselmis suecica</em></td>
<td></td>
<td>50 mg/ml (Biomass/solvent)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Desmodesmus communis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Chlorella vulgaris</em></td>
<td>50</td>
<td>dichloromethane:methanol (1:1)</td>
<td>1 h/stirring/37 °C</td>
<td>25</td>
<td>(Jeon et al., 2013)</td>
</tr>
<tr>
<td><em>Arthrospira platensis</em></td>
<td>1</td>
<td>chloroform/methanol (2:1)</td>
<td>NA</td>
<td>20</td>
<td>(Baunillo et al., 2012)</td>
</tr>
<tr>
<td><em>Pavlova</em> sp</td>
<td>10</td>
<td>Water/methanol/ethyl acetate (10:24:48)</td>
<td>NS</td>
<td>44.7</td>
<td>(Cheng et al., 2011a)</td>
</tr>
<tr>
<td><em>Chlorococcum</em> sp</td>
<td>4</td>
<td>Hexane, hexane/isopropanol (3:2)</td>
<td>450/800/25</td>
<td>6.8</td>
<td>(Halim et al., 2011)</td>
</tr>
<tr>
<td><em>Synechocystis PCC 6803</em></td>
<td>15</td>
<td>Chloroform/methanol/water (1:2:0.8)</td>
<td>24 h/stirring/room temperature</td>
<td>4.2</td>
<td>(Sheng et al., 2011b)</td>
</tr>
<tr>
<td><em>Schizochytrium limacinum</em></td>
<td>1</td>
<td>Water/chloroform/methanol (4:6:12)</td>
<td>NA</td>
<td>57</td>
<td>(Johnson &amp; Wen, 2009)</td>
</tr>
<tr>
<td>Species</td>
<td>Concentration</td>
<td>Solvent System</td>
<td>Yield</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td><em>Aurantiochytrium</em> sp.</td>
<td>28-32</td>
<td>Methanol/chloroform/water (2:2:1)</td>
<td>55</td>
<td>(Jakobsen et al., 2008)</td>
<td></td>
</tr>
<tr>
<td>strain T66</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanol/chloroform/water</td>
<td>28.6</td>
<td>(Lee et al., 1998)</td>
<td></td>
</tr>
<tr>
<td><em>Phaeodactylum</em> tricornutum</td>
<td>10</td>
<td>Ethanol</td>
<td>6.3</td>
<td>(Fajardo et al., 2007)</td>
<td></td>
</tr>
<tr>
<td><em>S. mangrovei</em> IAo-1</td>
<td>100-200</td>
<td>Chloroform/methanol (2:1)</td>
<td>33.2</td>
<td>(Leano et al., 2003)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soxhlet: methylene chloride:methanol (2:1)</td>
<td>11.9</td>
<td>(Guckert et al., 1988)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Batch 1: chloroform/methanol/50 mM phosphate buffer (35:70:28)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Batch 2: n-hexane/IPA/distilled water (70:47.7:3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Botryococcus</em> braunii</td>
<td>0.12</td>
<td>Chloroform/methanol (2:1), hexane/isopropanol (3:2), dichloroethane/methanol (1:1), dichloroethane/ethanol (1:1), acetone/dichloromethane (1:1)</td>
<td>28.6</td>
<td>(Lee et al., 1998)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soxhlet: 180/NS/NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Batch 1: 2160/NS/NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Batch 2: overnight/NS/NS</td>
<td></td>
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</tr>
</tbody>
</table>

...
1.4.2. Lipid extraction from wet biomass

Drying microalgae biomass prior to lipid extraction accounts for 90% of the total production energy cost in dry algal lipid extraction for biodiesel production. Microalgae biomass drying is an energy intensive process, more than 25% of energy required for this process can be saved by using wet algal biomass (Du et al., 2015). There are few studies on microalgae lipid extraction using wet biomass. Yao et al. investigated the use isopropanol as a potential solvent in extracting lipids from *Nannochloropsis* sp. wet biomass and achieved 70% of lipid extraction (Yao et al., 2012). Dejoye, et al. developed a new environmentally safer method, called simultaneous distillation and extraction process for lipid extraction from wet microalgae *Nannochloropsis oculata* and *Dunaliella salina*. This method used alternative solvents such as d-limonene, α-pinene and p-cymene. The lipid recovery yields were compared with standard soxhlet extraction and Bligh & Dyer method and observed similar lipid yields (Dejoye Tanzi et al., 2013). Yang, et al. proposed the use of ethanol as an organic solvent for lipid extraction from wet microalgae *Picochlorum* sp. This study investigated the effect of extraction temperature and time and the
influence of solvent biomass ratios on extraction efficiency and lipid class, with results indicating that the extracted lipid was comparable with Bligh & Dyer method (Yang et al., 2014). Another recent study confirms the suitability and efficiency of ethanol as an extraction solvent for lipid extraction from some microalgae (Yang et al., 2015). Sathish, et al, developed and modified a lipid extraction procedure from wet microalgae biomass using acid and base hydrolysis. This method was capable of extracting 79% of total transesterified lipids from *Chlorella* and *Scenedesmus* sp. (Sathish et al., 2015; Sathish & Sims, 2012).

Recently, oil extraction from wet biomass and direct biodiesel conversion called direct (in-situ) transesterification, has been investigated (Park et al., 2015). Chen et al, developed a novel method for microalgal biodiesel production using *Chlamydomonas* sp. JSC4 with 68.7 wt% water content as the feedstock. This method involves microwave disruption, partial dewatering (via combination of methanol treatment and low-speed centrifugation), oil extraction, and transesterification without removal of solvent and they achieved 97% biodiesel conversion (Chen et al., 2015a). A recent study by Kim et al, investigated the effect of factors influencing the direct biodiesel conversion from wet *Nannochloropsis salina* biomass. The reaction conditions such as temperature, reaction time, and solvent and acid quantity were optimised and 90% of total conversion was obtained (Kim et al., 2015).

### 1.4.3. Microalgae cell disruption for lipid extraction

A large number of lipid extraction procedures are used for microalgal biomass and their extraction efficiencies have been documented. All microalgae are single cell organisms having an individual cell wall. Furthermore, some species of microalgae have high lipid levels but they are located inside a tough cell wall. The presence of tough cell walls of microalgae increase the lipid extraction cost, since a cell disruption step is required for effective lipid extraction. Cell disruption is an energy consuming process but an important step in liberating intracellular lipid molecules from microalgae (Lee et al., 2012). A number of cell disruption methods are available for lipid extraction from microalgae. Different microalgae have different disruption propensities and as a result, no single method of disruption can be universally applied to the various microalgae species (Halim et al., 2012b).

Cell disruption methods (Figure. 1.3.) generally used at the laboratory scale are classified according to the working procedure by which microalgal cells are disrupted,
for example mechanical and non-mechanical (Chisti & Moo-Young, 1986; Günerken et al., 2015; Middelberg, 1995). Mechanical methods include bead mill, press, high-pressure homogenisation, ultrasonication, autoclave, lyophilisation, and microwave, while non-mechanical methods often involve lysing the microalgal cells with acids, alkalis, enzymes, or osmotic shocks (Chisti & Moo-Young, 1986; Günerken et al., 2015).

![Classification of cell disruption methods](image)

**Figure. 1.3. Classification of cell disruption methods. Modified from (Chisti & Moo-Young, 1986).**

### 1.4.3. Effect of cell disruption methods on lipid extraction

Microalgal cell wall disruption in a non-specific manner is generally achieved by mechanical methods using solid-shear forces. The basic principle of bead milling involves physically grinding the microalgal cells against the solid surfaces of glass beads in a violent agitation (Chisti & Moo-Young, 1986). Some key factors include high throughput process, high biomass loading, good temperature control, easy scale up procedures, low labour intensity and high disruption efficiency, which in combination make bead milling a suitable method of implementing large scale applications (Günerken et al., 2015; Jahanshahi et al., 2002). The method has been used for years to disrupt microorganisms. The size of beads used in the disruption
process is important. The optimal diameter of the beads for bacterial cell disruption is 0.1 mm and 0.5 mm for yeast and other unicellular organisms. The cell disruption efficiency can be further improved by using zirconia-silica, zirconium oxide or titanium carbide made beads. This is because of their hardness and density. In addition, the separation of beads from the agitated solution is easily achieved due to the high density of beads (Lee et al., 2012c).

High pressure homogenisation is an effective and simple cell disruption method. In this process microalgal suspension pumped through narrow orifice of a valve under high pressure. Cell disruption is achieved by high-pressure impact (shear forces) of the accelerated fluid jet on the stationary valve surface as well as hydrodynamic cavitation from the pressure drop induced shear stress (Chisti & Moo-Young, 1986). Some recent studies observed that high working pressure and cycle number have a positive effects on cell disruption efficiency (Halim et al., 2012b). In addition, microalgae biomass concentration and species have major effect on specific energy consumption (Halim et al., 2013). High speed homogenization is a simple and effective method by which a stirring device which can rotate at high rpm with in a stator-rotor assembly. The stator-rotor are usually made of stainless steel with a variety of designs. High speed homogenization achieves cell disruption via hydrodynamic cavitation, generated by stirring at high rpm, and shear forces at the solid-liquid interphase. Hydrodynamic cavitation occurs when the impeller tip reaches approximately 8500 rpm due to the local pressure decreases nearly down to the vapour pressure of the liquid (Gogate & Pandit, 2008; Kumar & Pandit, 1999).

Microalgal cells can be disrupted by an ultrasonication process that is transmission of sonic waves. An ultrasonicator converts the electrical energy into mechanical vibrations. The probe intensifies the mechanical vibrations, resulting in the formation of pressure waves in the liquids. This mechanism forms significant amounts of cavities (bubbles) in the liquid, which expand during the low pressure phase and collapse violently during the high pressure phase. This phenomenon is referred as “cavitation”, which releases pressure and heat. Although the cavitational collapse lasts only for a few microseconds, the cumulative effect of bubbles is to release high levels of energy into the liquid (Chisti & Moo-Young, 1986).

Generally the non-mechanical cell disruption methods involve use of chemicals, enzymes and osmotic shock (Monks et al., 2013). These methods primarily
depend on the interaction between chemicals or enzymes and cell walls of the microalgalae, which modify the outer membrane layer and release the intracellular components. Enzymatic cell disruption is a desirable process due to biological specificity, mild operating conditions, low energy requirements, low capital investment. The use of enzymes normally circumvents the need to use aggressive physical conditions such as high shear stress and elevated temperatures (Choi et al., 2010; Harun & Danquah, 2011). The discovery of the enzyme lysozyme led interest in the application of lytic enzymes in cell lysis. During enzymatic cell lysis, enzymes bind to cell membranes at specific sites and hydrolyse specific bonds leading to degradation of cell membrane (Choonia & Lele, 2013; Fu et al., 2010; McKenzie & White Jr, 1991). A variety of chemical agents such as antibiotics, chelating agents, chaotropes, detergents, solvents, hypochlorites, acids and alkali can cause cell disruption. The efficiency of the cell disruption depends on the selectivity of the chemical agent and composition of the microorganisms (Jalalirad, 2013; Middelberg, 1995).

Lee et al., (2010) assessed the effect of prior mechanical cell disruption on lipid extraction for Botryococcus sp., Chlorella vulgaris, and Scenedesmus sp. using chloroform and methanol (2:1 v/v) as an extraction solvent. Among the methods used (autoclave, bead beating, microwave, sonication, and osmotic shocks), the microwave method was resulted in the higher lipid extraction yields in all three microalgal species. Another study by Zheng et al., (2011) investigated the effect of different mechanical and non-mechanical cell disruption methods for release of lipids from a marine species of Chlorella vulgaris. Overall, grinding in liquid nitrogen was identified as the most effective method in terms of disruption efficiency.

A similar study was conducted by Halim et al, (2012), in which four cell disruption methods were compared to determine their lipid extraction efficiency. Among the four methods (Bead beating, ultrasonication, acid treatment and high-pressure homogenisation), high-pressure homogenisation was the most effective in disrupting Chlorococcum cells. Summary of recent studies on microalgal cell disruption and their lipid extraction efficiencies are given in table. 1.4.
Table 1.4. Extraction of lipids improved by cell disruption methods.

<table>
<thead>
<tr>
<th>Microalgae</th>
<th>Cell disruption method</th>
<th>Maximum lipid yield (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Schizochytrium</em> S31</td>
<td>Osmotic shock</td>
<td>48.7</td>
<td>(Byreddy et al., 2015)</td>
</tr>
<tr>
<td><em>Thraustochytrium</em> AMCQ55-5</td>
<td></td>
<td>29.1</td>
<td></td>
</tr>
<tr>
<td><em>Chlorella vulgaris</em></td>
<td>Ultrasonication</td>
<td>55</td>
<td>(dos Santos et al., 2015)</td>
</tr>
<tr>
<td>Scenedesmus sp</td>
<td>Freeze drying + Microwave</td>
<td>29.6</td>
<td>(Guldhe et al., 2014)</td>
</tr>
<tr>
<td><em>Chlorella vulgaris</em></td>
<td>pressure-assisted ozonation</td>
<td>27</td>
<td>(Huang et al., 2014)</td>
</tr>
<tr>
<td>Mixed culture</td>
<td>Microwaves</td>
<td>33.7</td>
<td>(Florentino de Souza Silva et al., 2014)</td>
</tr>
<tr>
<td><em>Chlorella vulgaris</em></td>
<td>H₂O₂ + FeSO₄</td>
<td>17.34</td>
<td>(Steriti et al., 2014)</td>
</tr>
<tr>
<td>Nannochloropsis sp</td>
<td>Microwave</td>
<td>38.3</td>
<td>(Wahidin et al., 2014)</td>
</tr>
<tr>
<td><em>C. vulgaris</em></td>
<td>Ultrasonication</td>
<td>52.5</td>
<td>(Araujo et al., 2013)</td>
</tr>
<tr>
<td>Chlamydomonas reinhardtii</td>
<td>Osmotic shock</td>
<td>NA</td>
<td>(Yoo et al., 2012b)</td>
</tr>
<tr>
<td><em>Microcystis sp</em></td>
<td>Sonication</td>
<td>53.89</td>
<td>(Supriya &amp; Ramachandra, 2012)</td>
</tr>
<tr>
<td><em>C. vulgaris</em> (SAG 211-12)</td>
<td>Grinding+microwaves+ sonication</td>
<td>9.82</td>
<td>(Sostaric et al., 2012)</td>
</tr>
<tr>
<td><em>Synechocystis</em> PCC 6803</td>
<td>Microwave+pulsed electric fields</td>
<td>9-13</td>
<td>(Sheng et al., 2012)</td>
</tr>
<tr>
<td><em>Chlorella vulgaris</em></td>
<td>Microwaves</td>
<td>NA</td>
<td>(Prommuak et al., 2012)</td>
</tr>
</tbody>
</table>
1.5. Lipases

Lipase have emerged as an important biocatalyst with potential applications in many industries including organic synthesis, paper manufacturing, oleochemistry, dairy, cosmetics, perfume, biosensors, and detergents (Hasan et al., 2006). Lipases catalyse the breakdown of fats into free fatty acids and glycerol at the water-oil interface. Lipase also synthesise ester from free fatty acids and glycerol at the hydrophobic interface. Lipases often exhibit high chemo-, region- and enantioselectivity at different conditions contributing to their utility as industrial biocatalysts (Gotor-Fernández et al., 2006).

1.5.1. Marine lipases

The marine ecosystem and marine microorganisms in particular represents an enormous pool of potential enzyme candidates for industrial and biotechnological applications. Marine microbes are an important source of bioactive compounds (Soliev et al., 2011). The demand for the novel enzymes is also increasing, due to their utility in the production of chemically and structurally important molecules. Marine microorganisms are a potential source for the isolation of enzymes with novel properties, because of their stability and their potential for wider applications than
those of enzymes isolated from plant or animal sources (Bull et al., 2000; Lam, 2006). The potential demand for enzymes in industrial applications is increasing every year. Marine environments provide unique genetic structures and life habitats such as nutrient rich regions, nutrient limited regions, high salinity, high pressure, low temperature and special light conditions. Microorganisms must adapt to survive in the marine environment and so new biodiversity is created (Kennedy et al., 2008; Zhang & Kim, 2010).

Lipases are found in all forms of life, includes bacteria, fungi, plants and animals (Wang et al., 2008). Microorganisms are potent sources of enzymes by comparison with plants and animals, because of the availability of their wide variety of catalytic activities, the high yields possible, relative ease of genetic manipulation, regular supply due to the absence of seasonal fluctuations and rapid growth of microorganisms (Iftikhar et al., 2010). Microbial lipases are normally more stable than the other lipase sources which makes them more suitable for industrial applications (Peng et al., 2014). Fungi are the most studied source for the production of commercial lipases. Some of the common lipase producing fungi belong to the following genus, *Rhizopus* sp., *Aspergillus* sp., *Penicillium* sp., *Geotrichum* sp., *Mucor* sp., and *Rhizomucor* sp. *Candida antarctica* and *candida rugosa* lipases are most extensively studied fungal species (Gopinath et al., 2013; Singh & Mukhopadhyay, 2012). Lipase production depends on the carbon source, nitrogen source, pH and temperature of the growth medium (Cihangir & Sarikaya, 2004). Unusual lipase producing microorganisms can be isolated from the extreme environments, such as sites contaminated with oil, dairy waste, vegetable oil waste and industrial waste (Sztajer et al., 1988).

1.5.2. Isolation of lipase producing microbes and screening methods

The increasing demand for lipases in industrial applications has led the identification of novel lipases with unique properties. Screening for specific lipase activity and new lipases is facilitated by the development of rapid, reliable, specific, selective, and sensitive analytical methods for evaluating their catalytic activity (Stoytcheva et al., 2012). Quantifying lipase activity can be challenging since lipases are water soluble enzymes but act on water insoluble substrates (Kanchana et al., 2011). Methods based on using chromogenic and fluorogenic substrate are amongst the simplest and most widely used methods for screening for lipase activity (Hasan et
A preliminary plate method is normally used in the initial screening for lipase producing strains. The medium in the agar plates are supplemented with lipid substrate (olive oil or tributyrin) and microorganisms are streaked on agar plate and periodically observed (Singh et al., 2006). Microbes that are producing lipase can make a halo in the plate and then these strains are selected for lipase production in the liquid medium (Cardenas et al., 2001; Hasan et al., 2009).

Lipases hydrolyse ester bonds and liberate free fatty acids that can be titrimetrically measured by neutralizing the released free fatty acids by manually adding NaOH or by a pH stat method (Borkar et al., 2009). In spectrophotometric methods, substrate are designed to give a colour compound upon enzyme hydrolysis. Lipase activity can be measured in microbial culture supernatant by using p-nitrophenyl esters of various chain lengths. The lipolytic activity on carboxylic ester leads to the release of yellow coloured p-nitrophenol that can be monitored quantitatively by spectrophotometry (Winkler & Stuckmann, 1979). Spectrofluorometric methods are rapid, simple and sensitive. Substrates used in this method are acylglycerol analogs coated with fluorophores such as dansyl, resorufin, or 4-methylumbelliferone (MUF). These analogues are released by lipolytic activity, which can be measured fluorometrically (Li et al., 2014a).

Chromatographic techniques can be used to measure lipase activity by quantifying the released free fatty acids from triacylglycerols. Most general techniques use thin-layer chromatography, gas-liquid chromatography, HPLC or gas chromatography- mass spectrometry (Hao et al., 2007; Stoytcheva et al., 2012). Lipase activity estimation by HPLC was developed by Maurich et al 1999, using beta-naphthyl or para nitrophenol esters. Substrate were incubated with the enzymes and released naphthol was quantified by reverse phase HPLC. Gas chromatography is used to determine the products released by the enzymatic reaction (Stoytcheva et al., 2012).

Since lipases are inducible enzymes, the medium composition, particularly the carbon source, influences lipase production. Most microbial lipases are extracellular lipases. Lipase production can be induced by adding lipase substrates into the media, such as triacylglycerols, fatty acids, hydrolysable esters, tweens, bile salts, and glycerol (Gupta et al., 2004). In addition, the nitrogen source and some important micronutrients also play a crucial role in the production of lipases. Lipase production levels can be increased by the optimisation of culture condition, generally optimised by changing
one variable at a time while keeping other variables constant. However, this systematic approach is time consuming and doesn’t effectively explore the interaction between variables. A more versatile approach is the Plackett-Burman (PB) method, which is an efficient and widely accepted approach for the optimisation of multiple culture variables (Mazzucotelli et al., 2015).

1.5.3. Extraction of intracellular lipases

Enzymes that are synthesized and remained inside the cells are called intracellular enzymes. To extract intracellular enzymes, the cells have to be broken by mechanical or non-mechanical methods to release the products. Some cell disruption methods are sonication, homogenisation, bead beating, French press, or chemical and enzymatic methods (Safi et al., 2014b). Jermsuntiea et al, extracted the intracellular lipase from Mortierella alliacea by homogenising with glass beads and investigated its substrate selectivity. The novel lipase preferentially hydrolysing monoacylglycerols containing long-chain unsaturated fatty acids and phosphatidylcholine (Jermsuntiea et al., 2011). Perez et al, isolated and purified a novel halophilic lipase from Marinobacter lipolyticus SM19 by ultrasonic treatment. This lipase was employed for hydrolysing fish oil and preferentially hydrolysed EPA from triglycerides over DHA (Pérez et al., 2011).

1.5.2. Lipase based methods for concentration of omega-3 fatty acids

Lipases are able to catalyse unique reactions such as hydrolysis, ethanolysis or transesterification of triglycerides. Fatty acids are naturally distributed in the glyceride ‘backbone’ of several marine oils, and these unique properties of lipases are very useful both in omega-3 concentration from fish oil and for the production of structured lipids (Ando et al., 1992; Tengku-Rozaina & Birch, 2014). Lipase based methods can improve the quality of fish oil concentrates by causing less damage to EPA and DHA during processing, which impacts the sensory quality and stability of the final products (Venkateshwarlu et al., 2004). EPA and DHA have multiple double bonds in the cis-configuration and these are very prone to oxidation, polymerization or isomerisation. Concentrated omega-3 oils can contain high levels of products such as polymers and trans fatty acids due to oxidative degradation (Kralovec et al., 2010). An effective way of concentrating omega-3 fatty acids (EPA and DHA) is selective hydrolysis or esterification of shorter chain fatty acids and their separation from the omega-3 fatty acids.
Chapter 1

acid enriched glycerides (Carvalho et al., 2009; Kralovec et al., 2010). Many commercial lipases available from fungi and bacteria discriminate against omega-3 fatty acids in that they preferentially hydrolyse shorter chain fats (Haraldsson, 1999). Saturated and monounsaturated fatty acids are partially selectively cleaved from triglycerides and long-chain omega-3 fatty acids primarily remain in the glyceride portion as residual mono-, di- and triacylglycerols. The resistance of omega-3 fatty acids to lipase hydrolysis by lipases is related to the presence of cis double bonds that causes steric hindrance and results in bending of the fatty acid chains, which brings the terminal methyl groups close to the ester bond (Okada & Morrissey, 2007). A more direct approach is selective hydrolysis of omega-3 fatty acids. However, no omega-3 selective lipases have been characterised, although fish digestive juice may contain such a lipase (Halldorsson et al., 2004). A more recent study also observed the presence of a lipase with preferential EPA and DHA selectivity from fish waste (Kurtovic & Marshall, 2013). A novel lipase isolated from the halophilic bacterium Marinobacter lipolyticus SM19 showed high efficiency in the enrichment of EPA from fish oil. These results indicate that selective concentration of omega-3 fatty acids from marine oils using lipases is possible through selective EPA and/or DHA hydrolysis.

Table 1.5. Main patents relating production of omega-3 fatty acids processing using lipases from the last 10 years.

<table>
<thead>
<tr>
<th>S. NO</th>
<th>Year</th>
<th>Title</th>
<th>Patent Number</th>
<th>Inventors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2015</td>
<td>Separation of omega-3 fatty acids</td>
<td>WO2015024055 A1</td>
<td>Colin James Barrow, Taiwo Olusesan Akanbi</td>
</tr>
<tr>
<td>3</td>
<td>2013</td>
<td>Process for production of oil or fat containing highly</td>
<td>EP 2578691 A1</td>
<td>Hideo Ikemoto, Nobushige Doisaki, Yasuo Umehara</td>
</tr>
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</table>
unsaturated fatty acid using lipase

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<tr>
<td>4</td>
<td>2013</td>
<td>Process for separating polyunsaturated fatty acids from long chain unsaturated or less saturated fatty acids</td>
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<tr>
<td></td>
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<td>EP 2585570 A1 Gudmundur G. Haraldsson, Bjorn Kristinsson</td>
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<tr>
<td>5</td>
<td>2013</td>
<td>Method for enrichment of eicosapentaenoic acid and docosahexaenoic acid in source oils</td>
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<tr>
<td></td>
<td></td>
<td>WO 2013043641 A1 Subramaniam Sathivel, Huaixia Yin</td>
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<td>6</td>
<td>2012</td>
<td>Enrichment of marine oils with omega-3 polyunsaturated fatty acids by lipase-catalysed hydrolysis</td>
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<tr>
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<td></td>
<td>WO2012087153 A1 Kahveci Derya, Xu Xuebing</td>
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<tr>
<td>7</td>
<td>2011</td>
<td>High-Purity Purification Method for Omega-3 Highly Unsaturated Fatty Acids</td>
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<td></td>
<td></td>
<td>US 20110091947 A1 Gap Jin Kim, Hong Joo Son, Wu Song Whang, Yoon Mo Koo, Jin Il Kim, Jin Hyo Yang</td>
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<tr>
<td>8</td>
<td>2011</td>
<td>Processes to generate compositions of enriched fatty acids</td>
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<tr>
<td></td>
<td></td>
<td>WO 2011067666 A1 Inge Bruheim, Svein Rye</td>
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<tbody>
<tr>
<td>9</td>
<td>2011</td>
<td>Method for producing oil containing polyunsaturated fatty acid using lipase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA 2800674 A1 Hideo Ikemoto, Nobushige Doisaki, Yasuo Umehara</td>
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<tbody>
<tr>
<td>10</td>
<td>2010</td>
<td>Triglycerides with high content of unsaturated fatty acids</td>
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<td></td>
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<td>WO 2010115764 A2 Per Munk Nielsen, Jesper Brask, Steffen Ernst</td>
</tr>
</tbody>
</table>
Chapter 1

11 2010 | A polyunsaturated fatty acid (PUFA) enriched marine oil comprising eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and a process of production thereof | EP 2147088 A1 | Patrick Adlercreutz, Ann-Marie Lyberg

12 2010 | Production method of oil or fat containing polyunsaturated fatty acid-containing triglyceride | US 7767427 B2 | Kengo Akimoto, Motoo Sumida, Kenichi Higashiyama, Shigeaki Fujikawa

13 2010 | A polyunsaturated fatty acid (PUFA) enriched marine oil comprising eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and a process of production thereof | EP 2147088 A1 | Patrick Adlercreutz, Ann-Marie Lyberg


16 2009 | Method for production of condensed polyunsaturated fatty acid oil | EP 2006389 A9 | Nobushige Doisaki, Kiyomi Furihata, Hiroyuki Kawahara
There are numerous research articles and patents published in relation to the production of omega-3 fatty acids from marine oils using lipases. Table 1.6 summarises the patents filed in the last ten years on the production of omega-3 fatty acids using lipase catalysed reaction. Linder et al, hydrolysed salmon oil using a sn-1, 3 specific lipase to concentrate PUFA 39.20 mol% to 43.05 mol% (Linder et al., 2002). Another study by Gámez-Meza et al. 2003, investigated the enzymatic hydrolysis of sardine oil and concentration using urea complexation. They also compared the effect *Pseudomonas* lipase hydrolysis with chemical hydrolysis using KOH, ethanol and hexane. This study concluded that the immobilized lipase with 10% protein was the most effective system by which to achieve the best purity of omega-3 fatty acids (86.58%) with an acceptable yield (78.0%). Although chemical hydrolysis yielded higher omega-3 recovery (90.5%), the purity was lower (83.13%). Moreover, the lipase based method resulted in easier separation of the products using a packed-bed reactor (Gámez-Meza et al., 2003). A recent study optimised the hydrolysis of anchovy fish oil and the concentration of omega-3 fatty acids using TL 100 lipase, from which 24% DHA was concentrated in the glycerol portion under optimised conditions (Akanbi et al., 2013). Kahveci and Xu, reported the repeated hydrolysis of salmon fish oil after distillation using *Candida rugosa* lipases in a 1 L stirred tank reactor resulted in PUFA content of 38.7mol%, double of the initial level (Kahveci & Xu, 2011). These approaches show the potential of lipase based methods to improve the nutritional value of marine oils. Compared to existing chemical processes, lipase based approaches are more environmentally friendly. Further research is required to obtain more selective lipases and to optimise suitable production and immobilisation methods.
1.6. Research aim and objectives

This research was focused on downstream processing of lipid and lipase from thraustochytrids for the concentration of omega-3 fatty acids. The main objectives were:

1. To establish a rapid lipid quantification method for the screening and identification of thraustochytrid strains from marine samples collected from Queenscliff, Victoria.
2. To determine which enzymatic activities are present in these thraustochytrid in house isolates.
3. To develop methods for the extraction of total lipids and lipases from selected thraustochytrids.
4. To investigate lipase production from thraustochytrid in a stirred tank reactor.
5. To develop a novel enzymatic method for concentrating omega-3 fatty acids from marine oils, including thraustochytrid oils.
Chapter 2: A quick colorimetric method for total lipid quantification in thraustochytrids
3.1. Introduction

Biodiesel is a renewable fuel of commercial interest, driven in part by the rising cost of fossils and negative environmental impacts associated with conventional fuel sources (Sadeghinezhad et al., 2014). First generation biodiesel was produced from biomass, vegetable oils, animal fats and waste oils by transesterification (van Eijck et al., 2014). The use of edible oils for first-generation biodiesel has resulted in the undesired effect of increasing global competition for food crops (Kumar & Sharma, 2015; Pinzi et al., 2014). Second-generation biofuels utilise non-competing crops such as jatropha, karanja, jojoba, mahua as well as the use of waste cooking oil, grease, and animal fats. While the use of “inedible” crops reduces competition with food crops, second-generation biofuels still compete for arable land, which ultimately negatively impacts the world food supply and can contribute to the destruction of soil resources. In addition, these feedstock cannot meet current energy demands (Nautiyal et al., 2014; Zhu et al., 2014). Sustainable alternatives must be found that can provide the large fuel volumes required, while not compromising the use of agricultural land for food production. Microalgae have attracted attention as a means of addressing rapidly growing demand for feedstock in biodiesel production (Baganz et al., 2014; Bellou et al., 2014; Chen et al., 2015b). Microalgae are easy to cultivate and can be grown on non-arable land, as well as accumulate more lipids as a percentage of their biomass than plants (Challagulla et al., 2015).

Production of biodiesel from heterotrophic microalgae requires expensive nutrients, particularly glucose as a carbon source (Slade & Bauen, 2013). Glucose constitutes 80% of the total medium cost and up to a third of total fermentation production costs (Li et al., 2007). To reduce process costs and assist in the commercialisation of biodiesel production from heterotrophic microalgae, sustainable low-cost carbon sources are required. One approach is to couple microalgae cultivation with the use of inexpensive carbon sources such as carbon rich waste water, or use glycerol derived from the biodiesel industry (Di Caprio et al., 2015; Xin et al., 2010). Production costs can also be decreased by discovering new microalgae with improved lipid productivity (Ahmad et al., 2015). An important reason for the slow development in potential microalgae strain isolation and identification is due to lack of quick and high-throughput assays (Cheng et al., 2011b). Existing techniques used for the screening and quantification of microbial lipids include gravimetric, chromatographic
and fluorescence dye methods (Bligh & Dyer, 1959; Han et al., 2011; Ren et al., 2015). Analysis of microbial lipids by gravimetric method is usually effective, but has limitations. The gravimetric method is time-intensive, requires the use of toxic chemicals and utilises relatively high levels of biomass. Spectrophotometric approaches can use a fluorescent dye, Nile Red, for lipid quantification. However, the sensitivity of this method is poor and microbial cell components such as proteins, pigments and some other environmental factors may interfere with lipid quantification using these methods (Cheng et al., 2011b). A disadvantage of using fluorescent dye is it can photo-bleach during exposure to light (Govender et al., 2012). Microbial lipid quantification using triethanol-amine copper salts is an alternative method, however, analyses of fatty acids in the ultraviolet region results without any colour development (Chen & Vaidyanathan, 2012).

Colorimetric sulfo-phospho-vanillin (SPV) method for lipid quantification firstly introduced by Charbo and Charonnat, 1937 for lipid quantification in human cerebrospinal fluid samples (Vatassery et al., 1981). Later, this method was modified for quantification of lipids in various samples include serum, foods and ecological samples (Haskins et al., 2010; Turlo et al., 2010). Van handel modified SPV methods into micro scale for total lipids quantification in single mosquitos (Van Handel, 1985).

Thraustochytrids, marine heterokonts and classified as oleaginous microorganism, have been identified as a potential candidate for feedstock in the heterotrophic production of lipids (Chen et al., 2015d). Extensive screening from marine habitats has led to the discovery of strains which produce more than 50% of their biomass as lipids (Raghukumar, 2008). In this study, we developed a colorimetric sulfo-phospho-vanillin (SPV) method (initially developed for the serum lipids quantification (Charbol & Charonnat, 1937)) for quantifying of lipids in thraustochytrids and other oils containing materials. The accuracy of the SPV method was compared with gravimetric and gas chromatography analysis. This method is useful for rapid screening of microalgal strains for biodiesel production.

### 3.2. Materials and Methods

#### 2.2.1. Chemicals used

All chemicals and medium components used in this study were procured from Sigma-Aldrich, Australia. Instant ocean sea salts were procured from Aquarium
Systems Inc., USA. *Schizochytrium* oil (batch number 11071602J) was obtained from Ocean Nutrition Canada, Canada.

### 2.2.2. Cultivation of thraustochytrids

A total of four thraustochytrids, *Schizochytrium* sp. S31 (ATCC 20888), *Thraustochytrium* sp. PRA 296, *Thraustochytrium* sp. AMCQS5-5 and *Schizochytrium* sp. DT3 (*in-house* isolates) were selected for the current study. *Thraustochytrium* S31 and PRA 296 were procured from the ATCC and used as standard cultures, whereas AMCQS5-5 (JX993841) and DT3 (KF682125) were isolated from Australian marine samples (Gupta et al., 2013). Seed culture was prepared in GYP medium containing: glucose 0.5 %, yeast extract 0.2 %, peptone 0.2 % in 50 % artificial seawater (Instant Ocean sea salts) and incubated at 150 rpm, 25 °C for 48 h. The inoculum (5 %) was later transferred into production media containing glycerol (1 %), yeast extract (0.2 %) and peptone (0.2 %) and incubated at 150 rpm, 25 °C for 120 h. The cultures were harvested by centrifugation at 8000 rpm for 10 min, freeze dried and stored at -20 °C for further use.

#### 2.2.3. Standard curve preparation

For preparing a standard curve, commercial *Schizochytrium* oil was dissolved in chloroform (10 mg in 10 ml) to a final concentration 1 mg mL⁻¹. For the preparation of the standard curve, different concentrations (15-90 μg) of lipid samples were taken in clean glass tubes. The tubes were incubated at 60 °C for 10 min to evaporate the chloroform. 100 μl of distilled water was added to each tube. Lipid quantification was done by SPV reaction.

#### 2.2.4. Quantification of thraustochytrid lipids by SPV

Vanillin phosphoric acid reagent was prepared by dissolving 0.120 g of vanillin in 20 ml of distilled water and the final volume was adjusted to 100 ml with 85 % phosphoric acid. This reagent was stored in the dark until further use. SPV reagent was prepared fresh, which resulted in high activity with lipid samples. A known amount of thraustochytrid dried biomass was suspended in distilled water and added to each test tube (0.2-1 mg). Tubes were incubated at 100 °C for 10 min and cooled for 5 min in an ice bath. Vanillin-phosphoric acid reagent (5 ml) was added to each tube and
incubated in a shaker incubator at 200 rpm at 37 °C for 15 min. Absorbance was measured at 530 nm.

The major steps involved in this method are: (a) phospho-vanillin reagent preparation using phosphoric acid and vanillin, (b) addition of concentrated sulfuric acid to a sample that contains lipids of interest, and heating of the mixture, (c) addition of phospho-vanillin reagent, and (d) measurement of absorbance at 530 nm (Knight et al., 1972). Fig. 2.1 illustrates in details the steps involved in the SPV method.

Figure 2.1. Schematic diagram of SPV method. Concentrated sulphuric reacts with unsaturated lipids microalgae and forms a carbonium ion. Phosphoric acid and vanillin reacts and form a phosphate ester, which increases the reactivity of carbonyl group. Carbonium ion and carbonyl group (from phospho vanillin reaction) reaction results in formation of a stable colour compound.

2.2.5. Lipid extraction

Lipid was extracted and quantified from freeze dried biomass every 48 h according to a previously published protocol (Lewis et al., 2000) with some
modifications. Freeze-dried biomass (10 mg) was added to 600 μl solvent (chloroform and methanol in 2:1 ratio) and the sample vortexed for 2 min, followed by centrifugation at 13,000 rpm for 15 min. This extraction process was repeated three times. The three supernatants were collected and dried at 50 °C. The lipid percentage was measured gravimetrically.

All the experiments were performed in triplicates. Results are represented as mean values and standard deviation is < 10%.

2.3. Results and discussion

2.3.1. Biomass and lipid production

Production of biodiesel from inexpensive carbon sources will reduce oil production costs. Glycerol is an economical carbon sources for microbial fermentation (Leite et al., 2015) and has been tested as a carbon source for growing thraustochytrids (Gupta et al., 2013; Scott et al., 2011). After 5 days, the biomass obtained were 938 mg L⁻¹ d⁻¹ in DT3, 950 mg L⁻¹ d⁻¹ in S31, 740 mg L⁻¹ d⁻¹ in AMCQS5-5 and 256 mg L⁻¹ d⁻¹ in PRA-296 (Table 1). *Schizochytrium* sp. S31 and DT3 produced similar biomass yields, whereas *Thraustochytrium* sp. AMCQS5-5 and PRA-296 exhibited lower biomass productivity, depending on how efficiently the strain utilised the carbon source to produce biomass. However, lipid productivity was documented to be higher in DT3 at 287.96 mg L⁻¹ d⁻¹ when compared to other strains. The next highest biomass was produced by S31 (227.05 mg L⁻¹ d⁻¹) followed by AMCQS5-5 (158.36 mg L⁻¹ d⁻¹) and PRA-296 (51.71 mg L⁻¹ d⁻¹) (Table 1).

Table 2.1. Biomass and lipid production in glycerol medium by thraustochytrids.

<table>
<thead>
<tr>
<th>Item</th>
<th>DT3</th>
<th>S31</th>
<th>AMCQS5-5</th>
<th>PRA-296</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomass (g L⁻¹)</td>
<td>4.69 ± 0.09</td>
<td>4.75 ± 0.1</td>
<td>3.7 ± 0.01</td>
<td>1.28 ± 0.005</td>
</tr>
<tr>
<td>Biomass productivity (mg L⁻¹ d⁻¹)</td>
<td>938 ± 0.19</td>
<td>950 ± 0.02</td>
<td>740 ± 0.002</td>
<td>256 ± 0.001</td>
</tr>
<tr>
<td>Average lipid content (mg L⁻¹)</td>
<td>1439 ± 92</td>
<td>1135 ± 68</td>
<td>791 ± 49</td>
<td>258 ± 15</td>
</tr>
<tr>
<td>Lipid productivity (mg L⁻¹ d⁻¹)</td>
<td>287 ± 28</td>
<td>227 ± 17</td>
<td>158 ± 11</td>
<td>51 ± 4</td>
</tr>
</tbody>
</table>
2.3.2 Microalgae oil quantification by SPV method

The colorimetric method was first validated to generate the calibration curve with known quantities of *Schizochytrium* oil. A known quantity of lipid standard in the range of 15-90 μg was taken in clean test tubes and placed in a hot air oven at 60 °C to evaporate chloroform, followed by the SPV reaction protocol described in the experimental section. Lipid content was measured by plotting absorbance at 530 nm and lipid concentration. A high correlation coefficient was obtained with $R^2$ value 0.9939 (Fig. 2.2). This demonstrates the high accuracy of the SPV method in quantifying lipids in aqueous samples.

![Graph](image.png)

Figure. 2.2. Standard curve representing relationship between *Schizochytrium* oil concentration and increase in the absorbance. The lipid concentrations were from 15 to 90 μg. Standard curve representing relationship between *Schizochytrium* oil concentration and increase in the absorbance.

Interference by other cellular and medium components such as protein (BSA), carbohydrate (Glucose) and glycerol did not occur with the SPV reagent (Fig. 2.3), which is consistent with a previous study (Izard & Limberger, 2003). These medium compounds were used as false positives. Thus, other compounds in the medium showed no effect on the absorbance value.

40
Chapter 2

Figure 2.3. Comparison of various lipids and other compounds with sulfo-phospho-vanillin reaction. Triolein yielded intense colour compared with other lipid standards. Compounds with no bonds in their structure do not react with the SPV reagent.

The basic principle underlying the SPV method is a three-step reaction. When the lipids are heated in the presence of sulphuric acid at boiling temperature (low temperature leads to lower reaction rates), a carbonium ion is formed. An aromatic phosphate ester is formed from the reaction of vanillin and phosphoric acid. The activated carbonyl group of phospho-vanillin reagent reacts with the carbonium ions to form a stable pink colour, which has a maximum absorbance at 530 nm (Knight et al., 1972). Colour formation depends mainly on the molecular structure of fatty acids present in the lipids. The molecules which do not possess double bonds do not detectably react with sulfo-phospho-vanillin reagent. Colour intensity varies from one lipid to another due to differences in fatty acid structure. It was observed that the colour intensity of the triolein was greater than that of the polyunsaturated fatty acids EPA and DHA (Fig. 2). The presence of multiple double bonds in EPA and DHA did not yield more colour due to steric hindrance near these double bonds. Saturated fatty acids such as palmitate and stearate did not react with sulfo-phospho-vanillin reagent (Frings & Dunn, 1970; Knight et al., 1972). Among the plant oils tested in this study, olive oil
resulted in higher levels of colour formation than did canola and sunflower oil, probably due to the presence of more than 70% oleic acid in olive oil. In general, higher levels of unsaturated fatty acids lead to higher absorbance, although the presence of multiple double bonds in a single fatty acid (polyunsaturated) may lead to the formation of intense colour (Knight et al., 1972). The presence of various fatty acids in microbial lipids limits the accuracy of the method. This can be rectified by selecting a suitable oil with the same fatty acid content as a reference. Previous studies have reported the quantification of bacterial and microalgal lipids with reference to triolein and canola oil, respectively (Izard & Limberger, 2003; Mishra et al., 2014). We used commercial *Schizochytrium* oil as reference for quantification of lipids in thraustochytrids.

2.3.3. Lipid quantification in thraustochytrid whole cells via SPV method

The established colorimetric method was applied to the quantification of lipids in whole thraustochytrid cells. A known quantity of dried biomass of all four thraustochytrid cultures was suspended in distilled water at a concentration of 20 mg ml\(^{-1}\). Aliquots of 10-50 μl were taken in clean test tubes and the final volume was made up to 100 μl with distilled water. Fig. 3 shows the correlation coefficient between biomass concentration and absorbance with an \(R^2 > 0.99\). All four cultures showed an increase in the intensity of pink colour formation with respect to the increasing biomass concentration (Fig. 2.4). Lipid content in all four cultures was quantified by converting the absorbance into mass using a reference curve with a low standard deviation. Colour change from low concentration to high concentration of biomass (lipid) in four different thraustochytrids shown in Fig. 2.5.
Figure 2.4. Profile of colorimetric detection of thraustochytrid whole cell lipids. The relationship between the concentration of biomass and absorbance at 530 nm for DT3 ($R^2 = 0.992$), S31 ($R^2 = 0.999$), AMCQS5-5 ($R^2 = 0.993$) and PRA-296 ($R^2 = 0.998$).
Chapter 2

Figure. 2.5. Change of lipid colour after reaction with sulfo-phospho-vanillin reagent. Increase of colour represents the correlation between the concentrations of lipid in thraustochytrids. (a) DT3, (b) S31, (c) AMCQS5-5 and (d) PRA-296.

Strain development and lipid quantification are major milestones on the path to sustainable production of biodiesel from the microbial sources. A rapid lipid quantification technique is required to screen potential lipid producing strains from environmental samples. In this study, we used a colorimetric method (SPV) to quantify the total lipids in an aqueous medium containing thraustochytrids, a potential lipid producer. The lowest amount of biomass used for quantifying the lipid content was 50 μg. This enables us to quantify the lipids from the thraustochytrids cells without an extraction step. Moreover, the colorimetric method not only quantified lipid content accurately, but also required less than one hour to complete. In contrast, gravimetric quantification of lipids, extraction and GC analysis are more time and labour intensive.

2.3.4. Validating the accuracy of the SPV method

Gravimetric quantification of lipids from the four selected thraustochytrids was used in order to validate the accuracy of the SPV method. Thraustochytrid lipids were
extracted using a conventional solvent extraction method with modifications (Lewis et al., 2000). The total lipid percentages determined gravimetrically from the four thraustochytrids DT3, S31, AMCQS5-5 and PRA-296, were 33.3, 25.8, 22.8 and 18.0, respectively. Lipid percentages determined also by the SPV method showed similar quantities in all thraustochytrids. Lipid contents determined by SPV, gravimetric methods and gas chromatography were compared (Table 2.2). The lipid profiles determined by gas chromatography were 27.3, 21.8, 20.3 and 18.4, respectively. In two thraustochytrid strains, AMCQS5-5 and PRA 296, the lipid percentages were the same as those obtained using the SPV method. However, in DT3 and S31 there was small (1.9-2.6 %) difference in the lipid percentages. These results obtained from standard gravimetric method for lipid quantification compared with the SPV method in quantifying the total lipids from thraustochytrid whole cells, and the variation was less than 3%, which confirms the suitability of SPV method in screening microbial samples.

Table 2.2. Comparison of thraustochytrid lipid content by SPV, gravimetric and GC methods.

<table>
<thead>
<tr>
<th>Thraustochytrid strains</th>
<th>Lipid percentage by SPV method</th>
<th>Lipid percentage by gravimetric method</th>
<th>Variation</th>
<th>Lipid percentage by gas chromatography</th>
</tr>
</thead>
<tbody>
<tr>
<td>DT3</td>
<td>33.3 ± 1.2</td>
<td>30.7 ± 0.59</td>
<td>2.6</td>
<td>27.3 ± 1.3</td>
</tr>
<tr>
<td>S31</td>
<td>25.8 ± 0.9</td>
<td>23.9 ± 0.86</td>
<td>1.9</td>
<td>21.8 ± 0.84</td>
</tr>
<tr>
<td>AMCQS5-5</td>
<td>22.8 ± 1.1</td>
<td>21.4 ± 0.78</td>
<td>1.4</td>
<td>20.36 ± 0.9</td>
</tr>
<tr>
<td>PRA-296</td>
<td>18.0 ± 1.4</td>
<td>20.2 ± 0.62</td>
<td>2.2</td>
<td>18.47 ± 0.81</td>
</tr>
</tbody>
</table>

Microalgal lipids are useful for biodiesel production. Isolation and identification of the high lipid-producing microalgae is a key step in achieving economically sustainable biodiesel production. Quantification of lipids from microalgae must be performed frequently in microbial screening and other research areas, such as in the screening of genetically engineered microbes for high lipid production and so higher throughput accurate methods are important.
2.4. Conclusion

In conclusion, this study demonstrated the use of the sulfo-phospho-vanillin reaction to quantify lipid accumulation in thraustochytrids. The SPV method resulted in similar lipid content determination in thraustochytrid whole cells as did gravimetric/gas chromatography analysis. Triolein contains only mono-unsaturated fatty acids and the colour intensity was greater than for polyunsaturated fatty acids with multiple double bonds. Carbohydrates, proteins and glycerol did not interfere with the SPV reaction. SPV is a rapid method that requires low levels of chemicals and so is useful for the screening of microalgal cultures for biodiesel production.
Chapter 3: Comparison of cell disruption methods for improving lipid extraction from thraustochytrid strains
Chapter 3

2.1. Introduction

The limited availability of fossil fuels and increasing greenhouse gases emission has led to interest in alternative biomass derived energy. As an alternative fuel, biodiesel is promising (Azeem et al., 2016). Biomass derived biofuels are classified as solid (bio-char), liquid (ethanol, vegetable oil, and biodiesel), and gaseous (biogas, biosyngas and biohydrogen) fuels (Mubarak et al., 2015). Biodiesel is renewable and a relatively clean energy in terms of carbon dioxide generation and greenhouse gas emissions. Biodiesel accounts for 10% of total biofuel production globally and its estimated production is about 6 billion litres/year (Nogueira, 2011).

The present approach to biodiesel (defined as the monoalkyl esters of long-chain fatty acids) production involves transesterification of plant oils such as soybean oil, sunflower oil and rapeseed oil with methanol using alkali catalysts (Daroch et al., 2013; Meher et al., 2006). The use of plant oils for biodiesel production has been associated with some drawbacks such as high viscosity, low volatility and deposition in combustion chambers (Lin et al., 2011). Also, the use of edible oils for biodiesel production has led to an increase in the price of oils, initiating a food versus fuel debate regarding biofuel sustainability. Feedstock useful for biofuel include soapstocks, acid oils, tallow oils, used cooking oils, various animal fats, non-edible plant oils and microbes including algae (Chisti, 2007; Silitonga et al., 2013).

Microalgae are promising vehicles for the production of biodiesel and possess advantages such as higher growth rate and productivity, grow in various environments (fresh, brackish or salt water), do not compete for land, and have high oil productivity (20-50% by dry weight basis) compared to conventional crops (Singh et al., 2011). Microalgal lipid content differs between microalgal species. Microalgal lipid composition and fatty acids profiles depends on culture media composition (carbon source and nitrogen source), temperature, pH and aeration (Halim et al., 2012a). The process of lipid extraction from microalgae is an energy intensive and expensive because the use of solvents and recovery methods in downstream processing. Selection of efficient microalgae species and suitable lipid extraction methods are important for commercial biodiesel production (Griffiths & Harrison, 2009; McMillan et al., 2013). Biodiesel production from microalgae involves four major stages that are cultivation, cell harvest, lipid extraction and finally conversion of lipids into biodiesel (Lee et al., 2010b; Schnurr & Allen, 2015). Therefore, a suitable lipid extraction
Chapter 3

technique is a prerequisite for microalgal lipid extraction. Lipid extraction efficiency is dependent on the polarity of the solvent and combination of solvent mixture (Lee et al., 2010b; Lee et al., 1998; Lewis et al., 2000). The combination of a polar and non-polar solvent mixture can in some cases extract more lipids from microalgae (Ramluckan et al., 2014; Ryckebosch et al., 2012b). For example, the Bligh and Dyer method uses chloroform and methanol for lipid extraction from a range of biological samples (Ramluckan et al., 2014). The use of chloroform and ethanol in a 1:1 ratio provided maximum lipid extraction from Chlorella sp. (Ramluckan et al., 2014), whereas, a combination of dichloromethane and ethanol increased lipid extraction efficiency by 25% in the same organism (R et al., 2015).

Microalgae cell walls block the release of lipid present inside the cells. To get higher product recovery and quality lipids with lower operating costs from microbial cells, a suitable cell disruption method is required. Cell disruption enhances the release of intracellular lipids from microalgae by improving the access of the extracting solvent to fatty acids (Halim et al., 2012b). Cell disruption methods used in microalgae lipid extraction are classified as chemical and mechanical. Chemical cell disruption methods involve use of acids, alkalis, enzymes or osmotic shocks and mechanical methods use microwave, ultrasonication, bead mill, drying, or supercritical fluid extraction. The choice of method influences lipid extraction yields from a range of microalgae (Lee & Han, 2015; Lee et al., 2010b; McMillan et al., 2013; Zheng et al., 2011).

The aim of this study was to compare various organic solvents and cell disruption methods for effective lipid extraction from a new strain of thraustochytrids (a newly isolated strain from the Queenscliff region, Victoria, Australia). This strain was used as a representative microalga in this work due to its ability to accumulate high levels of lipids (Gupta et al., 2013). In addition, microalga biomass was used for different extraction methods that were successfully used for efficient algal lipid extraction in previous studies. Finally, the conversion of lipids to fatty acid methyl ester (FAME) was performed to determine yield and fatty acid distribution after extraction.

3.2. Experimental Section

3.2.1. Chemicals
All the chemicals used in this study were of analytical grade. Medium components such as glucose, yeast extract and mycological peptone (Sigma-Aldrich, USA) and sea salt (Instant Ocean, USA) were used for biomass production while solvents such as acetone, ether, hexane (Merck, Australia), methanol and ethyl acetate from Fischer and Honeywell, Australia.

3.2.2. Strain selection and biomass production

*Schizochytrium* sp. S31 (ATCC 20888) was procured from American Type Culture Collection (ATCC) and used as standard culture. Thraustochytrids used in this study were maintained on GYP medium consisting (g L⁻¹): glucose 5, yeast extract 2, mycological peptone 2, agar 10 and artificial seawater 50% at 25 °C and sub-cultured after 15 days.

*Thraustochytrium* sp. AMCQS5-5 (an in house isolate; Genbank accession number JX993841), was grown in a medium containing (g L⁻¹): glucose 5, peptone 2, yeast extract 2 and artificial sea water 50% for inoculum preparation with shaking at 150 rpm for 2 days at 25 °C. The medium was autoclaved at 121 °C for 20 min. Inoculum (5% v/v) was used to inoculate production medium (100 ml contained in 500 ml flask) and incubated for 5 days in a shake flask at 25 °C and 150 rpm. The resultant biomass was harvested by centrifugation (4000 rpm for 15 min) and was freeze-dried and kept at -20 °C until further use.

The thraustochytrids grown in culture medium were harvested at the interval of 24 h up to 120 h. Optical density at 600 nm and dry cell weight (DCW) was measured at 24 h intervals. A calibration curve was plotted between OD and dry cell weight. Results are presented as mean ± SD of duplicates repeated twice.

The biomass and lipid productivity was calculated from the formula mentioned below:

\[
\text{Productivity} = \frac{\text{Biomass or Lipid content}}{T_1 - T_0}
\]

Where, \(T_1 = \text{Final day of biomass harvesting and } T_0 = \text{Initial day of incubation}\)

3.2.3. Lipid extraction from thraustochytrids by organic solvents

The nine solvents, chloroform, dichloromethane, diethylether, ethanol, heptane, hexane, isopropanol, methanol and toluene at ratios of 1:1, 1:2 and 2:1 were tested for maximising extraction of total lipids. For solvent extraction, 50 mg of freeze
dried biomass of thraustochytrid was blended with 3 ml of various solvents. The mixture was vortexed for 2 min and the sample was then centrifuged at 4000 rpm for 15 min. Supernatant (organic phase) was carefully collected in the pre-weighed glass vials and the solvent was evaporated under nitrogen gas at room temperature. Lipid content (% dry weight basis) was determined gravimetrically. To determine the optimal organic solvent mixture for lipid extraction from thraustochytrids, different ratios of the best three single solvents were investigated. For chloroform-methanol, the Bligh and Dyer method was followed (Bligh & Dyer, 1959).

3.2.4. Cell disruption for lipid extraction

Freeze dried biomass was blended with 3 ml of chloroform and methanol (2:1) and disrupted by means of different cell disruption methods as detailed below. After each treatment, lipid extraction from thraustochytrids was done according to Gupta and co-workers (Gupta et al., 2012c). After centrifugation (4000 rpm for 15 min), the upper layer was collected and dried under nitrogen gas. Lipid content (% dry weight basis) was determined gravimetrically.

3.2.4.1. Grinding with liquid nitrogen

A sample of freeze dried thraustochytrid biomass (50 mg) was taken in the ceramic mortar. About 10-15 ml liquid nitrogen was added and the sample was allowed to thaw and grinded with pestle for 2 min. After grinding the lipid was extracted using organic solvents.

3.2.4.2. Bead vortexing

Thraustochytrid cell suspension (50 mg) was taken in glass tube (35 ml) and 3 ml of solvent and 1 ml of beads (zirconia beads, size 0.4-0.6 mm, Klausen Pty Ltd, Australia) were added and contents were vortexed for 20 min, using a vortex in 30 second bursts. Samples were kept on ice in between the bursts and lipid was extracted using organic solvents.

3.2.4.3. Sonication

Thraustochytrid cell biomass (50 mg) was suspended in 3 ml of solvent in a 15 ml centrifuge tube. Sample was sonicated at 20 kHz, 40% amplitude and the pulse was
40 seconds on and 20 seconds off with total working time of 20 min (Sonics, USA). Sample tubes were kept in ice during the sonication process to prevent overheating.

3.2.4.4. Osmotic shock

Thraustochytrid suspension was disrupted using an osmotic shock method. 50 mg of thraustochytrid biomass was suspended in 3 ml of 10% NaCl solution (mass concentration 16.6 Kg/m$^3$, 0.6 Kg of NaCl) and vortexed for 2 min and incubated for 48 h at room temperature, followed by solvent extraction. Cell disrupting pressure was calculated by the Morse equation:

\[(\text{Morse Equation}) \Pi = iMRT\]

where $\pi$ is cell disrupting pressure generated due to osmotic shock (units in atm or kPa), $i$ is van ’t Hoff factor, $M$ is molar concentration of NaCl, $R$ is 0.0821 L atm/K mol, and $T$ is absolute temperature in °K at which the osmotic shock was performed. Osmotic shock was calculated using an online osmotic pressure calculation hosted by Georgia State University.

3.2.4.5. Water bath

3 ml samples containing 50 mg of biomass taken in 15 ml centrifuge tube was placed in preheated water bath (Ratek Instruments Pty Ltd, Australia) to induce thermolysis. Samples were kept in a water bath for 20 min at 90 °C. Three tubes were treated simultaneously as replicates without shaking.

3.2.4.6. Shake mill

Thraustochytrid cell suspension was disrupted in plastic sample bottle using shake mill (SPEX Mill 8000M, USA). The biomass and bead ratio was 3:1 ratio (Zirconia beads 0.4-0.6 mm) at maximum speed (1060 cycle/min) and exposed for 5 min.

All the cell slurries were observed under the microscope using differential interference contrast (Axio-imager, Zeiss, Germany) to check the disruption of thraustochytrid cells. Microbial smear was prepared on the glass slide, air dried and observed under a microscope. All experiments were performed three times.
3.5. Fatty acids methyl esters (FAMEs) analysis

Fatty acids were converted to methyl esters by acid-catalysed transesterification according to a published method (Han, 2010). 1 ml toluene was added to the glass tubes followed by the addition of 200 µl of internal standard, methyl nonadecanoate (C19:0) and 200 µl of butylated hydroxytoluene (BHT). Acidic methanol (2 ml) was also added to the tube and kept for overnight incubation at 50 °C. Fatty acid methyl esters (FAMEs) were extracted into hexane. The hexane layer was removed and dried over sodium sulphate. FAMEs were concentrated using nitrogen gas. The samples were analysed by a GC-FID system (Agilent Technologies, 6890N, Santa Clara, CA, USA). The GC was equipped with a capillary column (SGE, BPX70, 30 m × 0.25 mm, 0.25 µm thickness). Helium was used as the carrier gas at a flow rate of 1.5 ml min⁻¹. The injector was maintained at 250 °C and a sample volume of 1 µl was injected. Fatty acid peaks were identified on comparison of retention time data with external standards (Sigma-Aldrich, St. Louis, MO, USA) and corrected using theoretical relative FID response factors (Ackman, 2002). Peaks were quantified with Chemstation chromatography software (Agilent Technologies, Santa Clara, CA, USA). Results are presented as mean ± SD of triplicates.

3.3. Results and Discussion

3.3.1. Biomass and lipid production

Schizochytrium sp. S31 showed highest biomass productivity at 0.81 g L⁻¹ d⁻¹ and Thraustochytrium sp. AMCQS5-5 at 0.64 g L⁻¹ d⁻¹ at the end of 5 days (Table 1). The lipid productivity of Schizochytrium sp. S31 and Thraustochytrium sp. AMCQS5-5 were 100.74 mg L⁻¹ d⁻¹ and 64.2 mg L⁻¹ d⁻¹, respectively. Selection of suitable microalgae strain with adequate biomass and oil productivity is important for cost effective biodiesel production. Our results on biomass and lipid productivity are in agreement with previous findings by Vello et al. 2014 (0.30 g L⁻¹ day⁻¹ and 34.53 to 230.38 mg L⁻¹ day⁻¹) that demonstrated the suitability of Chlorella strain as a promising candidate for biodiesel production (Vello et al., 2014).
Table 3.1. Biomass, lipid contents and productivity of thraustochytrids.

<table>
<thead>
<tr>
<th>Properties</th>
<th>Thraustochytrid strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Schizochytrium sp. S31</td>
</tr>
<tr>
<td>Dry weight (g L⁻¹)</td>
<td>4.06</td>
</tr>
<tr>
<td>Biomass productivity (g L⁻¹ d⁻¹)</td>
<td>0.88</td>
</tr>
<tr>
<td>Average lipid content (mg L⁻¹)</td>
<td>503.7</td>
</tr>
<tr>
<td>Lipid productivity (mg L⁻¹ d⁻¹)</td>
<td>100.74</td>
</tr>
</tbody>
</table>

Lipid content and fatty acid profile of a microorganism is dependent on the growth conditions (Rodolfi et al., 2009; Salama et al., 2013). Medium composition influences the percentage of total lipid and type of fatty acids in the microbe. For example, the addition of tween 80 in the production medium led to the accumulation of oleic acid in the thraustochytrids (Taoka et al., 2011). A recent study reported the effect of seasonal variation and nitrogen limitation in the total lipid production and fatty acid composition of *Nannochloropsis oculata*. They observed an increased accumulation (up to 90%) of saturated and mono-unsaturated fatty acids (Olofsson et al., 2014). Since the aim of this work was to find a suitable disruption method for lipid extraction, higher biomass productivity was not pursued further.

### 3.3.2. Lipid extraction from thraustochytrid by organic solvents

The lipid extraction by organic solvents was examined to confirm the lipid-extraction characteristics of thraustochytrids. Fatty acids present in the lipid govern the polarity, based on the principle “like dissolves like”, thus a suitable solvent should be identified for total lipid extraction, however a universal solvent cannot be applied to all microbes with different fatty acid composition. Total lipid extraction yields vary primarily with solvent polarity (Phukan et al., 2011).

To understand the efficacy of organic solvents in lipid extraction from *Schizochytrium* sp. S31, nine solvents and their combinations (based on high lipid yield) were selected, since the lipid extraction is highly dependent on the polarity of the solvents and their ratios (Ramluckan et al., 2014). The effect of single solvent as
well as the combinations of the best 3 solvents with respect to the lipid extraction from *Schizochytrium* sp. S31 is shown in Figure 1. Among the single organic solvents, maximum lipid (12.5%) was extracted with hexane, followed by 11% in heptane and 9.7% in chloroform. However, among the combination of solvents, the mixture of chloroform: methanol (2:1) showed maximum lipid extraction (22%), followed by chloroform and hexane (2:1) with 13.4% (Fig. 3.1). Chloroform and hexane showed comparatively low efficacy in lipid extraction indicating that a range of polar to non-polar lipids were present in *Schizochytrium* sp. S31. A similar trend was observed during lipid extraction from *Chlorella* sp. (Ramluckan et al., 2014). A mixture of hexane:heptane (1:1) extracted the least amount of lipid (2.2%). Higher lipid yields with chloroform: methanol indicates the presence of more polar and neutral lipids in the algae. It was observed that the percentage of polar and neutral lipids were approximately 78% and non-polar lipids were 22% in some algae (Kale, 2013). Combination of polar and non-polar solvents could extract more lipids than individual solvents (Lewis et al., 2000; Ryckebosch et al., 2012b). However, a study (Shen et al., 2009) contradicts this conclusion, indicating that the combination of hexane and ethanol could not extract more lipid than hexane alone from *Scenedesmus dimorphus* and *Chlorella protothecoides*. This suggests the efficiency of lipid extraction depends on the algal species and their lipid compositions. Also, results obtained from chloroform and methanol (2:1) indicate the presence of more polar and neutral lipids in algae (Ramluckan et al., 2014). Mixtures of chloroform:methanol extract hydrocarbons, carotenoids, chlorophyll, sterols, triglycerides, fatty acids, phospholipids and glycolipids (dos Santos et al., 2015; Singh et al., 2013).
Figure 3.1. Lipid extraction percentage from dry biomass using various solvents from *Schizochytrium* sp. S31. The symbols represent Chl: Choloroform, Hex: Hexane, Hep: Heptane, Met: Methanol.

### 3.3.3. Comparison of lipid extraction methods

Six methods were evaluated in order to determine the efficiency of cell disruption methods for total lipid extraction from thraustochytrids. The effectiveness of the cell disruption methods were quantified using lipid yield percentages. Cell disruption breaks the cells and improves the accessibility to the intracellular components for extraction (Wang et al., 2015). Figure 2 shows the percentage of total lipids extracted as a function of cell disruption methods. All the cell disruption methods used in this study were able to disrupt thraustochytrid cells, although lipid yield varied.
Chapter 3

Figure 3.2. Effect of different cell disruption methods for lipid extraction from thraustochytrids. Average lipid extraction of each method was reported as % of total lipids extracted.

Thraustochytrid cells subjected to disruption resulted in rupture of the cell walls and release of intracellular components (Figure 3.3a and b). It was observed that lipid content of *Schizochytrium* sp. S31 was higher than that of *Thraustochytrium* sp. AMCQS5-5. Maximum lipid was extracted from both *Schizochytrium* sp. S31 (48.7%) and *Thraustochytrium* sp. AMCQS5-5 (29.1%) cells using osmotic shock (Figure 2). Grinding, sonication, shake mill and water bath treatments extracted 44.6%, 31%, 30.5% and 20.8% of lipids, respectively. Bead vortexing resulted in 25% lipid extraction from *Thraustochytrium* sp. AMCQS5-5 cells, whereas other methods (water bath, grinding, shake mill and sonication) resulted in lower yields. Osmotic shock resulted in a 2.2-folds increment in lipid extraction from *Schizochytrium* sp. S31 and a 2.8-folds increase from *Thraustochytrium* sp. AMCQS5-5, compared to control. A similar study for a *Chlorella* sp. indicated that osmotic shock was an effective method for extracting lipids (Prabakaran & Ravindran, 2011). This method also consumes less energy than traditional methods. In a recent study, when *Chlamydomonas reinhardtii* cells were incubated in a high osmotic environment, it led to a 2-folds improvement in lipid extraction (Yoo et al., 2012). Table 3.2 summarises some cell disruption methods reported for lipid extraction from microalgae. Due to differences in cell wall structure,
not all microalgae respond the same to pretreatment. For example, Lee et al. (2010) observed that microwave treatment was optimum for the disruption of *Botryococcus* sp., *Chlorella vulgaris* and *Scenedesmus* sp. cells. Another study showed that grinding with liquid nitrogen facilitated higher levels of lipid extraction from *Chlorella vulgaris* (Zheng et al., 2011). Available literature suggests that cell disruption methods improve lipid extraction from microalgae, and efficiency depends on microalgae species, age of the culture and composition of cell wall. Therefore, results obtained from one species cannot be generalised to all other species (Halim et al., 2012b).

Figure. 3.3. Effect of cell disruption on thraustochytrids cells. Thraustochytrid cells before (A) and after (B) cell disruption (scale bar 20 μm).

Table 3.2. Comparison of cell disruption methods employed to extract total lipids from different microalgae.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Cell disruption methods used</th>
<th>Efficient method</th>
<th>Organism used</th>
<th>Lipid content (%)</th>
<th>Reference</th>
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<td>1</td>
<td>Autoclaving</td>
<td>Microwaves</td>
<td><em>Botryococcus</em> sp.</td>
<td>28.6</td>
<td>(Lee et al., 2010b)</td>
</tr>
<tr>
<td></td>
<td>Bead beating</td>
<td></td>
<td><em>Chlorella vulgaris</em></td>
<td>11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Microwaves</td>
<td></td>
<td><em>Scenedesmus</em> sp.</td>
<td>11.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sonication</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Osmotic shock</td>
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</tbody>
</table>
There are many lab scale cell disruption methods discussed in the literature, however, only a few mechanical methods either alone or with the intervention of enzymes/chemicals can be scaled up for industrial applications. For instance, bead mill, high pressure homogenizer and Hughes press are used extensively at large scale, which reduce unit operation steps compared to chemical and enzymatic methods (Chisti & Moo-Young, 1986; Schutte & Kula, 1990). An osmotic shock method was implemented in thraustochytrid cell disruption and lipid extraction, to reduce the energy consumption and production cost. During the osmotic shock treatment, the resulting wastewater can be recycled through reverse osmosis technology (Arnal et al., 2005). The same method has been applied at pilot-scale for enhancing the release of ectoine (Sauer & Galinski, 1998). A recent study by Jayaranja and Rekha (2015) indicated that osmotic shock was a suitable method for maximising the extraction of intracellular products, and this method can also be industrially scaled up (Kar &

<table>
<thead>
<tr>
<th>2</th>
<th>Sonication</th>
<th>Sonication</th>
<th>Chlorella sp.</th>
<th>20.1</th>
<th>(Prabakaran &amp; Ravindran, 2011)</th>
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<tr>
<td></td>
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<td>Microwave</td>
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<td></td>
<td>Bead beating</td>
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<tr>
<th>3</th>
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<th>Grinding</th>
<th>Chlorella vulgaris</th>
<th>29</th>
<th>(Zheng et al., 2011)</th>
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<tr>
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<th>Schizochytrium sp. S31, Thraustochytrium sp. AMCQS5-5</th>
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<th>This study</th>
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<tr>
<td></td>
<td>Bead vortexing</td>
<td></td>
<td></td>
<td>29.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Osmotic shock</td>
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<td></td>
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<tr>
<td></td>
<td>Water bath</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Sonication</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Shake mill</td>
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<tr>
<th>5</th>
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<th>Grinding</th>
<th>Chlorella vulgaris</th>
<th>29</th>
<th>(Zheng et al., 2011)</th>
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<tr>
<th>6</th>
<th>Grinding</th>
<th>Osmotic shock</th>
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<th>48.7</th>
<th>This study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bead vortexing</td>
<td></td>
<td></td>
<td>29.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Osmotic shock</td>
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<tr>
<td></td>
<td>Water bath</td>
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</tr>
<tr>
<td></td>
<td>Sonication</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Shake mill</td>
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</table>
The advantages and disadvantages of selected investigated methods are summarised in Table 3.3.

Table 3.3. Advantages and disadvantages of the investigated cell disruption methods.

<table>
<thead>
<tr>
<th>Cell disruption methods</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manual grinding</td>
<td>Quickest and efficient</td>
<td>- Localised heating caused denaturation of molecules</td>
</tr>
<tr>
<td></td>
<td>- 2 min process</td>
<td></td>
</tr>
<tr>
<td>Bead vortexing</td>
<td>- Can be established easily and relatively effective</td>
<td>- High heat generation, Incomplete cell lysis</td>
</tr>
<tr>
<td>Osmotic shock</td>
<td>- Lower energy consumption</td>
<td>- Generation of waste salt water</td>
</tr>
<tr>
<td></td>
<td>- Easier scale-up</td>
<td>- Time consuming</td>
</tr>
<tr>
<td>Water bath</td>
<td>- Maximum disruption</td>
<td>- Increases the viscosity</td>
</tr>
<tr>
<td></td>
<td>- Easy in handling at lab scale</td>
<td>- Energy intensive</td>
</tr>
<tr>
<td>Sonication</td>
<td>- Faster extraction</td>
<td>- Damage chemical structure of molecules</td>
</tr>
<tr>
<td></td>
<td>- suitable for all cell type</td>
<td></td>
</tr>
<tr>
<td>Shake mill</td>
<td>- Rapid method</td>
<td>- High energy intensive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- High heat generation</td>
</tr>
</tbody>
</table>

3.3.4. Fatty acid composition of extracted lipid

The fatty acid profiles of the lipids extracted following different cell disruption methods from thraustochytrids are presented in Figure 4a and 5a. Major fatty acids such as myristic acid (28.1%), palmitic acid (27.3%), palmitoleic acid (20.7%) and oleic acid (12.8%) were detected in *Schizochytrium* sp. S31 (Figure 3.4a) after osmotic shock disruption. Total saturated, monounsaturated and polyunsaturated fatty acid contents after osmotic shock facilitated extraction were 58.7%, 34.6% and 4.8%, respectively, making it a potential feedstock for biodiesel production (Figure 3.4b). The other prominent fatty acids based on grinding, sonication and shake mill identified in the lipid extracts were saturated (49-57%), mono-unsaturated (31-35%) and polyunsaturated fatty acids (2-18%). Saturated and monounsaturated fatty acids are useful major components for microalgal biodiesel production because of their
relatively high oxidative stability (Cao et al., 2014). The general properties of biodiesel such as viscosity, specific gravity, cetane number, iodine value, and low temperature performance metrics are determined by the structure (length and unsaturation) of fatty acid esters (Hoekman et al., 2012).

Figure. 3.4a. Effect of different cell disruption methods on fatty acid profiles of *Schizochytrium* sp. S31.
Figure 3.4b. Effect different cell disruption methods on SFA, MUFAs and PUFAs of *Schizochytrium* sp. S31.

Figure 3.5a. Effect of different cell disruption methods on fatty acid profiles of *Thraustochytrium* sp. AMCQS5-5.
Chapter 3

Figure 3.5b. Effect of different cell disruption methods on SFA, MUFAs and PUFAs of Thraustochytrium sp. AMCQS5-5.

In Thraustochytrium sp. AMCQS5-5, palmitic acid (31.6%) and docosahexaenoic acid (31.5%) were the major fatty acids (Figure 3.5a), with other polyunsaturated fatty acids (C20:4n6, C20:5n3, C22:5n6 and C22:5n3) ranging from 2-9%, when cells were disrupted using osmotic shock. Saturated, monounsaturated and polyunsaturated fatty acid contents were 35.5%, 7.4% and 51%, respectively (Figure 3.5b). The highest polyunsaturated fatty acid percentages were extracted by grinding and sonication methods, 67% and 57%, respectively. Only shake mill resulted in a significant percentage of monounsaturated fatty acids extraction (37.5%). Most methods resulted in different lipid yields, but no difference in the fatty acid profiles for extraction from Thraustochytrium sp. AMCQS5-5 (Figure 3.5b). If the omega-3 fatty acids could be separated from other fatty acids in the Thraustochytrium sp. AMCQS5-5 extract, then these fatty acids, particularly DHA, could be used as nutritional products and offset the cost of biofuel production in this strain (Ryckebosch et al., 2012a; Singh et al., 2015b).

3.3.5. Prediction of biodiesel properties

We analysed fatty acid profiles of two different thraustochytrid strains to determine the suitability of these microalgae for biodiesel production. The fatty acid
values were taken as an input in predicting the biodiesel properties by using an open access software "Biodiesel Analyzer© Ver. 1.1" (Talebi et al., 2013). Some of the important parameters of biodiesel are cetane number (CN), Iodine value (IV) and oxidative stability (OS), which determine the combustion behavior, quality of biodiesel, stability and performance, respectively (Islam et al., 2013). The CN and IV values in *Schizochytrium* sp. S31 (47.4 and 98.61) when compared to *Thraustochytrium* sp. AMCQS5-5 (44.01 and 157.44) were observed. According to ASTM D6751-12, the standard values were 47-51 (CN) and 120 g I$_2$/100g maximum, indicating suitability of selected strain S31 for biodiesel, however, its further characterization will be a follow up study. The OS value of 6.5 for *Schizochytrium* sp. S31 was higher than 1.65 for *Thraustochytrium* sp. AMCQS5-5 suggesting oxidation stability decreased with the increase of polyunsaturated fatty acid content. Other properties such as saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), degree of unsaturation (DU), saponification value (SV), long chain saturated fatty (LCDF), cold filter plugging point (CFPP), cloud point (CP), allylic position equivalent (APE), bis-allylic position equivalent (BAPE), oxidation stability (OS), higher heating value (HHV), kinematic viscosity (μ), and density (ρ) were analysed as summarised in Table 4.

Table 3.4. Predicted biodiesel properties of given thraustochytrids strains.

<table>
<thead>
<tr>
<th>Properties</th>
<th>Units</th>
<th>Strain S31</th>
<th>Strain AMCQS5-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated fatty acid</td>
<td>% (m/m)</td>
<td>59</td>
<td>44</td>
</tr>
<tr>
<td>Monounsaturated fatty acids</td>
<td>% (m/m)</td>
<td>35</td>
<td>4.6</td>
</tr>
<tr>
<td>Polyunsaturated fatty acids</td>
<td>% (m/m)</td>
<td>14</td>
<td>33</td>
</tr>
<tr>
<td>Degree of unsaturation</td>
<td></td>
<td>63</td>
<td>70</td>
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<tr>
<td>Saponification value</td>
<td>mg KOH/g oil</td>
<td>233.98</td>
<td>164.73</td>
</tr>
<tr>
<td>Iodine value</td>
<td>g I$_2$/100 g</td>
<td>98.61</td>
<td>157.44</td>
</tr>
</tbody>
</table>
### Chapter 3

#### Cetane number

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<tbody>
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<td>min</td>
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#### Long chain saturated factor

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#### Cold filter plugging point

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#### Cloud point

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#### Allylic position equivalents

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#### Bis-Alllylic position equivalents

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#### Oxidation stability

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#### Higher heating value

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#### Kinematic Viscosity

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#### Density

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<th>Kg/m³</th>
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<td>0.94</td>
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### 3.3.6. Energy analysis

Energy consumption of the investigated cell disruption methods was carried out to establish their potential as large scale processes. The comparative estimated energy consumptions and processing times of the investigated cell disruption methods are presented in Table 5. In comparison, water bath and sonication methods resulted in highest energy consumption, 2400 MJ Kg⁻¹ dry mass and 1200 MJ Kg⁻¹ dry mass, respectively. Shake mill energy consumption was estimated to be 690 MJ Kg⁻¹ dry mass. The osmotic shock method consumed modest energy (4.8 MJ Kg⁻¹) and highest lipid recovery, and so was the preferred method. NaCl present in the lysate solution exerted osmotic pressure which was estimated to be 4.21 KPa, using an online osmotic pressure calculator. Another study showed that the osmotic pressure of 1.9 KPa was enough to break the microbial cells (Kar & Singhal, 2015). It has been demonstrated that energy consumption for the microwave (28 MJ Kg⁻¹) and ultrasound (44 MJ Kg⁻¹) methods enhanced lipid extraction from *Chlorella* sp. (Olofsson et al., 2014), which shows that osmotic shock consumed less energy than either of these methods.
Table 3.5. Energy consumption comparison.

<table>
<thead>
<tr>
<th>Cell disruption methods</th>
<th>Lipid yield (%)</th>
<th>Energy consumption (MJ Kg⁻¹ dry mass)</th>
<th>Processing time (min)</th>
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<tbody>
<tr>
<td>Control</td>
<td>22.04</td>
<td>Nil⁵</td>
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<tr>
<td>Manual grinding</td>
<td>44.6</td>
<td>ND⁶</td>
<td>2</td>
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<tr>
<td>Bead vortexing</td>
<td>22.8</td>
<td>48</td>
<td>20</td>
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<tr>
<td>Osmotic shock</td>
<td>48.7</td>
<td>4.8⁷</td>
<td>2</td>
</tr>
<tr>
<td>Water bath</td>
<td>20.8</td>
<td>2400</td>
<td>20</td>
</tr>
<tr>
<td>Sonication</td>
<td>31.05</td>
<td>1200</td>
<td>20</td>
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<tr>
<td>Shake mill</td>
<td>30.5</td>
<td>690</td>
<td>5</td>
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</table>

Thraustochytrids mass concentration of 16.6 kg/ m³ was used for energy analysis. Where Nil⁵ represents no energy was consumed; ND⁶ represents physical effort cannot be quantified; ⁷Osmotic pressure by virtue of salt addition.

3.4. Conclusion

In this study, we have shown that some cell disruption methods, particularly osmotic shock, result in both different oil yields and variation in the percentage of saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids in the extracted oil from thraustochytrid species.
Chapter 4: Optimised cell disruption methods for lipase extraction from Australian thraustochytrids
4.1. Introduction

The marine environment has a unique biodiversity and is a good source of enzymes with unusual properties with potential utility in biotechnology (Lee et al., 2010a). Considerable effort has gone into screening marine extracts for novel bioactive compounds, with unique structural classes being discovered (Kurtovic & Marshall, 2013; Vester et al., 2014). Marine microorganisms are particularly useful for bioactive compounds discovery since they can be fermented and so offer ease of scaleup versus other sources (Sana, 2015). Among marine microbes, thraustochytrids are one of the most scaleable strains since they are commerically useful for the production of omega-3 oils and carotenoids. These heterotrophic microorganisms also are a potentially useful source of new enzymes (Gupta et al., 2012a; Gupta et al., 2013). Thraustochytrids can accumulate approximately 40% docosahexaenoic acid (DHA) as a percentage of total lipids (Liu et al., 2014b). Thraustochytrids have been used as animal feed, aquaculture feed and poultry feed in addition to their use in omega-3 and carotenoid production (Milledge, 2012; Sprague et al., 2015). Thraustochytrids have been reported to produce degradative enzymes such as lipases, peptidases, phosphatases and cellulases, although the utility of their enzymes has not been fully investigated (Bongiorni et al., 2005; Kanchana et al., 2011).

Cell disruption is an important downstream processing step as it impacts extraction yields and therefore the cost of bioactive compound production (Lee et al., 2012c). Various methods—physical, chemical and mechanical—are available for disrupting microbial cells, with mechanical methods generally used for large-scale processing (Günerken et al., 2015). The selection of a suitable method of cell disruption for extracting intracellular products depends on cell wall strength, intracellular location of products, stability and the final use of the recovered products (Klimek-Ochab et al., 2011). For example, the effect of chemical and mechanical cell disruption methods was investigated for extraction of intracellular β-D-galactosidase (Puri et al., 2010).

Lipases are hydrolytic enzymes that catalyse the hydrolysis or synthesis of esters and possess positional as well as fatty acid selectivity (Ferreira-Dias et al., 2013; Singh & Mukhopadhyay, 2012). Lipases isolated from marine organisms generally showed maximum activity at low temperatures and have unusual fatty acid specificity (Vester et al., 2014). Lie and Lambertsen, observed that the crude enzyme mixture collected
from cod intestine showed an unusual fatty acid selectivity, preferentially hydrolysed omega-3 fatty acids (Lie & Lambertsen, 1985). Similar fatty acid selectivity has also been observed from the crude digestive juice from salmon and rainbow trout collected from the pyloric area of the stomach (Halldorsson et al., 2004). However, in each of these cases no omega-3 lipase has been characterised. A recent study reported that a novel lipase isolated from the marine microbe *Marinobacter lipolyticus* SM19 showed a high preference for hydrolysing eicosapentaenoic acid (EPA) from fish oil (Pérez et al., 2011).

We report here the development of a cell disruption method for release of intracellular lipases from thraustochytrids. The efficiency of different cell disruption methods for the release of intracellular proteins and their effect on lipolytic activity was investigated. To the best of our knowledge this is the first report on optimisation of cell disruption methods for lipase extraction from thraustochytrids.

### 4.2. Materials and methods

#### 4.2.1. Chemicals

All chemicals such as *p*-nitrophenyl palmitate (*p*-NPP), Tween 80, and medium components (yeast extract and peptone) were procured from Sigma-Aldrich, USA. Acid washed glass beads from Thomas Scientific, Australia and Sea salts from Instant Ocean, USA. The API ZYM kit was procured from BioMérieux, Australia Pty. Ltd.

#### 4.2.2. Strain selection and cultivation

Recently isolated strains designated as AMCQS5-3 (Genbank accession numbers JX993839), AMCQS5-4 (accession numbers JX993840), AMCQS5-5 (accession numbers JX993841), AMCQS1-9 (accession numbers JX993843) and AMCQ-4b27 (accession numbers JX993842) were used for screening lipase activity (Gupta et al., 2013). Two microbial cultures, *Schizochytrium* S31 (S31) and PRA 296, were procured from the American type of culture collection (ATCC).

Thraustochytrids were maintained in a GYP medium containing glucose (5 g L⁻¹), peptone (2 g L⁻¹) and yeast extract (2 g L⁻¹) and artificial sea water (50% v/v) at 20 °C. Stock cultures were subcultured onto fresh plates at regular intervals. All thraustochytrids were grown in modified Vishniac’s Medium (0.1% glucose, 0.01% yeast extract, 0.01% peptone, 0.1% gelatine hydrolysate, 1.2% agar in seawater, pH
for enzyme screening (Raghukumar, 2002). The incubation conditions were 20 °C and 150 rpm agitation until the cells reach the late exponential growth phase (96 h). Before harvesting, cells were observed under a microscope for growth.

### 4.2.3. Enzymatic profiling of isolates

The thraustochytrid cells were harvested at 5000 rpm for 15 min and washed with sterile artificial sea water. Cells were suspended in artificial sea water and 65 μl of sub aliquots (corresponds to 4.0 x 10^5 cells) inoculated into (19 microcupules except in control) API ZYM strips and incubated at 20 °C overnight. After incubation, 30 μl of API ZYM A and B were added to the microcupules and incubated for 5 min. Enzyme activities were measured using a colorimetric scale (Tiquia, 2002).

### 4.2.4. Lipase production

Thraustochytrid were grown in medium containing peptone (500 mg L⁻¹), yeast extract (500 mg L⁻¹) and Tween 80 (1% v/v) (Arafiles et al., 2011). The production medium was pre-adjusted to pH 6.0 using 0.1 M HCl. A volume of 50 ml medium contained in a 250 ml Erlenmeyer flask was inoculated with a loop full of culture from the plate. The flask was incubated at 20 °C with shaking at 150 rpm for 48 h. Inoculum (5% v/v) was used for the production of biomass. The production culture was incubated for 120 h under the same conditions. The biomass was harvested by centrifugation (5000 rpm, 10 min). Harvested biomass was resuspended in distilled water to remove the Tween 80, and this process was repeated three times (Li et al., 2001). The biomass was kept at -80 °C overnight and freeze-dried (Christ, Germany) for 24 h for further use.

### 4.2.5. Cell disruption methods for lipase release

#### 4.2.5.1. Bead vortexing

A sample of freeze-dried biomass (100 mg) was suspended in 1 ml of extraction buffer (100 mM Tris pH 7.2) with EDTA (10 mM) and NaCl (100 mM). One ml of acid washed glass beads (425-600 μm, Klausen Pty Ltd, Australia) were added and the contents were vortexed for 10 min, in 30 second bursts. Ground biomass was centrifuged at 12,000 rpm for 60 min at 4 °C. The supernatant was analysed for lipase activity and protein levels as described below.
4.2.5.2. Grinding with liquid nitrogen

A sample of freeze-dried biomass (100 mg) was suspended in 10-15 ml of liquid nitrogen and ground with a pestle for 2 min. 1 ml of extraction buffer was added and the resultant biomass was collected and centrifuged at 12,000 rpm for 60 min at 4 °C. The supernatant was analysed for lipase activity.

4.2.5.3. Sonication

A sample of freeze-dried biomass (100 mg) was suspended in 1 ml of extraction buffer. The suspension was sonicated at 20 kHz, 40% amplitude and the pulse was 40 seconds on 20 seconds off through a time-range of 5-25 min (Sonics, USA). After every 5 min, cells were observed under a microscope to check the level of disruption. During sonication, the sampling tubes were kept in ice-bath to avoid heat-mediated denaturation of crude enzyme. The suspension was centrifuged at 12,000 rpm for 60 min at 4 °C. The supernatant was analysed for lipase activity.

4.2.5.4. Homogenization

Homogenization was performed using a rotor-stator type homogenizer (Unidrive 1000, CAT Scientific, USA). A sample of freeze-dried biomass (100 mg) was suspended in 1 ml of extraction buffer and the suspension was subjected to homogenization at 8500 rpm/min for 5-25 min at 5 min intervals. After every 5 min, the suspension was observed under the microscope. The suspension was centrifuged at 12,000 rpm for 60 min at 4 °C. The supernatant was analysed for protein concentration and lipase activity.

4.2.6. Microscopic observation of the native and disrupted thraustochytrid cells

The cell slurries were observed under a microscope using differential interference contrast (Axio-imager, Zeiss, Germany) to check for disruption. Microbial smears of normal and disrupted cells were prepared on the glass slide and air-dried

4.2.7. Lipase and protein assay

Lipase activity was measured from the supernatant obtained after the cell disruption using $p$-nitrophenol palmitate pNPP as a substrate, following a previously described method with some modifications (Winkler & Stuckmann, 1979). The lipase substrate solution was prepared by adding solution A consisting of 30 mg of pNPP in 10 ml of isopropanol and the solution B consisting of 0.4% Triton X-100, 0.1% Gum
Arabic and 50 mM Tris-HCl buffer, pH 7.0. Solution A (10 ml) was added to solution B (90 ml) drop by drop with stirring. The substrate solution (2.9 ml) and 0.1 ml of enzyme solution was incubated at 37 °C for 10 min and absorbance was measured at 410 nm. One unit (IU) of lipase activity was defined as one nmol of pNPP released per minute under the standard assay conditions.

Protein concentration was estimated from the supernatant using the Bradford method with bovine serum albumin as the standard (Bradford, 1976). All the enzyme and protein assays were done in four replicates and the standard error (SE) calculated using Microsoft excel software.

4.3. Results and discussion

In this study, seven thraustochytrids isolates were screened for 19 different enzyme activities. An API ZYM kit was used that contained enzyme strips that consisted of 20 microcupules with 19 different chromogenic substrates and one control. Table 4.1 shows the wide spectrum of enzyme activities that were exhibited by the thraustochytrids isolated from Australian marine water. The new isolates were isolated from two different sample collection trips. The investigated thraustochytrid strains exhibited high esterase, lipase, acid and alkaline phosphatase activities. The peptide degrading enzyme leucine arylamidase was produced by all strains, except AMCQS5-4. Maximum lipase activity was observed in AMCQS1-9. Other peptidases such as cysteine arylamidase and trypsin were produced in all isolates, except AMCQS5-4 and AMCQS1-9. PRA 296 and AMCQS1-9 showed α-chymotrypsin activity. High β-galactosidase and α-glucosidase activities were observed in the strains PRA 296 and AMCQS5-5.
Table 4.1a. Enzymatic activities of thraustochytrids isolated from Australian marine waters (First collection trip, October 2011).

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(Indicate what is shown in red, green and straw yellow colour)

*Samples were collected by Dr A Gupta and Dr T Thyagrajan from Barwon region, Victoria, Australia.
**Table 4.1b. Enzymatic activities of thraustochytrids isolated from Australian marine waters (Second collection trip, September 2013).**

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<td></td>
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<tr>
<td>α-fucosidase</td>
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<td></td>
<td></td>
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</tbody>
</table>

**Footnote:** Semi-quantitative screening of thraustochytrid for different enzymatic activities was done using a commercial enzyme kit. Thraustochytrid cells were inoculated into the strips and observed for colour formation due to the enzymatic action. (Green) high activity, (yellow) medium activity; (red) low activity; (no colour) no activity. GenBank accession numbers for isolates given in Appendix.

Cluster analysis revealed that the enzymatic activities of thraustochytrid strains were homogenous, with 86% similarity (Fig. 4.1). Bongiorni et al. also observed a range of enzyme activity in some thraustochytrids (Bongiorni et al., 2005).

**Others bioprocessing lab members Dr A Gupta, Dr T Thyagrajan, Dr D Singh and Dr S Sonkar were involved in the collection of various isolates from the Queenscliff region, Victoria.**
Figure. 4.1. Cluster analysis of thraustochytrids based on various enzyme activities. Cluster analysis was done using XLSTAT version and reveals that degree of similarity was 86% based on presence/absence in their enzymatic profiles.

Lipase production was inhibited by high concentrations of glucose in the medium (Kanchana et al., 2011). To improve lipase production levels, thraustochytrids were grown in a Tween 80 containing medium. Intracellular lipase activity was measured using a colorimetric enzyme assay. The lipase activity from S31 (39 IU/g) was higher than that observed for the other strains used in the current study. The maximum lipase activities observed for the thraustochytrid isolates were 34 IU/g for AMCQ-4b27 and 36 IU/g for AMCQS1-9 (Table 4.1). 18r DNA sequence analysis identified these strains as a *Thraustochytrium* sp (AMCQ-4b27) and a *Schizochytrium* sp (AMCQS1-9) (Gupta et al., 2013).
Table 4.1. Lipase activity of thraustochytrids new isolates and ATCC cultures. Lipase activity was determined by p-NPP assay using whole cell as catalyst.

<table>
<thead>
<tr>
<th>Thraustochytrid strains</th>
<th>Lipase activity (IU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In house isolates</strong></td>
<td></td>
</tr>
<tr>
<td>AMCQS5-3</td>
<td>26±2.0</td>
</tr>
<tr>
<td>AMCQS5-4</td>
<td>21±1.0</td>
</tr>
<tr>
<td>AMCQS5-5</td>
<td>32±2.0</td>
</tr>
<tr>
<td>AMCQS1-9</td>
<td>36±1.6</td>
</tr>
<tr>
<td>AMCQ-4b27</td>
<td>34±4.2</td>
</tr>
<tr>
<td><strong>ATCC</strong></td>
<td></td>
</tr>
<tr>
<td>S31</td>
<td>39±3.7</td>
</tr>
<tr>
<td>PRA 296</td>
<td>28±2.0</td>
</tr>
</tbody>
</table>

4.3.1. Cell disruption for lipase extraction

Cell disruption is a key factor in the extraction of intracellular enzymes from microbial cells. Different methods are available in the literature to solubilize the intracellular proteins that depend on the location of enzymes, its application and stability (Kurtovic & Marshall, 2013). The thraustochytrids strains examined had a range of enzymes activities, with some lipase activity. We assessed the effect of cell disruption techniques on lipase extraction from two thraustochytrid strains. The impact of different cell disruption methods on enzyme yield varies with enzyme stability and cell type, so that a balance between maximum cell degradation effectiveness and minimum protein denaturing needs to be achieved.

The effect of cell disruption methods on lipase extraction from thraustochytrid was studied via quantifying protein content released, lipase extraction and also by direct microscopic observation (Fig. 4.2 a-d). Undisrupted cells of AMCQS1-9 and AMCQ-4b27 remained intact (Fig. 4.2a and b), whereas, cells subjected to disruption ruptured to release intracellular components (Fig. 4.2c and d).
Figure. 4.2. Microscopic observation of thraustochytrid after cell disruption. Undisrupted/Intact cells of AMCQS1-9 and AMCQ-4b27 (a and b), and disrupted cells of AMCQS1-9 and AMCQ-4b27 (c and d) by sonication reaction conditions (20 KHz, 40% amplitude, 15 min) were observed after the treatment using differential interference contrast.

4.3.1.1. Bead vortexing

Thraustochytrid cells AMCQ-4b27 and AMCQS1-9 were disrupted by bead vortexing. The lipase activity and specific activity after cell disruption by bead vortexing are shown in Fig. 4.3. More protein was obtained from AMCQ-4b27 cells (130 μg/ml) than from AMCQS1-9 (63 μg/ml), however, lipase activity was greater in protein extracted from AMCQS1-9 cells (0.045 IU/μg versus 0.125 IU/μg, respectively). The effectiveness of bead vortexing versus other cell disruption methods
appears to be cell type dependant. Becerra et al, studied extraction of β-galactosidase from *Kluyveromyces lactis* by disruption with glass beads. They also observed the volumetric and specific activity were minimum when compared with sonication and other methods (Becerra et al., 2001).

**Figure. 4.3.** Effect of bead vortexing on thraustochytrid cell disruption and lipase activity. Bead vortexing of thraustochytrid cells resulted in more protein release from AMCQ-4b27 than AMCQS1-9. S1-9 cells were relatively more susceptible for high enzyme activity than AMCQ-4b27 cells (in all case SE <10%).

4.3.1.2. Grinding with liquid nitrogen

Grinding with liquid nitrogen is a simple and quick method for disrupting microbial cells (Zheng et al., 2011). Grinding with liquid nitrogen was faster than other methods for cell disruption. The total protein extracted by this method was higher than that obtained by other methods, for AMCQ-4b27 (156 μg/ml) and AMCQS1-9 (107 μg/ml). Fig. 4.4 showed that grinding with liquid nitrogen led to a higher degree of cell disruption, consistent with higher protein yields. Lipase activity of AMCQ-4b27 (25 IU/ml) was higher than that for AMCQS1-9 (18 IU/ml). However, specific activity from AMCQ-4b27 (0.160 IU/μg) was lower than that for AMCQS1-9 (0.168 IU/μg). Lipase from AMCQ-4b27 more readily degraded with temperature than lipase from AMCQS1-9. Our results are consistent with the study investigated the effect of different chemical and physical methods for extraction of β-D-galactosidase, where
grinding with river sand is effective in enzyme extraction compared with other methods (Puri et al., 2010).

![Activity vs Protein yield and Specific activity](image)

**Figure. 4.4. Effect of grinding on thraustochytrid cell disruption and lipase activity.** AMCQS-4b27 cell were more susceptible to cell disruption compared with AMCQS1-9. Grinding with liquid nitrogen resulted in more protein released than did other methods.

### 4.3.1.3. Sonication

Sonication was previously shown to be an effective method for releasing intracellular bioactive compounds (Liu et al., 2013). In order to understand the optimal sonication time for maximum protein release and lipase activity, we varied the sonication treatment time. The impact of sonication exposure time on thraustochytrid cells was investigated. Enzyme released by sonication can be calculated as:

\[ R = R_m (1 - e^{-kt}) \]

Where \( R \) and \( R_m \) are the released enzyme activity and maximum enzyme activity that can be released, respectively (U/mg/min), \( k \) is the disruption constant for sonication and \( t \) is the time of sonication in minutes (Doulah, 1977). Lipase release from thraustochytrid cells was investigated at an acoustic power of 20 kHz and 40 % amplitude for 5 to 25 mins. Figure 4.5 [(a) and (b)] shows higher levels of protein extraction as sonication time increased for thraustochytrids AMCQ-4b27 and AMCQS1-9 cells. However, lipase activity increased to 15 minutes but plateaued after that, so that the lipase concentration in extracted protein decreased after 15 minutes.
Chapter 4

extraction (Figure 4.5). The maximum lipase activity obtained from AMCQ-4b27 was 47 U/ml and from AMCQS1-9 was 42 U/ml. This indicates that either heat produced during sonication may have denatured lipase, or that lipase extracted more readily than other protein (Farkade et al., 2006). Decreased lipase activity when acoustic power was increased beyond 40% amplitude is consistent with heat-induced degradation. A recent study indicated deactivation of *Yarrowia lipolytica* lipase upon increasing sonication time (Kapturowska et al., 2012).

**Fig. 4.5 (b)**

![Graph showing lipase activity, protein yield, and specific activity against sonication time](image)

**Fig. 4.5 (b)**

![Graph showing lipase activity, protein yield, and specific activity against sonication time](image)
Figure 4.5. Effect of sonication on thraustochytrid cell disruption and lipase activity. Time course effect of sonication on the protein release and lipase activity from AMCQ-4b27 and AMCQS1-9.

4.3.1.4. Homogenization

During homogenization, microbial cells enter into a rotor-stator creating shear force that disrupts the cells (Lee et al., 2012c). After 25 min homogenization 133 μg/ml protein was extracted from AMCQ-4b27 cells and 75 μg/ml protein from AMCQS1-9 cells. As expected, protein content increased with increasing number of cycles (Halim et al., 2012b). Lipase activity increased substantially in AMCQ-4b27 (25 IU/ml) and AMCQS1-9 (37 IU/ml) until 20 min of homogenization and started declining thereafter [Fig. 4.6 (a) and (b)], likely due to heat deactivation of the lipases. The decrease in lipase activity after 20 min of homogenization can be attributed to enzyme inactivation from the heat produced during the process. Overall lipase activity was relatively low when compared to sonication, indicating that the combination of shear forces and surface tension during disruption process increases lipase degradation (Lee Ying Yeng et al., 2013; Taubert et al., 2000).

Fig. 4.6 (a)

Fig. 4.6 (b)
4.3.2. Comparison of different cell disruption methods for lipase extraction

To release intracellular microbial products, such as lipases, efficient cell disruption methods are required. However, these should not denature the compounds of interest. In this study the four different cell disruption methods, bead vortexing, grinding with liquid nitrogen, sonication and homogenization, were compared for impact on protein and lipase recovery from thraustochytrid extraction (Table 4.2). Sonication resulted in maximum lipase yield from AMCQ-4b27 (0.903 IU/μg) and AMCQS1-9 (1.44 IU/μg). Lower specific activities were obtained using bead vortexing (0.045 IU/μg and 0.125 IU/μg), grinding with liquid nitrogen (AMCQ-4b27 0.160 IU/μg and AMCQS1-9 0.168 IU/μg) and homogenization (AMCQ-4b27 0.211 IU/μg and AMCQS1-9 0.556 IU/μg). Extracts from strain AMCQS1-9 showed approximately 1.5 times higher lipase activity than those from AMCQ-4b27. Overall, sonication resulted in the highest lipase activity, followed by homogenization, grinding with liquid nitrogen and bead vortexing.
Table 4.2. Disruption of thraustochytrid cells for lipase extraction.

<table>
<thead>
<tr>
<th>Cell disruption method</th>
<th>AMCQ-4b27 Lipase activity (IU/ml)</th>
<th>AMCQ-4b27 Protein released (μg/ml)</th>
<th>AMCQS1-9 Lipase activity (IU/ml)</th>
<th>AMCQS1-9 Protein released (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bead vortexing</td>
<td>6±0.7</td>
<td>130±2.1</td>
<td>8±1.0</td>
<td>63±2.0</td>
</tr>
<tr>
<td>Grinding with liquid nitrogen</td>
<td>25±3.0</td>
<td>156±2.4</td>
<td>18±0.5</td>
<td>107±1.6</td>
</tr>
<tr>
<td>Sonication</td>
<td>47±0.2</td>
<td>52±3.0</td>
<td>42±3.7</td>
<td>29±1.3</td>
</tr>
<tr>
<td>Homogenization</td>
<td>25±5.0</td>
<td>126±17</td>
<td>37±4.0</td>
<td>73±9.0</td>
</tr>
</tbody>
</table>

4.4. Conclusion

Thraustochytrids contain a range of potentially useful enzyme activities, including lipase activity. Cell disruption methods were used to increase the extraction yield of intracellular lipase from thraustochytrid cells. We conclude that AMCQ-4b27 cell were sensitive to protein release than the AMCQS1-9. Moreover, AMCQS1-9 has yielded high total specific lipase activity than the AMCQ-4b27. Sonication resulted in the higher lipase activity yield, with strain AMCQS1-9 yielding more lipase activity than did strain AMCQ-4b27. Lipase activity yield did not completely correlate with protein extraction yield, probably due to partial lipase degradation under conditions of high temperature or sheer.
Chapter 5: Tween 80 influences the production of intracellular lipase by *Schizochytrium* S31 in a stirred tank reactor
5.1. Introduction

Lipases (triacylglycerol lipases, EC 3.1.1.3) belong to the hydrolase family of enzymes that catalyse the hydrolysis of triglycerides into free fatty acids and glycerol (Schreck & Grunden, 2014). However, under certain conditions, they are also able to catalyse synthetic reactions (Martins et al., 2014). In addition, lipases have some important functional properties such as substrate selectivity, regio-selectivity and high enantio-selectivity (Gupta et al., 2007; Yuan et al., 2014). These properties make them potential catalyst in numerous industrial processes, such as food, detergent, chemical, and pharmaceutical industries (Sangeetha et al., 2011; Yuan et al., 2014). Lipases are ubiquitous enzymes widely distributed in plants, animals and microorganisms (Mazzucotelli et al., 2015). Among them, microbial lipases have displayed a broad range of substrate specificities. This property might have evolved through the access of microbial lipases to different carbon sources (Hasan et al., 2006; Salihu & Alam, 2015). There is a growing demand for the novel lipases from a microbial sources, because of their ease in production and access to genetic manipulations. Also because they work under mild reaction conditions without requirement of any co-factors (Pérez et al., 2011).

The health benefits of omega-3 fatty acids have been evaluated by a number of clinical studies (Barrow, 2010; Nichols et al., 2014). There is strong evidence for the efficacy of omega-3 fatty acids in the prevention of sudden death from cardiovascular disease and rheumatologic conditions (Abuajah et al., 2015; Bhangle & Kolasinski, 2011; De Caterina, 2011). The anti-cancer activity of omega-3 fatty acids, particularly against colorectal cancer, has recently been shown (Cockbain et al., 2012). Infant brain and eye development is influenced by DHA, which has led to the addition of DHA to the infant formulae. Learning capacity of school going children has been shown to increase by taking DHA enriched food supplements (Qawasmi et al., 2013; Wu et al., 2015). Oily marine fish are the primary source for the industrial production of omega-3 fatty acids. These fish derived omega-3 fatty acids from their diet, primarily from phytoplankton. Researchers have implemented different methods for concentrating omega-3 fatty acids (Rubio-Rodríguez et al., 2010). A promising green approach for concentration of omega-3 fatty acids is the use of lipases. Generally the enzymatic methods are faster than other methods and can be carried out under milder conditions then methods involving chromatographic separation, molecular distillation or urea
complexation (Kahveci et al., 2010; Kralovec et al., 2010). Commercially available lipases have been exploited for omega-3 fatty acid concentration (Akanbi et al., 2013; Casas-Godoy et al., 2014; Fernández-Lorente et al., 2011; Sharma et al., 2013; Valverde et al., 2013). However, selectivity of fatty acid hydrolysis is poor and no lipase selectivity hydrolyse DHA and EPA, a property that would be useful in selecting these fatty acids from fish or microbial oils.

Thraustochytrids produce lipases and because of their high productivity of omega-3 fatty acids, particularly DHA, they are potential sources of omega-3 selective lipases, although none have yet been characterised (Bongiorni et al., 2005; Kanchana et al., 2011). We recently reported lipase activity from a strain of Schizochytrium named S31 (Table 4.2). In this work we optimised culture conditions to enhance lipase production from Schizochytrium sp. S31 in a bioreactor and optimised the extraction of this lipase using sonication. Using a recently developed p-nitrophenyl esters (p-NP) assay (Nalder et al., 2014) we showed that lipase extracted from S31 preferentially hydrolysed DHA over EPA, an unusual selectivity for lipases.

5.2. Materials and methods

5.2.1. Microorganism and maintenance of culture

Schizochytrium S31 (ATCC 20888) was procured from the American type culture collection (ATCC). The stock culture was maintained on GYP medium consisting of glucose 5, yeast extract 2, mycological peptone 2, agar 10 g L\(^{-1}\) and artificial seawater 50% at 25 °C and sub-cultured after 15 days. All medium components and chemicals including p-NP were obtained from Sigma, Australia. The p-NP fatty acid esters of linoleic acid (C18:2), α-linolenic acid (C18:3), eicosapentaenoic acid (C20:5) and docosahexaenoic acid (C22:6) were synthesised based on a recently published protocol (Nalder et al., 2014).

5.2.2. Schizochytrium S31 growth and lipase production

Schizochytrium S31 was grown in a medium containing peptone 0.5, yeast extract 0.5 g L\(^{-1}\) and Tween 80 (1% w/v; as sole carbon source). 50 ml of medium in a 250 ml Erlenmeyer flask was inoculated with a loop full of culture from the plates. This culture flask was incubated for 48 h at 20 °C and shaken at 150 rpm. A 48 h old 5% seed culture (v/v) was used to inoculate sterilised production medium (100 ml in
500 ml Erlenmeyer flask) containing peptone 0.5, yeast extract 0.5 g L\(^{-1}\) and Tween 80 for the production of lipase. The flasks were incubated in a rotary shaker for up to 9 days. Samples were withdrawn aseptically at regular time intervals and then dried and weighted and lipase activity determined. For dry weight determination, the entire content of the liquid culture was transferred to a pre-weighed centrifuge tube and harvested by centrifugation at 3500 rpm for 10 min and the supernatant was discarded. Harvested cells were washed thoroughly with phosphate buffer (pH 7.0) and then freeze-dried for 24 h. The biomass weight was determined gravimetrically. Tween 80 concentrations were estimated based on a colorimetric method (Cheng et al., 2011b). A standard curve was prepared by varying the Tween 80 concentration and the unknown sample concentration was determined using the standard curve. Temperature (15 to 30 °C) and initial pH (5-8) of the medium were optimised to maximise lipase activity. All experiments were carried out in triplicates.

Fermentation of thraustochytrids was carried out in a 2 L stirred tank reactor (New Brunswick, USA) with a working volume of 1.4 L at 25 °C with aeration of 0.5 vvm and agitation of 150 rpm. Varying concentration of Tween 80 (0.5 to 1.5%, v/v) was used to optimise biomass, lipase and lipid production in the bioreactor. Samples were withdrawn at regular time intervals and analysed for residual substrate. Changes in pH were also monitored during the course of fermentation.

5.2.3. Estimation of Schizochytrium S31 growth

The growth of the organism was estimated by taking 1-ml aliquot periodically and measuring the optical density (OD) at 600 nm in a UV-visible spectrophotometer (Shimadzu1601, Japan) with medium as a blank. The OD value was converted into cell mass concentration (mg L\(^{-1}\)) using a standard curve.

5.2.4. Optimisation of cell disruption through sonication

Cell disruption of Schizochytrium S31 biomass was performed as previously described (Byreddy et al., 2015). Thraustochytrid cell biomass (50 mg) was suspended in 3 ml of solvent in a 15 ml centrifuge tube. Sample was sonicated at 20 kHz, 40% amplitude and the pulse was 40 sec on and 20 sec off with total working time of 20 min (Sonics, Newtown, CT, USA). Sample tubes were kept on ice during the sonication process to prevent overheating. Sonication conditions such as time and
acoustic power were optimized. The sonicator used in this study was a horn type with 500 W power output and 20 KHz.

5.2.5. Lipase assay and protein estimation

Lipase activity was measured in the supernatant obtained after cell disruption using \( p \)-nitrophenol palmitate (pNPP) as a substrate, following a previously described method with some modifications (Winkler & Stuckmann, 1979). The lipase substrate solution was prepared by adding solution A consisting of 30 mg of pNPP in 10 ml of isopropanol and the solution B consisting of 0.4% Triton X-100, 0.1% Gum Arabic and 50 mM Tris-HCl buffer, pH 7.0. Solution A (10 ml) was added to solution B (90 ml) drop by drop with stirring. The substrate solution (2.9 ml) and 0.1 ml of enzyme solution was incubated at 37 °C for 10 min and absorbance was measured at 410 nm. One unit (IU) of lipase activity was defined as one nmol of \( p \)-nitrophenol released per minute under the standard assay conditions. The relative activity of crude enzyme is calculated as a percentage activity divided by the most hydrolysed substrate (designated 100% for each substrate).

Protein concentration was estimated from the supernatant using the Bradford method with bovine serum albumin as the standard (Bradford, 1976). All enzyme and protein assays were done in four replicates and the standard error (SE) calculated using Microsoft excel software.

5.2.6. Lipid extraction

Lipid was extracted and quantified from freeze-dried biomass every 48 h according to a previously published protocol (Lewis et al., 2000) with some modifications. Freeze-dried biomass (10 mg) was added to 600 \( \mu \)L solvent (chloroform and methanol in 2:1 ratio) and the sample vortexed for 2 min, followed by centrifugation at 13,000 rpm for 15 min. This extraction process was repeated three times. The supernatants were collected in a pre-weighed vials and dried in an oven at 50 °C. The lipid percentage was measured gravimetrically. All the experiments were performed in triplicate.

5.3. Results and discussion

5.3.1. Optimisation of culture conditions for lipase production
Lipase production is influenced by the type of carbon source used, temperature and initial pH of the culture medium (Orlando Beys Silva et al., 2005). Tween 80 has been successfully used as a carbon source for the production of lipases from various microbes (BoekeMa et al., 2007; Li et al., 2001; Sharma et al., 2014). In this study, Tween 80 was used as the sole carbon source and its concentration was optimised to maximise biomass and lipase productivity (Fig. 5.1). Tween 80 (1%) resulted in the highest level of biomass (0.9 g L⁻¹) and lipase (39 IU/g) productivity by Schizochytrium S31. Similar results were found previously for Acinetobacter radioresistens (Li et al., 2001). Lipase production did not improve when higher tween 80 concentration (1.5%) was used in the medium, although the reason for this is unclear. Remaining experiments were performed with 1% tween 80.

![Figure 5.1. Effect of Tween 80 concentration on lipase production by Schizochytrium S31 at shake flask, pH 6.0 and incubation temperature 25 °C.](image)

Initial pH and temperature are known to influence lipase production (Priji et al., 2015). In Schizochytrium S31 maximal lipase activity was observed at pH 6 (38 U/g) for the pH range of 5 to 8. Similar results were reported for lipase production from Thraustochytrium sp. AH-2 and TZ when the medium pH was pre-adjusted to 6 (Kanchana et al., 2011). The effect of incubation temperature on lipase production was investigated for the four temperatures 15 °C, 20 °C, 25 °C and 30 °C at pH 6 and agitation 150 rpm, with lipase levels being highest at 25 °C (data not shown). Our
results are comparable with lipase production from *Colletotrichum gloeosporioides* where maximum lipase activity at 25 °C (Balaji & Ebenezer, 2008).

### 5.3.2. Production of lipase in stirred tank reactor

Growth kinetics of *Schizochytrium* S31 was studied in terms of biomass production, lipid accumulation in cells, and lipase production. After 48 h acclimatisation in the medium, dry cell weight went from 0.16 g L$^{-1}$ to 0.2 g L$^{-1}$, with the log phase beginning after this time, up to 120 h when growth slows and the stationary phase begins (Fig. 5.2). *Schizochytrium* S31 cells grew well in a fermentation medium containing Tween 80 (1%) as sole carbon source. Fig. 5.2 shows the time course of the investigated parameters at different growth phases and also showed that lipase and lipid production were growth associated. Biomass increased with incubation time and was maximal (0.9 g) after 5 days of incubation. Lipase production reached a maximum of 39 U/g in the late exponential phase, then the lipase activity declined (10.7 U/g) up until 9 days of incubation, perhaps due to product inhibition. Our results are in agreement with other researchers who observed the maximum lipase activity of *Yarrowia lipolytica* KKP 379 in late exponential phase (after 72 h) (Fabiszewska et al., 2014). Another study conducted by Kanchana et al., (2011), found that the maximum lipase activity was observed in thraustochytrids between 4-7 days of incubation (Kanchana et al., 2011). The pH and substrate concentration of the medium decreased as the incubation time increasing, which may be due to the hydrolytic activity of lipase on Tween 80 releasing oleic acid for utilisation. In the bioreactor, lipase production increased with increasing biomass concentration as a result of increasing Tween 80 concentration from 0.5 to 1 %. In addition, improved control of environmental factors in the vessel resulted in increased enzyme production, compared with that achieved in flasks.
Figure. 5.2. Fermentation profile for the production of lipase by *Schizochytrium* S31 in a stirred tank reactor (Tween 80 containing medium was used as sole carbon source, pH 6.0 and temperature 25 °C).

Figure. 5.3. Biomass, lipid and DHA production reached a maximum after five days of incubation. After that the DHA percentage decreased more rapidly than total lipids.

To investigate lipid content and fatty acid composition, biomass was collected and analysed at different time intervals up to 9 days. DHA content increased with total
lipid content and reached a maximum of 5.4% on day 5. Biomass, lipid and DHA all decrease after day 5, although DHA decreases more rapidly than does total lipid (Fig. 5.3). Lipid is used for energy and membrane synthesis during starvation (Thompson Jr, 1996) and DHA is associated with many cellular functions. DHA appears to be used preferentially in the late stages, which also indicates that lipase is acting on DHA during this stage (Fig. 5.2 and 5.3). The fatty acid composition of oil from *Schizochytrium* S31 grown in Tween 80 medium is given in Fig. 4, with growth in glucose medium as a control. The oleate in Tween 80 was directly accumulated into cells (Fig. 5.4). A similar pattern was observed in *Thraustochytrium aureum ATCC* 34304, where Tween 80 in the medium increased the oleic acid content in lipid (Taoka et al., 2011). A corresponding decrease in the percentages of other fatty acids was observed in Tween 80 medium (Fig. 5.4). There was also changes in the ratio of fatty acids when Tween 80 medium was used, with relative decreases in C16 (26%), C18 (70%) relative to DHA.

![Figure 5.4](image)

**Figure. 5.4.** Fatty acid composition of *Schizochytrium* S31 was compared between lipid extracted from biomass grown GYP medium and that from grown in Tween 80 medium.

5.3.3. Disruption of *Schizochytrium* S31 using sonication
In order to investigate the fatty acid selectivity of lipase from *Schizochytrium* S31, sonication was used to release the intracellular lipase. The effect of sonication on *Schizochytrium* S31 was evaluated by microscopic observation and cells were shown to be fully broken at high acoustic power. Table 5.1 shows lipase activity extracted from thraustochytrid cells at an acoustic power of 20 kHz and 40 % amplitude for intervals of 5 to 25 mins. Lipase activity increased to 15 minutes but plateaued after that, indicating partial lipase inactivation with increased time over 15 minutes. Since sonication releases intracellular enzymes but also degrades, optimising time and power are important for maximising lipase extraction (Kadkhodaee & Povey, 2008; Mawson et al., 2011; Nguyen & Le, 2013).

Table 5.1. Effect of sonication on protein release and lipase activity from *Schizochytrium* S31.

<table>
<thead>
<tr>
<th>Cell disruption methods</th>
<th>Lipase activity (IU/ml)</th>
<th>Protein concentration (μg/ml)</th>
<th>Specific activity (IU/μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sonication</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 min</td>
<td>22.8±1.4</td>
<td>43±1.5</td>
<td>0.512</td>
</tr>
<tr>
<td>10 min</td>
<td>32.5±1.6</td>
<td>60±1.8</td>
<td>0.533</td>
</tr>
<tr>
<td>15 min</td>
<td>37.8±1.5</td>
<td>82±2.1</td>
<td>0.451</td>
</tr>
<tr>
<td>20 min</td>
<td>26.3±1.3</td>
<td>105±9</td>
<td>0.247</td>
</tr>
</tbody>
</table>

Fig. 5.5 (a) and (b) show the effect of sonication on lipase activity from *Schizochytrium* S31 at different levels of sonication time and power. As the sonicator power increased from 30% to 70%, the total proteins released increased from 53 to 122 μg/ml and the most lipase activity was observed at 50% power (64 IU/ml). Total proteins released after sonication treatment was 3 folds higher at 50 % (0.75 IU/μg) sonication power than 70% (0.25 IU/μg) (Fig. 5a). Our results are in agreement with previous studies showing that the amount of protein release from microbial cells increased linearly with increasing sonication time and power (Apar & Özbek, 2008; Iida et al., 2008). The highest protein extraction levels from *Schizochytrium* was observed after 15 min of sonication time at 70% of sonication power. However, both total lipase activity and specific lipase activity decreased at 70% power, with extended time causing additional lipase activity degradation. A recent study by Kapturowska et
al, 2013, demonstrated the negative effects of extended sonication time on lipase activity from *Yarrowia lipolytica* KKP 379 (Kapturowska et al., 2012).

![Graph a](image1)

![Graph b](image2)
Figure. 5.5. Cell disruption optimisation of *Schizochytrium* S31 a) as a function of sonication power, b) time duration. (Sonication power 70% for 10 mins was subjected to 100 mg biomass for the lipase extraction).

**5.3.4. Hydrolysis of *p*-Nitrophenyl esters**

Understanding and controlling lipase fatty acid selectivity is important in the modification of fats and oils for high value products and in the production of structured lipids (Qin et al., 2014). The substrate selectivity of S31 crude lipase activity was determined using the standard assay in the presence of 8 mM of the different *p*-NP esters of various chain lengths: *p*-NP acetate (C2); *p*-NP butyrate (C4); *p*-NP caprylate (C8); *p*-NP decanoate (C10), *p*-NP laurate (C12), *p*-NP myristate (C14), *p*-NP palmitate (C16), *p*-NP stearate (C18), *p*-NP-EPA and *p*-NP-DHA. Lipase extracted from *Schizochytrium* S31 showed a broad range of substrate activity (Fig. 6). Maximum activity was observed towards short chain length fatty acids, followed by medium chain length fatty acids. An unusual selectivity was observed towards *p*-NP-DHA, when compared with *p*-NP-EPA. This type of unusual fatty acid selectivity was previous observed for a fish digestive lipase, although this lipase was never characterised (Halldorsson et al., 2004). However, a study investigated the fatty acid selectivity of five commercial lipases using *p*-NP esters found that all lipase showed preference to EPA over DHA (Nalder et al., 2014). This unusual selectivity might be due to the requirement of the *Schizochytrium* to process DHA more than EPA, since EPA is present in only trace amounts in the oil from this strain, whereas DHA levels are relatively high, in contrast to most microalgae.
Chapter 5

5.4. Conclusion

*Schizochytrium* S31 was grown in a bioreactor using Tween 80 as the carbon source, which resulted in high biomass and oleic acid levels. Lipase extraction by sonication was optimised and the fatty acid selectivity of the extracted lipase investigated using *p*-NP fatty acid esters. The lipase extract preferentially hydrolysed shorter chain fatty acids, however, DHA was preferentially hydrolysed over EPA. This preferential hydrolysis of DHA is unusual and may be industrially useful. This selectivity may be a result of this *Schizochytrium* species having much higher levels of DHA than EPA, and so DHA processing is more important for the organism growth and function.

Figure 5.6. Substrate specificity of lipase from *Schizochytrium* S31 (*p*-nitrophenyl esters of different chain lengths were incubated with crude enzyme).
Chapter 6: Bead milling for lipid recovery from thraustochytrids cells
6.1. Introduction

Marine fish and microalgal oils are rich sources of omega-3 fatty acids. The health benefits of omega-3 oil supplements have been documented in numerous studies (Ellulu et al., 2015). Methods available for the concentration of omega-3 fatty acids from fish and microbial oils include urea complexation, chromatography, distillation, low temperature crystallisation, supercritical fluid extraction and enzymatic methods (Rubio-Rodríguez et al., 2010). The primary advantages of enzymatic methods are operation under mild reaction condition and less or no use of chemicals which can degrade the oxidatively sensitive structures.

Omega-3 fatty acid production from microalgae is potentially useful due to their ability to accumulate up to 30-70% of their dry weight as lipid (Adarme-Vega et al., 2014; Martins et al., 2013). Microalgae can be used to produce bulk ingredients for applications in pharmaceuticals, cosmetics, nutraceuticals, animal feeds and functional foods (Yen et al., 2013). Thraustochytrids are marine heterotrophic protist commonly found in marine environments, and are classified under Labyrinthulomycetes (Gupta et al., 2012). They have rapid growth rates and are capable of producing significant amounts of PUFA, including DHA (Gupta et al., 2013). Thraustochytrids have recently been identified as a potential microalgae for omega-3 fatty acid and carotenoids production (Singh et al., 2015a). A range of studies have also demonstrated the suitability of thraustochytrids for biodiesel production (Chen et al., 2015c; Gupta et al., 2015; Gupta et al., 2013).

Specific compounds produced from microbial sources, such as lipids, proteins, carotenoids and carbohydrates, must be extracted and purified using downstream processes prior to their respective applications (Wijffels et al., 2010). Production of intracellular metabolites from microalgae involves extensive processing steps including microalgae cultivation, harvesting, extraction and product recovery. The selection of suitable technologies depends on the characteristics of the algae and the stability of the product (Guldhe et al., 2014; Pahl et al., 2013). Research is ongoing into the development of improved downstream processing techniques for microalgae lipid recovery, a step which is a substantial contributor to total costs for algal production systems (Delrue et al., 2012). Following harvesting, cell disruption is an important step for the extraction of microalgae produced compounds. Different cell disruption methods have been applied to a range of microalgae, including
Chapter 6

ultrasonication, enzymatic treatment, high-pressure homogenisation, alkali treatment, microwaves and pulsed electric field (Grimi et al., 2014; Safi et al., 2014a), however, some of these methods have resulted in low extraction yields of the products of interest.

Bead mill, a homogeniser used for the grinding of ceramics and paints, has been used for the mild disruption of microorganisms for the release of proteins (Schwenzfeier et al., 2011). Recent advancements in temperature control in microalgae cell suspensions make bead milling a promising technique for microalgae component recovery (Doucha & Livanský, 2008; Günerken et al., 2015). The operating principle of bead milling is based on rapid grinding of thick microbial cell suspensions in the presence of beads. The operating mode can be either batch (recirculation) or continuous (single passage through milling chamber) depending on the feedstock used. Parameters which influence cell disruption efficiency include feedstock residence time (suspension feed rate in continuous operation, or process time in batch operation) agitator speed, agitator design, milling chamber design, biomass concentration, bead diameter, bead density and bead filling (Doucha & Livanský, 2008). It is hypothesized that the suspended cells are disrupted in the bead collision zones by compaction or shear forces with energy transfer from the beads to cells (Gunerken et al., 2015). Bead milling has been successfully applied in Saccharomyces sp and Chlorella sp cell disruption for bioactive recovery (Balasundaram & Pandit, 2001; Balasundaram et al., 2012).

In this study, we optimised thraustochytrid cell disruption using bead mill for maximising lipid extraction yields. The cells of Schizochytrium were spherical and filled with numerous lipid bodies. The process was optimised for lipid yield, energy consumption and processing time. The extracted oil was characterised and further employed for enzymatic hydrolysis for the concentration of omega-3 fatty acids, particularly DHA.

6.2. Materials and Methods

6.2.1. Chemicals

All chemicals used in this study were of analytical grade. Medium components such as glucose, yeast extract, mycological peptone (Sigma-Aldrich, USA) and sea salt (Instant Ocean, USA) were used for biomass production. Schizochytrium S31 (ATCC 20888) was procured from the American Type Culture Collection (ATCC) and used
as a standard culture. Solvents such as chloroform, methanol, hexane (from Merck), acetic acid and diethyl ether (from Fischer and Honeywell, Australia) were used in the lipid class analysis and gas chromatography. TLC standards (from Nu-Chek Prep, USA) were used to identify each lipid class.

**6.2.2. Thraustochytrids biomass cultivation**

Thraustochytrids used in this study were maintained on GYP medium consisting of glucose 5, yeast extract 2, mycological peptone 2, agar 10 g L\(^{-1}\) and artificial seawater 50%, at 25 °C and sub-cultured after 15 days. Two strains, *Schizochytrium* sp. S31 (ATCC) and *Schizochytrium* sp. DT3 (an in-house isolate), grown in glycerol media, showed high biomass and lipid productivity (Gupta et al., 2013). Seed medium (1% glucose, 0.2% yeast extract, 0.2% mycological peptone, ASW 50%, pH 6.5) and production medium (4% Glycerol, 1% yeast extract, 0.1% mycological peptone, ASW 50%, pH 6.5) were autoclaved at 121 °C for 20 min. Seed medium (50 ml) was inoculated from agar plates and grown for 2 days in a shake flask at 25 °C, at 150 rpm. Inoculum (5%) was used to inoculate production medium and incubated under the same conditions for 5 days. Biomass was harvested by centrifugation (10,000 × g, 15 min) and washed twice with distilled water, then freeze-dried for 24-48 h. Freeze-dried biomass was stored at -20 °C for further use.

**6.2.3. Cell disruption by bead milling**

The Dyno-Mill (bead mill; Model No. 130857) from Klausen Pvt. Ltd., Germany was used for cell disruption. This mill is well suited to applications in research and development. The Dyno-mill grinding chamber is positioned horizontally with a chamber volume 79.6 ml. Algal suspension is fed through a funnel mounted on the grinding chamber. The beads are accelerated by a motor connected via a central shaft, causing the grinding effect. The bead mill contained an integrated cooling system to prevent overheating. A cooling jacket and cooling coil are integrated into the grinding chamber and supplied by an external water bath to extract heat, and maintain a constant temperature below 20 °C throughout processing. The cell suspension residence time in the chamber is controlled via the pump speed. The beads and product are separated at the end of the chamber by a sieve. Beads used in this study were (0.4-0.6 and 0.8-1.0 mm) zirconium oxide, which are reported to exhibit maximum efficiency in cell disruption (Lee et al., 2012a). As per manufacturer advice
the beads were filled to 54% v/v in the chamber. Keeping these parameter stable, the effect of biomass concentration, bead size, RPM and processing time were investigated. In total, four different cell concentrations in distilled water were used; 1%, 3%, 5%, and 7%. Samples were collected at 2 min intervals up to 8 mins and analysed for total lipid yield and fatty acid profiles.

6.2.4. Microscopic observation

In addition to using lipid yield as a parameter for validating the effectiveness of bead mill treatment on thraustochytrid cells, the direct effect on the cells was observed under a differential interference contrast microscope (Axio-imager, Zeiss, Germany). Microbial smears were prepared on glass slides, and air dried prior to observation.

6.2.5. Lipid extraction

Fatty acid extraction was performed in accordance with Gupta and co-workers (Gupta et al., 2012c) with some modifications. 10 mg of freeze dried biomass was blended with 0.6 ml of solvent. Biomass and solvent were vortexed and then centrifuged at 4000 rpm for 15 min. Supernatant was placed in pre-weighed glass vials and solvent was evaporated under nitrogen. Lipid content (% dry weight basis) was determined gravimetrically.

6.2.6. Lipase catalysed hydrolysis of thraustochytrid oil

Lipase-catalysed hydrolysis of oil was carried out using a modification literature method (Kahveci & Xu, 2011). 500 mg of thraustochytrid oil, a known amount of lipase (3000 U, EC 232-619-9, Candida rugosa; Sigma-Aldrich, St. Louis, US) and 1.8 ml of Tris-HCl buffer (50 mM, pH 7.0) were taken in 25 ml round bottom flasks and heated to 45 °C at 300 rpm stirring. Substrates were stirred for 30 min and lipase was added to initiate hydrolysis. Samples from the reaction mixture were withdrawn at specific time intervals over the course of the 1-4 h reaction. The enzyme hydrolysis reaction was scaled up with 2 g of thraustochytrid oil with optimised enzyme concentration. All experiments were conducted in triplicates with the mean values reported.
6.2.7. Thin Layer Chromatography for lipid class analysis

Both the hydrolysed and unhydrolysed portions of thraustochytrid oil were analysed by capillary chromatography with a flame ionization detector (Iatroscan MK5, Iatron Laboratories Inc., Tokyo, Japan). The Iatroscan settings were: air flow rate; 200 ml/min, hydrogen flow rate; 160 ml/min and scan speed of 30 s/scan. Under these conditions, the chromarods were cleaned by scanning twice before applying samples. 1 μL of each lipid fraction in hexane was spotted onto the rods with the aid of an auto pipette along the line of origin on the rod holder and developed for 22 min in a solvent tank containing hexane/diethyl ether/acetic acid (60:17:0.2, vol/vol/vol). TLC standards were used to identify each lipid class.

6.2.8. Gas Chromatography analysis for fatty acid composition

Hydrolysed and unhydrolysed thraustochytrid oil compositions were analysed by gas chromatography (Agilent 6890) with a flame ionisation detector (FID), equipped with a Supelcowax 10 capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness; Supelco). Fatty acids were converted to methyl esters by acid-catalysed trans-esterification with minor modifications (Christie & Han, 2010). 1 ml toluene was added to the glass tubes followed by the addition of 200 μl of internal standard, methyl nonadecanoate (C19:0) and 200 μl of butylated hydroxytoluene (BHT). Acidic methanol (2 ml) was also added to the tube and kept for overnight incubation at 50 °C. Fatty acid methyl esters (FAMEs) were extracted into hexane. The hexane layer was removed and dried over sodium sulphate. FAMEs were concentrated using nitrogen gas. Helium was used as the carrier gas at a flow rate of 1.5 ml min⁻¹. The injector was maintained at 250 °C and a sample volume of 1 μl was injected. Fatty acid peaks were identified on comparison of retention time data with internal standard (Sigma-Aldrich, St. Louis, MO, USA) and corrected using theoretical relative FID response factors (Ackman, 2002). The peaks were quantified with chemstation chromatography software (Agilent Technologies, Santa Clara, CA, USA).

6.3. Results and discussion

6.3.1. Thraustochytrid cultivation
Chapter 6

Thraustochytrids are marine microalgae known for their ability to produce large quantities of lipid, including a high ratio of omega-3 fatty acids, particularly DHA. The carbon source used in the culture medium has a significant effect on biomass and lipid productivity (Abedini Najafabadi et al., 2015). In this study, we used production medium with glycerol as a carbon source for thraustochytrids cultivation, since glycerol is a low cost carbon source that thraustochytrids grow well on (Gupta et al., 2013; Li et al., 2015). In this study the highest biomass and lipid production achieved were 12.23 ± 0.4 gL⁻¹ and 29% ± 1.9 for Schizochytrium sp. S31 and 14.65 ± 0.54 g-L⁻¹ and 35% ± 2.1 for Schizochytrium sp. DT3.

6.3.2. Cell disruption by bead mill

Bead milling has been utilised for cell disruption and extraction of intracellular metabolites from microorganisms such as yeasts (Middelberg, 1995), bacteria (Schütte et al., 1983), filamentous fungi (Baldwin & Moo-Young, 1991) and even microalgae such as Chlorella vulgaris (Postma et al., 2015). Nevertheless, the use of bead milling in lipid extraction from marine microalgae thraustochytrids has not been previously reported. Important process parameters such as biomass concentration, bead size, RPM and process time were optimised in the present study to improve the lipid extraction from thraustochytrids specifically. The experimental process from thraustochytrid cell disruption to enzyme concentration used in the present study is depicted in Fig. 6.1.
Chapter 6

Figure 6.1. Schematic diagram of experimental process followed from thraustochytrid cell disruption to enzymatic omega-3 fatty acids concentration.

The extent of cell disruption depends on residence time, shear forces, type of microorganism, and cell concentration etc. During experimental stage, biomass concentration, processing time, bead size (0.4-0.6 and 0.8-1.0 mm zirconium oxide) and processing speed were optimised and the bead load (54%) was kept constant based on manufacturers advice. Various biomass concentrations (1%, 3%, 5% and 7%) and processing times (in minutes 2, 4, 6 and 8) were investigated. Thraustochytrids cells disrupted by bead mill were observed under a microscope to validate the cell disruption. Fig. 6.2 shows typical microscopic images of disrupted cells at optimised bead mill conditions.

Figure 6.2. Thraustochytrid cell disruption (40 X magnification) employing bead mill at 5% biomass concentration, bead size 0.4-0.6 mm, 4500 rpm and 4 min processing time.

The rate of cell disruption will increase with increasing stirring speed, however, simultaneous increase in biomass load may decrease this effect. The degree of cell disruption increased with increasing bead filling volume in the grinding chamber, up to about 70% where interference among the grinding elements prevents the establishment of effective cell disruption (Doucha and Livansky, 2008). As shown in Fig. 6.3, lipid recovery increased up to biomass concentrations of 5% and then
decreased, probably due to increased viscosity above 5% (Oh et al., 2009). Our results are in agreement with a previous study in which the high cell density of *Chlorochococum* was found not to increase cell disruption (Doucha & Livanský, 2008), but are inconsistent with a study showing that yeast mass concentration during bead milling had no significant effect on cell disruption (Baldwin & Moo-Young, 1991; Mogren et al., 1974).

Figure 6.3. Optimisation of lipid extraction by bead mill as function of biomass concentration. Four different biomass concentrations include 1 g, 3 g, 5 g and 7 g were used.

Lipid yield increased between 1500 rpm and 4500 rpm while further increase in rpm had no effect on lipid yield (Fig. 6.4). Doucha & Livanský, 2008) found that the agitator speed (rpm) between 4500 rpm and 5500 rpm resulted in maximal cell disintegration in *Chlorococum* cells. Increasing milling time increased lipid recovery at all biomass concentrations. Maximum lipid recovery was achieved with 5% biomass, 4500 rpm and 4 min processing time, with no increase in lipid recovery after 4 minutes since thraustochytrid cells were completely disrupted at that time (Fig. 6.2). A recent study using bead milling to extract *Chlorella* cells resulted in 90-95% cell disruption in 4 min of bead milling (Postma et al., 2015).
In order to understand the effect of bead size on thraustochytrid cell disruption and lipid yield, we investigated two different bead sizes, 0.4-0.6 mm and 0.8-1.0 mm. The maximum lipid yield (40.5% ± 0.9) was achieved with 0.4-0.6 mm beads compared to 38.6 ± 1.2 with 0.8-1.0 mm beads. The optimum bead sizes was previously indicated to be 0.5-0.7 mm for yeast, 0.25-0.5 mm for bacteria and 0.3-0.7 mm for *C. vulgaris* (Schütte et al., 1983) (Doucha & Livanský, 2008; Lee et al., 2012a).

At low biomass concentration 10 Kg m\(^{-3}\) and 8 min processing time, the highest energy consumption was 528 MJ Kg\(^{-1}\) biomass and lipid yield was 31.45%. Maximum lipid recovery was observed with 50 Kg m\(^{-3}\) and 4 min processing time, yielding 40.12%, with an estimated energy consumption of 264 MJ Kg\(^{-1}\). The disruption efficiency was quantified as a measure of lipid recovery and had the highest efficiency at 50 Kg m\(^{-3}\) and 4 min processing time with low energy consumption compared to other parameters (Table 6.1). Lee et al, 2010, reported the energy consumption of bead milling on three different microalgae *Botryococcus, Chlorella* and *Scenedesmus* is equivalent to 504 MJ kg\(^{-1}\) of the dry mass.

Table 6.1. Energy consumption analysis for different mass concentrations and lipid yields. *Schizochytrium* sp. 31 biomass was used in different mass concentration and energy was calculated where the maximum lipid yield was achieved.
### 6.3.3. Lipid profile analysis

The optimised bead mill processing parameters were applied to lipid extraction from two thraustochytrid species, resulting in 49.6% lipid as a percentage of dry weight, with 39.8% DHA in the extracted lipid, for *Schizochytrium* sp. DT3. Extraction of *Schizochytrium* sp. S31 resulted in lipid (39.06%) as a percentage of dry weight, with 31.4% DHA in the extracted lipid (Fig. 6.5). Our results are in agreement with a recent study on lipid and DHA production from *Aurantiochytrium limacinum* SR21, which achieved maximum lipid and DHA accumulation using glycerol as the carbon source (Li et al., 2015). A similar study conducted by Gupta et al., (2015) investigated the suitability of *Schizochytrium* sp. DT3 for lipid production from enzyme saccharified hemp, and reported DHA accumulation of 38% (Gupta et al., 2015). *Schizochytrium* sp. DT3 oil contained more DHA than did strain S31 and so was investigated further for the lipase concentration of DHA.

<table>
<thead>
<tr>
<th>Mass concentration (Kg m⁻³)</th>
<th>Energy consumption (MJ Kg⁻¹ mass)</th>
<th>Lipid yields (%)</th>
<th>Process time (min)</th>
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<tbody>
<tr>
<td>10</td>
<td>528</td>
<td>31.45</td>
<td>8</td>
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<tr>
<td>30</td>
<td>396</td>
<td>35.87</td>
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<td>4</td>
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<tr>
<td>70</td>
<td>396</td>
<td>38.4</td>
<td>6</td>
</tr>
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6.3.4. Selective hydrolysis of *Schizochytrium* DT3 oil using *Candida rugosa* lipase

Before hydrolysis, oil extracted from the DT3 strain was confirmed to be essentially in the triacylglycerol form (Fig. 6.6a). *Candida rugosa* lipase (CRL) was used for DHA concentration since it is known to preferentially hydrolyse saturated (SFAs), then mono-unsaturated fatty acids (MUFAs), with low activity towards polyunsaturated fatty acids (PUFAs) from TAGs (Kahveci et al., 2010; McNeill et al., 1996). Enzyme concentrations from 1000 U-4000 U were tested with 500 mg of thraustochytrid oil where oil was incubated with different enzyme concentrations and samples were collected at specific time interval and analysed for hydrolysis. Fig. 6.7 shows that the percentage of oil hydrolysed increased with increase in enzyme concentration, particularly with 3000 and 4000 U enzyme resulting in more than 50% of hydrolysis after 3 hrs. Enzyme concentrations of more than 3000 U did not increase the level of thraustochytrid oil hydrolysis, possibly because of substrate limitation. A similar trend was observed in the hydrolysis of crude palm oil by *Candida rugusa* lipase (You & Baharin, 2006). Enzymatic hydrolysis with *Candida rugosa* lipase
resulted in free fatty acid increase over time (Fig. 6.8), with corresponding increases in diacyl and monoacyl glycerolds (Fig. 6.6b). Fifty percent hydrolysis of TG occurred in 3 hours. *Candida rugosa* lipase preferentially hydrolysed myristic acid (C14:0) and palmitic acid (C16:0), with DHA and DPA being only slightly hydrolysed (Fig. 6.9).

Figure 6.6. Capillary chromatography (iatrascan) profile of thraustochytrid oil a) before and b) after hydrolysis by *Candida rugosa* lipase at 40 C.
Figure 6.7. Optimisation of enzyme concentration. 500 mg of thraustochytrid oil was incubated with varying enzyme concentrations and samples were withdrawn at different time intervals.

Figure 6.8. Lipid class analysis of thraustochytrid oil after hydrolysis with *Candida rugosa* lipase at different time intervals.
Chapter 6

Figure. 6.9. AG and FFA are separated hydrolysed oil and analysed by gas chromatography. Short chain fatty acids were preferentially hydrolysed from TAG and DPA and DHA significantly concentrated at glyceride portion.

In the triacylglycerol portion myristic acid was reduced from 10.2% to 4.3% and palmitic acid from 35.3% to 10.24%. DPA in the triacylglycerol portion increased from 19.2% to 29.14%, with DHA increasing from 39.8% to 55.45%. CRL has been shown previously to preferably hydrolyse short and medium chain fatty acids while increasing omega-3 levels in the remaining triacylglycerides (Kahveci & Xu, 2011 (Nalder et al., 2014). This fatty acids selectivity is due to the deep tunnel like active site which is buried inside the protein structure. Under optimised conditions in this study the omega-3 content of thraustochytrid oil was enriched to 88.7%, 1.4 folds above initial levels. The fatty acid profiles obtained were as expected, from fatty acid chain length selectivity of Candida rugosa lipase.

6.4. Conclusion

Schizochytrium sp. DT3 is a renewable source of omega-3 fatty acids, particularly DHA. We optimised a bead mill based cell disruption method for maximising lipid extraction with lower energy consumption. We proposed a simple enzymatic process for the concentration of DHA from marine microalgae
Schizochytrium sp. DT3 oil using Candida rugosa lipase. This lipase at 3000 U hydrolysed 50% of thraustochytrid oil in 3 h, increasing DHA levels by 1.4 fold.
Chapter 7: Phospholipase A1 as a catalyst to enrich omega-3 fatty acids in anchovy and algal oil
7.1. Introduction

Consumption of fish oils has been shown to have health benefits, mainly in cardiovascular events, based on several prospective and retrospective studies (Lavie et al., 2009; Molfino et al., 2014). Most of the benefits of fish oil were attributed to the polyunsaturated omega-3 (ω-3) fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Udani & Ritz, 2013). The American Heart Association recommends consumption of 1g/day of omega-3 fatty acids to patients with coronary heart diseases (Kralovec et al., 2012). Omega-3 fatty acids constitute approximately 30% of the total fatty acids in natural fish oils. Concentrated esters of omega-3 fatty acids have been formulated to deliver higher amounts of EPA and DHA per dose, particularly in pharmaceutical products where 80% EPA and DHA concentrates are used (Molfino et al., 2014). However, conversion to ethyl esters followed by fractional distillation and urea concentration is damaging to these oxidatively sensitive omega-3 fatty acids. Also, re-esterification to triacylglycerides requires further damaging processing of these oils, and also results in statistical distribution of fatty acids on the glycerol backbone (Catal et al., 2013).

To overcome heat induced degradation, lipases were employed to selectively hydrolyse omega-3 fatty acids. Triglycerides from natural fish oils are complex in composition (Miller et al., 2008), besides containing several kinds of fatty acids, mainly saturated, monounsaturated and polyunsaturated, the fatty acids are also non-uniformly distributed on the glycerol backbone. $^{13}$C NMR spectral studies on anchovy fish oils show that DHA in more abundant at sn-2 than at sn-1 and -3 positions, while EPA is nearly statistically distributed across all three positions (Akanbi et al., 2013). Five lipases were tested for specificity in the hydrolysis of fish oil, using fatty acid esters as a model system. Discrimination for EPA and DHA was observed with fatty acid esters but not with fish oils (Lyberg & Adlercreutz, 2008). Another study on lipase mediated fish oil hydrolysis suggested that hydrolysis is biased towards the chemical nature of the fatty acid rather than to their positional abundance (Akanbi et al., 2013).

Statement of Contribution to Research for this Chapter: The research outlined in the chapter was carried out jointly with Mr. Tushar Ranjan Moharana (PhD student) and Dr Madhusudhana Rao at CCMB, Hyderabad, India, with funding from an Australia-India strategic research grant. The experiments data shown in Figures 7.2B, 7.3B, 7.5B, 7.6 and 7.7 were performed by Mr. Moharana and the data shown in Figures 7.1,7.2A, 7.3A, 7.4, 7.5A, 7.8, 7.9 and Table 1 were performed by me.
Lipases have been extensively used for selective concentration of ω-3 fatty acids from fish oils either by fish oil hydrolysis or selective esterification (Akanbi et al., 2013; Kosugi & Azuma, 1994; Kralovec et al., 2010; Shimada et al., 1997). Lipases possess some important properties such as selectivity towards chain length and position of fatty acid in a triglyceride and also discriminate between fatty acids with single and multiple double bonds. These properties of lipases make them as suitable candidates for enzymatic concentration of omega-3 fatty acids (Shahidi & Wanasundara, 1998). Most lipases preferentially hydrolyses saturated and mono-unsaturated fatty acids from triacylglycerides and strongly discriminate against omega-3 fatty acids, due to the presence of double bonds that enhance the steric hindrance at lipase active site (Casas-Godoy et al., 2014; Halldorsson et al., 2004). Most lipases preferentially hydrolyse EPA over DHA, probably due to the presence of an additional double bond located closer to the ester bond in DHA (Akanbi et al., 2013; Shimada et al., 1998). In another study, pancreatic lipase was observed to preferably hydrolyse docosapentaenoic acid (DPA) over EPA and DHA (Akanbi et al., 2014).

Phospholipase A1 specifically hydrolyses the fatty esters at the sn-1 position and release 2-acyl lysophospholipid and a fatty acid. Functions of PLA1 are not clearly established and some PLA1 were reported to show lipase-like activity (Mishra et al., 2009; Richmond & Smith, 2011). Lipases that show phospholipase activity have been extensively investigated, but not the reverse (Aloulou et al., 2014). The catalytic mechanism between lipases and phospholipases is identical; however, the specificity emerges from the active site properties. Studies have indicated that lipases with a short lid and a short β9 loop are more suitable to accommodate polar phospholipids (Carrière et al., 1998). PLA1 was tested for its specificity on triglycerides in this study and the activity profiles were compared with those of a lipase from Bacillus subtilis. To investigate the ability of a phospholipase in regioselective hydrolysis of triacylglycerols, we choose PLA1 since it is likely to be selective for the sn-1, 3 position in triacylglycerols.

Phospholipase A1 is a recombinant enzyme manufactured by Novozymes by the fusion of a lipase gene from Thermomyces lanuginosus and a phospholipase gene from Fusarium oxysporum. This enzyme was observed to hydrolyse both phospholipid and triglycerides with a single active site (Fernandez-Lorente et al., 2008; Mishra et al., 2009). The phospholipase activity of this enzyme is applied in the commercial...
degumming of vegetable oils and for modifying phospholipids (Sheelu et al., 2008; Yang et al., 2008). The lipase activity of PL A1 has been applied in organic synthesis and in the synthesis of structured lipids (Sheelu et al., 2008). PLA1 also successfully immobilised on different nanoparticles to further improve its catalytic properties (Liu et al., 2012; Mishra et al., 2009; Wang et al., 2010). Therefore, in this study, we attempted to use phospholipase A1 for the concentration of omega-3 fatty acids from anchovy fish oil. We investigated the chain length selectivity of phospholipase A1 using p-NP esters. The ability of Phospholipase A1 to concentrate omega-3 fatty acids into the glyceride portion was investigated by examining the preferential fatty acid hydrolysis using gas chromatography and $^{13}$C NMR spectroscopy.

**7.2. Material and Methods**

**7.2.1. Chemicals**

Bleached Anchovy oil was supplied by Ocean Nutrition Canada (Canada). The major fatty acid composition of native oil was determined by Gas chromatography analysis (Akanbi et al., 2013). Phospholipase A1 (Lecitase Ultra®) and gas chromatography standards were procured from Sigma-Aldrich (Castle Hill, Australia) and hexane (Merck). All the buffers were made using analytical grade chemicals. All solvents used were either analytical grade or higher. Gum Arabic and phospholipase A1 (PLA1) and methyl nonadecanoate were purchased from Sigma Aldrich. TLC plates were purchased from Merck.

**7.2.2. Hydrolysis of anchovy oil**

Reaction mixture containing 50 mM Tris, 25 mM CaCl$_2$, 5% (w/v) gum Arabic at pH 8.00 and 5% (v/v) anchovy oil were micellised by sonication. 100 unit of PLA1 was added and the reaction was carried at 37 °C at constant stirring under nitrogen. Rate of reaction was monitored by pH stat (Ω Metrohm 718 STAT Tritino) by titrating against 1 M NaOH. 2 ml of sample were withdrawn at every time point and free fatty acid was separated from glyceride by solvent extraction and the purity of both the fractions were analysed by TLC. Solvent was evaporated under nitrogen gas and fractions stored in an airtight polypropylene tube at -20 °C for further analysis. Oxygen was removed by blowing nitrogen whenever the sample was exposed to air.
7.2.3. **Thraustochytrid oil hydrolysis by phospholipase A1**

Thraustochytrid lipid extraction and analysis was described in section 6.2.5, 6.2.7 and 6.2.8. Phospholipase hydrolysis was performed based on the method described in section 6.2.6 with some modifications. 500 mg of thraustochytrid oil and 1.8 ml of Tris HCL buffer (50 mM, pH 7.0) were taken in 25 ml round bottom flask and heated up to 45 °C and 300 rpm stirring. Substrates were stirred for 30 min and Phospholipase A1 (2500 U/g oil) added to initiate the hydrolysis. Samples were collected periodically and analysed.

7.2.4. **IATROSCAN analysis**

Both the unhydrolysed and hydrolysed portions of the fish oil were analysed by capillary chromatography with flame ionization detector (Iatroscan MK5, Iatron Laboratories Inc., Tokyo, Japan). The Iatroscan settings were: air flow rate; 200 ml/min, hydrogen flow rate; 160 ml/min and scan speed of 30s/scan. Under these conditions, the chromarods were cleaned by scanning twice before applying samples. One microliter of each lipid fraction in hexane was spotted onto the rods with the aid of an auto pipette along the line of origin on the rod holder and developed for 22 minutes in a solvent tank containing hexane/diethyl ether/acetic acid (60:17:0.2, vol/vol/vol). TLC standards purchased from Nu-Chek Prep were used to identify each lipid class (Akanbi et al., 2013).

7.2.5. **Methylation and GC analysis**

5 μL (both free fatty acid and glyceride) of sample was methylated by using acetyl chloride in methanol as described by Christie et al 2010 (Christie & Han, 2010). Hydrolysed and unhydrolysed fish oil was analysed using gas chromatography (Agilent 6890) with flame ionisation detector (FID), equipped with a Supelcowax 10 capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness; Supelco). Fatty acids were converted to methyl esters by acid-catalysed esterification with minor modifications. 1 ml toluene was added to the methylation tubes followed by the addition of 200 μl (5mM) of internal standard, methyl nonadecanoate (C19:0) and 200 μl (1mM) of butylated hydroxytoluene (BHT). 2 ml acidic methanol (prepared by adding 10% acetyl chloride in methanol drop wise on ice bath) was also added to the tube and kept for overnight incubation at 50°C. Fatty acid methyl esters (FAMEs) were
extracted into hexane. The hexane layer was removed and dried over sodium sulphate. FAMEs were concentrated using nitrogen gas and analysed by gas chromatography. Helium was used as the carrier gas at a flow rate of 1.5 ml min\(^{-1}\). The injector was maintained at 250°C and a sample volume of 1 µl was injected. Fatty acid peaks were identified on comparison of retention time data with external standards (Sigma-Aldrich, St. Louis, MO, USA) and corrected using theoretical relative FID response factors (Ackman, 2002). Peaks were quantified with Chemstation chromatography software (Agilent Technologies, Santa Clara, CA, USA).

### 7.2.6. Analysis of positional distribution of fatty acid by NMR

300 µl of glyceride were dissolved in 1 ml of CDCl\(_3\) (99.8% pure). NMR spectra were collected by using Bruker 600MHz NMR machine. Peak corresponding to different fatty acid at different position were assigned as described by (Suárez et al., 2010). Relative quantification was done by comparing the area under each peak. Quantitative \(^{13}\)C NMR spectra of the unhydrolysed oils were recorded under continuous \(^1\)H decoupling at 24°C. In order to quantify the residue of each fatty acid at different positions, peak area ratios were analysed by integration and presented in percentages (Akanbi et al., 2013).

### 7.3. Results and discussion

#### 7.3.1. Lipase activity of Phospholipase A1

PLA1 used in this study was isolated from *Thermomyces lanuginosus* (Lecitase\(^{\text{TM}}\)) and is a recombinant enzyme preparation. This enzyme has been explored primarily for the degumming of vegetable oils (Yang et al., 2006). Some studies include the use of PLA1 lipase activity for the resolution of mandelate esters and the regioselective hydrolysis of carbohydrate esters (Mishra et al., 2009). Lipase-like activity of PLA1 was reported earlier. However its ability to hydrolyse triglycerides is only marginal in the vegetable oil degumming processes. PLA1 was previously characterised for substrate selectivity and it was observed that this enzyme does hydrolyse C2-esters (Mishra et al., 2009). Initially the lipase activity of PLA1 was tested using \(p\)-nitrophenyl esters of various chain length fatty acids. Some of the \(p\)-NP esters were synthesised based on published methods (Nalder et al., 2014; Neises & Steglich, 1978). Fig. 7.1 shows that PLA1 was able to hydrolyse esters of fatty acid
Chapter 7

chain lengths from C4–C20 with comparable efficiency. The activity was highest for C10 and lowest with C4 ester (40% of C10). The presence of unsaturation, single or multiple double bonds, did not impact the activity appreciably. However, esters of DHA were not efficiently hydrolysed. A similar chain length study was performed for lipase from Bacillus subtilis (BSL) and it was found that C8 chains were most efficiently hydrolysed (Dartois et al., 1992).

![Figure 7.1 Phospholipase A1 activity towards p-NP esters with different acyl chains. Activity of PLA1 on p-NP-decanoate was taken as 100%.](image)

Lipase activity of phospholipases and vice versa was studied in few cases. The ratio of lipase to phospholipase activity of lipases or phospholipases varies widely and the ratio is not only related to the structure of the lipase but also to the reaction system (Yang et al., 2008). For example, PLA1 from Thermomyces lanuginosus used in this study shows hydrolytic activity against both triacylglycerides and phospholipids and is used to degum oils (Sheelu et al., 2008). PLA1 shows predominately phospholipase activity at reaction temperatures above 40 °C. Pancreatic phospholipases hydrolyse phospholipids in the aqueous phase, while lipase from Fusarium oxysporum hydrolyses phospholipids in the oil phase (Yang et al., 2008).

7.3.2. Hydrolysis of Anchovy oil by PLA1

Initially hydrolysis of anchovy oil was tested in an oil-in-water emulsion and the extent of hydrolysis was monitored using Iatroscan. Fig.7.2A shows the scan
before and after hydrolysis of the oil by PLA1. To obtain more quantitative kinetics of the hydrolysis, anchovy oil hydrolysis was monitored by a pH Stat, while maintaining a constant pH, using 5% Gum Arabic as emulsifier. Initially we measured the rate of hydrolysis of anchovy oil by PLA1 and BSL (Fig.7.2B). Both enzymes were able to hydrolyse the oil to a comparable extent. At 37 °C the reaction reached 45% hydrolysis and then plateaued.

Figure. 7.2. Time course of hydrolysis of anchovy fish oil by PLA1 and BSL. A, Iatroscan of unhydrolysed (light trace) and hydrolysed (darker trace) fraction of anchovy oil by PLA1. B, pH stat based determination of rate of hydrolysis of anchovy oil in the presence of PLA1 (closed circle) and BSL (open circle).

The fatty acid fraction was separated from the glyceride fraction, methylated and analysed by GC. Fig.7.3A shows the time course of release of various fatty acids
from anchovy oil by PLA1. Both saturated and monounsaturated fatty acids were extensively hydrolysed (approximately 40% hydrolysis), while the hydrolysis of both the omega-3 fatty acids EPA and DHA was relatively poor 15% and 4%, respectively. Identical experiments were also performed with BSL (Fig.3B). BSL also hydrolysed oil with similar efficiency. However, the extents of hydrolysis of various fatty acids were similar. While the extents of hydrolysis of unsaturated, monounsaturated and EPA were equal (50%), hydrolysis of DHA was marginally less at 30%. The fatty acid products obtained after 3 h hydrolysis are shown in Fig.7.4. The data suggests that PLA1 discriminates against EPA and DHA, while BSL shows marginal discrimination against DHA. A significant five-fold higher discrimination of DHA by PLA1 compared to BSL was observed.
Chapter 7

Figure. 7.3. Time course of hydrolysis of anchovy oil and fatty acid distribution in the hydrolysate. Anchovy oil was subjected to hydrolysis by PLA1 (A) and BSL (B). The reaction product at various times was subjected to methylation and GC analysis. Percent hydrolysis of each fatty acid was calculated based on its hydrolysis at tₓ as a fraction of tₒ. Saturated (○), Monounsaturated (●), EPA (◇), DHA (▲), Total hydrolysis (■).

PLA1 specifically hydrolyses sn-1 acyl esters from phospholipids producing FFAs and lysophospholipase. PLA1 enzymes normally exhibit very little lysophospholipase or lipase activity. However, some PLA1 enzymes exhibit some neutral lipase activity. PLA1 enzymes may be descendants of neutral lipases, and several PLA1 sequences show substantial sequence similarity to the well characterised pancreatic, hepatic and endothelial lipases (Aoki et al., 2007; Richmond & Smith, 2011). Although PLA1 enzymes are found in a wide variety of cells and tissues, only a small number of PLA1 enzymes have been cloned and their substrate preferences have not been well studied (Richmond & Smith, 2011). Several structural and protein engineering studies on lipases have focused on the architecture of the active site and its substrate preferences. The hydrophobicity and hydrophilicity balance of the β5, β9 loop and the lid domain of lipases play a selective role in preferring a triglyceride or a phospholipid (Aoki et al., 2007; Tiesinga et al., 2007). The lid domain of guinea pig pancreatic lipase related protein 2 (GPLRP2) is reduced in size and consequent exposure of hydrophilic residues enables the active site to accept phospholipids and larger galacto lipids (Carrière et al., 1998). Human pancreatic lipase that does not show phospholipase activity was not able to accommodate the phospholipid molecule. Sequence alignment of porcine pancreatic lipases indicates that Val260 is critical for interaction with lipids (Van Kampen et al., 1998).

PLA1 from Thermomyces lanuginosus, used in this study, discriminated against DHA and partly against EPA when hydrolysing anchovy oil. In similar experiments using a lipase from Bacillus subtilis there was no observed selectivity against EPA and DHA. Anchovy oil has DHA preferentially located at the sn-2 position (60%), compared to the sn-1, 3 positions (40%). If PLA1 preferentially hydrolyse at the sn-1, 3 position in TG oils then more DHA than EPA should be present in the unhydrolysed fraction. This was observed in our experiments (Fig.7.4). In our study PLA1 poorly hydrolysed DHA at all positions, and preferably hydrolysed EPA.
BSL preferentially hydrolysed DHA at the sn-2 position, while preferentially hydrolysing EPA at sn-1, 3.

7.3.3. \(13\text{C}\) NMR studies of the anchovy oil hydrolysis by PLA1 and BSL

\(13\text{C}\) NMR spectra of complex triglycerides, such as fish oils, can provide positional information on the various fatty acids in the oil. This information enables the study of the positional specificity of hydrolysis by PLA1 and BSL and also to obtain quantitative information on the extent of hydrolysis of fatty acids at each position. Initially we acquired the positional information of various fatty acids in anchovy oil. Table 7.1 provides the details of positional distribution of fatty acids in Anchovy oil. The ratio of abundance of various fatty acids at sn-1, 3 and sn-2 positions was observed to be saturated 1.64, monounsaturated 3.06, EPA 4.2 and DHA 0.6. A ratio of 2 suggests the fatty acids are equally distributed. A ratio of 0.6 for DHA indicates it is predominantly present at the sn-2 position. The ratio is high (4.2) for EPA indicating its preferential distribution at sn-1, 3 position, compared with the distribution of DHA.

Table 7.1. Positional distribution of various fatty acids in anchovy oil.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Distribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(sn-1,3)</td>
</tr>
<tr>
<td>Saturated</td>
<td>22.9</td>
</tr>
<tr>
<td>Mono unsaturated</td>
<td>18.7</td>
</tr>
<tr>
<td>Stearidonic acid (STA)</td>
<td>6.2</td>
</tr>
<tr>
<td>EPA</td>
<td>14.9</td>
</tr>
<tr>
<td>DHA</td>
<td>3.9</td>
</tr>
</tbody>
</table>

Anchovy oil hydrolysis by PLA1 and BSL was allowed to proceed until 30% of the oil was hydrolysed and then the unhydrolysed portion of the oil was extracted and \(13\text{C}\) NMR spectra were obtained. This was repeated with unhydrolysed anchovy oil. Figure 7.5 shows the \(13\text{C}\) NMR spectra of hydrolysed and unhydrolysed anchovy oil subjected to PLA1 (Fig. 7.5A) and BSL (Fig. 7.5B) treatment. The overlay of hydrolysed and unhydrolysed spectra for both PLA1 and BSL clearly demonstrates that BSL preferentially hydrolys EPA and DHA more than does PLA1. Fatty acids
remaining in the unhydrolysed portion of the oils with PLA1 and BSL are shown in Fig. 7.4. Saturated and monounsaturated fatty acids were preferentially hydrolysed by both the enzymes while omega-3 fatty acids were discriminated against. BSL also preferentially hydrolysed EPA over DHA.

Figure 7.4. Percent hydrolysis of various fatty acids (from Fig. 7.3) at 32% hydrolysis of anchovy oil by PLA1 (light) and BSL (dark). The relative proportions at other time points were also similar.
Chapter 7

Figure. 7.5. 13C NMR spectra of the unhydrolysed oil fraction before (blue) and after (red) 30% hydrolysis of anchovy oil by PLA1 (A) and BSL (B). Fatty acids and their positions were marked. Sat (saturated), mono (monounsaturated), STA (stearidonic acid), EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid).

The percent accumulation data shown in Fig. 7.6A indicates that EPA at sn-1 was more efficiently hydrolysed by BSL than by PLA1. BSL hydrolysed EPA at sn-1 but not DHA at sn-1 or at sn-2, while PLA1 had no preference for EPA over DHA. These results indicate that the discrimination against omega-3 fatty acids by PLA1 is a result of the chemical nature of the fatty acid and not its position on the TG. To verify this observation, we have performed hydrolysis of a structured triglyceride, 1, 3-dioleoyl-2-hexadecanoic glyceride, using PLA1 and BSL and estimated the fatty acid products by GC (Fig. 7.7). If PLA1 shows absolute preference for fatty acids at the sn-
1, 3 position, the hydrolysis of hexadecanoic acid at sn-2 should be much lower than that of oleic acid. The difference between the specificity of hydrolysis by PLA1 and BSL was also negligible, indicating that PLA1 did not show any specificity towards the sn-1,3 positions. However, there is a distinct preference against DHA in anchovy oil, indicating the fatty acid specificity of this enzyme.

Figure 7.6. Percent accumulation of different fatty acid classes in TG fraction after 30% hydrolysis of anchovy oil by PLA1 (dark) and BSL (light). A, Percent accumulation of each fatty acid is calculated as \( \frac{UHF_{A_x} - TotalFA_x}{TotalFA_x} \times 100 \). Each of the fatty acid fractions were calculated based on peak area in the NMR spectra. B, Percent accumulation data replotted with positional information.
Figure 7.7. Lipase hydrolysis of structured triglyceride 1, 3-dioeoyl-2-myristic triglyceride was subjected to hydrolysis by PLA1 (Light) and BSL (Dark) and the released fatty acids were quantified by GC analysis.

To assess the positional specificity of these hydrolases we have employed a structured glyceride with fixed positional distribution of fatty acids. Using 3-dioeoyl-2-myristic glyceride, a structured TG, neither PLA1 nor BSL exhibited any position specific hydrolysis under our reaction conditions, with both fatty acids being equally hydrolysed. However, with anchovy oil the hydrolysis by PLA1 clearly shows bias against DHA, indicating fatty acid selectivity against polyunsaturated fatty acids. In a previous study five lipases were tested for their specificity by using fish oil and methyl esters of EPA, DHA and palmitic acid. All lipases discriminated against EPA and DHA when presented as methyl esters. However, lipase from Thermomyces has shown discrimination against DHA, particularly in the early stage of the hydrolysis reaction. A study observed that Candida rugosa lipase was most efficient in the enrichment of DHA in the glyceride fraction (Lyberg & Adlercreutz, 2008). In another study lipase from Candida rugosa discriminated omega-3 fatty acids from other fatty acids during hydrolysis of sardine oil (Okada & Morrissey, 2007). These studies indicate that the specificity in hydrolysis of natural oils by lipases or phospholipases is strongly dependent on the temperature and duration. Also, positional selectivity can found fatty
acid selectivity analyses in natural triacylglycerides, since positional distribution is not always analysed in selectivity studies.

Our study and similar studies with PLA1 lipase from *Thermomyces lanuginosus* suggests that hydrolysis is due to fatty acid selectivity more than to regioselectivity (Akanbi et al., 2013). A related previous study showed that substrate binding was dependent on the relative volume of the substrate to the volume of the active site (Kamal et al., 2015). Thirty eight lipases were investigated in silico, for their affinity to structured TGs with different chemical and positional compositions. This study indicated that binding affinity differences between various substrates with lipases is complex, but helped to identify active site positions that are critical to binding. This information could be useful for designing site saturation mutagenesis to identify amino acid substitutions that would enhance hydrolysis of specific fatty acids.

Few studies have successfully identified amino acid positions that are important in binding TG and phospholipids (Carrière et al., 1998). Further studies are required to enable the design of enzymes that can specifically hydrolyse omega-3 fatty acids.

### 7.3.4. Thraustochytrid oil hydrolysis by phospholipase A1

Phospholipase A1 is an engineered carboxyl ester hydrolase, produced from *Thermomyces lanuginosus/Fusarium oxysporum* by genetically modified *Aspergillus oryzae*. Phospholipase A1 possess activity towards both phospholipids and triglycerides. (Mishra et al., 2009). Research has focused on its phospholipase activity and little information is available on the production of structured glycerides using phospholipase A1 from oils (Liu et al., 2012; Liu et al., 2011). DT3 oil was hydrolysed to determine phospholipase A1 activity towards concentration of omega-3 fatty acids. Hydrolysis was gradually increased with incubation time and reached 50% at 150 min. Fig. 7.8 illustrates the percentage of hydrolysis of DT3 oil by phospholipase A1.
Figure 7.8. Lipid class analysis of thraustochytrid oil after hydrolysis with phospholipase at different time intervals.

Fatty acids profiles of glycerol and free fatty acids of hydrolysed DT3 oil were analysed by gas chromatography and results are shown in Fig. 7.9. After 150 min of phospholipase A1 hydrolysis, a significant amount of short chain fatty acids and monounsaturated fatty acids were cleaved from the glycerol backbone and concentrated in the free fatty acids portion. In the triacylglycerol portion, the major saturated fatty acids were reduced, myristic acid was reduced from 10.2% to 5.3% and palmitic acid from 35.2% to 16.24%. EPA was equally distributed in the free fatty acid and glyceride portions and DHA was significantly enriched in the glyceride portion, indicating a strong selectivity for EPA over DHA (Fig. 7.9). The DHA concentration was enriched by 1.3 fold compared to native oil. These selectivity is similar to those observed for hydrolysis of fish oil using lipase TL100 (Akanbi et al., 2013).
7.4. Conclusion

The position selective hydrolysis of PLA1 was tested for the hydrolysis of anchovy oil. Fatty acid chain length selectivity of PLA1 was investigated using p-NP esters of chain length C2 to C22, including the long chain PUFAs, EPA and DHA. For anchovy oil hydrolysis SFAs and MUFAs were preferentially hydrolysed by PLA1 and omega-3 fatty acids were discriminated against. Of the omega-3 fatty acids, EPA was more preferentially hydrolysed than was DHA. Lipase from *Bacillus subtilis* did not show discrimination of fatty acids to the same extent as PLA1. Hydrolysis of the structured TG, 1, 3–dioleoyl-2-hexadecanoic glyceride, supported that the discrimination property of PLA1 was primarily due to the chemical nature of the fatty acid rather than its position in the triacylglyceride. PLA1 is a potential catalyst for selective enrichment of omega-3 fatty acids in triacylglycerides.

Phospholipase A1 was used to catalyse the hydrolysis of thraustochytrid DT3 oil to enrich DHA in glyceride portion, with short chain fatty acids being preferentially cleaved from the triacylglyceride. This results show that phospholipase A1 can be used to concentrate DHA from triacylglycerides.
Chapter 8. Summary and future aspects
The marine ecosystem is a large relatively unexplored pool for the isolation of potential compounds and materials for industrial and biotechnological applications. Marine microorganisms remain a major source for scientific discovery. The number of microorganisms isolated and characterised for their potential metabolites is increasing each year. Researchers have isolated bioactive compounds (lipids, enzymes, polysaccharides, proteins and pigments) from a range of marine inhabitants. The demand for novel enzymes is also increasing, particularly for the production of chemically and structurally important molecules with industrial applicability. Naturally marine environments provide unique genetic diversity and life habitats such as nutrient rich regions, nutrient limited regions, high salinity, high pressure, low temperature and light conditions. Microalgae lipid production is an area that is being explored, although the cost of production is high, partly due to downstream processing costs. Thus, cost-effective downstream processing methods play an important role in achieving commercial scale production of microalgae derived metabolites.

The main objective of this study is the isolation and selection of suitable thraustochytrid strains for lipid production, the development of lipid extraction and omega-3 enzymatic concentration methods applicable to the production of thraustochytrid derived oil. One aim was to develop a rapid lipid quantification method to screen newly isolated thraustochytrids. Another aim was to optimise lipid and lipase extraction methods. A third aim was to concentrate omega-3 fatty acids from thraustochytrids oil using lipases.

The first aim of the study was to develop a rapid colorimetric method for lipid quantification from thraustochytrid cells. The conventional lipid quantification methods are laborious and time consuming. Initially, to evaluate the colorimetric method, different lipid samples (plant oils, pure triolein, DHA and EPA) were quantified. A straight line showed the range of linearity of the method with different samples. In addition, the false positives from non-lipid components (glucose, glycerol and proteins) did not interfere with lipid quantification. In this study, four different thraustochytrids strains, two of them new isolates, were quantified for lipid content and the results compared with those obtained using a conventional gravimetric method. The results obtained from both methods were similar within experimental error.
Chapter 3 describes the effect of different solvents and cell disruption methods on lipid extraction from thraustochytrids. A total of nine solvents; chloroform, dichloromethane, diethyl ether, ethanol, heptane, hexane, isopropanol, methanol and toluene and their combinations, were investigated for lipid extraction. Maximum lipid extraction for single solvents were hexane (12.5%), heptane (11%) and chloroform (9.7%). However, a solvent mixture of chloroform and methanol (2:1) showed maximum lipid extraction of 22%, followed by chloroform and hexane (2:1) with 13.4%. Furthermore, different cell disruption methods were evaluated to improve lipid extraction from *Schizochytrium* S31 and *Thraustochytrium* AMCQS5-5. Cell disruption resulted in increased lipid extraction from both *Schizochytrium* S31 and *Thraustochytrium* AMCQS5-5, and maximal lipid extraction was obtained using osmotic shock, with 48.7% lipid extracted from *Schizochytrium* S31 and 29.1% lipid from *Thraustochytrium* AMCQS5-5. The other methods, grinding (44.6%), sonication (31%) and shake mill (30.5%) extracted higher lipids than the control method (22%).

The fatty acid profiles of the lipids extracted from thraustochytrids were investigated using GC. GC analysis revealed the suitability of S31 fatty acid profiles for biodiesel production and AMCQS5-5 profiles for nutraceutical applications. In addition, in silico analysis of fatty acid profiles confirms the potential of S31 lipid for biodiesel production. The energy consumption was evaluated for the methods investigated in this study to aid in determining their potential use in large scale processing. The osmotic shock method was found to be the most economical method compared to other methods for industrially scaled-up.

Thraustochytrids have been documented for polyunsaturated fatty acids production, particularly DHA, but little information is available about their enzymatic activities. The first part of this study (Chapter 4) describes screening of enzymatic activities from thraustochytrid strains using the API ZYM kit. The isolates investigated in this study were collected form two different collection trips. The investigated thraustochytrid strains exhibited high esterase, lipase, acid and alkaline phosphatase activities and the maximum lipase activity was observed in AMCQS1-9 followed by AMCQS-4b27. In addition, the effect of different cell disruption methods on protein release and lipase activity was evaluated from AMCQS1-9 and AMCQS-4b27 strains. In this study, four different cell disruption methods, bead vortexing, grinding with liquid nitrogen, sonication and homogenisation, were compared for impact on protein and lipase recovery from thraustochytrids. Strain AMCQ-4b27 cells were more
sensitive to protein release than those from AMCQS1-9 and sonication was found to be the suitable method for lipase yield from AMCQ-4b27 (0.903 IU/µg) and AMCQS1-9 (1.44 IU/µg). Lipase activity extracted from strain AMCQS1-9 was approximately 1.5 fold more than that from AMCQ-4b27.

Lipase production is influenced by the culture conditions. Chapter 5 demonstrated the optimisation of Schizochytrium S31 culture conditions for enhancing lipase production in a stirred tank reactor. In addition, sonication parameters were optimised to improve lipase extraction yields. Initially, Tween 80 concentration was optimised and a Tween 80 concentration of 1% gave the maximum biomass and lipase productivity. The optimum temperature was 25 oC and the optimum pH was 6. The process was scaled-up in a 2 L stirred tank reactor to monitor biomass, lipid and lipase productions and utilisation of substrate and change in pH. To quantify lipid content, fatty acid composition and lipase activity, biomass was collected and analysed at different time intervals up to 9 days. Biomass and lipase production reached a maximum after 5 days of incubation. Based on the results obtained from Chapter 4, sonication was resulted in the highest level of lipase extraction from thraustochytrids. In this study, we optimised sonication parameters to enhance lipase extraction. Optimised sonication conditions resulted in a 5 fold increase in lipase activity extraction. Novel *Schizochytrium* S31 lipase was employed to hydrolyse different chain length p-NP esters. The extracted lipase preferentially hydrolysed shorter chain fatty acids, however, DHA was preferentially hydrolysed over EPA.

Chapter 6 describes the use of bead milling in thraustochytrid lipid extraction and enzymatic concentration of DHA from extracted lipids. In this study, thraustochytrids were grown in glycerol, a low cost carbon source for biomass and lipid production. The highest level of biomass and lipid produced were 12.23 ± 0.4 g L-1 and 29% ± 1.9 for *Schizochytrium* sp. S31 and 14.65 ± 0.54 g L-1 and 35% ± 2.1 for Schizochytrium sp. DT3, respectively. *Schizochytrium* S31 biomass was used for optimising lipid extraction using a bead mill. Important process parameters such as biomass concentration, bead size, RPM and process time were optimised. The highest lipid yield (40.5%) was obtained with biomass concentration (5%), bead size (0.4-0.6 mm), RPM (4500) and 4 min processing time. Fatty acid profiles of extracted lipid from the both strains was compared and DHA accumulation was observed more in strain DT3 (39.8%) than S31 (31.4%). *Candida rugosa* lipase was used to concentrate DHA in the glyceride portion. Initially, the lipase concentration was optimised and it
was found that 3000 U resulted in 50% oil hydrolysis in 3 hours. The presence of lipid classes FFA, DAG and MAG were observed by TLC analysis. Under optimised conditions the omega-3 fatty acid content of thraustochytrid oil was enriched to 88.7%, a 1.4 fold increase on initial levels.

We also used phospholipase A1, a carboxyl ester hydrolase, for concentrating omega-3 fatty acids from *Schizochytrium* DT3 oil extracted from optimised bead milling process. At 2.5 h of incubation, oil hydrolysis reached 50%, and the fatty acid profiles were analysed using GC. EPA was equally distributed in the free fatty acid and glyceride portions, while DHA was significantly enriched in glyceride portion, indicating a strong selectivity for EPA over DHA.

Chapter 7 demonstrated the application of phospholipase as a catalyst for enriching omega-3 fatty acids from anchovy fish oil. Initially we investigated the substrate selectivity of PLA1 using *p*-NP esters chain lengths of C2 to C22, including the long chain PUFAs, EPA and DHA. PLA1 showed preferential hydrolysis of EPA and discriminated against DHA. In anchovy oil hydrolysis, the saturated and mono-unsaturated fatty acids were hydrolysed from the glyceride portion. Of the omega-3 fatty acids, EPA was preferentially hydrolysed over DHA. We compared fish oil hydrolysis with the lipase from *Bacillus subtilis*, which did not discriminate between fatty acids to the same extent as PLA1. Furthermore, hydrolysis of the structured lipid 1,3-dioleoyl-2-hexadecanoic confirmed that the observed selectivity of PLA1 was due to fatty acid selectivity and not positional selectivity.

Microalgae derived biofuels and nutraceuticals are an alternative approach for sustainable production. However, there are several challenges to be addressed. The large scale production of lipids from microalgae is only possible with the isolation and selection of microalgae with high biomass and lipid productivities. This can be achieved by using robust techniques in isolation and selection of microalgae from varied habitats for commercial scale production. Although the lipid extraction methods explored in this thesis were optimised using dry biomass, it is necessary to explore extraction of wet biomass, since biomass drying is an expensive process. The development of enzyme assisted and nano particle based lipid extraction and the use of natural solvents such as terpenes and organic-nanoclays reduce the environmental impacts and waste generation during processing. Thraustochytrids appears to have
some potentially useful lipases, including lipases with DHA versus DPA selectivity, although the structures of these lipases still remain to be identified.
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