Isolation and characterisation of omega-3 fatty acids producing marine microbes

by

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B.E, M.Tech

Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Deakin University
May, 2014
I am the author of the thesis entitled

“Isolation and characterisation of omega-3 fatty acids producing marine microbes” submitted for the degree of Doctor of Philosophy

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Acknowledgements

From the beginning it was clear to me
this is not about me or what I can do,
rather this is about what God wants me to do and
how He will enable me to do it.

It would not have been possible to finish this doctoral thesis without the help and support of the kind of people around me. The journey of thesis writing has made me learn and explore more.

I would like to extend my deepest appreciation to my principal supervisor, Professor Colin J. Barrow for his supervision, guidance, unflinching encouragement, and support in various ways and for extending all the facilities throughout the work. I would also like to thank him for his time and patience towards the revisions of this thesis report.

My sincere gratitude goes to my associate supervisor Associate Professor Munish Puri for being the inspiration to carry out my thesis work. I would like to thank him for the valuable discussions towards the design of the experiments and publications of the work. He has really made me better researcher and in all a capable person. He enabled me to develop an understanding of the bioprocessing discipline.

I would also like to thank Dr. Jacqui L. Adcock, Dr. Jitaporn Vongsvivut, Dr. Madan Verma, Dr. Bo Wang and Dr. Andrew Sullivan for their time and expertise towards the use of various equipments and techniques important for my project work. Thanks to Dr. Serena Wilkens at National Institute of Water and Atmospheric Research (NIWA), New Zealand for the training program that benefitted my project. I would like to express my gratitude to all technical staff at LES, Waurn Ponds for their continuous support towards lab facilities. A special mention for Mrs. Elizabeth Laidlaw, whose tremendous energy always motivated me. I would like to thank Rachel Shanahan for her support during my conference travel and other administrative issues. I would also like to thank Deborah Scarce and Helen Nicholls Stary for their support.
I express my gratitude to Tim Nalder for his constructive comments on this thesis. I would like to express my heartfelt thanks to all my lab-mates and colleagues; Reinu, Selvi, Taiwo, Tim, Avinesh, Dilip, Parveen, Shailendra, Parminder and Lovis for their constant enthusiasm in the lab and insightful comments in lab presentations. I cannot end without thanking my friends; Sudhir, Santosh and his family, Ashwantha, Sanjana, Pratibha, Raju, Rajesh, Mohan and Sreejoyee for their continuous encouragement and being supportive.

Words fail to express my gratitude to my parents and brothers for always being there and supporting me in many ways throughout my life.

I would like to acknowledge the financial, academic and technical support of Deakin University, Waurn Ponds campus, particularly in the award of a PhD scholarship to pursue research in the areas of algal/marine biotechnology.

Lastly and most important, a special mention for the Almighty for the wisdom and perseverance that he has bestowed upon me during this research project and indeed throughout the life.

*Dedicated To Maharaj ji*
List of publications

Published


Other publications/Book chapter


Oral Presentations

1. **Gupta A.*,** Barrow C. J., Puri M., Crude carbon source enhances PUFAs ratios in marine microalgae screened from Australian habitat, 3rd International Conference on Algal biomass, biofuels and bio-products, 16-19 June 2013, Toronto, Canada.

Sequences deposited to GenBank database

1. Rhodotorula sp. AMCQ8A – JN695595
2. Rhodotorula sp. AMCQ10C – JN695596
3. Thraustochytrium sp. AMCQS5-3 – JX993839
4. Thraustochytrium sp. AMCQS5-4 – JX993840
5. Thraustochytrium sp. AMCQS5-5 – JX993841
6. Ulkenia sp. AMCQS4-6 – JX993842
7. Schizochytrium sp. AMCQS1-9 – JX993843
8. Thraustochytrium sp. AMCCQ6A13 – JX993844
9. Ulkenia sp. AMCQ5B25 – JX993845
10. Schizochytrium sp. DT3 – KF682125
11. Thraustochytrium sp. DT4 – KF682126
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<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>FID</td>
<td>Flame Ionisation Detector</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reverse phase HPLC</td>
</tr>
<tr>
<td>CDW</td>
<td>Cell dry weight</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume by volume</td>
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<tr>
<td>w/v</td>
<td>Weight by volume</td>
</tr>
<tr>
<td>w/w</td>
<td>weight by weight</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>AU</td>
<td>Absorbance Units</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in-situ hybridization technique</td>
</tr>
<tr>
<td>AFDD</td>
<td>Acriflavine direct detection method</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>Acetyl Co-A</td>
<td>Acetyl co-enzyme A</td>
</tr>
<tr>
<td>FAMEs</td>
<td>Fatty acid methyl esters</td>
</tr>
<tr>
<td>EPS</td>
<td>Extracellular polysaccharides</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
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</table>
Abstract

Numerous marine microbes are capable of accumulating large quantities of lipids, such as the long chain omega-3 polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These compounds have been shown to have a number of positive effects on human health and therefore are of interest for use in nutritional products and pharmaceuticals. Microbial lipids are also used in the animal feed industries, including aquaculture. Microbial lipids offer a natural and sustainable alternative to fish oil for the production of EPA and DHA.

An oleaginous yeast strain, *Rhodotorula* sp. AMCQ8A, was isolated from the marine water in the Queenscliff region, Victoria, Australia, and identified by 18s rRNA sequencing. The identification of this strain was confirmed by morphological analysis. Fourier transform infrared (FTIR) micro-spectroscopic analysis demonstrated the presence of lipids and fats (band 1745 cm\(^{-1}\)), including the presence of unsaturated fatty acids (band 3015-3005 cm\(^{-1}\)). The lipid to amide ratio obtained from FTIR was used to indicate the amount of lipid in cells for AMCQ8A. GC analysis showed that over 65% of the fatty acids from this yeast strain were C16:0 and C18:1, with only small amounts of omega-3 fatty acids present, indicating that this strain may not be a useful omega-3 producer and has a fatty acid profile more suitable for biofuel.

A second study used pollen-baiting and direct plating techniques to isolate 13 thraustochytrid strains. The accumulation of DHA in strains obtained from pollen-baiting varied greatly (11 to 41% of total fatty acids (TFA)), while those isolated via direct plating showed a narrowing range of DHA as a percentage of TFA (19 to 25%). Molecular identification using 18S rRNA sequencing showed that there were different species in the thraustochytrid group, including *Thraustochytrium* sp., *Schizochytrium* sp. and *Ulkenia* sp. A variety of carbon sources (hexoses, pentose, disaccharides and glycerol) were used for the fermentation of thraustochytrids to determine the efficiency of these strains in converting different carbon sources into lipids. Two of the selected thraustochytrid strains (AMCQS5-5 and DT3) could efficiently utilise glycerol as an alternative carbon source to glucose, resulting in increased biomass, lipid, DHA and carotenoids with glycerol as the carbon source. However, higher glycerol levels (10%, w/v) inhibited growth and lipid production. Crude sugar hydrolysate from hemp biomass (agricultural residues) was also trialled
as a sole carbon source for some of the thraustochytrid strains, and resulted in comparable biomass and fatty acid composition to that obtained with glucose, indicating that these strains can grow on lower cost carbon materials. Tween 80 and sodium thiosulphate added to the media as additives and resulted in increased biomass growth and lipid accumulation in one of the thraustochytrid strain.

The isolated thraustochytrids exhibited orange or pink colouration. Therefore the carotenoid content of these organisms was analysed by reverse-phase HPLC. Two of the coloured isolates were found to produce astaxanthin, zeaxanthin, canthaxanthin, echinenone and β-carotene. Growth on medium containing low percentages of glycerol enhanced the carotenoid content, while higher glycerol concentrations inhibited it. Through this work we have shown that some thraustochytrids isolated from the Victorian marine environment can produce EPA, DHA and carotenoids. These strains were able to grow on glycerol and sugars from hemp hydrolysis, as low cost alternatives to glucose. Further optimisation of strain growth, through both controlled fermentation and metabolic engineering approaches, could further increase yields of these useful fatty acids and carotenoids from these organisms.
Chapter 1: Introduction and Literature review
1.1 Introduction

The potential for identifying and extracting novel bio-actives valuable to the medical and food industries makes marine environments attractive to researchers. Exploring these environments is a challenging task given the magnitude of marine life complicating the investigation of oceans and other aquatic ecosystems (Karl 2007; Lee et al. 2010). Further adding to the difficulties is the continuing anthropogenic contamination of marine environments with heavy metals and other contaminants. Pollution of marine environments is directly responsible for their deterioration, resulting in decreased phytoplankton growth and reduced synthesis of omega-3 fatty acids (Kang 2011). A variety of fish species such as herring, mackerel, sardine and salmon are regarded as good sources of omega-3 fatty acids (Gunstone 1996). Due to the many shortcomings of fish-derived oil including undesirable taste and odour, diminishing supplies, objections by vegetarians, chemical processing methods and the presence of contaminants such as mercury, dioxins and polychlorinated biphenyls (Certik and Shimizu 1999; Hooper et al. 2006), research has progressed towards the exploitation of other marine species for the development of suitable omega-3-producing alternatives. Novel oleaginous microorganisms producing more than 25 % lipid on a dry cell weight basis can potentially be tailored to produce high amounts of omega-3 fatty acids, particularly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Barrow 2010; Dyal and Narine 2005).

The production of omega-3 fatty acids from thraustochytrids and other high-oil producing microorganisms is an area of research and commercial interest (Raghukumar 2008; Sijtsma and Swaaf 2004). Thraustochytrids belong to a marine group of eukaryotic protists called Labyrinthulomycetes; single celled organisms abundantly distributed in marine ecosystems. These mono-centric protists are receiving attention as a result of their ultrastructure and biochemical composition. Based on phylogenetic analysis thraustochytrids are related to the heterokont group of microorganisms and also some other phyla of marine microbes such as Chromophytes and Prymnesiophytes; although they are considered primarily as a group of their own (Chamberlain and Moss 1988). Thraustochytrid cells are of uniform spherical shape, ranging between 30-100 µm in diameter (Bremer 2000). In contrast to low lipid accumulating bacteria, they can produce oils with a high percentage of polyunsaturated fatty acids (PUFAs) and a potentially better yield of DHA and EPA through fermentation (Barrow 2010). As well as applications in
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Mariculture, DHA and EPA are prescribed in diets due to their clinical importance to human health (Lewis et al. 1999). Thraustochytrids could therefore emerge as an alternative to fish oil with the potential for commercialisation and may grow and accumulate lipids by utilising low cost substrates such as bio-diesel derived glycerol (Chi et al. 2007; Ethier et al. 2011; Pyle et al. 2008). Moreover, these heterotrophs do not require light for their growth such as in autotrophic microalgae, which provides some fermentation advantages particularly at scale. This review highlights some of the significant progress made in the last two decades and discusses the potential application of thraustochytrids for omega-3 fatty acid production in biotechnology. Areas covered include employing aspects of metabolic engineering and medium optimisation strategies with a discussion on the isolation and identification of thraustochytrids.

1.1.1 Omega-3 fatty acids

PUFAs constitute a large group of fatty acids that are long chain carbonic molecules which include ω-3 fatty acids (Lozac'h 1986). Omega (ω) refers to the methyl end of the fatty acid while the number ‘3’ refers to the number of carbon atoms at that position from the methyl end (Figure 1.1). When counting the first occurrence of double bonds from the carboxyl end, delta, ‘Δ’ is used to indicate the position. The molecular structure of omega-3 fatty acids consists of an even number of carbon atoms (16 to 24) with diverse saturations (3 to 6 double bonds). Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are two members of the ω-3 family. The prefixes ‘docosa’ and ‘eicosa’ are of Greek descent, meaning the 22 and 20 C-atoms present in DHA and EPA, which contain 6 and 5 cis-double bonds, respectively (Lozac'h 1986).
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Figure 1.1 Chemical structure of the ω-3 fatty acid: Alpha-linolenic acid.

H₃C — CH₂ — C ≡ C — CH₂ — C ≡ C — CH₂ — C ≡ C — (CH₂)₇ — COOH

Omega Carbon

Double bond is present between 3rd carbon (from ω-carbon) and 4th carbon

Alpha linolenic acid 18: 3; 9, 12, 15

The other ω-3 fatty acids are

(a) Eicosapentaenoic acid 20:5; 5, 8, 11, 14, 17

(b) Docosapentaenoic acid 22:5; 7, 10, 13, 16, 19

(c) Docosahexaenoic acid 22:6; 4, 7, 10, 13, 16, 19
1.1.1.1 Omega-3 fatty acids as nutraceuticals

Ongoing debate about appropriate levels of long-chain fatty acid consumption has led to some confusion among consumers. The food standards code for Australia does not prescribe specific quantities of ω-6 and ω-3 fatty acid content in food products (Food standards Code, Australia, 2008). The food standards code specifies only the amount of PUFAs and total saturated fat content, which is inadequate for consumers wishing to make informed decisions about their dietary intake. PUFAs derived from plant sources are consumed in higher abundance than those from fish. In particular plant ω-6 PUFAs are consumed in higher quantities than ω-3 PUFAs (Newton 1998). This over consumption of ω-6 relative to ω-3 oil has been linked to increased risk of cancer, diabetes, cardiovascular and neurodegenerative diseases (Simopoulos 2006). To restore a more balanced consumption ratio, the ratio of ω-3 fatty acids to ω-6 fatty acids should be increased. Ratios of ω-6 to ω-3 between 5:1 and 3:1 have been suggested as optimum for human consumption (Simopoulos 2008). A recent report by the National Health and Medical Research Council included references to the amount and types of fat consumed. The consumption of PUFAs at an average of 6% of total energy and not exceeding 10% was recommended, with a reduction in the consumption of saturated fatty acids to maintain levels at 8-10% of total energy intake (National Health and Medical Research Council, Australia 2003). In the revised versions of the same report, the recommended intake of long chain omega-3 fatty acids (DHA/EPA) in order to lower the risk of chronic diseases was 610 mg/day for men and 430 mg/day for women (NHMRC 2003) (National Health and Medical Research Council, Australia, 2005). Adequate to high dietary intake of EPA and DHA may help in the prevention of inflammatory diseases, including cardiovascular diseases, bowel diseases, cancer and arthritis (Wall et al. 2010).

The ability of EPA and DHA to decrease blood triglyceride levels and lower cardiovascular disease risk led to the FDA approval of the drug Lovaza™, which is an 85% EPA and DHA concentrate derived from anchovy oil. This drug had worldwide sales of more than $1 billion and is primarily prescribed for hypertriglyceridemia (Pronova Biopharma 2012).

The health benefits of DHA and EPA have been extensively evaluated through a wide range of clinical studies (Barrow and Shahidi 2007; Kang and Weylandt 2008; Takahata et al. 1998). Omega-3 fatty acids have been shown to help prevent heart
attack as well as decrease the overall risk of cardiovascular disease in general (Masson et al. 2007; Russo 2009). DHA is ‘physiologically essential’ for brain and eye development, especially in infants, which has led to the addition of DHA to most infant formulas and many other infant-related food products (Birch et al. 2007; Innis 2008).

The biochemical and biological functions of DHA were studied to better understand why it is required by the central nervous system. The accumulation of DHA in the neuronal membranes is a significant indicator of brain development and endurance (Kim 2008). It was observed that long chain PUFA play a crucial role in visual development, particularly in infants supplemented with diets rich in DHA and Arachidonic acid (AA) (Birch et al. 2007). Positive effects on visual functions and motor skills such as coin sorting and dynamic balance were observed in children with phenylketonuria when their diet was supplemented with DHA (Ryan et al. 2010).

DHA and EPA inhibit the proliferation of vascular smooth muscle cells, thus contributing to the prevention of atherosclerosis disease (Horrocks and Yeo 1999). A diet with inadequate DHA during infancy and childhood may lead to inhibited development of the brain (Innis 2008). A major contribution of lipid peroxidation products was reported in the DHA-stimulated apoptosis of cancerous cells (Siddiqui et al. 2008). The effect of dietary ω-3 fatty acids on human colon cancer cells is also positive, acting to hinder the escalation of the deadly carcinomas (Kato et al. 2002), while the type of fat supplement chosen had a significant effect, the benefits appeared insensitive to the quantity administered. This suppression of tumour-genesis can be accredited to the diminished action of the genes involved in the growth of the tumour. Aside from being considered an inhibitor of carcinomas, DHA was found to exhibit chemo-protective properties towards normal tissues, facilitated by protectins, a downstream product synthesised from DHA which may be of specific importance to sufferers of neuroblastoma and medulloblastoma (Gleissman et al. 2010).

DHA also plays a vital role in thraustochytrid cell life (Lewis et al. 1999). A study to investigate the biological significance of DHA in thraustochytrid cells concluded that DHA is conserved in the form of fatty acid energy reserves as triacylglycerols (TAGs) and utilised during starvation (Jain et al. 2007). A decline in total lipids was observed with the extension in the starvation phase.
1.1.1.2 Biosynthesis of omega-3 fatty acids

Animals, including humans, lack the Δ-12 and Δ-15 desaturase enzymes, which are essential for the synthesis of ω-3 and ω-6 fatty acids. As such, they are not capable of synthesising omega-3 fatty acids de novo and must acquire them from their diets. Humans and other animals can synthesise some DHA and intermediate products such as EPA through the bioconversion of α-linolenic acid (18:3ω-3), the rest is obtained from direct consumption of DHA itself (Innis 2003). α-Linolenic acid is primarily acquired in our diet from plant sources. Further metabolism of α-linolenic acid is characterised by the action of the Δ-6 desaturase enzyme for the unsaturation of the fatty acid, followed by the action of the elongase enzyme for the addition of two carbon atoms to the molecular chain, leading to action by Δ-5 desaturase to form EPA. Earlier studies revealed the occurrence of the desaturation process for long chain PUFAs takes place in the endoplasmic reticulum (Innis 2003). Interestingly, the contribution of Δ-4 desaturase in the final steps of DHA synthesis (22:5ω-3 to 22:6ω-3) was established in the microbial pathway. This part of the synthesis was later interpreted in a different manner after the discovery of 24:5ω-3 with the help of the elongation of 22 carbon atoms in the mammalian pathway (Sprecher et al. 1999). The 24:5ω-3 is then desaturated to yield 24:6ω-3 in the peroxisomes where DHA is formed by the partial oxidation process (Ferdinandusse et al. 2001). Discovery of the mechanism for DHA synthesis may aid in the treatment of peroxisomal disorders such as Zellweger syndrome in infants, using DHA-supplemented diets. The unsaturation (addition of a double bond) and elongation (addition of a 2-C unit) mechanisms in the conventional fatty acid synthesis of these fatty acids are shown in Figure 1.2. Besides the conventional fatty acid synthase (FAS) system in thraustochytrids for fatty acid synthesis, one distinct pathway named polyketide synthase (PKS) pathway (Figure 1.3) has been observed in Schizochytrium sp. In contrast to the FAS system, this pathway does not require molecular oxygen (Metz et al. 2001). The synthesis of short chain fatty acids (C14:0 and C16:0) in Schizochytrium sp. may follow the FAS system, but the long chain PUFAs were found to be synthesised through PKS system (Ratledge 2004). This pathway involves the use of 8 enzymes; 3-ketoacyl synthase, malonyl-CoA:ACP acyltransferase, acyl carrier protein, 3-ketoacyl-ACP reductase, acyltransferase, chain length factor, enoyl reductase and dehydrase or isomerase. Unlike the use of several elongase and desaturase enzymes in the FAS pathway, the PKS pathway exhibited dehydration
and isomerisation reactions involving fatty acyl intermediates for carbon chain elongation. The identification of a Δ-4 fatty acid desaturase in *Thraustochytrium* sp. (Qiu et al. 2001) suggested that the synthesis of docosahexaenoic acid (DHA) may result from the FAS system. A complete understanding of long chain PUFA synthesis in thraustochytrids is yet to be defined.

Two distinct pathways have recently been shown to be involved in the synthesis of PUFAs in *Thraustochytrium aureum* ATCC 34204, the PKS pathway reported in lower bacteria and the conventional FAS pathway with the active role of Δ-12 fatty acid desaturase (Tau Δ-12 des) (Matsuda et al. 2012). The Δ-4, -5 and -9 desaturases and elongase enzymes have been isolated from *Thraustochytrium aureum* and heterologously expressed in yeasts such as *Pichia pastoris* and *Saccharomyces cerevisiae* (Lee et al. 2008; Qiu et al. 2001). These desaturases are purported to be involved in the production of EPA and DHA and are the subject of a patent application (Burja et al. 2011). However, the absence of the Δ-12 desaturase enzyme in *Schizochytrium* sp. makes it incapable of converting the C16:0 and C18:0 synthesised by the FAS pathway to long chain unsaturated fatty acids such as DHA (Lippmeier et al. 2009). Hence, the long chain PUFAs are synthesised through a distinct PUFA synthesis pathway involving a multi-subunit PUFA synthase system (Hauvermale et al. 2006).
Figure 1.2 Biosynthesis of ω-3 fatty acids (Innis 2003).
Figure 1.3 PKS pathway in *Schizochytrium* sp. 3-ketoacyl synthase (KS), 3-ketoacyl-ACP reductase (KR), dehydrase or (DH) and enoyl reductase (ER). This figure has been reproduced from Ratledge (2004).
1.2 Microorganisms producing fatty acids

Numerous organisms including bacteria, yeast, filamentous fungi and microalgae have been reported to produce different types of valuable fatty acids (Table 1.1). These fatty acids include long chain ω-6 and ω-3 PUFAs such as Gamma linolenic acid, AA, DHA and EPA.

Table 1.1 PUFA (% Total fatty acids, TFA) produced by various microorganisms.

<table>
<thead>
<tr>
<th>Name of microorganism</th>
<th>Fatty acids (% TFA)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pythium debaryanam</td>
<td>γ-linolenic acid (10.4)</td>
<td>Shaw 1965</td>
</tr>
<tr>
<td>Mortierella alpina</td>
<td>Arachidonic acid (68.5-78.8)</td>
<td>Totani and Oba 1987</td>
</tr>
<tr>
<td>Mortierella alpinapeyron</td>
<td>Arachidonic acid (11)</td>
<td>Kendrick and Ratledge 1992</td>
</tr>
<tr>
<td>Saprolegnia parasitica</td>
<td>AA (19) / EPA (18)</td>
<td></td>
</tr>
<tr>
<td>Vibrio sp.</td>
<td>DHA and EPA</td>
<td>Ando et al. 1992</td>
</tr>
<tr>
<td>Pythium irregulare</td>
<td>EPA (25.2)</td>
<td>O’Brien et al. 1993</td>
</tr>
<tr>
<td>Candida guilliermondii</td>
<td>DHA (6.7) and EPA (2.8)</td>
<td>Gua and Ota 2000</td>
</tr>
<tr>
<td>Colwellia sp. and Shwenella sp.</td>
<td>DHA (0.7-8) / EPA (2-22)</td>
<td>Bowman et al. 1997, 1998</td>
</tr>
<tr>
<td>Pichia methanolica</td>
<td>DHA (32)</td>
<td>Aoki et al. 2002</td>
</tr>
<tr>
<td>Mortierella sp.</td>
<td>EPA</td>
<td>Jacobs et al. 2009</td>
</tr>
<tr>
<td>Mortierella sp.</td>
<td>AA (25.9-53.8)</td>
<td>Suzuki et al. 2010</td>
</tr>
<tr>
<td></td>
<td>GLA (4.3-4.7)</td>
<td></td>
</tr>
<tr>
<td>Cryptococcus curvatus</td>
<td>OA (39.6) / ALA (0.7)</td>
<td>Liang et al., 2010</td>
</tr>
<tr>
<td>Mortierella alliacea YN-15</td>
<td>ALA (33-41) / EPA (1.3-13)</td>
<td>Jermsuntiea et al. 2011</td>
</tr>
<tr>
<td>Rhodotorula glutinis</td>
<td>OA (44.7) / LA (12.7) / GLA (5.5)</td>
<td>Zhang et al., 2011</td>
</tr>
</tbody>
</table>
### Chapter 1

| **Rhodosporidium toruloides Y4** | OA (49.6)  
|                                | ALA (0.3)  
|                                | **Zhao et al., 2011** |
| **Rhodosporidium toruloides Y2** | OA (49.9)  
|                                | ALA (N.D)   
|                                | **Zhou et al., 2013** |
| **Rhodotorula glutinis**        | OA (46.5)  
|                                | LA (26.6)   
|                                | ALA (3.4)   
|                                | **Schneider et al., 2013** |

Foot note: OA- oleic acid, LA-linoleic acid, ALA-alpha linolenic acid, GLA-gamma linolenic acid, DGLA-dihomo gamma linolenic acid, AA-arachidonic acid, EPA-eicosapentaenoic acid and DHA-docosahexaenoic acid.

### 1.2.1 Other PUFAs

Plants were initially considered to be the main source of PUFAs, particularly GLA (Gamma linolenic acid), which is available commercially (Alonso and Maroto 2000). Alternative sources such as microbial production of other long chain PUFAs such as arachidonic acid (AA, 20:4ω-6), dihomo-γ-linolenic acid (DGLA, 20:3ω-6), and γ-linolenic acid (GLA, 18:3ω-6) have also been reported (Ratledge 2001). The first commercial oil produced by microbes was for GLA. GLA was extracted predominantly from lower fungi (Phycomycetes) as other sources such as *Pythium debaryanam* (fungus) had lower quantities of lipids (Shaw 1965). *Mortierella alpina* produced total lipid content as 26 % of biomass with 80 % of total fatty acids as AA. This fungus required long cultivation periods of more than two weeks, hindering its commercialisation (Totani and Oba 1987). However, AA (11 % of TFA) was reported to be produced in the fungus, *Mortierella alpinapeyron* (Kendrick and Ratledge 1992). Other fungi such as *Conidiobolus nanodes* and *Entomorphthora exitalis* produced both ω-3 and ω-6 PUFAs but the output of ω-6 content was significantly higher than that of ω-3 fatty acids (Kendrick and Ratledge 1992). *Mortierella alliacea* was reported to be the industrial producer of AA and with the supplementation of plant oils EPA production was also documented (Jermsuntiea et al. 2011). *Mortierella sp.* was found to be resistant to the high glucose concentrations (4-11 %) that elicited an improvement in AA production (Suzuku et al. 2010) and other PUFAs such as GLA and DGLA. To devise a cost effective process, some
recent studies demonstrated the use of wastewater with oleaginous yeasts for lipid accumulation in their cells. Lipid accumulation in *Rhodotorula glutinis* (27 % lipids) and *Cryptococcus curvatus* (25 % lipids) was observed using distillery wastewater as fermentation medium (Gonzalez-Garcia et al. 2013). Distillery wastewater was also used with the oleaginous yeast *Rhodosporidium toruloides* and resulted in 43.75 % of biomass accumulated as lipid (Ling et al. 2013). In the same species bioethanol wastewater and glucose were utilised for lipid accumulation resulting in 34.9 % lipids (Zhou et al. 2013). Low cost fish meal has also been employed for lipid production in *Lipomyces starkeyi* (Huang et al. 2011).

Some other studies have reported using co-culture of different strains of immobilised yeasts such as *Rhodotorula mucilaginosa* and *Pichia guilliermondii* (Zhao et al. 2011); *Rhodosporidium* sp. and *Saccharomycopsis* sp. (Gen et al. 2014) for single cell oil production. Lipid content was reported to be 53.2 and 60 % by Zhao and co-workers and Gen and co-workers, respectively. These organisms can be classified as oleaginous as they contain more than 20 % of their biomass as lipids.

**1.2.2 Omega 3 fatty acids: DHA and EPA**

Many bacterial strains, including *Shwenella* sp. and *Colwellia* sp., have been reported to produce DHA and EPA (Bowman et al. 1998; Bowman et al. 1997; Nichols and McMeekin 2002). Some fungal strains belonging to *Mortierella* sp. were also reported to be producers of EPA (Jacobs et al. 2009). Higher EPA production was achieved with the fungus *Pythium irregulare* in a 14L fermentor using lactose, rather than glucose, as a carbon source with an EPA output of 25 % of total lipid content. This represented the highest reported EPA output using fungi (O’Brien et al. 1993). It has been demonstrated that *Pichia methanolica* can be DHA-enriched if it is cultured in a DHA-supplemented medium or provided with various other nutrients (Aoki et al. 2002). Temperature culturing (12-25 °C) using *Mortierella* sp. under different temperature regime assisted in EPA production (Suzuku et al. 2010). *Arthrospira maxima*, a mesophilic strain of cyanobacteria, produced DHA (28 % of TFA) at 20 °C (Hu et al. 2011). However, bacteria are not considered viable for the production of PUFAs as they have lower lipid content (2-5 % of biomass). In addition, the presence and characteristics of some undesirable lipids hinder the use of bacteria as suitable PUFA producers. Moreover, there is some scepticism regarding the quality of bacterial EPA and DHA (Ratledge 2001). Although bacteria are not toxic to
animals, there is no record of the use of bacterial lipids for animal consumption. Marine microalgae have been found to be the best alternative to fish oil in terms of ω-3 fatty acids production with a low likelihood of exhibiting the taste, odour and instability linked to fish oils (Barclay et al. 1994). In fact, microalgae produced 1-2 times more ω-3 fatty acids than bacterial and fungal cultures.

1.3 Thraustochytrids: Occurrence and methods for isolation

Mangrove leaves were found to be a habitat where thraustochytrids thrive, as thraustochytrids feed on the leaves resulting in their degradation (Bremer 1995; Wong et al. 2008). Invertebrates such as nudibranchs and squids are also reported to be associated with thraustochytrids as the microbes reside in these invertebrates (Jones and O'Dor 1983; McLean and Porter 1982). However, the type of biological associations that exist between thraustochytrids and the invertebrates is yet to be explored (Rabinowitz et al. 2006). Researchers have isolated thraustochytrid strains from oceanic and coastal marine environments (Burja et al. 2006; Kimura et al. 1999; Yang et al. 2010) as well as low temperature environments (Zhou et al. 2010) and the colonial tunicate *Botryllus schlosseri* (Rabinowitz et al. 2006). Salinity is a vital environmental variable which may limit the occurrence of thraustochytrids as they can only tolerate salinity to a limited extent (Bremer 2000).

1.3.1 Pollen baiting and Direct plating

Baiting techniques and direct plating methods are used for the isolation of thraustochytrid strains (Figure 1.4) (Bremer 2000). Pollen-baiting is one method that is commonly used for isolating thraustochytrids (Burja et al. 2006; Jacobsen et al. 2007). Sterilised pollen grains are interspersed on the surface of the water sample containing antibiotics and antifungal agents and incubated for 1-2 weeks. Once thraustochytrid cells are observed to be growing on the pollen surface, they are spread plated on agar plates and selectively grown in liquid medium to obtain pure cultures (Wilkens and Maas 2012). The direct plating method includes the distribution of the samples on agar plates under aseptic conditions (Bowles et al. 1999; Bremer 2000).
Figure 1.4 Light microscopy of thraustochytrid cells, a – ATCC *Thraustochytrium* PRA 296 (scale bar: 20 μm), b – cluster of fresh zoospores on the surface of pine pollen (scale bar: 20 μm), c – single thraustochytrid cell (sporangium) attached to pine pollen (scale bar: 20 μm), d – several zoospores released from a mature sporangium (scale bar: 20 μm).
1.3.2 Antibiotics and antifungal supplemented medium

Bacterial and fungal contaminations should be avoided during the isolation of thraustochytrids as the isolation temperatures (18-25 °C) may favour the growth of contaminant microbes. Antibiotics such as penicillin and streptomycin (Burja et al. 2006; Quilodran et al. 2010) and rifampicin and antifungal drugs such as nystatin (Jackobsen et al. 2007; Wilkens and Maas 2012) and amphotericin B (Taoka et al. 2010) have been used in the agar plates and liquid medium to minimise the growth of bacterial and mycelial colonies.

1.3.3 Identification techniques

Biochemical and molecular techniques have been used to identify newly isolated marine organisms (Burja et al. 2006). Staining procedures using fluorochrome for direct detection based on morphological characteristics of the organism have been used (Raghukumar and Schaumann 1993). Species-level identification is based on 18s rRNA homology for establishing taxonomic relationships (Mo et al. 2002).

1.3.3.1 Biochemical identification

A fluorescent staining technique has been developed to give an estimation of thraustochytrids cell numbers which involves the reaction between fluorochrome (acrilavine), the sulphated cell wall and their nuclei (Raghukumar and Schaumann 1993). The cell wall fluoresces red and the nuclei green, differentiating them from other protists that fluoresce only green (Moss 1986). This technique has some limitations as there is an absence of cell wall material in the zoospores of most thraustochytrid species (Moss 1986) and thraustochytrid cells with a thin cell wall are undetectable. A thraustochytrid-specific detection method employing a fluorescence in-situ hybridization technique (FISH) has been established using an rRNA targeted ThrFL1 probe (Takao et al. 2007). This technique overcomes the aforementioned limitation of acrilavine direct detection (AFDD) due to the thinner wall of thraustochytrid cells (Moss 1986). The FISH technique involves the hybridisation between the probe ThrFL1 and rRNA particles which assists the detection of zoospores and young somatic cells. Sudan black staining has also been used for staining the thraustochytrid cells (Subramaniam and Chaubal 1990; Wong et al. 2008).
1.3.3.2 Molecular identification

The molecular identification of thraustochytrids is based on the 18s rRNA sequencing technique which helps define the taxonomic relationships of unknown isolates with known isolates. It involves PCR-based amplification of the 18s rRNA gene requiring a set of universal primers designed from known thraustochytrid strains and comparative phylogenetic analysis (Bongiorni et al. 2005; Mo et al. 2002; Yokoyama and Honda 2007). This provides a sensitive tool for determining relationships between thraustochytrid species. The multiple sequence alignment of the isolated 18s rRNA sequences and available sequences give a branching relationship between the new isolates and known thraustochytrid species. The use of neighbour-joining and maximum likelihood methods for determining taxonomic relationships is possible with the help of analytical software packages such as PHYLIP 3.5c and PHYLOWIN (Bongiorni et al. 2005). PHYLIP 3.5c is the Phylogeny interference package involving the use of 31 programs using molecular sequence data, distance matrix data, gene frequencies and tree plot data (Felsenstein 1993). The PHYLOWIN package assists the phylogenetic tree construction with the use of neighbour joining, maximum parsimony and maximum likelihood algorithms, including bootstrap analysis (Galtier et al. 1996).

1.3.4 Ultra structure of thraustochytrids

Thraustochytrids can accumulate greater than 50 % of their dry weight as lipids, of which more than 25 % is normally DHA (Bajpai et al. 1991; Raghukumar 2008; Sijtsma and Swaaf 2004; Singh and Ward 1997; Yaguchi et al. 1997; Yokochi et al. 1998). Transmission electron microscopy (TEM) has revealed that numerous lipid bodies are present in the cytoplasm of thraustochytrid cells, which was later confirmed by Sudan Black B staining (Subramaniam and Chauabal 1990). The ultrastructure study which used TEM illustrated that thraustochytrid cells are heterogeneous in size, approximately 6-21 µm in diameter with a granular cytoplasm containing oil bodies. An association of lipid bodies was observed with the branched, hollow membrane-like fine structures containing light and dark bands. The pattern of these fine structures has been described as the ratio of saturated and monounsaturated fatty acids to polyunsaturated fatty acids (Wong et al. 2008). These fine structures were hypothesised to be linked with lipid synthesis, although they lose their structure almost completely as the lipid bodies increase in size (Weete et al. 1997). A
The relationship between lipid body formation and endoplasmic reticulum (ER) in *Schizochytrium limacinum* SR21 was observed (Morita et al. 2006). Lipid bodies surrounded by ER have been extensively studied in the oleaginous fungus, *Mortierella ramanniana* (Kamisaka et al. 1999), whereas analogous research in the phylum Labyrinthulomycota (which includes thaustochytrids) has yet to be undertaken.

### 1.4 Classification of thaustochytrids

There has always been confusion among researchers regarding the taxonomic classification of thaustochytrids. The physical structures first established for species classification were subsequently abolished during the process of developing identification parameters for thaustochytrids, and has added to ambiguity regarding their structural and functional behaviour. During the early 1970s, electron microscopic studies of the ultrastructure of protists determined the phylogenetic relationship of thaustochytrids and discovered that they were loosely related to Oomycetes (Kazama, 1972; 1974). With the development of DNA sequencing methods and electron microscopic studies of ultrastructure, thaustochytrids were subsequently designated as a unique group.

Before being incorporated into Oomycetes, thaustochytrids had been included within *Phycomycetes* (algae-like fungi) (Barr 1981). Subsequently, thaustochytrids were included within the phylum Heterokonta within the Chromophyta (Stramenopiles) kingdom (Cavalier-Smith 1993; Honda et al. 1999). The genera included in this group are *Thraustochytrium*, *Schizochytrium*, *Japonochytrium*, *Aplanochytrium*, *Elina* and *Labyrinthula* (or *Labyrinthuloides* or *Labyrinthulomyxa*). *Labyrinthula* have been included in Labyrinthulaceae family, whereas the Thraustochytriaceae family includes *Thraustochytrium*. Hence, after numerous revisions, thaustochytrids were assigned as a distinctive and characteristic division of protists in which the members can be classified under the Thraustochytriiales order (Metz et al. 2010).
An example: Kingdom: Chromalveolata
Phylum: Heterokontophyta
Class: Labyrinthulomycetes
Order: Labyrinthulales
Family: Thraustochytriaceae
Genus: *Thraustochytrium* or *Schizochytrium*
Species: *Thraustochytrium aureum* or *Schizochytrium limacinum*

### 1.5 Production of omega-3 fatty acids

Nutrient conditions must be optimised for any new isolate to achieve maximum omega-3 fatty acid productivity (Shene et al. 2010). The fermentative production of omega-3 fatty acids and the parameters required is discussed in following sections.

#### 1.5.1 Cultivation medium and bioprocess engineering

Glucose is frequently used in microbial fermentation processes as it is a relatively cheap raw material compared to other simple carbohydrates (Singh et al. 1996). It is also readily utilised by most microorganisms. While monosaccharides support rapid growth and increase DHA yields in thraustochytrids, poor cell growth has been observed when using disaccharides and polysaccharides in the fermentation medium (Burja et al. 2006). This observation conflicted with findings that showed all mono-, di- and polysaccharides supported growth and high lipid content in *Thraustochytrium aureum* ATCC 34304 (Bajpai et al. 1991). Earlier, starch supplemented media produced higher DHA yields compared with those using glucose, sucrose, corn and canola oil, although the resulting biomass yields were similar (Li and Ward 1994). The importance of the chosen carbon source is highlighted. Alternating carbon sources such as crude glycerol, wastewater from barley distillation, soybean cake and liquid residues from beer and potato processing were investigated to make the process more economical, while not compromising the DHA output (Quilodran et al. 2009; Yamasaki et al. 2006; Zhu et al. 2008). The different carbon and nitrogen sources used with thraustochytrids for producing omega oils are listed in Table 1.2.
Table 1.2 Summary of different carbon and nitrogen sources used for omega-3 fatty acid production in different thraustochytrid strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Carbon source</th>
<th>Nitrogen source</th>
<th>% of TFA</th>
<th>Cell Mass (g L⁻¹)</th>
<th>Volume (mL)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>DHA</td>
<td>EPA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T. aureum 34304</td>
<td>Glucose/Fructose</td>
<td>Peptone</td>
<td>41-75</td>
<td>1.2-5.2</td>
<td>1.1-5.0</td>
<td>50*</td>
</tr>
<tr>
<td></td>
<td>Starch</td>
<td>Tryptone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Maltose</td>
<td>Yeast extract</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Linseed oil</td>
<td>Malt extract</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Monosodium glutamate</td>
<td></td>
<td></td>
<td></td>
<td>Bajpai et al. 1991</td>
</tr>
<tr>
<td>T. roseum 28210</td>
<td>Starch</td>
<td>(NH₄)₂SO₄ Monosodium glutamate</td>
<td>48.3-58.2</td>
<td>--</td>
<td>6.1-17.1</td>
<td>50* (Fed batch)</td>
</tr>
<tr>
<td>Schizochytrium sp. SR21</td>
<td>Glucose</td>
<td>(NH₄)₂SO₄ Corn steep liquor</td>
<td>33.3-38.6</td>
<td>--</td>
<td>21.9-59.2</td>
<td>3000&quot;</td>
</tr>
<tr>
<td>S. limacinum SR21</td>
<td>Monosaccharides Oleic acid Linseed oil</td>
<td>Yeast extract Con steep liquor</td>
<td>6.0-43.1</td>
<td>&lt;1</td>
<td>12</td>
<td>10*</td>
</tr>
<tr>
<td>S. mangrovei G-13</td>
<td>Glucose</td>
<td>Yeast extract Peptone</td>
<td>28</td>
<td>--</td>
<td>14</td>
<td>2000&quot;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Bowles et al. 1999</td>
</tr>
<tr>
<td>Strain</td>
<td>Carbon Source</td>
<td>Nitrogen Source</td>
<td>Glucose (%)</td>
<td>Yeast Extract (%)</td>
<td>Polypeptone (%)</td>
<td>Productivity (g/L)</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>------------------------</td>
<td>--------------------------</td>
<td>-------------</td>
<td>-------------------</td>
<td>-----------------</td>
<td>---------------------</td>
</tr>
<tr>
<td><em>S. limacinum</em> KH 105</td>
<td>Glucose</td>
<td>Yeast extract</td>
<td>34.9</td>
<td>1.6</td>
<td>11.5</td>
<td>50*</td>
</tr>
<tr>
<td><em>Schizochytrium</em> sp. F26-b</td>
<td>Glucose</td>
<td>Yeast extract</td>
<td>31.8</td>
<td>1.2</td>
<td>3.5</td>
<td>200*</td>
</tr>
<tr>
<td><em>Thraustochytrium</em> sp.</td>
<td>Glucose</td>
<td>Yeast extract</td>
<td>16-32</td>
<td>--</td>
<td>10-26</td>
<td>30*</td>
</tr>
<tr>
<td>ONC-18</td>
<td></td>
<td>Glutamic acid</td>
<td></td>
<td>16-32</td>
<td>--</td>
<td>10-26</td>
</tr>
<tr>
<td><em>Schizochytrium</em> sp. KH 105</td>
<td>Glucose</td>
<td>Waste water for barley</td>
<td>25.8</td>
<td>--</td>
<td>~26</td>
<td>2000*</td>
</tr>
<tr>
<td>distillery</td>
<td></td>
<td>distillery</td>
<td></td>
<td>25.8</td>
<td>--</td>
<td>~26</td>
</tr>
<tr>
<td><em>Thraustochytrid strain</em> 12B</td>
<td>Glucose</td>
<td>Yeast extract peptone</td>
<td>40.1-47.5</td>
<td>0.9</td>
<td>15.4-30.9</td>
<td>10*</td>
</tr>
<tr>
<td><em>S. limacinum</em> SR21</td>
<td>Crude glycerol</td>
<td>Yeast extract</td>
<td>33.6</td>
<td>--</td>
<td>22.1</td>
<td>50*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ammonium acetate</td>
<td></td>
<td>33.6</td>
<td>--</td>
<td>22.1</td>
</tr>
<tr>
<td><em>S. mangrovei</em> Sk-02</td>
<td>Coconut water</td>
<td>Yeast extract</td>
<td>20</td>
<td>--</td>
<td>28</td>
<td>100*</td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
<td></td>
<td></td>
<td>20</td>
<td>--</td>
<td>28</td>
</tr>
<tr>
<td><em>Schizochytrium</em> sp. G13/2S</td>
<td>Glucose</td>
<td>Ammonium sulphate</td>
<td>~40</td>
<td>-</td>
<td>63.3</td>
<td>1000*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Fed batch)</td>
<td></td>
<td>~40</td>
<td>-</td>
<td>63.3</td>
</tr>
<tr>
<td><em>Aurantiochytrium</em> sp. T66</td>
<td>Glucose</td>
<td>Monosodium glutamate</td>
<td>28-52</td>
<td>1.4</td>
<td>25-100</td>
<td>1800*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>28-52</td>
<td>1.4</td>
<td>25-100</td>
</tr>
<tr>
<td><em>S. limacinum</em> SR21</td>
<td>Crude glycerol</td>
<td>Yeast extract</td>
<td>18.26-53.05</td>
<td>--</td>
<td>2.5-8</td>
<td>50*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Peptone</td>
<td></td>
<td>18.26-53.05</td>
<td>--</td>
<td>2.5-8</td>
</tr>
</tbody>
</table>
### Chapter 1

<table>
<thead>
<tr>
<th>Strain/Species</th>
<th>Carbon Source</th>
<th>Nitrogen Source</th>
<th>Optimal Concentration</th>
<th>pH Optimal</th>
<th>Other Conditions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aurantiochytrium mangrovei MP2</strong></td>
<td>Glucose</td>
<td>Yeast extract</td>
<td>3</td>
<td>25.4</td>
<td>50*</td>
<td>Wong et al. 2008</td>
</tr>
<tr>
<td><strong>S. limacinum OUC88</strong></td>
<td>Glucose</td>
<td>Soybean cake hydrolysate</td>
<td>4.08 (g L⁻¹)</td>
<td>25.92</td>
<td>100*</td>
<td>Zhu et al. 2008</td>
</tr>
<tr>
<td><strong>S. limacinum SR21</strong></td>
<td>Glycerol</td>
<td>Corn steep solids, Ammonium acetate</td>
<td>14.8-18.6</td>
<td>24.6-36.2</td>
<td>50*</td>
<td>Chi et al. 2009</td>
</tr>
<tr>
<td><strong>Thraustochytriidae sp. M12-X1</strong></td>
<td>Beer residues</td>
<td>Yeast extract, Monosodium glutamate</td>
<td>39.5-61.7, 7.4-11.3</td>
<td>2.3</td>
<td>100*</td>
<td>Quilodran et al. 2009</td>
</tr>
<tr>
<td><strong>Schizochytrium sp. HX-308</strong></td>
<td>Glucose</td>
<td>Yeast extract</td>
<td>35-60</td>
<td>~60</td>
<td>Batch study</td>
<td>Ren et al. 2009</td>
</tr>
<tr>
<td><strong>S. limacinum SR21</strong></td>
<td>Sweet sorghum juice</td>
<td>--</td>
<td>34.28</td>
<td>0.61</td>
<td>9.4</td>
<td>Liang et al. 2010</td>
</tr>
<tr>
<td><strong>Schizochytrium sp. HX-308</strong></td>
<td>Glucose</td>
<td>Yeast extract</td>
<td>35.8-48.4</td>
<td>72-92.7</td>
<td>35 L (Fed batch) #</td>
<td>Qu et al. 2011</td>
</tr>
<tr>
<td><strong>Schizochytrium sp. M209059</strong></td>
<td>Glucose</td>
<td>Monosodium glutamate</td>
<td>48.9</td>
<td>71</td>
<td>1000 L (Fed batch) #</td>
<td>Ren et al. 2010</td>
</tr>
<tr>
<td><strong>Aurantiochytrium limacinum SR21</strong></td>
<td>Glucose</td>
<td>Corn steep liquor</td>
<td>-</td>
<td>40.3</td>
<td>3.5 L#</td>
<td>Rosa et al. 2010</td>
</tr>
<tr>
<td><strong>S. limacinum SR21</strong></td>
<td>Crude glycerol</td>
<td>Yeast extract, Peptone</td>
<td>24.86-31.09</td>
<td>4-15</td>
<td>4500° (Continuous)</td>
<td>Ethier et al. 2011</td>
</tr>
</tbody>
</table>
### Chapter 1

<table>
<thead>
<tr>
<th>Organism</th>
<th>Carbon Source</th>
<th>Nitrogen Source</th>
<th>Lipid (g/L)</th>
<th>Fatty Acids (g/L)</th>
<th>Volume (L)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aurantiochytrium limacinum</em> SR21</td>
<td>Glycerol</td>
<td>Yeast extract</td>
<td>~70</td>
<td>~15</td>
<td>61.8</td>
<td>2 L (Fed batch)</td>
</tr>
<tr>
<td><em>Aurantiochytrium</em> sp. KRS101</td>
<td>Spent yeast (with glycerol)</td>
<td>-</td>
<td>34.2</td>
<td>0.35</td>
<td>31.8</td>
<td>250 (Fed batch)</td>
</tr>
<tr>
<td><em>Schizochytrium</em> sp. S31</td>
<td>Glycerol</td>
<td>Yeast extract</td>
<td>11.9-57.6</td>
<td>-</td>
<td>~45</td>
<td>5 L #</td>
</tr>
<tr>
<td><em>Schizochytrium</em> sp. S31</td>
<td>Glycerol</td>
<td>Yeast extract</td>
<td>22.9-44.3</td>
<td>-</td>
<td>14.8-151.4</td>
<td>7.5 and 30 L (Fed batch) #</td>
</tr>
<tr>
<td><em>Schizochytrium</em> sp. S31</td>
<td>Glycerol</td>
<td>Yeast extract</td>
<td>29.4-36</td>
<td>-</td>
<td>117.9-129.3</td>
<td>50 L (Fed batch) #</td>
</tr>
<tr>
<td><em>Aurantiochytrium</em> sp. KRS101</td>
<td>Palm empty fruit bunches</td>
<td>-</td>
<td>~40</td>
<td>-</td>
<td>34.4</td>
<td>5 L #</td>
</tr>
<tr>
<td><em>Aurantiochytrium</em> sp. KRS101</td>
<td>Glucose</td>
<td>Yeast extract</td>
<td>25.2-35.8</td>
<td>-</td>
<td>13.8-22.1</td>
<td>200 (Fed batch)</td>
</tr>
<tr>
<td><em>Aurantiochytrium</em> sp. TC20</td>
<td>Glucose</td>
<td>Yeast extract</td>
<td>38.9-48.4</td>
<td>0.4</td>
<td>56-71</td>
<td>1.6 L (Fed batch) #</td>
</tr>
<tr>
<td><em>Thraustochytriidae</em> sp. TN5</td>
<td>Glucose</td>
<td>Sodium glutamate</td>
<td>38.8</td>
<td>-</td>
<td>14</td>
<td>1000 (Fed batch) #</td>
</tr>
<tr>
<td></td>
<td>Maltose</td>
<td>Ammonium chloride</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Shake flask cultivation, #Bioreactor, TFA-Total fatty acids*
Some studies used glycerol in the medium while working on *Schizochytrium limacinum* SR21 and other thraustochytrid strains (Chi et al. 2007; Ethier et al. 2011; Pyle et al. 2008), whereas complex carbon sources such as sweet sorghum juice (Liang et al. 2010) were used for improving DHA production. Liquid residues obtained from the brewing industry and potato chip processing units have also been used to produce DHA (Quilodran et al. 2010). Other complex carbon sources such as cellulosic biomass, empty palm fruit bunches and organic waste from brewery industry have also been used in thraustochytrid fermentation to produce polyunsaturated fatty acids (Hong et al. 2012; Hong et al. 2013; Ryu et al. 2013).

Biodiesel-derived glycerol has the potential to minimise production costs because of its own low price when compared to other commercially available carbon sources (Pyle et al. 2008). However, crude glycerol obtained from the biodiesel industry requires pre-treatment to remove soap that may retard cell growth and lead to low DHA yields (Chi et al. 2007; Ethier et al. 2011; Liang et al. 2010; Pyle et al. 2008). Glycerol has potential application as a low cost carbon source for the production of omega-3 oils in thraustochytrids for food and pharmaceutical purposes. Aside from glycerol, carob pulp syrup has been used as a low cost carbon source for the production of DHA in the microalga *Crypthecodinium cohnii*, yielding higher biomass and DHA productivity than when compared to glucose as the carbon source (Mendes et al. 2007).

Inorganic nitrogen in the form of ammonium sulphate may be used as a sole nitrogen source, but glutamate, aspartate, their amides and alpha alanine can result in a higher biomass yields (Goldstein 1963). This is consistent with the research of Jacobsen and co-workers (2008) in which the effect of nitrogen in the form of monosodium glutamate as the sole nitrogen source was studied and observed to increase biomass growth and lipid accumulation relative to inorganic nitrogen growth. Nitrogen depletion from the fermentation medium elevated the lipid accumulation from 13 to 55 % w/w of the dry biomass of *Aurantiochytrium* sp. strain T66. Initiation of the lipid accumulation may be attributed to inadequate levels of oxygen and by limiting the nitrogen and phosphorous sources in the medium used for ω-3 fatty acid production. With this strategy, a high lipid content of 54-63 % w/w of dry weight of the *Aurantiochytrium* sp. cells was observed (Jakobsen et al. 2008). Abundant carbon and low nitrogen in the fermentation medium therefore results in high levels of oil accumulation in thraustochytrids. Due to the minimal levels of nitrogen, protein and
nucleic acid synthesis is limited and the carbon source is converted into storage lipids. A higher C:N ratio was found to support higher DHA production in thraustochytrids (Burja et al. 2006; Bowles et al. 1999; Yokochi et al. 1998). Glucose concentrations in the range of 6-10 % resulted in increased levels of DHA (Wong et al. 2008), whereas more than 10 % was reported to retard the growth of thraustochytrids (Yokochi et al. 1998).

Nutrient sources which may result in higher production of ω-3 fatty acids in thraustochytrids included polyoxyethylene sorbitan monooleate (Tween 80), acetyl-CoA and nicotinamide adenine dinucleotide phosphate (NADPH). Tween 80 increased biomass growth and total lipid content in <em>Thraustochytrium aureum</em> ATCC 34304 without modifying the DHA content (Taoka et al. 2011). The rise in the total lipid content was accredited to the presence of oleic acid in the form of oleate from the Tween 80. Furthermore, new strategies using acetyl-CoA and NADPH in the form of ethanol and malic acid, respectively, were implemented during the fermentation process to improve DHA yield. An increase from 35 to 60 % DHA content has been reported with the addition of malic acid (enhancing NADPH) at the rapid lipid accumulation phase (Ren et al. 2009). However, added NADPH was ineffective in increasing total lipid accumulation, but did alter the fatty acid composition whereas; the use of acetyl-CoA resulted in an increase of 35 % of lipid accumulation with only a minor change in DHA content. The combination of acetyl-CoA and NADPH may offer both high lipid accumulation and increased yield of DHA.

### 1.5.2 Factors affecting omega-3 fatty acid production

Like the chemical constituents of fermentation media, the physical conditions also modify the production of PUFAs. Fermentation conditions have been altered to enhance the production of PUFAs in thraustochytrids (Bajpai et al. 1991; Wu and Lin, 2003). In testing for optimal PUFA yield, chemical parameters such as carbon and nitrogen sources in different concentrations and physical parameters such as initial pH, temperature and inoculum age were adjusted. While temperature was found to have no notable effect, all other parameters were found to affect fatty acid production in <em>Thraustochytrium aureum</em> ATCC 34304 (Bajpai et al. 1991). However, it has also been reported that thraustochytrids can utilise the 20-30 °C temperature range for growth and lipid accumulation (Perveen et al. 2006). With the aim of high
Chapter 1

DHA productivity, a temperature shift regime resulted in a DHA content of 52% of total fatty acids. A decline in temperature from 30 to 20 °C yielded high levels of DHA making 30 °C the optimal culture temperature for the highest biomass growth (Zeng et al. 2011). A low culture temperature may affect the lipid synthesis in the marine microbes isolated from low temperate regions (Fang et al. 2000). DHA production in thraustochytrid strains varies (Burja et al. 2006; Huang et al. 2003; Unagul et al. 2005) depending on the environment they have been isolated from. The variation of conditions in different environments may contribute to variable production of omega-3 fatty acids, ranging from strain to strain.

The biomass yield achieved for thraustochytrids was highest at neutral pH (Kumon et al. 2005) though no significant change was observed in the fatty acid profile. Similarly, salinity plays an important role in thraustochytrid fermentation. Salinity up to 50% (v/v) is known to yield high biomass (Yokochi et al. 1998). Artificial sea water (ASW) has been extensively used for providing salinity in the medium. However, there was a report of using only sodium chloride as salt source (Ganuza et al. 2008). Salinity depends on the type of habitat where the thraustochytrid has been isolated. Moreover, it may significantly affect the lipid content, biomass and fatty acid profile (Perveen et al. 2006).

The transformation of saturated fatty acids into unsaturated fatty acids was promoted when oil producing strains were grown in oxygen-rich environments (Raghukumar 2008). However, in another study biomass was grown at 4-8% oxygen saturation and this was lowered to 1% during oil accumulation, resulting in higher DHA yields (Bailey et al. 2003). A two stage oxygen supply system was introduced based on oxygen transfer coefficient yielding high concentrations of DHA (Qu et al. 2011). The volume of the liquid medium was also assessed and it was found that the dissolved oxygen was affected, thus resulting in a lower DHA yield (Zhou et al. 2010). A large volume of liquid medium (more than 40 mL in 250 mL flask) resulted in a low DHA yield.

Various statistical designs have been reported for enhancing fatty acid production that can assist in the optimisation of DHA yields by using various models such as fractional factorial, central composite design and RSM (Wu and Lin 2003). Response surface methodology (RSM) was found to be suitable for optimising the effect of
multiple factors on fermentation yields (Kalil et al. 2000), as the experimental value was comparable to the predicted yields with 97 % closeness.

The production of omega-3 fatty acids in large-scale bioreactors has been reported elsewhere (Bajpai et al. 1991; Chi et al. 2009; Hong et al., 2011; Jakobsen et al. 2008). The growth of thraustochytrid strains in a bioreactor differ significantly, depending on the strain characteristics. Thraustochytrium roseum was found to grow better in a flask than in a stirred tank bioreactor (Ilda et al. 1996). However, Schizochytrium sp. SR21 exhibited significantly better growth under bioreactor conditions (Nakahara et al. 1996). Of 68 isolates comprising the genera Schizochytrium and Thraustochytrium, 54 were found to produce DHA in the range of 22-80 % of total C20 to C22 content of the cells (Burja et al. 2006). These observed DHA yields are consistent with the findings of Huang et al. (2003) who reported 53 % DHA of total fatty acids present in the thraustochytrids isolated from cold environments. Some thraustochytrid isolates with DHA content are summarised in Table 1.3.
Table 1.3 Thraustochytrid strains isolated from different environments and their respective DHA (% Total fatty acids, TFA) content.

<table>
<thead>
<tr>
<th>Strain</th>
<th>DHA (% of TFA)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Thraustochytrium aureum</em> ATCC 34304</td>
<td>51</td>
<td>Bajpai et al. 1991</td>
</tr>
<tr>
<td><em>Schizochytrium</em> sp. ATCC 20888</td>
<td>32</td>
<td>Barclay et al. 1994</td>
</tr>
<tr>
<td><em>T. roseum</em> ATCC 28210</td>
<td>46-49</td>
<td>Li and Ward 1994</td>
</tr>
<tr>
<td><em>Schizochytrium</em> sp. SR21</td>
<td>34</td>
<td>Nakahara et al. 1996</td>
</tr>
<tr>
<td><em>S. limacinum</em> MYA 1381</td>
<td>34</td>
<td>Yokochi et al. 2003</td>
</tr>
<tr>
<td><em>Schizochytrium</em> mangrovide Sk-02</td>
<td>28</td>
<td>Unagul et al. 2005</td>
</tr>
<tr>
<td><em>Thraustochytrium</em> sp. ONC-T18</td>
<td>31.5</td>
<td>Burja et al. 2006</td>
</tr>
<tr>
<td>Thraustochytrid strain 12B</td>
<td>40.1-47.5</td>
<td>Perveen et al., 2006</td>
</tr>
<tr>
<td><em>Schizochytrium</em> sp. G13/2S</td>
<td>43-47</td>
<td>Ganuza and Izquierdo 2007</td>
</tr>
<tr>
<td><em>Aurantiochytrium</em> sp. T66</td>
<td>28-52</td>
<td>Jakobsen et al., 2008</td>
</tr>
<tr>
<td><em>Schizochytrium</em> sp. HX-308</td>
<td>60</td>
<td>Ren et al. 2009</td>
</tr>
<tr>
<td><em>Thraustochytriidae</em> sp. C41</td>
<td>18.4-27.1</td>
<td>Quilodran et al. 2009</td>
</tr>
<tr>
<td><em>Thraustochytriidae</em> sp. M12-X1</td>
<td>31.5-61.7</td>
<td></td>
</tr>
<tr>
<td><em>Schizochytrium</em> sp. M209059</td>
<td>48.9</td>
<td>Ren et al., 2010</td>
</tr>
<tr>
<td><em>Aurantiochytrium</em> sp. BL10</td>
<td>29</td>
<td>Yang et al. 2010</td>
</tr>
<tr>
<td><em>Thraustochytriidae</em> sp. Z105</td>
<td>32</td>
<td>Zhou et al. 2010</td>
</tr>
<tr>
<td><em>Aurantiochytrium</em> sp. KRS101</td>
<td>38.2</td>
<td>Ryu et al., 2013</td>
</tr>
<tr>
<td><em>Aurantiochytrium</em> sp. TC20</td>
<td>38.9-48.4</td>
<td>Lee Chang et al., 2013</td>
</tr>
<tr>
<td><em>Thraustochytriidae</em> sp. TN5</td>
<td>38.8</td>
<td>Shene et al., 2013</td>
</tr>
</tbody>
</table>
Growth-dependent lipid accumulation was achieved by introducing a nitrogen source in the medium in the form of ammonia (300-400 mg L\(^{-1}\)) in a fed batch system using \textit{Schizochytrium} sp. G13/2S (Ganuza et al. 2008). This resulted in the highest growth rate irrespective of the degree of lipid accumulation. Ganuza and co-workers also demonstrated the use of sodium chloride as the only salt source in a defined medium. Another study explored the lipid accumulation as a result of nitrogen limitation in the fermentation medium (Jakobsen et al. 2008). A two stage growth process for the culture of \textit{Schizochytrium} sp. SR21 was employed and enabled the optimisation of the parameters separately for maximal biomass growth and lipid accumulation (Chi et al. 2009).

With the change in fermentation process, a continuous fermentation was designed to study lipid accumulation in \textit{Schizochytrium} sp. G13/2S in different glucose (7-40 g L\(^{-1}\)) and glutamate (4-6 g L\(^{-1}\)) concentrations (Ganuza and Izquierdo 2007). This process stimulated more lipid accumulation as compared to the batch fermentation where lipid accumulation ceased due to stalled cell growth. A fed batch system using \textit{Aurantiochytrium} sp. KRS101 that resulted in a DHA productivity of 40 % of TFA was recently reported (Hong et al. 2011). Another modified fed-batch strategy using \textit{Aurantiochytrium} sp. KRS101 was reported to increase levels of palmitic acid (with respect to biodiesel production) and DHA yield (towards PUFA production), where distinctive yield coefficients were used (Kim et al. 2013).

A recurrent oxygen feeding method was employed to maintain 50 % DO level in the fermentation environment that achieved high biomass of 61.76 g L\(^{-1}\) and 65.2 % of biomass as lipid content, which also improved the DHA content up to 70 % of the total fatty acids (Huang et al. 2012). A recent study involved \textit{Aurantiochytrium} sp. KRS101 in a stepwise fermentation strategy using spent yeast as the sole nutrient source which supported good biomass growth and lipid productivity (Ryu et al. 2013). The direct trans-esterification method was found to be more effective in achieving high fatty acid yields (Zuniga et al. 2012).
1.6 Lipid extraction methods

When referring to a classic example of a lipid extraction technique, the method by Bligh and Dyer (1959) is usually the first to be cited, largely due to its simplicity and use of common solvents such as chloroform, methanol and water mixtures. In the Bligh and Dyer method, the lipids remain in the chloroform layer while the methanol layer contains the non-lipid components. This method has been modified by researchers to increase the actual PUFA yield from microbes by combining lipid extraction with trans-esterification, followed by recovery using a hexane and chloroform mixture (Burja et al. 2007; Lewis et al. 2000). The quantification of PUFAs is complex due to their tendency to undergo oxidation. To avoid this, various modified methods using different solvent mixtures and disruption techniques based on sonication have been reported. Direct saponification and trans-esterification, the acid Bligh and Dyer method coupled with trichloroacetic acid and the miniature Bligh and Dyer method were some of the modified extraction methods reported recently (Burja et al. 2007). Such methods yielded higher amounts of lipids from *Thraustochytrium* and *Schizochytrium* species. Among these, the miniature Bligh and Dyer method was found to be the easiest, whereas the direct esterification method had limitations such as low fatty acid recovery. Often, modifications involving variables such as the time period of trans-esterification and volume of the trans-esterification mixture are required to enhance the extraction of fatty acids (Burja et al. 2007).

A modified trans-esterification method using sodium hydroxide in methanol and boron trifluoride in methanol was used to extract lipids from fish oil (Armenta et al. 2009). Direct trans-esterification of freeze-dried thraustochytrid cells was performed and analysed from a calibration graph of standards. Both of these methods were found to be efficient in extracting PUFAs from fish oil and thraustochytrid cells.

A solid phase extraction method was designed for fast and quantitative separation of lipid classes from microbial oils (Pinkart et al. 1998). The method proved to be a straightforward, high yielding and cost-effective technique for lipid class evaluation, and has been applied for PUFA separation (Burja et al. 2007).

The use of microbial samples for fatty acid analysis may be problematic and ultimately inaccurate unless the process performed is thorough. The main steps in the process involve extraction, the conversion of fatty acids into fatty acid methyl esters
(FAMEs), GC optimisation and the usage of appropriate internal and external standards for determining concentrations in unknown samples (Masood et al. 2005; Schreiner 2005). An improved protocol was derived with the addition of acetyl chloride on a dry ice bath, followed by trans-esterification performed at room temperature and an analysis of GC data using relative response factors. This procedure was found to be applicable in the recovery of the ω-3 fatty acids DHA and EPA (Xu et al. 2010).

Another technique involves heat treatment and protease digestion of freeze dried thraustochytrid cells. The use of heat and an enzyme in the lipid extraction was a novel concept to be implemented in the lipid extraction process (Jakobsen et al. 2008).

1.7 Quantification of fatty acids

Various analytical and separation techniques have been reported for the quantification of fatty acids. These include urea fractionation (Abu-Nasr et al. 1954), thin layer chromatography (TLC) and preparative scale gas chromatography (Hardy and Keay 1967). This was followed by high performance liquid chromatography (HPLC) methods with some modifications such as reverse phase HPLC using C18 column and silver nitrate HPLC (Aveldano et al. 1983; Ozcimder and Hammers 1980; Scholfield 1979). However, silver nitrate HPLC was found to be unsuitable for mono and di-unsaturated fatty acids, as inadequate resolution is achieved due to the confounding effects of the number of double bonds and carbon chain length. A method involving low temperature fractional crystallization of unwanted lipids was reported but was not applicable to highly unsaturated fatty acids (Ackman 1981).

Other chromatography techniques such as silica gel TLC (Shantha and Ackman 1991) and open column chromatography (Hayashi and Kishimura 1993) were applied to large scale preparations (Medina et al. 1995) following urea fractionation. Super critical fluid extraction technology associated with the separation of PUFAs by gas chromatography was reported in late 1990s (Walker et al. 1999). Some limitations of these techniques should be noted, namely i) low PUFA yield, ii) exhaustive preparation and iii) scale-up difficulty (Cartens et al. 1996; Medina et al. 1995). Coupling of gas chromatography to mass spectrometry (GC-MS) is now widely used for the rapid separation and identification of lipids since it is highly
specific for fatty acid separation and identification (Ren et al. 2009; Zeng et al. 2011).

1.8 Other metabolites produced by thraustochytrids

Thraustochytrids are known to produce various secondary metabolites such as carotenoids, sterols, steroids and surfactants (Fan and Chen 2007; Lewis et al. 2001). In addition to this, they are also a potential source of enzymes and extracellular polysaccharides.

1.8.1 Carotenoids

Thraustochytrids are a promising source of carotenoids such as astaxanthin, zeaxanthin, canthaxanthin, echinenone, phoenicoxanthin and β-carotene (Burja et al. 2006; Carmona et al. 2003). Thraustochytrid strain KH-105 was reported to be a β-carotene and xanthophyll (astaxanthin and canthaxanthin) accumulator (Aki et al. 2003). Carbon and nitrogen sources are very important nutrient factors for carotenoid production. Low glucose concentration resulted in high β-carotene production, whereas high glucose concentration produced high canthaxanthin content (Aki et al. 2003). Xanthophyll production was found to respond in the opposite manner with respect to nitrogen concentrations. It was also observed that moderate nitrogen concentrations of 0.3 and 6 % supported the production of astaxanthin and canthaxanthin, respectively (Aki et al. 2003). Other substrate such as distillery waste as the only nitrogen source in the culture medium have been used for the production of DHA and xanthophylls yielding 3.4 g L⁻¹ and 7.7 mg L⁻¹, respectively (Yamasaki et al. 2006). Astaxanthin and canthaxanthin exhibit antioxidant and chemo-protective properties, making them potential candidates for food additives that may act against the development of neurodegenerative disorders (Yuan et al. 2011). Various methods, using the combination of organic solvents and ultrasonic baths, have been examined to determine carotenoid content present in the Thraustochytrium strain ONC-T18 (Armenta et al. 2006). Semi-fragile cell walls in thraustochytrid strain KH-105 aided the downstream processing of the pigments with effective recovery using a solvent such as acetone. A mutation strategy was furthermore carried out to enhance the astaxanthin productivity of S. limacinum by treating the cells with N-methyl-N’-nitro-N-nitroso guanidine (NTG) resulting in intense colour development in the colonies (Chatdumrong et al. 2007).
As β-carotene acts as a precursor for vitamin A, sufficient intake of β-carotene can help to prevent diseases caused by vitamin A deficiency, including blindness, immune dysfunction and skin disorders (Fierce et al. 2008). More than 80% of β-carotene is synthesized by chemical processes. The chemosynthetic pathway produces relatively more trans-stereoisomers of β-carotene than from microbial processes. Trans-stereoisomers are less competent antioxidants and therefore less desirable for medical applications (Del Campo et al. 2007; Shaish et al. 2006). Carotenoids produced via microorganisms are widely accepted and considered to be a safe drug component. The halo-tolerant marine microalga *Dunaliella* sp. is considered to be a large carotenoid accumulator. However, high carbon dioxide consumption, low production efficiency, poor control and the high cost of land has limited the expansion of mass cultures of *Dunaliella* for β-carotene production (Ye et al. 2008). Thraustochytrids can be used as an alternative for carotenoid production.

1.8.2 Squalene and sterols

Another important metabolite with anti-oxidant properties is squalene, an intermediate triterpene in the cholesterol synthesis pathway. This compound protects the cells from free radicals and reactive oxygen species and is known as a chemoprotective agent against colon, lung and skin carcinogenesis in animal-studies and breast cancer in humans (Smith 2000; Warleta et al. 2010). It has been used to treat different ailments (Aguilera et al. 2005; Ramirez-Torres et al. 2012). Various thraustochytrids including *Aurantiochytrium* sp., *Thraustochytrium* sp., *Ulkenia* sp. and *Schizochytrium* sp. have been reported as potential producers of squalene and sterols (Lee Chang et al. 2012; Li et al. 2009; Nakazawa et al. 2012).

1.8.3 Enzymes

Since thraustochytrids are found in abundance on degraded mangrove leaves and marine sediments, it was expected that they were able to produce extracellular enzymes to digest these materials. Thraustochytrids do produce a range of enzymes such as proteases, lipases, esterases, acid and alkaline phosphatases, cellulases and xylanases (Raghukumar 2008). The extracellular cellulolytic activity was studied in 19 strains of thraustochytrids using carboxymethyl cellulose (CMC) as the substrate, with 14 strains confirming cellulase activity (Nagano et al. 2011). A three-fold increase was observed in the extracellular lipase activity of two thraustochytrid
strains AH-2 and TZ with the help of various optimisations of the culture conditions (Kanchana et al. 2011).

1.8.4 Extracellular polysaccharides

Thraustochytrids are reported to produce extracellular polymeric substances or polysaccharides (EPS) that include sugars as the major component (39-53 %) with the presence of proteins, lipids, uronic acids and sulphates (Jain et al. 2005). Specifically, glucose formed the main fraction of sugars in EPS followed by galactose, mannose and arabinose. The molecular weight of EPS was determined as 94 kDa (Kilo Dalton) and 320 kDa. However, it has been observed that many bacterial EPS possess a 3-dimensional structure (Dogsa et al. 2005). Different structures have been reported in microbes, including large net-like structures of hyaluronan (carbohydrate polymer), an EPS from *Streptococcus equi* (Gamini et al. 2002), fibrous structures from *Bacillus* sp. CP912 (Yun and Park 2003) and elastic coiled structures from *Erwinia chrysanthemi* spp. (Ding et al. 2003). EPS can act as anti-tumour and antiviral agents and can also be used in the cosmetic and food industries (Sutherland 1998). EPS are found to play an important role in the cell life of thraustochytrids. They may protect thraustochytrids from desiccation, assist in cell adherence to the marine substrata and serve as an energy source during periods of starvation (Jain et al. 2005). *Schizochytrium* sp. was reported to produce the highest EPS yield among different thraustochytrid strains (Lee Chang et al. 2014). The EPS may also afford thraustochytrids protection against metal and toxin contamination (Colaco et al. 2006).

1.9 Thraustochytrids and their potential

DHA and EPA currently used as food supplements and medicines are primarily obtained from fish oil. These oils are accumulated in fish through their diet of PUFA-rich zooplanktons and microalgae (Bell et al. 1986; Saito et al. 1995). However, the concentration of EPA and DHA from fish oil is expensive and can result in environmentally unfriendly waste products from urea complexation (Zuta et al. 2003). Also, the changing of the ratio of EPA to DHA while concentrating fish oil is costly and requires harsh fractional distillation. Microbial sources such as thraustochytrids could provide direct concentrates and naturally high EPA and/or DHA oils, particularly high DHA and low EPA concentrates (Spolaore et al. 2006). DHA from thraustochytrid (*Schizochytrium* sp.) has been used in foods like butter,
cheese, yoghurt and cereals (Ward and Singh 2005). In addition, thraustochytrid-derived PUFAs has been implemented in aquaculture feeds as well (Song et al. 2007). In addition, thraustochytrids are potential sources of novel drugs and secondary metabolites (Raghukumar 2008).

1.10 Commercial production of omega-3 fatty acids

It is estimated that the global market of nutraceuticals is around $110-150 billion with 6-8 % of annual growth (Ferreri 2013) and based on consumer demand, it is expected to exceed US$243 billion by 2015 (Schieber 2012). Moreover, DHA fortified infant formulas represent an estimated 70 % of the total $9 billion global infant formula business (Barrow et al. 2007). As discussed earlier, there is a need for alternatives to fish oil. Microbial oil can be an alternative with potential for the production of oils rich in EPA and DHA. The ratio of EPA and DHA can potentially be altered through fermentation and genetic modifications to satisfy a market need to a higher ratio of one over the other. The commercial production of microbial omega-3 fatty acids has been achieved in some cases (Table 1.4). The estimated value of global expenditure on omega-3 fortified food and beverage products, dietary supplements and pet products was $13 billion in 2011 (Facts 2011). Martek Biosciences Corp. (now owned by DSM) has been producing single cell oils on a commercial scale and these are primarily added to infant formula (Barrow 2010; Ratledge 2004). Martek Biosciences Corp. is one of the leading establishments in global microbial oil market. DHASCO™ is produced from Crypthecodinium cohnii which contains DHA up to 40 % of total fatty acids (TFA) and is used exclusively in infant formulas. They also have utilised the Schizochytrium sp. in fed batch mode that produced 170-210 g L⁻¹ cell dry weight (CDW) with the DHA yield of 40 g L⁻¹ using non-chloride salts (Bailey et al. 2003). Thraustochytrid isolates and their mutants have been patented by Martek Biosciences US patent 8207363 (Apt et al. 2012). Ulkenia sp. has also been tailored to produce DHA by Suntory Ltd., Nagase and Co. Ltd. and Nagase Chemtex Corporation (Tanaka et al. 2003). The 2010 sales figures of Martek Biosciences Corp. was $450 million, out of which only $17.05 million was accounted for by food and beverage sales; the rest included the sales of infant formulas and dietary supplements (Elaine Watson 2011).
Table 1.4 Different algal sources used for commercial production of the omega-3 oils.

<table>
<thead>
<tr>
<th>Source</th>
<th>Brand name</th>
<th>% Total fatty acids (TFA)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>14:0</td>
<td>16:0</td>
</tr>
<tr>
<td>C. cohnii</td>
<td>N/A</td>
<td>16.6</td>
<td>16.9</td>
</tr>
<tr>
<td>S. limacinum</td>
<td>SR21</td>
<td>3</td>
<td>49</td>
</tr>
<tr>
<td>Schizochytrium sp.</td>
<td>DHA-O</td>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td>Schizochytrium sp.</td>
<td>DHA-S</td>
<td>9.3</td>
<td>21</td>
</tr>
<tr>
<td>C. cohnii</td>
<td>DHASCO</td>
<td>10-20</td>
<td>10-20</td>
</tr>
<tr>
<td>Schizochytrium sp. ONC-T18</td>
<td>N/A</td>
<td>13.9</td>
<td>26.1</td>
</tr>
<tr>
<td>Schizochytrium sp. ATCC 20888</td>
<td>DHA-Gold</td>
<td>10.1</td>
<td>23.7</td>
</tr>
<tr>
<td>Ulkenia sp.</td>
<td>DHA45-oil</td>
<td>-</td>
<td>35</td>
</tr>
</tbody>
</table>

Fatty acid profile represented as % of total fatty acids; 14:0 myristic acid, 16:0 palmitic acid, 18:0 stearic acid, 18:1 oleic acid, 20:5 EPA, 22:6 DHA
DHA-enriched oil is useful for infant formula, but for food fortification oil containing both EPA and DHA is preferable (Barrow and Shahidi 2007). In this context, Martek Corp., now owned by DSM, recently launched DHA-O, new oil produced from their *Schizochytrium* sp. that has been modified to produce an approximate 2:1 ratio of DHA to EPA. This oil is specified as containing at least 22.5 % DHA and 10 % EPA. They succeeded in increasing EPA yields in their *Schizochytrium* sp. (Martek Press Release, Apr. 5, 2011).
1.11 Aims of the study

The primary source of omega-3 fatty acids consumed by humans is fish oil. Microbial-derived omega-3 fatty acids can potentially replace fish oil as a renewable alternative. The primary objective of this study was to isolate novel oleaginous marine microbes from the Victorian marine environment and culture them to produce DHA and EPA.

The research aims of this project were as follows:

1. The first aim of the project was to collect marine samples from the Victorian environment and isolate oleaginous microbes. The isolates were screened and identified on the basis of fatty acid production with microscopic analysis and molecular identification.
2. The second aim of the project was to assess the ability of selected isolates to grow on inexpensive carbon source such as glycerol and sugar hydrolysate.
3. The third aim was to evaluate the effect of media supplements such as Tween 80 and sodium thiosulphate on omega-3 fatty acid production levels.
4. The final aim was to evaluate carotenoid production from colour producing isolates.
Chapter 2: Isolation and identification of new omega-3 producing marine *Rhodotorula* strains of yeast
2.1 Introduction

The marine environment is attractive to researchers due to the diversity of microorganisms, the majority of which have not yet been described (Delong 2007). These organisms potentially contain a range of functional and valuable compounds, including enzymes, antibiotics, therapeutic metabolites and lipids (Lee et al. 2010). Marine organisms represent a great phylogenetic diversity containing unique genetic information and important bio-actives useful for human development. Some of these organisms contain high amounts of lipids and can be important sources of omega-3 fatty acids. Among oleaginous organisms, yeasts have relatively fast growth rates with high levels of lipid accumulation and so have potential as commercial sources of oils and fats (Saenge et al. 2011).

Marine microorganisms have advantages over plant-based production of lipids due to their superior growth rates, higher oil content and easier scale-up; while maintaining a similar triacylglycerol composition to plant oils (Li et al. 2008). Marine microbes such as bacteria, microalgae, yeasts and other fungi have been reported to produce medicinally important oils (Fuentes-Grunewald et al. 2012; Li et al. 2010; Sijtsma and Swaaf 2004; Zhao et al. 2010). These microbes are termed as ‘oleaginous organisms’ and are capable of accumulating lipids in their cells at ≥20% of their dry weight; which differs between strains and species (Meng et al. 2009). Some strains of *Rhodotorula* have been shown to be capable of rapidly producing high biomass with high lipid content (Almazan et al. 1981). This is particularly true of *R. mucilaginosa* which was isolated from the skin of marine fish (Li et al. 2010; Saenge et al. 2011). Some oleaginous yeasts, including *Rhodotorula* species, are capable of using a variety of complex carbon sources including cassava starch, inulin, tubers, distillery waste water and bioethanol wastewater (Gen et al. 2014; Gonzalez-Garcia et al. 2013; Ling et al. 2013; Zhao et al. 2011; Zhou et al. 2013). *Rhodotorula* sp. is also known to produce colour pigments such as β-carotene, which has been demonstrated to possess anti-carcinogenic and antioxidant properties, and is widely used in aquaculture (Astorg 1997).

Alpha-linolenic acid (ALA) is the essential fatty acid which is primarily acquired through diet as ALA cannot be produced by the human body. In humans, dietary ALA is a biosynthetic precursor to EPA and DHA and can be derived from plant sources such as canola oil, flaxseed oil, linseed and rapeseed oils, walnut, and leafy
green vegetables (Colaco et al. 2006; Innis 2003). However, relatively low consumption levels and low levels of conversion ALA to DHA in humans has led to supplementation of DHA and EPA through nutritional supplements or fortified foods (Anderson and Ma 2009; Burdge and Calder 2005; Innis 2007).

The aim of this study was to discover new yeasts from the Southern Victorian marine environment with the ability to produce high levels of PUFAs or carotenoids. Strains were screened for PUFA producing capabilities and then isolated, subjected to microscopic analysis and identification using molecular biological techniques. Fourier transform infrared spectroscopy (FTIR) was also used to study the accumulation of unsaturated fatty acids within the microbial cells.

2.2 Materials and Methods

2.2.1 Chemicals used

All chemicals including fatty acid methyl ester standards (C23:0) used in this study were procured from Sigma-Aldrich (St. Louis, MO, USA) and Merck Chemicals (Frankfurter Strabe, Darmstadt, Germany) and were of analytical grade. For the molecular identification of yeast, molecular biology-grade chemicals were used. The PCR clean-up kit was procured from Promega (Madison, WI, USA) and the DNA sequencing kit was from Applied Bio-systems (Foster city, CA, USA).

2.2.2 Sampling site

Different samples of seawater and sediments were collected from the low-tide region (Elevation 16 ft.; S 38°16.204’, E 144°38.212’) at Queenscliff, Victoria, Australia in August 2010 (Figure 2.1, Figure 2.2 and Figure 2.3). The samples were placed in 50 mL falcon tubes to which 300 mg L⁻¹ of penicillin and streptomycin were added. Samples were placed in an ice box and used within 72 h.
Figure 2.1 Sample collection site (The Queenscliff, Victoria 3225, Australia).
Figure 2.2 Preparations for the sample collection.

Figure 2.3 The sampling site; Marine Centre, Queens cliff, Victoria: (Elevation 16 ft.; S 38°16.204’, E 144°38.212’).
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2.2.3 Screening and isolation of the marine yeasts

Seawater samples (3 mL) and the sediment (1 g) were suspended in 20 mL YPD medium with the following composition: 1 g L\(^{-1}\) yeast extract, 1 g L\(^{-1}\) peptone, and 2 g L\(^{-1}\) glucose, containing penicillin and streptomycin (300 mg L\(^{-1}\) each). The antibiotics were pre-filtered using a 0.22 µm sterile filter and added to the medium after it had been autoclaved and allowed to settle to room temperature. After an appropriate dilution of the cell cultures, the medium was placed on YPD plates with the above mentioned antibiotic concentration and the plates were incubated at 25 °C for 5 days. Growth was monitored at 24 h intervals. Different colonies were taken from the petri dish and re-grown in a 50 mL Erlenmeyer flask with 10 mL of fresh medium. Strains showing red colouration were selected for further investigation. Pure colonies were obtained by the streak plate method.

2.2.4 Cultivation of marine yeasts

For optimisation, isolates labelled as AMCQ8A and AMCQ10C (loop full of cells) were transferred into 50 mL basal medium consisting: 30 g L\(^{-1}\) glucose, 10 g L\(^{-1}\) yeast extract, 30 g L\(^{-1}\) NaCl, 0.7 g L\(^{-1}\) KCl, 10.8 g L\(^{-1}\) MgCl\(_2\), 5.4 g L\(^{-1}\) MgSO\(_4\), and 1 g L\(^{-1}\) CaCl\(_2\) prepared with sea water in a 250 mL flask and kept in an orbital shaker at 25 °C at 150 rpm. The growth of the cell culture was determined by the optical density (OD) at 600 nm using a UV-Vis spectrophotometer (Shimadzu Corporation, Kyoto, Japan) at regular time intervals. The cells in the culture were collected and washed twice with sterile saline solution after centrifugation at 5,000 x g and 4 °C. Cell dry weight (CDW, g L\(^{-1}\)) of the biomass was measured by placing the cell pellets at 80 °C until their dry weight was constant. The microbial growth rates were studied in triplicates. AMCQ8A was also grown in nutrient broth (NB) containing: 5 g L\(^{-1}\) peptone, 3 g L\(^{-1}\) yeast extract, 5 g L\(^{-1}\) NaCl in distilled water and was used as control in FTIR study.

2.2.5 Microscopy studies

The isolates were observed using the differential interference contrast (DIC) or Nomarski microscopy using an Axio-imager.A1 microscope (Zeiss, Oberkochen, Germany). Yeast cells were fixed on a slide, air dried and Gram stained before observation under the microscope. For Gram staining a thin smear of the isolate was prepared and heat fixed under aseptic conditions. Crystal violet was used on the smear and left for 30 seconds. The slide was washed with distilled water and then
flooded with Gram’s iodine for 60 seconds. The iodine was washed with 95 % ethanol drop by drop. The slide was then washed with distilled water. Safranine was applied for 30 seconds and blot dried before microscopic observation.

2.2.6 Scanning electron microscopy (SEM)

Colonies from agar plates were fixed with a 5 % formalin solution and suspended in 2-3 mL of sterile distilled water before the cells were freeze dried. A small flake of freeze dried cells was mounted onto carbon tape on an aluminium stub and air dried, after which 60 nm of gold was deposited on its surface using a sputter coater (BAL-TEC SCD 050, Leica Microsystems, Wetzlar, Germany). The cells were examined under a Supra 55 VP scanning electron microscope (Carl Zeiss AG, Oberkochen, Germany) at an accelerating voltage of 10 kV using a secondary electron detector.

2.2.7 DNA extraction from isolates

The total genomic DNA of the yeast strains AMCQ8A and AMCQ10C was isolated and purified as done by Sambrook et al. (1989) with modifications. Two day old culture (1.5 mL) grown in YPD medium was centrifuged at 8,000 x g for 10 min at 4 °C. The resulting cell pellet was dissolved in 200 µL of lysis buffer (0.25 M Tris-HCl pH 8.2, 0.1 M EDTA, 2 % sodium dodecyl sulphate and 0.1 M NaCl) and gently mixed. DNA was extracted with 200 µL of phenol, chloroform and isoamyl alcohol (25:24:1) and centrifuged at 8,000 x g for 5 min at 4 °C. The upper layer was collected in a microcentrifuge tube, to which 3 M sodium acetate (10 µL) and ethanol (265 µL, 95 % v/v) were added before centrifugation at 8,000 x g for 30 min. The extracted DNA was washed with ethanol (100 µL, 70 % v/v) and centrifuged at 8,000 x g for 5 min at 4 °C. The tube was kept at 37 °C for 2-3 min to evaporate residual ethanol. Extracted DNA was dissolved in distilled water (40 µL) and kept at -20 °C for further use.

2.2.8 PCR amplification and 18s rRNA sequencing

DNA was amplified by polymerase chain reaction (PCR) on a Thermal cycler (Eppendorf Master Cycler, Foster city, CA, USA). The amplification of the extracted DNA in yeasts used the universal primers: F-5’-TACCTGGTTGATCCTGCCAG-3’ and R-5’ CCTTCCGCAGGTTCACCTAC-3’. A 25 µL PCR reaction mixture composed of 200 µM of master mix (ready to use solution containing Taq DNA polymerase, dNTPs, MgCl₂, Promega, Madison, WI, USA) and 1.25 µM of forward
and reverse primers to amplify 200 ng of DNA template. The conditions for the PCR amplification were as follows: initial denaturation (3 min at 95 °C), final denaturation (1 min at 94 °C) with annealing (1 min at 50 °C), extension at 72 °C for 1 min followed by final extension at 72 °C for 10 min. PCR was run for 35 cycles. The amplified DNA was purified from the agarose gel using the Wizard PCR kit (Promega, Madison, WI, USA) according to the manufacturer’s instructions, which was as follows. DNA bands were excised from the agarose gel under UV light with a scalpel and kept in pre-weighed micro-centrifuge tubes. Membrane binding solution (10 μL per 10 mg of gel slice) was added and incubated at 50-60 °C until the gel slice was completely dissolved. The dissolved gel mixture was transferred to the mini-column assembly (SV mini-column + collection tube) and incubated at room temperature for 1 min. The assembly was centrifuged at 13,000 x g for 1 min. The supernatant was discarded and the mini-column was re-inserted into the collection tube. An aliquot (700 µL) of membrane wash solution was added to the mini-column and centrifuged at 13,000 x g for 1 min. Supernatant was discarded and the mini-column was re-inserted into collection tube. The washing was repeated with 500 µL membrane wash solution and centrifuged at 13,000 x g for 1 min. The collection tube was emptied and the column assembly was re-centrifuged for 1 min to allow evaporation of any residual ethanol. The mini-column was transferred to a clean 1.5 mL centrifuge tube and 20-30 µL of nuclease free water was added to the mini-column. The assembly was incubated at room temperature for 1 min and centrifuged at 13,000 x g for 1 min. The mini-column was discarded and collected DNA was stored at -20 °C for further use. DNA concentration was measured by Nanodrop 1000 Spectrophotometer (Thermo scientific, Wilmington, DE, USA). The purified DNA of yeast isolates was sequenced using a DNA sequencer (Applied Bio systems, Foster city, CA, USA).

2.2.9 Phylogenetic analysis

The 18S rRNA sequences of isolates were aligned with sequences retrieved from the Genbank databases using the Basic Local Alignment Search Tool (BLAST) software (Altschul et al. 1990). The sequences sharing over 98 % similarity were considered and multiple alignments were performed using Clustalx and a phylogenetic tree was constructed using the clustalw2 tool (Larkin et al. 2007). AMCQ8A and AMCQ10C sequences were deposited to GenBank with the accession numbers JN695595 and JN695596, respectively.
2.2.10 FTIR microspectroscopy

Spectroscopic analysis was carried out on four isolates. To prepare the cell samples for FTIR microspectroscopic measurements, the cell concentration in the medium was determined by a cell-counting method using a Bright-Line™ haemocytometer (Sigma-Aldrich, St. Louis, MO, USA). The original culture suspension was subsequently diluted to ~4 x 10^6 cells per mL in 30 g L\(^{-1}\) isotonic saline solution. The cells were then washed twice with isotonic saline solution using a microcentrifuge (Heraeus Fresco17 model, Thermo Scientific, Wilmington, DE, USA). After the final wash, the cell precipitate was fixed and preserved in 30 g L\(^{-1}\) isotonic saline solution containing 5 % formalin. Then 500 µL of the cell suspension was deposited by cyto-centrifuge (Cytospin-III; Thermo Fisher Scientific, Waltham, MA, USA) to produce mono-layers of cells on IR reflective glass slides (MirrIR slides, Kevley Technologies, Chesterland, OH, USA). The films were then kept dried in a desiccator for 30 min prior to FTIR spectral data collection.

FTIR microspectroscopic measurements were conducted using a Varian FTS 7000 series FTIR spectrometer coupled to a model UMA600 microscope (Varian Inc., Palo Alto, CA, USA) with a 64 x 64 element focal plane array (FPA) detector and a 36x objective lens. The acquisition parameters were set for the mid-IR region (4000-400 cm\(^{-1}\)) in reflectance mode. Blackman-Harris 3-Term apodization, power-spectrum phase correction and a zero-filling factor of 2 were set as default acquisition parameters using Stingray software (Varian Inc., Palo Alto, CA, USA). FTIR spectra were recorded in a mapping mode using an 8 cm\(^{-1}\) spectral resolution and 64 co-added scans, to acquire a set of 64 x 64 recorded spectra per map. Data processing including baseline correction and vector normalization was performed using the OPUS 6.0 software suite (Bruker Optik GmbH, Ettlingen, Germany).

2.2.11 Fatty acid extraction and Gas chromatography (GC) analysis

Lipids were extracted from the freeze-dried cells (10 mg) with chloroform and methanol (2:1) (Lewis et al. 2000). The sample was vortexed and centrifuged, the supernatant collected and the solvent evaporated under a stream of nitrogen. Lipid content (% dry weight) was determined gravimetrically. Fatty acids were converted to fatty acid methyl esters (FAMEs) by acid-catalysed trans-esterification following the method of Christie and Han (2010) with minor modifications. Briefly, the lipid extract was dissolved in 1 mL toluene, and 200 µL of internal standard (methyl
nonadecanoate, 5 mg mL\(^{-1}\)) and 200 µL of butylated hydroxytoluene (BHT, 1 mg mL\(^{-1}\)) were added. To this, 2 mL of hydrogen chloride in methanol (prepared by adding 5 mL acetyl chloride drop wise to 50 mL of methanol on ice) was added, the solutions mixed and incubated overnight at 50 °C. FAMEs were extracted into hexane. The hexane layer was collected and dried over sodium sulphate. Solvent was evaporated under a stream of nitrogen. The samples were analysed using an Agilent 6890 gas chromatography unit with a flame ionisation detector (Agilent Technologies, Santa Clara, CA, USA). The GC was equipped with a capillary column (Supelcowax 10, 30 m x 0.25 mm, 0.25 µm film thickness). Helium was used as the carrier gas at a flow rate of 1.5 mL min\(^{-1}\) (constant flow). The injector was maintained at 250 °C and a sample volume of 1 µL was injected with a 50:1 split ratio. The oven was programmed from 140 °C (5 min hold) to 240 °C (10 min hold) at a rate of 4 °C min\(^{-1}\). Fatty acid peaks were identified by comparison of retention time data with external standards (Sigma-Aldrich, St. Louis, MO, USA). Peaks were quantified with Chemstation chromatography software (Agilent Technologies, Santa Clara, CA, USA) and corrected using theoretical relative FID response factors (Ackman 2002). Results are presented as mean ± SD of samples prepared in duplicate and analysed twice.

2.3 Results and Discussion

2.3.1 Isolation of marine microbes

A total of 54 yeast strains were obtained from seawater and sediments. The best growing isolates were selected based on their growth in the basal medium prepared by the addition of natural seawater collected from the sample collection site. From these, intense red coloured yeast colonies (pure and profuse) were obtained both from the seawater and sediment samples collected. Four yeast isolates were selected after antibiotic-assisted screening and named AMCQ1D, AMCQ8A, AMCQ10C and AMCQ12C (Figure 2.4). Upon microscopic examination, small, non-motile, almost rounded budding cells were observed (Figure 2.5). Gram staining revealed a dark violet colour in the isolates showing gram positive characteristics. Intact yeast cells are known to exhibit a violet colour under gram staining (Bianchi 1965). The preliminary observation based on colony and cell morphology suggested that the isolates may be pigmented yeast, due to their colour-producing ability. Yeasts of the genus *Rhodotorula* are widely distributed in the environment and easily identified by
their distinctive orange/red colonies (Li et al. 2010). However, no reports have been published on the isolation of *Rhodotorula* sp. from Victoria region, Australia.

![Figure 2.4 Pure isolates on agar plates.](image)

![Figure 2.5 Differential interference contrast (DIC) images of various marine isolates, AMCQ1D, AMCQ8A, AMCQ10C and AMCQ12C (Scale bar 10 µm).](image)
2.3.2 Biomass growth determination

The highest biomass growth (18.88 g L$^{-1}$ CDW) was obtained from the yeast isolate AMCQ8A within 72 h of incubation, whereas low biomass growth (4.38 g L$^{-1}$ CDW) was recorded for the isolate AMCQ10C (Figure 2.6). Two other yeast isolates (AMCQ12C and AMCQ1D) were also found to grow, but much more slowly than strain AMCQ8A. Biomass growth of 10-13 g L$^{-1}$ (CDW) has been reported for *R. mucilaginosa* TJY15a when grown on various carbon sources such as sucrose, glucose, and xylose (Li et al. 2010). The use of crude glycerol supplemented with Tween 20 resulted in high biomass production (5.47 g L$^{-1}$) with *R. glutinis* TISTR 5159 (Saenge et al. 2011). The growth and biomass accumulation of isolate AMCQ8A was higher than that reported for related *Rhodotorula* strains (Almazan et al. 1981; Li et al. 2010; Saenge et al. 2011). The unsaturated fatty acid content was found to be 63-75 % of total fatty acid (TFA) for the investigated isolates. The use of other carbon sources such as cassava starch, inulin and extract of Jerusalem artichoke tubers has been reported using co-culture of yeasts cultures (Gen et al. 2014; Zhao et al. 2011). The biomass reported in the studies using these carbon sources ranged from 12.2 to 20 g L$^{-1}$, which is comparable to our report (18.88 g L$^{-1}$ CDW). Yeast cultures such as *Rhodosporidium toruloides*, *Rhodotorula glutinis* and *Cryptococcus curvatus* were also reported to have utilised low cost substrates such as distillery and bioethanol waste waters, producing approximately 4-6 g L$^{-1}$ of biomass of which 25-44 % was lipid (Gonzalez-Garcia et al. 2013; Ling et al. 2013; Zhou et al. 2013). This demonstrates the versatility of oleaginous yeasts to grow well under a variety of culture conditions. Yeast isolate AMCQ8A was chosen for further study based on its fast growth and relatively high lipid content (Figure 2.6).
Figure 2.6 Biomass growth and lipid content of the marine yeast isolate AMCQ8A.

2.3.3 Microscopic analysis

Differential interference contrast images were observed for the yeast isolate AMCQ8A. Perforated structures were observed in the isolate with cell size ranging from 4-8 µm. Gram staining revealed a dark violet colour in the isolates showing gram positive characteristics (data not shown) as previously observed for yeast (Shinde et al. 2008). SEM images (Figure 2.7) revealed scars on the surface of the yeast cells. The presence of scars in *Rhodotorula* sp. is attributed to the cell reproduction cycle which results in a condensed cell wall (Jacob 1992).
2.3.4 DNA extraction and phylogenetic analysis

DNA fragments of 15 kb in size were extracted from the isolates with respect to the 1 kb DNA ladder (1 kb to 15 kb size). The DNA concentration obtained from AMCQ8A and AMCQ10C was found to be 305.9 and 413.9 ng µL⁻¹, respectively. The extracted DNA was amplified by PCR and a band of DNA was observed in the agarose gel (Figure 2.8). The DNA consisted of 2000 bp based on comparison with standards (100 bp to 12 kb size) (Figure 2.8). The molecular identification of yeast isolates (AMCQ8A and AMCQ10C) was carried out based on 18S rRNA gene sequence analysis. Phylogenetic analysis revealed that many available sequences exhibit evolutionary closeness with the isolates obtained in this study. Based on the evolutionary distance between the yeast strains, the phylogenetic search indicated that the isolate AMCQ8A is closely related to *R. mucilaginosa* L10-2 (Genbank accession number EF218987.1) (Figure 2.9). The similarity of the 18S rRNA gene sequence of AMCQ8A was 99 and 98 % identical to the sequences of *R. mucilaginosa* L10-2 and *Pucciniomycotina* sp. ZB-HB (Genbank Accession number FJ538170.1), respectively. The other yeast strain, AMCQ10C was found to be closely related to *R. mucilaginosa* PTD3 (Genbank accession number EU563926.1). Hence, the yeast strains were identified as *R. mucilaginosa* AMCQ8A and
AMCQ10C. Further molecular characterisation of the isolates is required to establish if they are new species. Some *Rhodotorula* sp. and other yeast strains such as *Cryptococcus albidus*, *C. curvatus*, *Lipomyces lipofera*, *L. starkeyi*, *Rhodotorula glutinis*, *Sporobolomyces roseus*, *Trichosporon pullulan* and *Yarrowia lipolytica* have recently been reported to accumulate large amount of lipids in their cells (Cui et al. 2012; Meng et al. 2009).

Figure 2.8 Agarose gel electrophoresis of the amplified DNA. Lane 1 denotes DNA ladder (100 bp to 12000 bp); Lane 2 and 3 – AMCQ8A; Lane 4, 5 and 6 – AMCQ10C.
Figure 2.9 Phylogenetic relationship of the 18s rRNA sequences of marine yeast (AMCQ8A and AMCQ10C) based on their similarity to closely related sequences. ClustalW program was applied to construct the tree using neighbour joining method. The numbers show the evolutionary distance between the strains.

2.3.5 FTIR analysis

In FTIR spectroscopy types of bonds, and thus different functional groups, absorb the infrared (IR) radiation of different wavelengths, which is represented in the form of an IR spectrum is plotted as wave number (x-axis) versus absorbance (y-axis) (Socrates 2001). The obtained spectral features therefore indicate the specific functional groups present within the analyte. Figure 2.10 shows the FTIR spectra of the yeast isolates used in this study. The depicted FTIR bands are characteristic of those observed for many biological samples and microorganism and are attributed to the major macromolecular components including protein, lipid, carbohydrate and nucleic acid (Branan and Wells 2007; Corte et al. 2011; Zeroual et al. 1994). In the high-wave length region, the spectra of every isolate establish a broad and strong ν(O-H) stretching band centred at ~3300 cm⁻¹ arising from the water molecules inside the cells. The observed triplet bands in the range of 3000-2800 cm⁻¹ are attributed to C-H stretching modes from lipids and proteins (Jackson and Mantsch 1993). The protein moiety is particularly prominent in the low-wavelength region, as indicated by the strong amide I and II bands at 1650 and 1545 cm⁻¹, respectively. The sharp band at 1745 cm⁻¹ represents ν(C=O) stretches of ester functional groups from lipids and fatty acids and is therefore indicative of the total lipids present in the cells.
(Guillen and Cabo 1997). In order to compare lipid production in each isolate, the ratio of band area under the total lipid and protein (amide II) bands were calculated and plotted (Figure 2.11). Maximum lipid production on the basis of the ratio of the band area of total lipids and amides (1.1807) was observed in AMCQ8A grown in 30 g L$^{-1}$ glucose medium. The minimum lipid production (0.0459) was observed for AMCQ8A in a simple nutrient medium without glucose. This indicated that the carbon source in the growth medium is crucial for lipid production (Li et al. 2007).

Figure 2.10 FTIR microspectroscopic spectra of marine yeast isolates.
2.3.6 Fatty acid production

The fatty acid composition analysis demonstrated the presence of polyunsaturated fatty acids, confirming the results of FTIR microspectroscopy examination. Based on the GC analysis, over 90 % of the total fatty acids produced by the yeast strains were C16:0, C18:0, C18:1 and C18:2 which is consistent with reported studies using oleaginous yeasts in co-culture with other yeast (Gen et al. 2014; Zhao et al. 2011). The most prominent fatty acids present in AMCQ8A were oleic acid (~45.4 % of TFA) and linoleic acid (~24.7 %) (Figure 2.12). Oleic acid and linoleic acid were found to be more abundant in AMCQ1D (~55.6 %), AMCQ10C and AMCQ12C (~30 %) as compared to AMCQ8A. This fatty acid profile is in qualitative and quantitative agreement with the fatty acid profiles of other lipid-producing yeast such as Rhodosporidium sp. (Li et al. 2007). Moreover, the presence of oleic acid (54.7-63.5 % of TFA) and linoleic acid (5.7-11.3 % of TFA) in Rhodotorula mucilaginosa TJY15a strain with the use of various carbon and nitrogen sources has recently been reported (Li et al. 2010; Zhao et al. 2010). The oleic acid content observed in this study was comparable to that produced by the Rhodotorula mucilaginosa TJY15a strain. ALA was present in all isolates (~0.8-5.5 %), with levels similar to those
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reported for related strains (Saenge et al. 2011; Yen and Zhang 2011). Fatty acid levels increased with fermentation time for AMCQ8QA, particularly the levels of unsaturated fatty acids (Figure 2.13). ALA was not observed in R. mucilaginosa TJY15a. Also, a low amount of ALA (only 1 % of TFA) was found in oleaginous yeast, Y. lipolytica (Li et al. 2008). This indicates that our strain is superior for the production of this fatty acid when compared to previously studied yeasts. ALA may have some cardiovascular benefits (Mozaffarian 2005), although it is likely that these benefits are related to its conversion to EPA and DHA. ALA is more acceptable to consumers versus EPA and DHA due to lower allergy concerns, no dietary restrictions (vegetarianism), lower risks of heavy metal contaminants and improved tolerability (Riediger et al. 2009).

![Figure 2.12 Fatty acid profile of all marine yeast isolates (% TFA= % of total fatty acids).](image)

Figure 2.12 Fatty acid profile of all marine yeast isolates (% TFA= % of total fatty acids).
2.3.7 Effect of different carbon source concentrations on growth and lipid production

The most commonly used carbon source for producing oil from microbes is glucose. While optimising the carbon source concentration (2-10 %, w/v) in the fermentation medium, an improvement (from 1.7 % to 6.21 %) in polyunsaturated fatty acid content was observed, notably ALA increased (Table 2.1). Also, improvements in C18:1 (from 7.96 to 35.71 %) and C18:2 (3.68 to 23.69 %) content was observed while using different concentrations of glucose. However, with high concentrations (10 %) of glucose in the fermentation medium, no improvement in biomass was observed. A sugar concentration of 7 % was found to yield the highest level of ALA (6.2 % of TFA). The ALA productivity could probably be enhanced further through controlled fermentation studies in a bioreactor.

Figure 2.13 Time course study of the fatty acid profile (% TFA) in AMCQ8A.
Table 2.1 Effect of different carbon concentration on unsaturated fatty acid profile of AMCQ8A (% of TFA).

<table>
<thead>
<tr>
<th>Carbon source concentration (%)</th>
<th>Biomass (g L⁻¹)</th>
<th>Unsaturated fatty acids (% TFA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C₁₈:₁</td>
</tr>
<tr>
<td>2</td>
<td>14.5±0.21</td>
<td>7.96±0.01</td>
</tr>
<tr>
<td>3</td>
<td>16.5±0.07</td>
<td>14.44±0.12</td>
</tr>
<tr>
<td>5</td>
<td>15.8±0.32</td>
<td>29.57±0.13</td>
</tr>
<tr>
<td>7</td>
<td>15.3±0.18</td>
<td>29.05±0.04</td>
</tr>
<tr>
<td>10</td>
<td>14.6±0.21</td>
<td>35.71±0.03</td>
</tr>
</tbody>
</table>

Some oleaginous yeast has been reported to accumulate high amount of lipids (% w/w) at similar levels to those observed in our study. Examples include *Rhodosporidium toruloides* (76.1 %), *L. starkeyi* (52.6 %), and *Trichosporon cutaneum* (59 %), based on extra glucose addition (15 g L⁻¹) to the medium (Hu et al. 2011; Kong et al. 2007; Li et al. 2006). AMCQ8A showed 35-60 % (w/w) lipid accumulation (as a percentage of biomass) in shake flasks. Lipid levels have been increased for some strains using controlled fermentation methods. For example, fed-batch fermentation of *R. toruloides* gave lipid accumulation of 48 % (w/w), and this was increased to 67.5 % (w/w) using a 15 L stirred tank fermenter (Li et al. 2007). In another study, *Yarrowia lipolytica* produced lipids (43 %, w/w) using continuous fermentation (Papanikolaou and Aggelis 2002). A 5 L fermenter utilised different concentrations of dissolved oxygen and resulted in 25-60 % lipid content (w/w) using *Rhodotorula glutinis* (Yen and Zhang 2011). Another *Rhodotorula* strain *R. mucilaginosa* TJY15a, showed lipid accumulation of 47.9 % (w/w, batch fermented), which increased to 52.9 % w/w when fed-batch fermentation was applied (Li et al. 2010).

Some studies have demonstrated the efficient consumption of agriculture waste carbon sources by different yeast strains. *Rhodotorula mucilaginosa* TJY15a was grown in co-culture (*Pichia* sp.) with inulin and tuber extract, and accumulated 53.2–56.6 % of lipids (Zhao et al. 2011). A similar co-culture study of another oleaginous yeast, *Rhodosporidium toruloides* with *Saccharomycopsis fibuliger* on cassava starch resulted in 60 % of lipid accumulation (Gen et al. 2014). These reports indicate that with the application of controlled fermentation strategies (fed batch and continuous
fermentations) including the utilisation of other low cost carbon sources, AMCQ8A could accumulate even higher amounts of lipids.

2.4 Conclusion

Two marine yeast isolates, AMCQ8A and AMCQ10C, were isolated and identified as *Rhodotorula* sp. and found to be closely related to *R. mucilaginosa*. FTIR microspectroscopy was used to show the presence of lipids in these yeasts with only AMCQ8A showing a clear peak of unsaturated fatty acids. AMCQ8A produced higher biomass than AMCQ10C in glucose medium. AMCQ8A also gave a higher lipid to amide ratio and greater levels of ALA than the other isolates investigated. More than 90% of TFA was recorded as C16:0, C18:0, C18:1 and C18:2 in all yeast strains. The accumulation of unsaturated fatty acids (C18:1, C18:2 and C18:3) in AMCQ8A increased with increasing glucose in the fermentation media. AMCQ8A may be a suitable candidate for single cell oil production due to its high growth, easy cultivation and high fatty acid content when compared to other isolates.
Chapter 3: Pollen-baiting facilitates the isolation of marine thraustochytrids with potential in omega-3 and biodiesel production
3.1 Introduction

Thraustochytrids are monocentric protists and are ubiquitous in marine habitats, particularly mangrove areas (Raghukumar 2002). These marine protists are known for accumulating high levels of the omega-3 fatty acid docosahexaenoic acid (DHA) (Gupta et al. 2012; Raghukumar 2008). Despite their abundance in marine ecosystems, they are difficult to isolate since they are relatively slow growing compared to other marine micro-organisms such as bacteria, yeast and fungi. These faster growing microbes tend to contaminate samples during the isolation process when traditional serial dilution and spread plating methods are used (Taoka et al. 2010; Wilkens and Maas 2012). Despite these contamination issues microorganisms of the genus *Thraustochytrium* have been isolated from a variety of locations, from as early as the 1950s (Watson and Ordal 1957). Thraustochytrids have been isolated from coastal environments such as cold temperate littoral, cool temperate littoral, temperate environments, sub-tropical mangrove areas and from different samples such as brackish and marine water, moist sediments, algae, shells and degrading mangrove leaves (Bowles et al. 1999; Lee Chang et al. 2012; Perveen et al. 2006; Rosa et al. 2011).

We found traditional methods (Vishniac 1956) of isolation difficult to apply due to rapid contamination by faster growing microbes. Thraustochytrid growth can be selected for, based on their ability to metabolise lignocellulosic substrates such as pine pollen grains. Direct plating involves spread-plating of sea water or inoculating the sediment onto an agar plate containing a combination of antibiotics and antifungal agents. Baiting techniques using hairs (with *Geodermatophilus obscurus*) and pollen grains (with *Actinoplanes* sp.) have been reported for the isolation of zoosporic actinomycetes (Garrity et al. 1996; Hayakawa et al. 1991). *Actinoplanes* sp. has been selectively isolated from pollen grains of *Pinus* sp. with the application of a chemotactic method (Garrity et al. 1996). In addition, 30 species of zoosporic fungi related to the Kingdoms of Chromista and Fungi have recently been isolated using different types of baits that included onion skin, corn leaves, cellophane, shrimp exoskeleton, snake skin, *Sorghum* sp. seed, some strands of blond hair from children and pine pollen grains (Nascimento et al. 2012).

Previously, the pollen-baiting method has been used by researchers for quantitative studies of zoosporic fungi from soil and water samples (DiLeo et al. 2010; Lee
The pollen-baiting technique involves the use of pollen grains, such as pine pollen, which is interspersed on the surface of the water sample, with antibiotics and antifungal agents.

Here we describe the isolation and identification of thraustochytrids isolated using a modified pine pollen-baiting method (Bremer 2000). We compare the use of direct plating and a pollen-baiting-assisted technique for the isolation of thraustochytrids.

3.2 Materials and Methods

3.2.1 Chemicals used

All chemicals used in this study were of analytical grade and obtained from Sigma-Aldrich (St. Louis, MO, USA) and Merck Chemicals (Frankfurter Strabe, Darmstadt, Germany). For 18S rRNA sequencing of thraustochytrids, molecular biology grade chemicals were used. The PureLink™ Genomic DNA Mini Kit was obtained from Invitrogen (Carlsbad, CA, USA). The PCR clean-up kit was procured from Promega (Madison, WI, USA) and the DNA sequencing kit from Applied Bio-systems (Foster city, CA, USA).

3.2.2 Location of the sampling site

Seawater samples and sediments containing degraded leaf material were collected from the temperate inter-tidal zone at Queenscliff, Victoria, Australia during February 2011. The GPS coordinates (S 38°16.202’, E 144°38.212’) and the temperature of the location (18-20 °C) were recorded. The samples were placed in sterile 50 mL falcon tubes containing 500 mg L⁻¹ of penicillin and streptomycin. The tubes were moderately agitated for uniform dispersal of the antibiotics. Sample tubes were placed in an ice box and transported to the laboratory and stored at 4 °C until further use.

3.2.3 Isolation procedure for thraustochytrids

The isolation procedure was performed within 24 hours of sample collection. The collected sea water samples were processed according to different isolation techniques described below.

3.2.3.1 Direct plating technique

Seawater (3 mL) and sediment (1 g) samples were suspended in 20 mL of yeast extract-peptone medium (YP) (1 g L⁻¹ yeast extract, 1 g L⁻¹ peptone, 500 mg L⁻¹ penicillin and streptomycin).
penicillin, 500 mg L\(^{-1}\) streptomycin, 50 mg L\(^{-1}\) rifampicin and 10 mg L\(^{-1}\) nystatin in 100 % sterile seawater. The antibiotics and antifungal agent were pre-filtered using a 0.22 µm sterile filter and immediately added to the autoclaved and cooled medium just before pouring of the agar plates. A portion of the suspension culture, with appropriate antibiotic dilutions, was spread plated on YP plates containing the above mentioned antibiotic concentration and incubated at 20 °C for 5 days. The leaf samples were washed with sterile sea water to remove any particulates, which can be a source of contaminants. The leaf sample was then inoculated onto the YP plates containing the same antibiotic concentrations. Growth on the plates was monitored at 24 hour intervals. Different colonies, identified by their morphology as thraustochytrid-like, were taken using a sterile loop and re-grown in a 50 mL Erlenmeyer flask with 10 mL of fresh medium. Spherical, uneven, and slimy colonies along with some translucent, orange color producing colonies were selected for further study. Pure colonies were obtained by the streak plate method.

3.2.3.2 Pollen baiting technique

The falcon tubes containing the seawater samples were pollen-baited with sterile pine pollen grains (Allife, Silverwater, NSW, Australia). The same concentrations of antibiotic and antifungal agents were added to the tubes as in the direct plating method. The tubes were incubated at 20 °C for 2 weeks. Sub-samples (10 μL) were removed from the water surface and observed under the microscope at 24 h intervals to check for the appearance of thraustochytrid colonies growing on the pollen grains. Tubes showing a thin film over the water level were checked for contamination under a microscope and when microorganisms other than thraustochytrids were observed, they were discarded. Subsamples (20 μL) of thraustochytrid cells and pine pollen were spread-plated on YP agar medium containing 1 g L\(^{-1}\) yeast extract, 1 g L\(^{-1}\) peptone, 10 g L\(^{-1}\) agar and antibiotic and antifungal agents in 100 % sterile seawater. Plates were incubated at 20 °C for one week. Thraustochytrid colonies were sub-cultured on YP agar medium containing antibiotics to obtain pure isolates.

3.2.4 Microscopy studies

The isolates were observed using differential interference contrast (DIC) and Nomarski microscopy using an Axio-imager. A1 microscope equipped with Axio- vision software (Zeiss, Oberkochen, Germany). Thraustochytrid cells were fixed on a slide and air-dried before observation under the microscope.
3.2.5 Fatty acid production

To determine the fatty acid profile all isolates were cultured in sterile (autoclaved) GYP medium containing 5 g L\(^{-1}\) glucose, 2 g L\(^{-1}\) yeast extract, 2 g L\(^{-1}\) peptone in 70 % artificial seawater (Instant Ocean Sea Salts, Aquarium Systems, Inc., Blacksburg, VA, USA). The liquid medium (50 mL) was inoculated with the isolates from the agar plates and incubated at 20 °C with a shaking (150 rpm). The cell suspension was centrifuged at 10,000 x g for 10 min to obtain a pellet. The thraustochytrid cell pellet was freeze-dried and stored at -20 °C before proceeding with fatty acid extraction. Results are presented as mean ± SD of samples prepared in duplicate and analysed twice.

3.2.6 Fatty acid extraction and GC analysis

This method is described in Chapter 2 (Section 2.2.11).

3.2.7 DNA extraction and 18S rRNA sequencing

A 3 mL sub-sample of a 7 day old thraustochytrid culture in YP medium was centrifuged at 8,000 x g for 10 min at 4 °C to obtain a cell pellet. The total genomic DNA of the isolates was isolated and purified using PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions as follows. The cell pellet was resuspended in 180 µL of PureLink genomic digestion buffer and 20 µL proteinase K and was incubated at 55 °C until the lysis was completed. After the lysis the rest of the extraction was performed at room temperature. RNase A (20 µL) was added and incubated for 2 min. The lysate was centrifuged at 17,000 x g for 5 min and the supernatant transferred to a fresh microcentrifuge tube. PureLink genomic binding buffer (200 µL) was added to the lysate and vortexed (1-2 sec) to yield a homogenous solution. An aliquot (200 µL) of ethanol (100 %) was added to the lysate and vortexed. The total lysate (~640 µL) was transferred to the spin column (with collection tube) and centrifuged at 10,000 x g for 1 min. The collection tube was discarded and the spin column was placed in new collection tube. An aliquot (500 µL) of wash buffer 1 was added to the column and centrifuged at 10,000 x g for 1 min. The spin column was transferred to new collection tube and 500 µL of wash buffer 2 was added to the spin column and centrifuged at 17,000 x g for 3 min. Sterile distilled water (20 µL) was added to the column and incubated for 1 min. The column was centrifuged at 17,000 x g for 1 min to obtain purified genomic DNA. The extracted DNA was kept at -20 °C for
DNA was amplified by polymerase chain reaction (PCR) on a Thermal cycler (Eppendorf Master Cycler, Foster city, CA, USA). The PCR strategy was modified from Mo et al. (2002). The amplification of 18S rRNA gene from the extracted DNA was performed with the following primers: F-5' CAACCTGGTTGATCCTGCCAGTA-3' and R-5' TCACTACGAAAACCTTGTTACGAC-3' (Burja et al. 2006). A 25 µL PCR reaction mixture containing 200 µM of master mix (ready to use solution containing Taq DNA polymerase, deoxynucleotide triphosphates (dNTPs), MgCl₂, Promega, Madison, WI, USA) and 1.25 µM of forward and reverse primers was used to amplify 200 ng of DNA template. The conditions for the PCR amplification were as follows: initial denaturation (3 min at 94 °C), final denaturation (45 s at 94 °C) with annealing (30 s at 64 °C), extension at 72 °C for 2 min followed by final extension at 72 °C for 10 min. PCR was run for 30 cycles. The amplified DNA was purified from the agarose gel using the Wizard PCR kit (Promega, Madison, WI, USA) according to manufacturer’s instructions as follows: The DNA bands were excised from the gel with new scalpel under UV light and kept in pre-weighed micro-centrifuge tubes. The weight of the tube with the gel was recorded. Membrane binding solution (10 µL) was added to 10 mg of gel slice and incubated at 50-60 °C until the gel slice was completely dissolved. The dissolved gel mixture was transferred to the mini-column assembly (SV mini-column + collection tube) and incubated at room temperature for 1 min. The assembly was centrifuged at 13,000 x g for 1 min. The supernatant was discarded and the mini-column was re-inserted into the collection tube. An aliquot (700 µL) of membrane wash solution was added to the mini-column and centrifuged at 13,000 x g for 1 min. The supernatant was discarded and the mini-column was re-inserted into another collection tube. The washing was repeated with 500 µL membrane wash solution and centrifuged at 13,000 x g for 1 min. The collection tube was emptied and the column assembly was re-centrifuged for 1 min to allow the evaporation of any residual ethanol. The mini-column was transferred to a clean 1.5 ml centrifuge tube and 20-30 µL of nuclease free water was added to the mini-column. The assembly was incubated at room temperature for 1 min and centrifuged at 13,000 x g for 1 min. The mini-column was discarded and collected DNA was stored at -20 °C for further use. DNA concentration was measured by Nano drop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The purified DNA of the selected isolates i.e. BAITING1, BAITING2, BAITING3, BAITING4,
PLATING4 and PLATING5 were sequenced using a DNA sequencer (Applied Biosystems, Foster city, CA, USA).

3.2.8 Phylogenetic analysis

The seven potential thraustochytrids isolated in the lab were selected for molecular characterization followed by phylogenetic analysis. PCR amplified 18S rRNA was sequenced and validated after BLAST analysis (Altschul et al. 1990) and found to be closely related to thraustochytrid species. All sequences were submitted to Genbank with accession numbers JX993839-JX993845. Further 18S rRNA sequences from 22 related species were outsourced/retrieved from Genbank databases and used as reference sequences for phylogenetic studies. The phylogenetic tree was constructed using MEGA5 software (Tamura et al. 2011). The evolutionary relationship was concluded by using the maximum likelihood method, based on the Kimura 2-parameter model (Kimura 1980). The bootstrap consensus tree established from 1000 replicates represents the evolutionary history of the analysed taxa (Felsenstein 1985). Branches analogous to partitions reproduced in less than 50 % bootstrap replicates are collapsed. Initial tree(s) for the experimental search were obtained as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used. Otherwise BIONJ method with MCL distance matrix was used. The tree is drawn to scale, with branch distances measured in the number of substitutions per site. The analysis involved 30 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1150 positions in the final dataset.

3.2.9 Effect of different carbon sources on the fatty acid profile of Thraustochytrium sp. BAITING3

Based on having the highest DHA to TFA ratio, the strain BAITING3 was selected for further study. Various carbon sources such as sucrose, galactose, fructose, β-lactose, xylose, starch, maltose and glycerol were used to grow the BAITING3 strain, and the fatty acid profiles were subsequently analysed. GYP medium (autoclaved) was used as a base and 0.5 % (w/v) of the respective carbon sources was added. Cultures were grown in 50 mL of the medium in a 250 mL flask. Fatty acid extraction and GC analysis was performed as detailed in section 3.2.6 above. Results are presented as mean ± SD of samples prepared in duplicates and analysed twice.
Note: The assigned names of the isolates used were changed to make it simple to understand the difference between plated and baited isolates (Table 3.1).

Table 3.1 Lab isolates and their assigned names.

<table>
<thead>
<tr>
<th>Lab isolates</th>
<th>Assigned names</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMCQ1A8</td>
<td>PLATING1</td>
</tr>
<tr>
<td>AMCQ1C7</td>
<td>PLATING2</td>
</tr>
<tr>
<td>AMCQ4B27</td>
<td>PLATING3</td>
</tr>
<tr>
<td>AMCQ5B25</td>
<td>PLATING4</td>
</tr>
<tr>
<td>AMCQ6A13</td>
<td>PLATING5</td>
</tr>
<tr>
<td>AMCQS5-3</td>
<td>BAITING1</td>
</tr>
<tr>
<td>AMCQS5-4</td>
<td>BAITING2</td>
</tr>
<tr>
<td>AMCQS5-5</td>
<td>BAITING3</td>
</tr>
<tr>
<td>AMCQS4-6</td>
<td>BAITING4</td>
</tr>
<tr>
<td>AMCQS1-9</td>
<td>BAITING7</td>
</tr>
<tr>
<td>AMCQS1-10</td>
<td>BAITING8</td>
</tr>
</tbody>
</table>

3.3 Results and Discussion

3.3.1 Isolation of thraustochytrids

Thirteen thraustochytrid strains were isolated from the low-tide region of Queenscliff, Victoria using direct plating and pollen–baiting isolation methods. Although the media was supplemented with several broad spectrum antibiotics and an antifungal agent, some bacteria and fungi were still found to grow on the agar plates. Pure thraustochytrid strains identified microscopically were obtained after serial sub-culturing. The selection of oleaginous strains was based on results of fatty acid profiling.

Thraustochytrids spores of varying sizes (10-50 µm) were observed by microscopy, with the zoospores moving erratically in the medium and the cells were found to be attached to the pollen surface (Figure 3.1; a and b). At low magnifications (5x and
10x) these cells appeared highly reflective, indicating the presence of oils. At high magnification a clear sporangium was observed, with progressive cleavage demonstrating that the cell was on the verge of releasing zoospores (Figure 3.1; c). Zoospore release is a characteristic feature of thraustochytrid cell division (Ulken et al. 1985). Direct plating resulted in the isolation of 5 strains (PLATING1, PLATING2, PLATING3, PLATING4, and PLATING5), whereas 8 strains (BAITING1, BAITING2, BAITING3, BAITING4, BAITING5, BAITING6, BAITING7, and BAITING8) were isolated using the pollen-baiting technique.

Figure 3.1 Microscopic images of various marine isolates, a) and b); Thraustochytrid cells attached to the surface of pine pollen with released zoospores (scale bar 20 µm), c); PLATING5; mature sporangium ruptured to release zoospores (scale bar 20 µm) and d); BAITING3 (scale bar 20 µm).
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The morphological characteristics of the thraustochytrid cells were observed by microscopy and we found mostly non-motile spherical cells for all isolates (Figure 3.1; d). We found three types of strains in this study belonging to *Thraustochytrium*, *Schizochytrium* and *Ulkenia* species. There were distinguishable morphological features for the different isolates in the terms of cell division which are illustrated in Figure 3.2. For the *Schizochytrium* species binary division was observed as previously reported (Honda et al. 1999). For *Thraustochytrium* and *Ulkenia* species binary division was not observed. Honda and co-workers suggested that thraustochytrids belonging to *Schizochytrium* sp. may have evolved in the evolution of new thraustochytrid families.

Figure 3.2 Morphology of in-house isolates (a, b and c); *Schizochytrium* sp. BAITING7, *Ulkenia* sp. PLATING4 and *Thraustochytrium* sp. BAITING3.
The direct plating technique involves spreading a seawater sample containing thraustochytrids onto agar plates. This method may increase the level of contamination by yeast, other fungi and antibiotic resistant bacteria. In this research, pollen-baiting was predominantly selective for thraustochytrids. We observed that the pollen-baiting method was better suited to the isolation of thraustochytrids from seawater or sediment samples. It has already been observed that pollen grains from coniferous plants such as pine trees are more prone to attract thraustochytrids than those of other plants (Goldstein 1960). We found that pine pollen grains acted as specific substrate that thraustochytrids would readily attach to.

Pine pollen is a rich source of nutrients and include carbohydrates in the form of starch (44 %) and sugars (4-10 %), protein (6-28 %), amino acids (14-22 %), lipids (1-20 %), flavonoids (2.5 %) and trace amounts of vitamins and sterols (Khan and Abourashed 2010). Specific major components include stearic acid, palmitic acid, ursolic acid, flavonoids (isorhamnetin glycoside, narcissin, and luteolin), pentacosane, sitosterol and 6-amino purine. A reason for pollen-baiting’s effectiveness has been proposed by Hayakawa and co-workers (1991), who postulated that the pollen grains are responsible for the release of saccharides and proteins that may attract the zoospores of actinomycetes. A similar theory may be applied to the attraction of thraustochytrid zoospores towards the pollen grains. Recently, a revised method using pollen grains from sweet gum (Liquidambar sp.) and brine shrimp larvae as bait has also been reported for the isolation of thraustochytrids (Rosa et al. 2011).

3.3.2 Fatty acid composition of new thraustochytrid isolates

All isolates exhibited a fatty acid profile composed primarily of saturated fatty acids (C16:0, C17:0 and C18:0), monounsaturated fatty acids (C18:1n9) and medium and long chain PUFAs (C18:2n6, C20:4n6, C20:5n3 and C22:6n3). EPA and DHA as a percentage of total fatty acids (TFAs) ranged from 5.6-12.8 % and 11-41.2 % (Figure 3.3). Of all the isolates, BAITING3 was found to have the greatest DHA content, comprising 41.2 % of TFA. The present study is consistent with that of Burja et al. (2006) and Huang et al. (2003), who showed that thraustochytrids from cold water environments can accumulate up to 35-50 % of TFA as DHA. BAITING3 accumulated EPA at an amount of 8.7 % of TFA. However, the highest EPA levels were recorded in PLATING5 (direct plating isolate) at 12.8 % of TFA. A recent
study observed the accumulation of long chain omega-3 PUFAs in some related strains, including *Schizochytrium* sp. (29 % TFA), *Thraustochytrium* sp. (40.5-47 % TFA), *Ulkenia* sp. (52.6 % TFA), and *Aurantiochytrium* sp. (37.8-69.1 % TFA) (Lee Chang et al. 2012).
Figure 3.3 Fatty acid profiles of the collected isolates. C16:0, palmitic acid; C18:0, stearic acid, C20:5n3, eicosapentaenoic acid; C22:5n6, docosapentaenoic acid; C22:6n3, docosahexaenoic acid. The hashed bars indicate plating isolates and solid bars indicate baiting isolates.
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The commercial strain *Schizochytrium limacinum* SR21 exhibited 30 % of TFA as DHA (Nakahara et al. 1996; Yokochi et al. 1998). Also, the saturated fatty acids, particularly C16:0 (49.7 %) and C18:0 (25.1 %) were produced at their highest levels by one of the pollen-based isolates. The findings of this study are in agreement with the observations of Bowles et al. (1999), in which isolates from temperate regions produced high levels of DHA with relatively low biomass production. Low growth rates in new strains of thraustochytrid were also observed in isolation studies from marine samples in Argentina (Rosa et al. 2011).

In the current study the fatty acid profile for omega-3 PUFAs was between 20-50 % of TFA depending upon the strain. BAITING3 accumulated C16:0 and C18:0 at about 40 % of TFA, considerably more than the commercial strain *Schizochytrium* sp. SR21 (Yaguchi et al. 1997). Furthermore, BAITING7 accumulated 74.7 % of TFA as even chain saturated fatty acids (C16:0 and C18:0). This is a relatively high amount when compared to other strains, such as the 52 % of TFA in *Aurantiochytrium* sp. (Lee Chang et al. 2012). DPA (C22:5n6) was found to be in the range of 6.8-17.9 % with PLATING3 accumulating the highest quantity. The odd chain saturated fatty acid (C17:0) was found to be present in two of the isolates, with the highest in PLATING3 (4.5 % of TFA). C18:1n9 and C20:4n6 were also detected in some isolates in the range of 2.7-4.1 % and 2.5-5.3 %, respectively. These fatty acid species were mainly found in strains isolated by direct plating. Direct plating isolates accumulated moderate levels of DHA (18.7-25.1 % of TFA). The pollen based isolates (except BAITING7 and BAITING8) showed higher DHA as a percentage of TFA, when compared to the direct plating isolates (Figure 3.4). Similarly, the DHA: EPA ratio was also found to be higher in the pollen baited isolates. A summary of the properties of these isolates is given in Table 3.2.
Figure 3.4 Relative DHA to TFA productivity for each strain.
Table 3.2 Summary data of the strains collected from the Queenscliff region, Victoria, Australia.

<table>
<thead>
<tr>
<th>Newly isolated strains</th>
<th>Colony description</th>
<th>Sporulation/Morphology</th>
<th>Cell size (μm)</th>
<th>TFA (%)</th>
<th>% TFA</th>
<th>DHA:EPA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Pollen Baiting</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>B1*</td>
<td>Vegetative, spherical</td>
<td>~30</td>
<td>27.8±1.8</td>
<td>7.9±0.06</td>
<td>40.2±0.05</td>
<td>2.7</td>
</tr>
<tr>
<td>B2*</td>
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<td>28.6±1.9</td>
<td>8.3±0.6</td>
<td>36.9±0.2</td>
<td>1.6</td>
</tr>
<tr>
<td>B3*</td>
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<td>41.5±0.3</td>
<td>3.8</td>
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<tr>
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<td>5.1±0.01</td>
<td>30.4±0.2</td>
<td>2.3</td>
</tr>
<tr>
<td>B7*</td>
<td>Cluster of zoospores released, spherical</td>
<td>~25</td>
<td>23±0.2</td>
<td>5.6±0.4</td>
<td>11.03±0.1</td>
<td>1.6</td>
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<tr>
<td>B8</td>
<td>Cluster of zoospores released, spherical</td>
<td>~25</td>
<td>19.9±0.3</td>
<td>6.1±0.2</td>
<td>13.6±0.6</td>
<td>5.1</td>
</tr>
<tr>
<td>Plating</td>
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</tr>
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<td>~20</td>
<td>20.5±0.3</td>
<td>11.6±0.45</td>
<td>18.8±0.97</td>
<td>4.4</td>
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<td>25.2±0.8</td>
<td>4.8</td>
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<td>~50</td>
<td>23.5±3.2</td>
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<td>22.6±0.6</td>
<td>6.0</td>
</tr>
<tr>
<td>P4*</td>
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<td>~20</td>
<td>18.1±1.7</td>
<td>7.3±0.4</td>
<td>16.7±0.6</td>
<td>2.0</td>
</tr>
<tr>
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<td>24.9±1.9</td>
<td>12.8±0.1</td>
<td>20.4±0.2</td>
<td>2.2</td>
</tr>
</tbody>
</table>

*Strains identified through 18S rRNA sequencing, B denotes BAITING, P denotes PLATING
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The lipid-rich biomass produced in this study could be an alternative substrate to plant sources for biodiesel production (Chisti 2008; Li et al. 2008) and aquaculture (Song et al. 2007). Strains that produce low levels of omega-3 fatty acids and higher levels of monounsaturated and saturated fatty acids are potential candidates for use in biodiesel production. The presence of approximately 75% of TFA in two isolates, BAITING7 and BAITING8, as saturated fatty acids (C16:0 and C18:0) indicates that these strains may be useful for biodiesel generation. PUFAs, due to their susceptibility to oxidation, are not suitable for use in biodiesel (Falk and Meyer-Pittroff 2004; Knothe 2007). Strains accumulating larger amounts of DHA are potentially useful for the production of this PUFA for food and nutritional supplement applications, including infant formula (Takahata et al. 1998). Strains that produce oil with a higher EPA to DHA ratio have potential for addition to food, where both EPA and DHA are desirable. If EPA and DHA can be selectively concentrated from the oil then these PUFA can constitute a value-added co-product, with the remaining oil being useful for biodiesel. Carotenoids could also be selectively isolated as a value added co-product, further reducing the cost of biodiesel production using these organisms.

3.3.3 Molecular identification of new thraustochytrid isolates

Seven isolates were selected for PCR amplification as shown in Figure 3.5. Lane 1 shows the 1 kb ladder (Bio-Rad, Hercules, CA, USA). The subsequent lanes showed the amplified DNA from different isolates.
Figure 3.5 Agarose gel showing different PCR products as a result of amplification. The band size of the ladder is mentioned on the left hand side. The PCR products were ~1.8 kb. The lanes denotes: Lane 2 and 3 (BAITING1), Lane 4 and 5 (BAITING2), Lane 6 and 7 (BAITING3), Lane 8 and 9 (BAITING4), Lane 10 and 11 (BAITING7), Lane 12 (PLATING4) and Lane 13 (PLATING5).
These seven isolates were identified based on 18S rRNA gene sequences and found to be closely related to other 18S rRNA sequences from thraustochytrid strains available in the GenBank database. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Figure 3.6). The isolates BAITING1, BAITING2, BAITING3 and PLATING5 were found to be closely related to the sequence of *Thraustochytrium aureum* (GenBank accession number AB022110). These isolates were designated as *Thraustochytrium* sp. (Figure 3.6). Two isolates BAITING4 and PLATING4 were phylogenetically related to *Ulkenia* sp. (GenBank accession number HQ228976 and HQ228958, respectively) and BAITING7 was found to be closely related to *Schizochytrium* sp. (GenBank accession number DQ367050). *Schizochytrium* sp. was renamed as *Aurantiochytrium* sp. (Yokoyama and Honda 2007).

The presence of arachidonic acid (C20:4n6) was found to be significant in identifying any strain as closely related to *Schizochytrium aggregatum* (Huang et al. 2003). Arachidonic acid accumulation was observed in isolates BAITING1, BAITING2 and BAITING3. However, in developing a chemotaxonomic grouping method (A to H) based on fatty acid and sterol profiles of 36 strains in order to minimise the difficulty of distributing the strains based solely on fatty acid profiles, Lee Chang and co-workers (2012) found a close relationship between chemotaxonomic and phylogenetic grouping of most of their 36 thraustochytrid strains. There was always a disagreement with the classification of thraustochytrid strains. However, with recent developments in genetic identification techniques and chemotaxonomy, an evolutionary relationship can be established (Honda et al. 1999; Yokoyama and Honda 2007).
Figure 3.6 Molecular phylogenetic analysis implementing the maximum likelihood method showing a close relationship between the isolates and other selected sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.
3.3.4 Effect of other carbon sources on one selected thraustochytrid isolate

To explore the potential growth of one of the new thraustochytrid isolates on carbon sources other than glucose, BAITING3 was selected. The isolate showed promising lipid content (33.3-50 %) and fatty acid profiles with the different carbon sources used, which included monosaccharides (galactose and fructose), disaccharides (sucrose, maltose and lactose), polysaccharide (starch) and glycerol (Figure 3.7). DHA was found in the range of 25.6-39 % of TFA with the best accumulation in medium containing glycerol. Feeding with xylose resulted in about DHA as 30 % of TFAs. Feeding with monosaccharide and polysaccharide sugars resulted in about DHA as 26 % of TFAs, with an increased level of palmitic acid (~35 %).
Figure 3.7 Effect of carbon source on the fatty acid profile of Thraustochytrium sp. BAITING3.
Thraustochytrids have previously been found to grow on different carbon sources such as glucose, fructose, galactose and sucrose (Shene et al. 2010). Glycerol has recently been used as the primary carbon source in the fermentation medium for thraustochytrid (Abad and Turon 2012; Chang et al. 2013a). Although, thraustochytrids can utilise monosaccharides resulting in good DHA yields (Burja et al. 2006), xylose (pentose sugar) was found to be better utilised than fructose, galactose (monosaccharide) and starch (polysaccharide) and resulted in higher levels of DHA. Fructose, galactose and starch were found to enhance the level of palmitic acid (Figure 3.7). The utilisation of a pentose sugar by one of the isolates in this study indicates that this isolate has the potential to metabolise lignocellulosic biomass for DHA production.

3.4 Conclusion

In summary, pollen-baiting and direct plating techniques were both found to be effective in isolating thraustochytrids from marine samples. However, pollen-baiting provided a simpler method than direct plating for the isolation of thraustochytrids from complex samples. Thirteen species of thraustochytrids were isolated, five by plating and eight from baiting from Queenscliff region, Victoria, Australia and seven selected isolates were identified as Thraustochytrium sp., Schizochytrium sp., and Ulkenia sp. Phylogenetic analysis indicated that plating and baiting did not result in the isolation of distinctly different organism genetic types. However, four of the organisms selected by baiting had significantly higher DHA as a percentage of TFAs (30-41 %) compared with the other organisms. More than 75 % SFAs were recorded in two isolates (BAITING 7 and BAITING8) with the application for biodiesel production. Further samples would need to be collected to determine if this is more than coincidence. The isolated producing the highest amount of DHA as a percentage of TFAs (BAITING3) and grown with a range of carbon sources. The strain grew well with a variety of carbon sources, but DHA productivity was highest with glucose or glycerol. The ability of this strain to utilise low cost carbon sources compared with glucose means that this strain could be potentially useful for commercial DHA production.
Chapter 4: Glycerol as a carbon source for the production of omega-3 fatty acids, carotenoids and biofuel in some Australian thraustochytrids
4.1 Introduction

Glucose has been used extensively in microalgal fermentation and often provides increased biomass yields and lipid productivity in comparison to other carbon sources (Bajpai et al. 1991; Beopoulous et al. 2009; Chen and Walker 2012). Microalgal lipids potentially provide a sustainable source of oil and offer an alternative for the production of third-generation biofuels. Microalgae also produce other metabolites, such as astaxanthin, lutein, arachidonic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are of high economic value. Maximising lipid productivity requires the optimisation of an array of nutrient conditions in the growth medium. This process must be repeated for newly isolated strains (Shene et al. 2010). Glycerol has been used as an alternative carbon source to replace glucose due to its abundance and relatively low cost compared with glucose. Biodiesel derived glycerol can be used as a carbon source to obtain value added secondary metabolites, including polyunsaturated fatty acids (PUFAs) (Abad and Turon 2012) thereby improving economic viability of the fermentation process.

Thraustochytrids, large-celled marine heterokonts classified as oleaginous microorganism, have been reported to utilise a range of substrates, such as glucose, galactose, fructose, mannose, sucrose (Shene et al. 2010; Yokochi et al. 1998), complex organic matter (Bongiorni et al. 2005) and more recently, cellulosic biomass (Hong et al. 2012), for the production of polyunsaturated fatty acids (PUFAs). These lipid-rich organisms are promising DHA producers (Lewis et al. 1999). DHA is an important omega-3 fatty acid due to its health benefits and use in infant formula (Johnson and Wen 2009). Thraustochytrids have the potential to produce other metabolites like carotenoids, such as astaxanthin, canthaxanthin, β-carotene, zeaxanthin and echinenone (Aki et al. 2003). Carotenoids have antioxidant activities and are therefore potentially beneficial to human health. For example, they may assist in the treatment of cancer and improve vision (Johnson 2002). The carotenoid zeaxanthin is endogenous in humans and is an important component of eye retina (Roberts et al. 2009).

Thraustochytrids are also known to produce enzymes, polysaccharides, and co-enzymes (Armenta et al. 2006; Raghukumar 2008). A commercial thraustochytrid strain, *Schizochytrium limacinum* SR21, has been used to produce PUFA by utilising biodiesel-derived glycerol, corn steep liquor, waste water from barley distilleries,
soybean cake, liquid residues from beer and potato processing, and sweet sorghum juice (Chi et al. 2007; Ethier et al. 2011; Liang et al. 2010; Pyle et al. 2008; Quilodran et al. 2009; Yamasaki et al. 2006; Zhu et al. 2008). In this study, the utilisation of glycerol as the sole carbon source for the production of value-added lipids and carotenoids by thraustochytrid is explored for strain AMCQS5-5 (a newly isolated strain from the Queenscliff region, Victoria, Australia). A second newly isolated strain, *Schizochytrium* sp. DT3 from Barwon Waters, Victoria, Australia is also investigated. The effects and optimum levels of glycerol and the C:N ratio were determined for maximum laboratory-scale production of biomass and lipids production. Selected coloured isolates were examined for carotenoid production. Sugar hydrolysate obtained from hemp (*Cannabis sativa*) was also used as a carbon source with AMCQS5-5 and DT3, and biomass growth and lipid accumulation were analysed.

4.2 Materials and Methods

4.2.1 Chemicals used

The chemicals used in this study were of analytical and HPLC grade. The other medium components such as glycerol, yeast extract, mycological peptone (Sigma-Aldrich, St. Louis, MO, USA) and sea salts (Aquarium Systems Inc., Blacksburg, VA, USA) were used for biomass production. Solvents such as acetone, dimethyl sulphoxide (DMSO), ether, hexane (from Merck, Frankfurter Strabe, Darmstadt, Germany), methanol and ethyl acetate (HPLC grade from Fischer and Honeywell, Australia) were used for carotenoid extraction and HPLC analysis for carotenoid identification and quantification. Carotenoid standards (astaxanthin, zeaxanthin, canthaxanthin, res/meso-astaxanthin, β-cryptoxanthin, echinenone) were obtained from CaroteNature (Ostermundigen, Switzerland) and β-carotene from Sigma-Aldrich (St. Louis, MO, USA) Australia.

4.2.2 Strain selection and biomass production

Thraustochytrid isolates designated as AMCQS5-3 (Genbank accession number JX993839), AMCQS5-5 (Genbank accession number JX993841) and AMCQS1-9 (Genbank accession number JX993843) were selected for this study based on their colour. *Schizochytrium* S31 (ATCC 20888) was procured from the American Type Culture Collection (ATCC) and used as a standard culture. A recently isolated
thraustochytrid strain, *Schizochytrium* sp. DT3 (Genbank accession number KF682125) was also employed in this study. The isolates used in this study were maintained on GYP medium consisting of the following 5 g L$^{-1}$ glucose, 2 g L$^{-1}$ yeast extract, 2 g L$^{-1}$ mycological peptone and 10 g L$^{-1}$ agar in 50 % artificial seawater at 25 °C and sub-cultured after 15 days. For inoculum preparation the thraustochytrid isolates were cultivated in a medium containing 2 g L$^{-1}$ yeast extract, 2 g L$^{-1}$ peptone and 50 % artificial seawater with shaking at 150 rpm at 25 °C for 2 days. The medium was autoclaved at 121 °C for 20 min. Seed medium (2 g L$^{-1}$ yeast extract, 2 g L$^{-1}$ mycological peptone, 50 % ASW, pH 6.5) and production medium (10 g L$^{-1}$ yeast extract, 1 g L$^{-1}$ mycological peptone, 50 % ASW, pH 6.5) was autoclaved and syringe filtered (0.22 µm) 5 g L$^{-1}$ glucose and 10 g L$^{-1}$ glucose added, respectively. Glucose and glycerol (1 %) were used as carbon sources in the production medium. Inoculum (5 %, v/v) was used to inoculate 95 mL of production medium and cultured for 7 days in a shake flask at 25 °C, and 150 rpm. The resultant biomass was harvested by centrifugation (10,000 x g for 15 min) and freeze-dried until required. Some of the in-house isolates exhibited an orange colouration and were taken for carotenoid extraction.

4.2.3 Growth at different glycerol and glucose concentrations

Different concentrations of glycerol and glucose (0.5, 1, 2, 4, 6, 8 and 10 %) were used in the fermentation medium to evaluate their effect on fatty acid production. The fermentation was performed at 25 °C with shaking at 150 rpm for seven days. The biomass growth was monitored by measuring the optical density (OD) at 600 nm every 24 h.

4.2.4 Growth of AMCQS5-5 and DT3 with different carbon source

*Cannabis sativa* (Hemp variety) was used in this study in the form of its cellulosic biomass. The drying of the biomass was performed at 70 °C and the size reduction was achieved with milling. Further sieving was performed to obtain a size of approximately 300 micron. This material was then pre-treated by the addition of 1 g sodium hydroxide and 100 mL of deionised water per 2 g of cellulosic biomass, followed by heating to 120 °C. The hydrolysis of pre-treated biomass was done at 50 °C for 72 h using cellulase from *Trichoderma reesei* (EC 3.2.1.4; 700 units, Sigma-Aldrich, St. Louis, MO, USA). The concentration of reducing sugars in the hydrolysate was determined using the Dinitrosalicylic (DNS) method (Miller 1959).
All the pre-treatment, enzyme hydrolysis and sugar estimation procedures were performed according to available literature (Abraham et al. 2013; Saha et al. 2005; Yamashita et al. 2010). The hydrolysate was cooled to room temperature, readjusted to pH 6.5 and used as the carbon source in the fermentation medium with 50% ASW.

4.2.5 Fatty acid production and cell dry weight

To determine fatty acid production levels the culture was harvested at the end of 7 days and centrifuged at 10,000 x g for 10 min to pellet the cells. The pellet was freeze-dried and stored at -20 °C until required for fatty acid extraction. The cell dry weight (CDW) was estimated after freeze-drying the thraustochytrid cells. Results are presented as mean ± SD of samples prepared in duplicates and analysed twice (n=4).

4.2.6 Fatty acid extraction, esterification and GC analysis

This method is described in Chapter 2 (Section 2.2.11).

4.2.7 Carotenoid extraction

Extraction was carried out using a modified method previously applied to algae and fungi (Armenta et al. 2006; Raghukumar 2008). To 25 mg of freeze dried biomass, 1 mL of DMSO (preheated to 55 °C) was added and incubated at 55 °C for 60 min, followed by centrifugation at 4,000 rpm for 15 minutes at 15 °C. The supernatant was then taken and stored at 15 °C in darkness. This cycle was repeated 3 to 4 times until the biomass became colourless. To extract carotenoids from the DMSO solution, ether and water (1:1) was used as a solvent system in a 1:2 ratio (DMSO to solvent system). This solvent mixture was centrifuged at 4,000 rpm for 15 min at 15 °C and kept at -20 °C for 10 min. The upper solvent layer was transferred into a new centrifuge tube while the bottom frozen DMSO layer was discarded. The upper layer was washed twice with water to remove traces of DMSO. The solvent was evaporated under nitrogen and an equivalent volume of acetone added to the sample before being stored at -20 °C. The final extract volume was noted for total carotenoid estimation. Carotenoids were identified and quantified by reverse phase-HPLC analysis.
4.2.8 HPLC analysis of carotenoids

The method for carotenoid analysis was adopted from Armenta and co-workers (2006). An Agilent 1200 Series HPLC system with an Agilent 1200 Series photodiode array detector (Agilent Technologies, Santa Clara, CA, USA) and fitted with a 5 µm Luna C18 reversed-phase column, 4.6 mm x 250 mm (Phenomenex, USA) and a security guard column C18, 3.0 mm x 4.0 mm, (Phenomenex, USA), was used for carotenoid analysis. Carotenoids were detected at 477 nm. This column was equilibrated with mobile phase A (methanol, ethyl acetate and water (88:10:2, v/v/v)) in a gradient mode at a flow rate 0.75 ml min⁻¹. This flow rate was maintained for 10 min. Mobile phase composition was changed to 2:50:48 (mobile phase B) between 10 and 30 min and the flow rate was adjusted to 1.5 mL min⁻¹. This stage was maintained for a further 5 min and then set for column washing with mobile phase A for 10 min. Calibration curves for each carotenoid standard (astaxanthin, zeaxanthin, canthaxanthin, res/meso-astaxanthin, β-cryptoxanthin, echinenone and β-carotene) were prepared for identification and estimation of each carotenoid present in the different extracts. 1 mL of the acetone extract was taken and evaporated under nitrogen gas. Then 1 mL of mobile phase A was added and the solvent was filtered (0.22 µm) before for HPLC analysis of the extracts.

4.3 Results and Discussion

4.3.1 Biomass growth curves for AMCQS5-5 and DT3

To achieve higher biomass concentrations, the marine isolates were grown in a fermentation medium containing glucose or glycerol for 7 days. Maximum OD values of 12.3 AU (absorbance units) and 10.1 AU were observed when growing Thraustochytrium sp. AMCQS5-5 with glycerol and glucose, respectively (Figure 4.1). When grown under the optimal conditions the stationery phase of AMCQS5-5 was reached after 96 and 120 h of incubation with glucose and glycerol, respectively. Schizochytrium sp. DT3 grew at a faster rate and reached the stationery growth phase after 96 h of incubation. The growth curve for DT3 is shown in Figure 4.2.
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Figure 4.1 Biomass growth curve of *Thraustochytrium* sp. AMCQS5-5 as a function of time.

Figure 4.2 Biomass growth curve of *Schizochytrium* sp. DT3 as a function of time.
The highest specific growth rate of the isolate AMCQS5-5 was measured at 0.023 h\(^{-1}\) at the end of an incubation period of five days. For DT3 it was 0.11 h\(^{-1}\) at the end of three days. Other studies of thraustochytrid-like strains and *Schizochytrium* species have shown growth rates up to 0.16 h\(^{-1}\) and 0.38 h\(^{-1}\), respectively (Min et al. 2012; Perveen et al. 2006). The use of glucose as the carbon source at different initial concentrations resulted in minor improvements in biomass growth for AMCQS5-5, ranging from 1.0 to 1.44 g L\(^{-1}\). Maximum biomass was obtained in cultures grown with a 2 % initial concentration of glucose (Figure 4.3).

Figure 4.3 Effect of initial glucose and glycerol concentration on growth of AMCQS5-5.
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Figure 4.4 Effect of initial glucose and glycerol concentration on growth of DT3.

Figure 4.5 Effect of sugar hydrolysate on the growth of *Thraustochytrium* sp. AMCQS5-5 and *Schizochytrium* sp. DT3.
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However, the effect of varying glycerol concentrations resulted in biomass growth ranging from 0.7 to 8.32 g L$^{-1}$ for AMCQS5-5. The highest cell dry weight was found to be 8.32 g L$^{-1}$ at 4 % glycerol concentration, where the C:N ratio was 2 and lowest biomass growth was observed at 10 % glycerol concentration (0.7 g L$^{-1}$). This showed a gradual decrease with increasing concentration after 4 % of glycerol, indicating that higher concentrations inhibited growth (Figure 4.3). When the ratio of yeast extract and peptone as the nitrogen source, with the concentration of glycerol maintained at 4 %, so that the C:N ratio was increased to 4, the cell dry weight increased to 11.84 g L$^{-1}$. Other studies have also shown increased biomass and lipid content with increasing C:N ratio (Yokochi et al. 1998; Zhu et al. 2007). In contrast, one study found that increasing the C:N ratio led to lower biomass and less lipid production (Huang et al. 2012), but this appeared to be strain specific for this relatively poor lipid producing strain. When *Schizochytrium* sp. DT3 was grown under different glucose and glycerol concentrations (2-10 %), the highest cell dry weight achieved was 4.31 and 16.31 g L$^{-1}$ at 8 % of glucose and glycerol concentrations, respectively (Figure 4.4). The high biomass observed for DT3, relative to that for AMCQS5-5, may be due to the type of thraustochytrid strain. It was also observed that the cell dry weight obtained with DT3 decreased when the strain was grown at higher carbon concentrations (above 8 %), which may be due to substrate inhibition at higher glycerol concentration (Figure 4.4). This is consistent with results from a commercial thraustochytrid strain, *S. limacinum* SR21, in which higher concentrations of glycerol were found to retard biomass growth (Chi et al. 2007; Ethier et al. 2011; Huang et al. 2012). These reports showed that crude glycerol can be a viable alternative carbon source to replace glucose (Huang et al. 2012). Moreover, crude glycerol has been used in the production of valuable products such as 1, 3-propanediol, citric acid, polyhydroxyalkanoates and lipids, including omega-3 fatty acids (Yang et al. 2012).

The strains AMCQS5-5 and DT3 utilised reducing sugars from the sugar hydrolysate (1 %) obtained from enzyme saccharified hemp biomass and showed similar biomass growth, when compared to growth in glucose (Figure 4.2 and Figure 4.5). Using these alternative carbon sources such as cellulosic biomass can decrease the cost of fermentation making production more economically viable.
4.3.2 Effect of glycerol concentrations on lipid and fatty acid composition

The lipid content was observed gravimetrically for the two selected strains of thraustochytrid (Figure 4.6 and Figure 4.7). As the carbon concentration increases there is an elevation in the lipid content of AMCQS5-5. The highest lipid accumulation was observed at cultures grown in 2 % of glycerol and 4 % of glucose. Higher carbon concentrations lowered the lipid content, indicating that growth inhibition was occurring. In contrast, the lipid content in DT3 increased gradually with increasing carbon concentration.

Figure 4.6 Lipid content (% dry wt. basis) of AMCQS5-5 at various carbon concentrations.
It has been reported that nutrient sources in the medium (glucose and glycerol) affected the fatty acid composition in other oleaginous microorganisms, such as *Isochrysis* sp. and *Rhodosporidium* sp. (Lin et al. 2007; Xu et al. 2012). In our study with AMCQS5-5, PUFA as a proportion of TFA was highest (61.6 %) with 4 % glucose concentration, while SFA was highest (46.8 %) with 6 % glucose (Table 4.1). Similarly, the highest SFA proportion of total fatty acids in DT3 was also observed at 49 % in 6 % glucose (Table 4.5). The PUFA content of TFA for DT3 was relatively high at 71.3 % in 2 % glucose (Table 4.5). There was little change in the amount of DHA present in AMCQS5-5 (Table 4.2) or DT3 (Table 4.6) when glucose concentration was altered. Higher glucose concentrations have previously been shown to not necessarily result in increased DHA production in thraustochytrids (Burja et al. 2006).
Table 4.1 Proportion of fatty acids in *Thraustochytrium* sp. AMCQS5-5 at different glucose concentrations.

<table>
<thead>
<tr>
<th>Glucose concentration (% w/v)</th>
<th>SFAs</th>
<th>MUFAs</th>
<th>PUFAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>42.8±0.4</td>
<td>2.4±0.1</td>
<td>54.3±1.2</td>
</tr>
<tr>
<td>1</td>
<td>38.1±0.2</td>
<td>1.8±0.2</td>
<td>59.5±0.2</td>
</tr>
<tr>
<td>2</td>
<td>37.8±0.1</td>
<td>2.2±0.1</td>
<td>59.5±0.4</td>
</tr>
<tr>
<td>4</td>
<td>35.2±0.3</td>
<td>2.5±0.1</td>
<td>61.6±0.3</td>
</tr>
<tr>
<td>6</td>
<td>46.8±0.3</td>
<td>2.3±0.1</td>
<td>50.5±0.6</td>
</tr>
<tr>
<td>8</td>
<td>39.1±0.2</td>
<td>2.01±0.1</td>
<td>58.1±0.4</td>
</tr>
<tr>
<td>10</td>
<td>40.8±0.1</td>
<td>0.8±0.01</td>
<td>57.7±0.3</td>
</tr>
</tbody>
</table>

SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids. Results are presented as mean ± SD of samples prepared in duplicates and analysed twice.
Table 4.2 Effect of variable glucose concentrations on the fatty acid profile of *Thraustochytrium* sp. AMCQS5-5.

<table>
<thead>
<tr>
<th>Glucose concentration (% w/v)</th>
<th>C16:0</th>
<th>C18:0</th>
<th>C18:1n9</th>
<th>C20:4n6</th>
<th>C20:5n3</th>
<th>C22:5n6</th>
<th>C22:6n3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>29.8±0.13</td>
<td>11.3±0.17</td>
<td>0.9±0.02</td>
<td>3.3±0.06</td>
<td>7.4±0.15</td>
<td>8.1±0.4</td>
<td>31.6±0.45</td>
</tr>
<tr>
<td>1</td>
<td>27.6±0.05</td>
<td>9.03±0.05</td>
<td>0.87±0.04</td>
<td>3.8±0.01</td>
<td>8.1±0.02</td>
<td>8.8±0.1</td>
<td>35.1±0.04</td>
</tr>
<tr>
<td>2</td>
<td>27.6±0.01</td>
<td>8.9±0.04</td>
<td>0.86±0.03</td>
<td>3.41±0.06</td>
<td>7.9±0.08</td>
<td>8.7±0.2</td>
<td>35.8±0.04</td>
</tr>
<tr>
<td>4</td>
<td>26.4±0.13</td>
<td>7.1±0.06</td>
<td>0.80±0.05</td>
<td>3.52±0.02</td>
<td>8.5±0.03</td>
<td>9.3±0.2</td>
<td>36.3±0.01</td>
</tr>
<tr>
<td>6</td>
<td>32.2±0.18</td>
<td>13.2±0.1</td>
<td>0.63±0.05</td>
<td>2.7±0.1</td>
<td>6.4±0.02</td>
<td>8.7±0.3</td>
<td>29.6±0.06</td>
</tr>
<tr>
<td>8</td>
<td>28.2±0.05</td>
<td>9.4±0.05</td>
<td>0.69±0.01</td>
<td>2.9±0.03</td>
<td>7.7±0.04</td>
<td>8.8±0.3</td>
<td>34.5±0.06</td>
</tr>
<tr>
<td>10</td>
<td>29.9±0.07</td>
<td>9.6±0.03</td>
<td>-</td>
<td>3.3±0.01</td>
<td>7.1±0.07</td>
<td>8.7±0.1</td>
<td>35.7±0.07</td>
</tr>
</tbody>
</table>

C16:0, Palmitic acid; C18:0, Stearic acid; C18:1n9, Oleic acid; C20:4n6, Arachidonic acid; C20:5n3, EPA; C22:5n6, DPA, C22:6n3, DHA. TFA: total fatty acid. Results are presented as mean ± SD of samples prepared in duplicates and analysed twice.
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For AMCQS5-5 the highest PUFA content was 62.9 % of TFA with 0.5 % of glycerol (Table 4.3). Increased glycerol did not result in increased PUFA as a percentage of TFA. Similar results were observed for DT3 (Table 4.3 and Table 4.7). One possible explanation is that higher glycerol concentrations increase viscosity, which could have an impact on the ability of these organisms to effectively utilise glycerol (Huang et al. 2012).

Table 4.3 Proportion of fatty acids in *Thraustochytrium* sp. AMCQS5-5 at different initial glycerol concentrations.

<table>
<thead>
<tr>
<th>Glycerol concentration (% , w/v)</th>
<th>SFAs</th>
<th>MUFAs</th>
<th>PUFAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>33.1±0.3</td>
<td>3.8±0.1</td>
<td>62.9±0.7</td>
</tr>
<tr>
<td>1</td>
<td>35.2±0.04</td>
<td>3.2±0.04</td>
<td>60.4±032</td>
</tr>
<tr>
<td>2</td>
<td>45.4±0.1</td>
<td>6.8±0.1</td>
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</tr>
<tr>
<td>4</td>
<td>48.8±0.09</td>
<td>3.2±0.04</td>
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<tr>
<td>6</td>
<td>43.5±0.1</td>
<td>7.4±0.1</td>
<td>48.6±0.2</td>
</tr>
<tr>
<td>8</td>
<td>38.1±0.04</td>
<td>8.3±0.04</td>
<td>53.3±0.07</td>
</tr>
<tr>
<td>10</td>
<td>39.2±0.2</td>
<td>13.5±0.2</td>
<td>47.2±0.5</td>
</tr>
</tbody>
</table>

SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids. Results are presented as mean ± SD of samples prepared in duplicates and analysed twice.
Table 4.4 Effect of variable glycerol concentrations on the fatty acid profile of *Thraustochytrium* sp. AMCQS5-5.

<table>
<thead>
<tr>
<th>Glycerol concentration (% w/v)</th>
<th>C16:0</th>
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<th>C20:4n6</th>
<th>C20:5n3</th>
<th>C22:5n6</th>
<th>C22:6n3</th>
</tr>
</thead>
<tbody>
<tr>
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<td>25.21±0.3</td>
<td>6.59±0.03</td>
<td>2.11±0.04</td>
<td>4.91±0.02</td>
<td>7.72±0.04</td>
<td>9.78±0.1</td>
<td>37.02±0.3</td>
</tr>
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<td>1</td>
<td>28.88±0.01</td>
<td>4.24±0.01</td>
<td>1.63±0.01</td>
<td>5.65±0.01</td>
<td>6.15±0.03</td>
<td>10.91±0.10</td>
<td>35.17±0.1</td>
</tr>
<tr>
<td>2</td>
<td>38.21±0.03</td>
<td>4.61±0.01</td>
<td>4.86±0.01</td>
<td>4.54±0.01</td>
<td>2.70±0.03</td>
<td>7.25±0.01</td>
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<td>40.78±0.05</td>
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<td>27.49±0.01</td>
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<tr>
<td>6</td>
<td>35.76±0.01</td>
<td>5.23±0.02</td>
<td>5.32±0.03</td>
<td>5.29±0.01</td>
<td>2.37±0.01</td>
<td>7.38±0.1</td>
<td>28.83±0.02</td>
</tr>
<tr>
<td>8</td>
<td>28.6±0.01</td>
<td>6.02±0.01</td>
<td>6.04±0.01</td>
<td>6.92±0.01</td>
<td>2.97±0.01</td>
<td>7.94±0.01</td>
<td>29.13±0.02</td>
</tr>
<tr>
<td>10</td>
<td>27.9±0.1</td>
<td>8.72±0.03</td>
<td>11.55±0.05</td>
<td>7.33±0.01</td>
<td>2.31±0.02</td>
<td>7.24±0.1</td>
<td>21.65±0.1</td>
</tr>
</tbody>
</table>

C16:0, Palmitic acid; C18:0, Stearic acid; C18:1n9, Oleic acid; C20:4n6, Arachidonic acid; C20:5n3, EPA; C22:5n6, DPA, C22:6n3, DHA. TFA: total fatty acid. Results are presented as mean ± SD of samples prepared in duplicates and analysed twice.
Table 4.5 Proportion of fatty acids in *Schizochytrium* sp. DT3 at different initial glucose concentrations.

<table>
<thead>
<tr>
<th>Glucose concentration (% w/v)</th>
<th>SFAs</th>
<th>MUFAs</th>
<th>PUFAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>26.1±1.3</td>
<td>2.6±0.1</td>
<td>71.3±0.2</td>
</tr>
<tr>
<td>4</td>
<td>36.8±0.6</td>
<td>5.0±0.4</td>
<td>58.3±0.1</td>
</tr>
<tr>
<td>6</td>
<td>49.0±3.2</td>
<td>4.0±0.1</td>
<td>47.0±0.9</td>
</tr>
<tr>
<td>8</td>
<td>47.9±0.8</td>
<td>5.9±0.7</td>
<td>46.2±1.0</td>
</tr>
<tr>
<td>10</td>
<td>48.7±0.8</td>
<td>4.0±0.7</td>
<td>47.3±0.1</td>
</tr>
</tbody>
</table>

SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids. Results are presented as mean ± SD of samples prepared in duplicates and analysed twice.
Table 4.6 Effect of variable glucose concentrations on the fatty acid profile of *Schizochytrium* sp. DT3.

<table>
<thead>
<tr>
<th>Glucose concentration (% w/v)</th>
<th>C16:0</th>
<th>C18:0</th>
<th>C18:1n9</th>
<th>C20:4n6</th>
<th>C20:5n3</th>
<th>C22:5n6</th>
<th>C22:6n3</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>19.5±0.01</td>
<td>1.0±0.02</td>
<td>1.3±0.07</td>
<td>2.1±0.03</td>
<td>5.0±0.04</td>
<td>19.7±0.1</td>
<td>43.4±0.01</td>
</tr>
<tr>
<td>4</td>
<td>27.3±0.19</td>
<td>1.3±0.04</td>
<td>2.8±0.2</td>
<td>1.3±0.01</td>
<td>3.6±0.02</td>
<td>15.5±0.04</td>
<td>37.8±0.07</td>
</tr>
<tr>
<td>6</td>
<td>35.2±1.47</td>
<td>1.5±0.12</td>
<td>2.4±0.01</td>
<td>0.9±0.01</td>
<td>2.6±0.08</td>
<td>12.2±0.2</td>
<td>31.0±0.55</td>
</tr>
<tr>
<td>8</td>
<td>33.9±0.06</td>
<td>1.5±0.01</td>
<td>3.5±0.32</td>
<td>0.8±0.060</td>
<td>2.6±0.2</td>
<td>11.7±0.21</td>
<td>30.6±0.45</td>
</tr>
<tr>
<td>10</td>
<td>34.4±0.09</td>
<td>1.6±0.05</td>
<td>2.4±0.39</td>
<td>1.0±0.02</td>
<td>3.3±0.05</td>
<td>11.6±0.03</td>
<td>31.1±0.01</td>
</tr>
</tbody>
</table>

C16:0, Palmitic acid; C18:0, Stearic acid; C18:1n9, Oleic acid; C20:4n6, Arachidonic acid; C20:5n3, EPA; C22:5n6, DPA, C22:6n3, DHA. TFA: total fatty acid. Results are presented as mean ± SD of samples prepared in duplicates and analysed twice.
Table 4.7 Proportion of fatty acids in *Schizochytrium* sp. DT3 at different initial glycerol concentrations.

<table>
<thead>
<tr>
<th>Glycerol concentration (% w/v)</th>
<th>SFAs</th>
<th>MUFAs</th>
<th>PUFAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>27.5±1.3</td>
<td>5.4±0.6</td>
<td>66.0±3.8</td>
</tr>
<tr>
<td>4</td>
<td>38.2±0.6</td>
<td>7.2±0.3</td>
<td>54.0±0.3</td>
</tr>
<tr>
<td>6</td>
<td>44.1±1.1</td>
<td>7.7±0.2</td>
<td>47.8±0.8</td>
</tr>
<tr>
<td>8</td>
<td>47.2±0.5</td>
<td>6.9±0.3</td>
<td>45.5±0.3</td>
</tr>
<tr>
<td>10</td>
<td>49.1±0.4</td>
<td>5.9±0.2</td>
<td>44.7±0.2</td>
</tr>
</tbody>
</table>

SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids. Results are presented as mean ± SD of samples prepared in duplicates and analysed twice.
Table 4.8 Effect of variable glycerol concentrations on the fatty acid profile of *Schizochytrium* sp. DT3.

<table>
<thead>
<tr>
<th>Glycerol concentration (%), w/v</th>
<th>% TFA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C16:0</td>
</tr>
<tr>
<td>2</td>
<td>19.9±1.12</td>
</tr>
<tr>
<td>4</td>
<td>27.8±0.23</td>
</tr>
<tr>
<td>6</td>
<td>32.3±0.73</td>
</tr>
<tr>
<td>8</td>
<td>34.6±0.04</td>
</tr>
<tr>
<td>10</td>
<td>36.4±0.19</td>
</tr>
</tbody>
</table>

C16:0, Palmitic acid; C18:0, Stearic acid; C18:1n9, Oleic acid; C20:4n6, Arachidonic acid; C20:5n3, EPA; C22:5n6, DPA, C22:6n3, DHA. TFA: total fatty acid. Results are presented as mean ± SD of samples prepared in duplicates and analysed twice.
For AMCQS5-5, palmitic acid (40.8 % of TFA) and DHA (37.02 % of TFA) were the major fatty acids present for 4 % glycerol and 0.5 % glycerol containing media, respectively. Fatty acid concentrations in both AMCQS5-5 and DT3 generally decreased with increasing glycerol concentrations. Saturated fatty acids were the major fatty acids observed at high glycerol concentrations (Table 4.4 and Table 4.8). Decreasing DHA levels with increasing glycerol concentrations in the media was previously observed for *S. limacinum* SR21 (Perveen et al. 2006; Pyle et al. 2008) and was attributed at least partially to insufficient oxygen supply due to increased viscosity at higher glycerol levels. Our finding that palmitic acid increased as a proportion of TFA relative to DHA at higher glycerol concentrations is consistent with the results of Pyle and co-workers (2008), but contrasts with the findings of Perveen et al. (2006) where DHA levels were higher than those of palmitic acid. The major fatty acids that were elevated in the two strains, other than DHA and palmitic acid, were stearic acid (0.9-13.2 % of TFA), oleic acid (0.9-11.6 % of TFA), arachidonic acid (0.8-7.3 % of TFA), EPA (2.3-8.5 % of TFA) and DPA (7.2-19.7 % of TFA). Chi and co-workers (2007) suggested that the higher levels of glycerol, up to 100 g L⁻¹, inhibited the production of DHA.

A higher C:N ratio has been demonstrated to enhance lipid accumulation in thraustochytrids (Burja et al. 2006). For both strains we observed that total TFA was higher when glycerol, rather than glucose, was the carbon source. For AMCQS5-5 the TFA ranged from 48.1 to 67.0 mg L⁻¹ with highest at 4 % glucose and 85 to 1112.4 mg L⁻¹ with highest in 4 % glycerol. For DT3, it ranged from 155.4 to 806 mg L⁻¹ with glucose and 702.8 to 6430.5 mg L⁻¹ with glycerol (Table 4.9 and Table 4.10). These results show that glycerol can enhance the production of fatty acids in these organisms. DHA production in AMCQS5-5 was found to be 24.3 mg L⁻¹ (4 % glucose) which is consistent with another study using 8 % glucose with *Thraustochytrium* sp. 12B (Taha et al. 2013). The DHA level (305.70 mg L⁻¹) was also higher in cultures grown in 4 % glycerol (Table 4.9). Other studies have reported higher DHA production using glucose and results appear to be dependent on the thraustochytrid strain used. DHA was observed at 120 mg L⁻¹ with *Thraustochytrium* ATCC 34304 (Taoka et al. 2011) and 0.78 g L⁻¹ with *Thraustochytriidae* sp. Z105 (Zhou et al. 2010). DT3 produced higher DHA yields than AMCQS5-5 (Table 4.10). In our study with the *Schizochytrium* sp. DT3, relatively high levels of 6.43 g L⁻¹ of TFA and 2.16 g L⁻¹ of DHA were achieved.
Table 4.9 Comparison between Total fatty acids (TFA) and DHA production in glucose and glycerol using AMCQS5-5.

<table>
<thead>
<tr>
<th>Carbon concentration (%)</th>
<th>Glucose</th>
<th>Glycerol</th>
<th>Glucose</th>
<th>Glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TFA (mg L⁻¹)</td>
<td>TFA (mg L⁻¹)</td>
<td>DHA (mg L⁻¹)</td>
<td>DHA (mg L⁻¹)</td>
</tr>
<tr>
<td>0.5</td>
<td>48.1±0.02</td>
<td>148.6±0.01</td>
<td>15.2±0.01</td>
<td>55.01±0.01</td>
</tr>
<tr>
<td>1</td>
<td>59.5±0.01</td>
<td>423.8±0.01</td>
<td>20.9±0.01</td>
<td>149.05±0.01</td>
</tr>
<tr>
<td>2</td>
<td>66.2±0.01</td>
<td>728.3±0.01</td>
<td>23.7±0.05</td>
<td>207.21±0.01</td>
</tr>
<tr>
<td>4</td>
<td>67.0±0.03</td>
<td>1112.4±0.02</td>
<td>24.3±0.2</td>
<td>305.70±0.01</td>
</tr>
<tr>
<td>6</td>
<td>45.2±0.1</td>
<td>636.5±0.01</td>
<td>13.4±0.2</td>
<td>183.64±0.01</td>
</tr>
<tr>
<td>8</td>
<td>56.1±0.1</td>
<td>527.2±0.3</td>
<td>19.4±0.02</td>
<td>153.70±0.2</td>
</tr>
<tr>
<td>10</td>
<td>52.7±0.03</td>
<td>85.0±0.3</td>
<td>18.9±0.2</td>
<td>18.56±0.3</td>
</tr>
</tbody>
</table>

Table 4.10 Comparison between Total fatty acids (TFA) and DHA production in glucose and glycerol using DT3.

<table>
<thead>
<tr>
<th>Carbon concentration (%)</th>
<th>Glucose</th>
<th>Glycerol</th>
<th>Glucose</th>
<th>Glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TFA (mg L⁻¹)</td>
<td>TFA (mg L⁻¹)</td>
<td>DHA (mg L⁻¹)</td>
<td>DHA (mg L⁻¹)</td>
</tr>
<tr>
<td>2</td>
<td>155.4±12.1</td>
<td>702.8±160.8</td>
<td>67.4±5.3</td>
<td>288.2±56.5</td>
</tr>
<tr>
<td>4</td>
<td>459.6±44.2</td>
<td>3129.8±45.9</td>
<td>173.7±16.4</td>
<td>1117.7±8.5</td>
</tr>
<tr>
<td>6</td>
<td>806.9±50.9</td>
<td>5729.0±705.5</td>
<td>250.0±11.3</td>
<td>2160.7±140.6</td>
</tr>
<tr>
<td>8</td>
<td>774.5±17.2</td>
<td>6180.7±297.0</td>
<td>237.2±1.7</td>
<td>1875.9±97.2</td>
</tr>
<tr>
<td>10</td>
<td>739.0±106.1</td>
<td>6430.5±106.1</td>
<td>229.9±15.5</td>
<td>1907.3±37.9</td>
</tr>
</tbody>
</table>
Thraustochytrids are known to grow on a variety of carbon sources (Shene et al. 2010). To investigate whether our strains could grow on sugar produced from cellulosic biomass we enzymatically hydrolysed hemp and used the resulting crude hydrolysate as a carbon source for growing AMCQS5-5 and DT3. Both strains were able to utilise the reducing sugars in the hydrolysate, which resulted in a similar fatty acid profile to that obtained with glucose (Figure 4.8). The DHA content as a percentage of TFA was about 38% in both strains. This indicates that both strains can be grown and provide a reasonable DHA yields on a potentially low cost carbon source produced from cellulosic biomass.

Figure 4.8 Effect of the sugar hydrolysate on the fatty acid profile of AMCQS5-5 and DT3; C16:0, Palmitic acid; C18:0, Stearic acid; C18:1n9, Oleic acid; C20:5n3, EPA; C22:5n6, DPA, C22:6n3, DHA. TFA: total fatty acid.
4.3.3 Effect of carbon source on biomass and carotenoid production in the selected strains

*Schizochytrium* sp. S31, *Thraustochytrium* sp. AMCQS5-3, *Thraustochytrium* sp. AMCQS5-5 and *Schizochytrium* sp. AMCQS1-9 were selected for biomass production and carotenoid extraction. Biomass (Figure 4.9) and carotenoid production (Figure 4.10) were found to be significantly higher in all cultures when fed with glycerol instead of glucose.

![Figure 4.9 Biomass production (cell dry weight) in selected thraustochytrid isolates fed with glucose and glycerol.](image)
Figure 4.10 Carotenoid production in selected thraustochytrid isolates fed with glucose and glycerol.

These results are in agreement with the findings of others, who reported an increase in biomass and lipid content for thraustochytrids when cultures were fed with glycerol and fish oil-derived raw glycerol instead of glucose (Scott et al. 2011). Use of glycerol as the carbon source resulted in increased carotenoid content for *Schizochytrium* sp. S31, from 16.8 to 20.28 µg g⁻¹, and from 5.23 to 10.16 µg g⁻¹ in *Schizochytrium* sp. AMCQS1-9. The greatest improvement in carotenoid content was observed in *Thraustochytrium* sp. AMCQS5-3 and AMCQS5-5. For both AMCQS5-3 and AMCQS5-5 a 3-fold increase was observed, where carotenoid content increased from 16-17 µg g⁻¹ to 50-60 µg g⁻¹ (Figure 4.10). Others have reported carotenoid content up to 50 µg g⁻¹ using glucose as the carbon source for thraustochytrid strain ONC-T18 (Armenta et al. 2006). Chatdumrong et al. (2007) generated a mutant of *S. limacinum* BR2.1.2 that produced carotenoid at 8-13 µg L⁻¹ using glucose as carbon source. Increases in biomass, lipid and carotenoid production using glycerol indicates the efficient regulation of enzymes involved in glycerol metabolism prior to entry into the glycolytic pathway (Scott et al. 2011). These processes enable enhanced supply of acetyl-CoA or nicotinamide adenine dinucleotide phosphate (NADPH) for lipid biosynthesis, which are the driving forces for oleaginicity in oleaginous microorganisms (Ratledge 2004). However, to the best of our knowledge, this is the first instance where an increase in carotenoid content was documented when glycerol was used to replace glucose as the carbon source for
thraustochytrid growth. TAG accumulation and carotenoid biosynthesis are inter-
connected, since both share common precursors; acetyl-CoA and NADPH. The role
of trisporic acid, a carotenogenesis activator under glycerol cannot be ruled out for
enhanced carotenoid production (Mantzouridou et al. 2008).

4.3.4 Carotenoid profiling of thraustochytrid strains

Existing literature suggests that vortexing of samples should be repeated until
carotenoid content is either completely extracted or the pellet becomes colourless
(Carmona et al. 2003; Quilodran et al. 2010). However, in practice the supernatant
becomes colourless after 3-4 extraction cycles, while the pellet retains some colour.
This suggests inefficient carotenoid extraction with vortexing. DMSO-mediated
extraction has been used extensively for carotenoid extraction and quantification
(Sedmak et al. 1990), and provides an alternate method for extracting carotenoids
from freeze dried biomass. A major improvement was observed in carotenoid
extraction from thraustochytrid biomass when the vortex method was replaced with
the DMSO approach (Figure 4.10 and Figure 4.11).

Figure 4.11 Carotenoid profiling of selected thraustochytrid isolates based on HPLC
analysis.

The high dipolar moments and dielectric constants of DMSO are the driving force
behind its powerful solvation capacity, enabling it to efficiently extract carotenoids
from microbial biomass. However, the extraction efficiency of DMSO is broadly
affected by the type of biomass used and the total carotenoid content in the cell (Sarada et al. 2006). Toxicity issues associated with DMSO mediated carotenoid extraction are a major concern for food applications and so methods using food grade solvents such as ethanol need to be developed.

All orange coloured thraustochytrid isolates have astaxanthin or canthaxanthin as the major carotenoids, with lower levels of echinenone. No other carotenoids were observed at significant levels in these thraustochytrids (Figure 4.11). The ATCC strain *Schizochytrium* sp. S31 has comparable astaxanthin and echinenone quantities of 29.7 and 33.21 µg g⁻¹, respectively. Some canthaxanthin (14.63 µg g⁻¹) is also present. β-carotene was not observed in the carotenoid profile, reflecting the efficient conversion of beta carotene into xanthophylls in the late phase of this culture. The presence of significant amounts of astaxanthin and echinenone reflect the rapid oxidation of β-carotene into echinenone followed by its oxidation/hydroxylation into astaxanthin via the canthaxanthin route (Gouveia et al. 1996). Thraustochytrid strains AMCQS5-3 and AMCQS5-5 showed similar growth patterns and carotenoid profiles. No astaxanthin was observed in the carotenoid profile of AMCQS5-3 and AMCQS5-5, although canthaxanthin was present in quantities of 38.13 and 29.31 µg g⁻¹, respectively. Echinenone was also present at 20.13 and 19.61 µg g⁻¹ respectively. Lastly, β-carotene was present at 10.24 µg g⁻¹ and 8.06 µg g⁻¹, respectively. These results are in agreement with the findings of Armenta and co-workers (2006), who reported similar carotenoid profiles in the thraustochytrid ONC-T18, showing canthaxanthin, echinenone and β-carotene with small quantities of astaxanthin. Based on the literature and our findings, it appears that canthaxanthin is the major carotenoid present in *Thraustochytrium* sp. (Aki et al. 2003; Armenta et al. 2006), whereas astaxanthin is the major carotenoid in *Schizochytrium* sp. (Yamasaki et al. 2006; Chatdumrong et al. 2007). The absence of astaxanthin from the carotenoid profile of these thraustochytrid strains indicates their inability to add hydroxyl groups to the canthaxanthin backbone and the inactivation of the β-carotene hydroxylase enzymes led to impaired biosynthesis of astaxanthin (Scaife et al. 2009) and to the accumulation of canthaxanthin. The absence of other hydroxylated carotenoid intermediates such as β-cryptoxanthin, zeaxanthin and 4-ketoexanthanin supports this assumption.
4.4 Conclusion

The experimental data demonstrated the efficient utilisation of glycerol as an alternative carbon source to glucose in the fermentation medium of newly isolated thraustochytrid strains from Victorian marine environment, Australia. The C:N ratio appears to be a crucial parameter in biomass growth and TFA production. Glycerol resulted in higher biomass growth and increased lipid and DHA productivity in both AMCQS5-5 and DT3, than when the strains were grown with glucose. Both strains also grew well on hemp hydrolysate containing reducing sugars. Carotenoid content was higher for all strains of thraustochytrid examined when glycerol rather than glucose was used as the carbon source, without impacting the relative amounts of each carotenoid type. Therefore, both strains have the potential to produce DHA and carotenoids using low cost carbon sources such as glycerol.
Chapter 5: Evaluation of additives to the fermentation medium for improving lipid production and PUFA accumulation
5.1 Introduction

Surfactants such as Tween 80 are amphiphilic compounds that minimise surface and interfacial tensions by accumulating between immiscible liquids. They also improve the movement, solubility and bioavailability of hydrophobic or insoluble inorganic compounds (Van Hamme et al. 2006). Biosurfactants interfere with physiological processes at the cellular level and play a functional role in improving the bioavailability of hydrophobic molecules. Biosurfactants can also possess antimicrobial activity (Singh et al. 2007; Van Hamme et al. 2006). These surface active substances, when added to fermentation medium, may affect the structural properties of microbial cells and subsequently impact secondary metabolite and lipid production (Benchekroun and Bonaly 1992; Nemec and Jernejc 2002). Tween 80 is an oleate ester of sorbitol and their anhydride copolymerised with ethylene oxide. It is an effective non-ionic surfactant that has been reported to have inducing effects on the release of enzymes from aerobic fungi cultures (Lee and Ha 2003; Long and Knapp 1991; Yazdi et al. 1990). Moreover, Tween 80 can positively affect degradative enzymes in aerobic and anaerobic microbial cultures (Helle et al. 1993; Lee and Ha 2003; Park et al. 1992). Some reports suggested that Tween 80 can be used as a carbon source or an additive to improve bacterial fermentation growth (Brar et al. 2005; Partanen et al. 2001). Tween 80 also enhances bacterial swarming (Escherichia coli and Azospirillum brasilense) on agar plates (Niu et al. 2005).

In a previous report, Tween 80 was found to alter the fatty acid composition and phospholipid content in a Streptococcus strain (Masaru et al. 1989). Supplementation with this non-ionic surfactant (Tween 80) in the fermentation medium enhanced oleic acid (a major hydrophobic part of Tween 80) accumulation in lipid for an Aspergillus niger strain (Nemec and Jernejc 2002). The effect of polyoxyethylene sorbate compounds (tweens) on colonial morphology, growth and ultrastructure of Mycobacterium paratuberculosis has also been studied previously (van Boxtel et al. 1990). The fatty acid composition of Lactobacillus sp. is impacted by supplementation with tweens (Nikkila et al. 1995). Researchers have recently reported the production of glycolipids in mycobacteria (Corynebacterium matruchotii) when Tween 80 was added in the culture medium (Wang et al. 2011).

A recent report demonstrated the use of Tween 80 as a stimulatory agent in the submerged fermentation of the mycelium of Pleurotus tuber-regium, where the
addition of Tween 80 improved the glucose consumption rate without altering the mycelial structure, while oleic acid levels were increased (Zhang and Cheung 2011). Xia (2013) recently reported the use of Tween 80 to produce exo-polysaccharides without any effect on the biomass growth of *Alcaligenes faecalis* ATCC 31749.

Tween 80 has been used as an anti-microbial agent in combination with other compounds against *Helicobacter pylori* (Figura et al. 2012), *Staphylococcus aureus* and *E. coli* (Zhang et al. 2009). It has been used as an important constituent in dosing carriers for pre-clinical *in vivo* studies in rats, improving compound permeability (Zhang et al. 2003). In addition, Tween exhibited some toxicity in rat thymocytes (Hirama et al. 2004).

Tween 80 has been used in thraustochytrid isolation and fermentation. Tween 80 was used in the basal medium for the isolation of thraustochytrids, which increased the number of isolates obtained from marine water samples (Taoka et al. 2008). The lipid content and the total fatty acid production were also found to be enhanced when 1% Tween 80 was supplemented in the fermentation medium of the isolated strains (Taoka et al. 2011). Taoka et al. (2011) suggested that the oleic acid in Tween 80 may have been used as a carbon source by the thraustochytrid strain, or that it may have been incorporated directly into the fatty acid profile.

In addition, Tween 80 has been used as an additive in the medium to enhance the production of lipase in bacterial fermentation (Boekema et al. 2007; Li et al. 2001). Tween 80 appears to be a natural lipase substrate that enhances lipase production. Li et al (2001) reported increased production of lipases in *Acinetobacter radioresistens* using Tween 80 as the primary carbon source in the medium. Similarly, Tween 80 was used as the sole carbon source for the production of thermostable lipolytic enzymes in various bacterial strains (Fakhereddine et al. 1998). Apart from bacterial systems, tween 80 has also been used as a substrate for lipase secretion in thraustochytrids (Damare and Raghukumar 2006). Tween 80 has also been reported to improve the overall performance of microbial fuel cells which may be due to an increase in cell membrane permeability (Wen et al. 2011).

Other additives such as thiosulphates (sodium thiosulphate, ammonium thiosulphate, silver thiosulphate) have been used previously to supplement the media for the growth of the fungi *Trichoderma* sp. (Wainwright and Grayston 1988) and *Fusarium* sp. (Jones et al. 1991), cyanobacteria *Synechocystis* sp. (Wang et al. 2002), some
fresh water microalgae like *Chlamydomonas reinhardtii* and *Pseudokirchneriella subcapita* (Hiriart-Baer et al. 2006) and marine microalgae like *Chlorella* (Feng et al. 2005). In fact, Feng et al. (2005) reported the use of sodium thiosulphate in combination with glucose, where glucose was found to increase the microalgal cell concentration and sodium thiosulphate enhanced fatty acid accumulation in the cells.

In our study, the effects of sodium thiosulphate and Tween 80 were examined as supplements in the fermentation medium for thraustochytrids. The impact of these additives on biomass growth, changes in fatty acid profile, and lipid production were examined.

### 5.2 Materials and Methods

#### 5.2.1 Chemicals used

Unless otherwise stated, all chemicals used in this study were analytical grade and obtained from Sigma-Aldrich, (St. Louis, MO, USA). Solvents were purchased from Merck Chemicals (Frankfurter Strabe, Darmstadt, Germany). Instant ocean sea salts, was obtained from Aquarium Systems Inc. (Blacksburg, VA, USA) and used for the preparation of artificial seawater (ASW).

#### 5.2.2 Culture maintenance

A new thraustochytrid strain AMCQS5-5 isolated in our lab was used for this study (GenBank accession number JX993841). It was maintained on sterile (autoclaved) GYP agar medium containing 5 g L⁻¹ glucose, 2 g L⁻¹ yeast extract, 2 g L⁻¹ peptone, 12 g L⁻¹ agar in 50 % artificial seawater (ASW). It was subcultured every 2 weeks.

#### 5.2.3 Inoculum preparation and fermentation medium

A solution of 1 % sodium thiosulphate and 1 % Tween 80 was filter sterilised using 0.44 µm syringe filters and added to the sterile glucose medium. The inoculum was prepared in GYP medium containing 5 g L⁻¹ glucose, 2 g L⁻¹ yeast extract, and 2 g L⁻¹ peptone in 50 % ASW, grown for 48 hours. A 5 % (v/v) inoculum was used in 50 mL of fermentation medium in a 250 mL Erlenmeyer flask. The fermentation medium contained 100 g L⁻¹ glucose, 20 g L⁻¹ yeast extract and 20 g L⁻¹ peptone in 50 % ASW and initial pH was maintained at 6.5. All experiments were performed in duplicates and analysed twice.
5.2.4 Observation of biomass growth in glucose medium containing sodium thiosulphate and Tween 80

The fermentation process was designed as described above. The shake flask study was performed at 25 °C with shaking at 150 rpm for 7 days. The biomass growth was monitored by measuring the optical density (OD) at 600 nm every 24 h. The OD was measured using a UV-Vis spectrophotometer (UV-1800, Shimadzu Corporation, Kyoto, Japan).

5.2.5 Freeze drying and cell dry weight determination

The isolate was grown in glucose medium with different additives and the biomass was harvested after seven days and centrifuged at 10,000 x g for 10 min to obtain a cell pellet. The medium was decanted and the pellet washed three times with distilled water, frozen at -20 °C and freeze dried at -60 °C equivalent to 0.03 mbar of atmospheric pressure. Dry weight of the cell pellet was recorded for the thraustochytrid samples. The freeze dried cells were stored at -20 °C before proceeding with the lipid extraction. Results are presented as a mean ± SD of samples prepared in duplicates and analysed twice.

5.2.6 Lipid extraction and fatty acid methyl esters production

Lipid was extracted and quantified from freeze dried biomass 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 in the oven at 50 °C. The lipid percentage was measured gravimetrically. Fatty acids were converted to fatty acid methyl esters (FAMEs) by acid-catalysed trans-esterification with minor modifications (Christie and Han, 2010). To this 500 μL toluene was added into glass vials followed by the addition of 10 μL internal standard (methyl nonadecanoate, C19:0) and 200 μL butylated hydroxytoluene (BHT, 1 mg mL⁻¹). Subsequently, 400 μL of acidic methanol (prepared by adding 5 mL acetyl chloride drop wise into 50 mL of methanol on ice) was added to the tube and incubated overnight at 50 °C. FAMEs were extracted using hexane. The hexane layer was collected and dried over sodium sulphate.
5.2.7 Gas chromatography analysis

FAMEs were concentrated under a nitrogen stream and analysed using gas chromatography with flame ionization detection (GC-FID) (Agilent Technologies, Model 6890N, Santa Clara, CA, USA), equipped with a capillary column (SGE, BPX70, 30 m x 0.25 mm, 0.25 μm thickness). The injector was maintained at 250 °C and a sample volume of 1 μL was injected with a 50:1 split ratio. Helium was used as the carrier gas and its flow rate was maintained at 1.5 mL min⁻¹. The oven was programmed from 140 °C (5 min hold) to 240 °C (10 min hold) at a rate of 4 °C min⁻¹. Fatty acid peaks were identified using Chemstation chromatography software (Agilent Technologies, Santa Clara, CA, USA) on comparison with retention times of external standards (St. Louis, MO, USA) and corrected using theoretical relative FID response factors (Ackman 2002). Results are presented as mean ± SD of samples prepared in duplicates and analysed twice.

5.3 Results and Discussion

5.3.1 Biomass growth determination

The dry cell weight obtained with control medium, sodium thiosulphate and Tween 80 supplemented media are given in Figure 5.1. The highest biomass growth was obtained at the end of day 3 with sodium thiosulphate and Tween 80 at 1.8 g L⁻¹ and ~4.5 g L⁻¹, respectively. Biomass gradually decreased after the day 3. This increase in the growth of Thraustochytrium sp. AMCQS5-5 with Tween 80 is in agreement with that previously observed for Thraustochytrium aureum (Taoka et al. 2011), where maximum cell dry weight of 3.4 g L⁻¹ was reported, compared with the observed maximum of 4.5 g L⁻¹ for our thraustochytrid strain.
Figure 5.1 Cell dry weight of AMCQS5-5 in glucose medium over time.

Several studies have reported increased biomass growth when Tween 80 was added to the culture medium (Brar et al. 2005; Figura et al. 2012; Partanen et al. 2001; van Boxtel et al. 1990;). The hydrolysis of Tween 80 by microbes releases oleic acid, which can act as a nutrient source for microbial growth and also as an elicitor (Chen et al. 2010; Taoka et al. 2011). The surfactants effect of Tween 80 might also impact cellular structure in a manner that improves growth (Nemec and Jernejc 2002). For example, it has been reported that Tween 80 may interfere with cell membranes and intracellular physiological properties without affecting cell viability (Hirama et al. 2004). Van Boxtel et al. (1990) reported a change to cell wall appearance in *Mycobacterium paratuberculosis* in the presence of Tween 80, where the cell walls appeared smooth. An effect of Tween 80 on the cell permeability of the thraustochytrid cells has not yet been established (Taoka et al. 2011).

Sodium thiosulphate has been reported to stimulate biomass production in fungi (Wainwright and Grayston 1988) and improve the glucose consumption rate of the mycelium *Pleurotus tuber-regium*, leading to enhanced biomass (Zhang and Cheung 2011). We observed a slight increase in biomass production for *Thraustochytrium* sp. AMCQS5-5 when sodium thiosulphate was added to the medium in combination with glucose, which is in agreement with a similar study using *Chlorella* sp. (Feng et
The benefits of sodium thiosulphate have been mainly attributed to its ability to protect microbial cells (reported in bacteria and cyanobacteria) from damage by reactive oxygen species which can be produced by the degradation of the glucose during its consumption by the cells (Maclean et al. 1996; Wang et al. 2002).

5.3.2 Effect of Tween 80 and sodium thiosulphate supplemented medium on lipid production in Thraustochytrium sp. AMCQS5-5

Lipid production per gram of dry biomass was observed in the control glucose medium to be a maximum of 37 mg g\(^{-1}\). When the medium was supplemented with sodium thiosulphate, or Tween 80, lipids increased in both cases, with maximal values of 97.1 mg g\(^{-1}\) for sodium thiosulphate and 96.6 mg g\(^{-1}\) for Tween 80 (Figure 5.2, Figure 5.3 and Figure 5.4). This clearly showed that supplementation of the medium with these additives improved lipid accumulation when compared to glucose alone. Similarly, lipid productivity in the control glucose medium reached a maximum of 159.7 mg L\(^{-1}\). Whereas with sodium thiosulphate the maximum lipid content was 175.2 mg L\(^{-1}\) and increased further to 425.3 mg L\(^{-1}\) with Tween 80 (Figure 5.2, Figure 5.3 and Figure 5.4). This lipid productivity also depended on the cell dry weight. Higher biomass with Tween 80 translated into higher lipid content, with maximum growth occurring from 72 to 96 h of incubation.
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Figure 5.2 Total lipids production for AMCQS5-5 in glucose medium.

Figure 5.3 Total lipids production for AMCQS5-5 in glucose medium supplemented with Tween 80.
A slight improvement in total lipid levels was reported in samples cultivated with 1 % Tween 80 for *Thraustochytrium aureum* (Taoka et al. 2011), which is similar to the observations in our study (Figure 5.3). Comparable observations were recorded for a culture of *Aspergillus niger* when it was grown in Tween 80-supplemented medium (Nemec and Jernejc 2002).

Studies in a mycobacterium culture concluded that Tween 80 improved cell permeability and the organism converted it into a series of organic compounds resulting in the formation of novel glycolipids (Wang et al. 2011). The supplementation of Tween 80 in the fermentation medium of thraustochytrids may also result in the bioconversion of available oleic acid in Tween 80 into lipids.

There are no previous reports on the use of sodium thiosulphate as an additive for thraustochytrid fermentation. However, it has been used with other microbes. Sodium thiosulphate in combination with the glucose was observed to be effective in promoting lipid accumulation in a *Chlorella* sp. (Feng et al. 2005). The fatty acid accumulation was found to depend on the proportion of glucose and thiosulphate in the medium. Similar observations were recorded for AMCQS5-5 with sodium thiosulphate when compared with the control (Figure 5.4), showing that sodium thiosulphate increased lipid accumulation. The concentrations of glucose and sodium
thiosulphate need to be optimised to sufficiently enhance lipid production in thraustochytrids.

5.3.3 Effect of Tween 80 and sodium thiosulphate supplemented medium on the fatty acid composition in *Thraustochytrium* sp. AMCQS5-5

The major fatty acids produced by *Thraustochytrium* sp. AMCQS5-5 grown in control, sodium thiosulphate, and Tween 80 supplemented media are presented in Figure 5.5, Figure 5.6 and Figure 5.7. These were palmitic acid, stearic acid, oleic acid, EPA, DPA (n-6) and DHA.

The saturated fatty acids (SFAs) palmitic acid (34-59.9 %) and stearic acid (6.4-24.1 %) were recorded as % of TFA for the control over the course of the fermentation (Figure 5.5). The SFA levels were highest at 24 hours and then decreased and stabilised as the thraustochytrid culture entered the exponential and stationery phases of its growth (Figure 5.5). DHA was in the range of 2.6 to 32.9 % of TFA in the control, with the major increase in DHA occurring between 24 and 48 hours. Oleic acids maximum was approximately 1.7 % of TFA. Maximal EPA and DPA (n-6) levels were 6.2 % and 8 % of TFA, respectively.

![Figure 5.5 Fatty acid profiles showing the major fatty acids for AMCQS5-5 in glucose medium.](image)

Figure 5.5 Fatty acid profiles showing the major fatty acids for AMCQS5-5 in glucose medium.
In Tween 80-supplemented medium the major fatty acids were palmitic acid (13.8-35.3 %), stearic acid (3.4-10.6 %), oleic acid (4.3-30.5 %), EPA (0.5-8.1 %), DPA (3.8-14.6 %) and DHA (13.3-23.2 %) (Figure 5.6). Similarly, in sodium thiosulphate supplemented medium the major fatty acids were palmitic acid (34.9-44.2 %), stearic acid (6.4-16.8 %), oleic acid (0.8-3.4 %), EPA (3-7.2 %), DPA (2.4-5.8 %) and DHA (9.1-29.1 %) (Figure 5.7).

The results show that fatty acid profile is related to incubation time. When the organism reaches the exponential growth phase, between 24 and 72 h, DHA levels increase and SFAs decrease. A similar pattern was observed for Thraustochytrium aureum ATCC 34304 where the stationary phase was recorded after 72 h (Taoka et al. 2011). This may be due to activation of the fatty acid synthase (FAS) that converts the available acetyl-CoA into SFAs, which are subsequently used to synthesise longer chain fatty acids via the PKS pathway (Taoka et al. 2011). In conjunction with growth enhancement due to Tween 80, this results in the accumulation of more lipids. Two distinct pathways for PUFA synthesis have been established in Thraustochytrium aureum ATCC 34304 previously (Matsuda et al. 2012).

![Figure 5.6 Fatty acid profiles showing the major fatty acids for AMCQS5-5 in glucose medium supplemented with Tween 80.](image-url)
Figure 5.7 Fatty acid profiles showing the major fatty acids for AMCQS5-5 in glucose medium supplemented with sodium thiosulphate.

In our study, higher proportions of palmitic and stearic acids were recorded in the control and no improvement was observed with addition of Tween 80 (Figures 5.5 and Figure 5.6). This is in contrast to previous studies with *Thraustochytrium aureum*, which showed slightly increased levels of these fatty acids with Tween 80 (Taoka et al. 2011). However, in a similar study with *Lactobacillus buchneri*, palmitic and stearic acids were observed to be higher when grown in medium without Tween 80 (Nikkila et al. 1995).

The amount of oleic acid was found to be higher in the Tween 80 supplemented medium than in the control (Figure 5.6). The thraustochytrid cells obtained after harvesting were washed three times with distilled water to remove any excess Tween 80, as it contains fatty acids that can complicate the profile. Therefore, the oleate observed in the culture is not from free Tween 80, but is from thraustochytrid cells when culture medium is supplemented with Tween 80. In the fatty acid profile of *Thraustochytrium aureum*, oleic acid increased when compared to the control, indicating the incorporation of oleate from Tween 80 into the cells (Taoka et al. 2011). It has been proposed that enzymes from *Thraustochytrium aureum* might have hydrolysed the ester bonds in Tween 80 releasing oleic acid, which is then incorporated into the fatty acid profile and increasing the content of oleic acid. This
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has been shown to occur with other microbes. For example, oleic acid from Tween 80 was incorporated into the cell membranes of *Bacillus thuringiensis* when cultivated with this surfactant (Brar et al. 2005). Furthermore, there was an improvement in the amount of oleic acid, from 2.6 to 18.5 % in the fatty acid profile of the mushroom mycelium *Pleurotus tuber-regium* (Zhang and Cheung 2011). They observed that addition of Tween 80 promoted lipid production in the mushroom in a similar manner to what was observed for *Thraustochytrium aureum*.

The level of DHA as a percentage of fatty acids in our organism when grown with Tween 80 was lower than in the control. However, overall DHA productivity was higher due to the increased lipid yield, which is consistent with the results of a previous study (Taoka et al. 2011).

The amount of SFAs, particularly palmitic and stearic acids, were higher in the medium containing sodium thiosulphate than for the control and Tween 80 supplemented media. The sodium thiosulphate-containing media also resulted in slightly higher levels of EPA, but decreases in DPA and DHA compared to the control (Figure 5.5 and Figure 5.7). However, DHA as a percentage of TFA was higher in the sodium thiosulphate supplemented medium than in that supplemented with Tween 80 (Figure 5.6 and Figure 5.7). The improvement in EPA levels in the sodium thiosulphate-supplemented media may be due to this additive protecting the cells against cell damage, as has been reported in bacterial systems (Mclean et al. 1996) but it needs further research to point out specific reason. The protective effect of sodium thiosulphate on the membrane lipid degradation induced by the metabolism of glucose has been reported in the cyanobacterium *Synechocystis* sp. PCC 6803 (Wang et al. 2002). This has also been confirmed in plants, where reactive oxygen species that are produced during photosynthesis can damage the cell membranes (Asada 1999).

5.3.4 The proportion of PUFAs and SFAs in Tween 80 and sodium thiosulphate supplemented media for *Thraustochytrium* sp. AMCQS5-5

The ratio of PUFAs to SFAs was found to be 1.8:1 and 0.8:1 with Tween 80 and sodium sulphate-supplemented media, respectively (Figure 5.8). As shown in Figure 5.8, Tween 80 promoted enhanced PUFA levels, in contrast to sodium thiosulphate, which increased SFA accumulation. This is in agreement with a recent study on *Thraustochytrium aureum*, in which the ratio of unsaturated fatty acids and SFAs
was enhanced with Tween 80 and promoted PUFA accumulation (Taoka et al. 2011). Nemec and Jernejc (2002) reported an increased unsaturated/saturated ratio in the lipid profile of *Aspergillus niger* grown in Tween 80-supplemented medium, demonstrating that this phenomenon is not limited to thraustochytrids. Similarly, the ratio of unsaturated and SFAs in the mycelial lipids of *Pleurotus tuber-regium* increased from 2.88 to 3.41 after the addition of Tween 80 (Zhang and Cheung 2011).

Figure 5.8 Proportion of polyunsaturated and saturated fatty acids for AMCQS5-5 in control, sodium thiosulphate and Tween 80-supplemented media.
5.4 Conclusion

Using our isolated *Thraustochytrium* sp. AMCQS5-5 the effect of sodium thiosulphate and Tween 80 on growth, biomass levels, lipid productivity and fatty acid composition was examined. The biomass obtained from media supplemented with sodium thiosulphate was similar to that of the control. The addition of Tween 80 enhanced total cell dry weight. Both additives promoted lipid production, although the fatty acid composition varied. When compared to the control, the fatty acid profile obtained from growth in sodium thiosulphate medium was similar, while that grown with Tween 80 medium showed lower levels of the major fatty acids (palmitic acid and DHA). Tween 80 did enhance total lipids levels, but this was primarily due to increased biomass rather than a higher percentage of lipids in the biomass. Tween 80 also promoted PUFA accumulation, when compared to the control and sodium thiosulphate fermentations. The concentration of sodium thiosulphate in the medium and the ratio of this additive to glucose still need to be optimised. Additionally, optimisation of the amounts and ratios of additives could be performed to further enhance fatty acid production and PUFA accumulation in thraustochytrids.
Chapter 6: Comparative study of carotenoid production in two thraustochytrid species using glucose and glycerol media
6.1 Introduction

A key area of focus for industrial biotechnology is the production of renewable biofuels from microbial sources. Currently the production of biofuels from microbial sources is relatively expensive. However, other microbial-derived compounds and biofuel co-products can be used to offset the high production costs of biofuel production (Borowitzka 2013). The production of valuable compounds, co-products and materials from microbial sources for use in food, pharmaceuticals and industrial materials, is in itself a large industry (Borowitzka 2013). Carotenoids (colour pigments) are major secondary metabolites produced by microbial cells and are commonly found in nature. Marine animals like salmonoids and crustaceans are known to be good sources of oxygenated carotenoids (Goodwin 1986). Natural pigments have been extracted from plants, insects, algae, cyanobacteria and fungi (Mortensen 2006). Various microbes have been reported to accumulate these pigments in their cell bodies, including prodigiosin producing gram negative bacteria (Lewis and Corpe 1964), β-carotene-producing yeasts, *Rhodotorula* sp., *Rhodosporidium* sp., *Sporabolomyces* sp., *Sporidiobolus* sp. (Buzzini et al. 2007), *Rhodotorula glutinis* (Malisorn and Suntornsuk 2008), different carotenoid-producing green algae, *Chlorococcum humicola* (Sivathanu and Palaniswamy 2012), β-carotene-producing microalgae, *Dunaliella* sp. (Ye et al. 2008), astaxanthin-producing freshwater algae *Haematococcus* sp. (Li et al. 2011), many pigment-producing thermo-tolerant microalga *Coelastrella* sp. (Hu et al. 2013) and fungus strains such as *Blakeslea* sp., *Monascus* sp., *Paecilomyces lilacinus* and macromycetes (Elbandy et al. 2009; Hajjaj et al. 2012; Mapari et al. 2005; Sun et al. 2012; Zhou and Liu 2010), and fucoxanthin-producing marine diatom, *Phaeodactylum tricornutum* (Kim et al. 2012). A *Chlorella* sp. was reported to produce zeaxanthin (Singh et al. 2013). A marine cyanobacterium, *Synechococcus* sp. producing β-carotene, zeaxanthin and chlorophyll has been suggested as a suitable candidate for genetic modification to enhance pigment production (Macias-Sanchez et al. 2007).

A recent review discussed numerous marine microbes known for producing pigments such as carotenoids and xanthophylls (Dewapriya and Kim 2014). Marine microalgae accumulate a variety of carotenoids such as β-carotene, astaxanthin, canthaxanthin, lutein and violaxanthin (Plaza et al. 2009). In fact, some pigments from marine
sources have been commercialised. β-Carotene from \textit{Dunaliella salina} is used in cosmetics and dietary supplement preparations (Guedes et al. 2011). \textit{Nannochloropsis gaditan} has been demonstrated as a potential source of pigments (Henriques et al. 2007). Other pigments from marine bacteria, such as prodiginines, carotenes, violacein and quinones also have commercial potential (Soliev et al. 2011).

Carotenoids are essential nutrient precursors playing a role in human health (Fernandez-Garcia et al. 2012). β-Carotene possesses anti-oxidative and immune modulatory properties that may assist in the prevention of several diseases such as cancer, cardiovascular disease, rheumatoid arthritis and neurodegenerative diseases (Dembinska-Kiec 2005). Astaxanthin is used in the cosmetic, nutraceutical and aquaculture industries. In humans it has potential for the prevention of cardiovascular disease and cataract formation, helps strengthening the immune system and also possesses anti-oxidant and anti-inflammatory activities (Guerin et al. 2003; Higuera-Ciapara et al. 2006). Astaxanthin from \textit{Haematococcus pluvalis} has been studied for its antihypertensive and neuro-protective effects and dietary astaxanthin has been investigated for its effects on blood pressure, stroke and vascular dementia in animal models (Hussein et al. 2006). Astaxanthin from \textit{H. pluvalis} has been approved for commercialisation for companies such as Algatechnologies Ltd. (Israel), AstaReal AB (Sweden), Cyanotech (USA) and U.S. Nutra (USA) (Campenni et al. 2013). Lutein and zeaxanthin support normal vision and skin health. They are deposited in the lens of the eye, macula lutea, decreasing the possibility of cataracts and age related macular degeneration (Alves-Rodrigues and Shao 2004; Roberts et al. 2009).

Thraustochytrids are known for the production of polyunsaturated fatty acids (PUFAs), but have also been explored for the production of various pigments; including astaxanthin, zeaxanthin, canthaxanthin, β-carotene, echinenone and phenicoxanthin (Aki et al. 2003; Armenta et al. 2006; Burja et al. 2006; Carmona et al. 2003; Quilodran et al. 2010).

In our study we compare the carotenoid producing potential of two thraustochytrid strains (\textit{Schizochytrium} sp. and \textit{Thraustochytrium} sp.), grown on two different carbon sources (glucose and glycerol). The primary aim of this work was to determine if these strains could grow effectively on glycerol as a replacement for glucose for carotenoids production.
6.2 Materials and Methods

6.2.1 Chemicals used

All the chemicals used for extraction of carotenoids were of analytical grade. For HPLC analysis, solvents used were procured from Sigma-Aldrich (St. Louis, MO, USA) and Merck chemicals (Frankfurter Strabe, Darmstadt, Germany). Carotenoid standards (astaxanthin, zeaxanthin, canthaxanthin, β-cryptoxanthin, echinenone) were procured from CaroteNature (Ostermundigen, Switzerland) while β-carotene was procured from Sigma-Aldrich (St. Louis, MO, USA).

6.2.2 Strain selection, identification and maintenance

The in house isolates *Schizochytrium* sp. DT3 and *Thraustochytrium* sp. DT4, that were newly isolated from Barwon mangrove site in Victoria during October 2012, were used in this study. The GPS positions (S 38°15.922’ E 144°29.771’) were recorded. The DT3 strain produced a pale orange colour on agar plates; while DT4 gave an orange colour, indicating the presence of carotenoids. These isolates were identified on the basis of 18s rRNA sequencing as described in Chapter 3 (Sections 2.7 and 2.8). The sequences obtained were submitted to the GenBank database. The cultures were maintained on GYP agar medium containing 5 g L⁻¹ glucose, 2 g L⁻¹ yeast extract, 2 g L⁻¹ mycological peptone, 12 g L⁻¹ agar in 50 % artificial seawater (ASW). Strains were subcultured every 2 weeks.

6.2.3 Growth at different glycerol and glucose concentrations

Seed medium (w/v, 2 g L⁻¹ yeast extract, 2 g L⁻¹ mycological peptone, ASW 50 %, pH 6.5) was inoculated from agar plates and grown for 2 days in a shake flask at 25 °C and 150 rpm. An inoculum of 5 %, v/v was used to inoculate 47.5 ml of production medium and cultured for 7 days in a shake flask at 25 °C and 150 rpm. Glucose and glycerol (w/v, 2, 4, 6, 8 and 10 %) was used individually in the fermentation medium to assess their effect on carotenoid production. Biomass growth was monitored to check contamination. The resultant biomass was harvested by centrifugation (10,000 x g for 15 min) and freeze dried until required. Cell dry weight was recorded. All experiments were performed duplicates and repeated once.
6.2.4 Carotenoid extraction

The freeze dried biomass (25 mg) was taken for carotenoid extraction. Acetone was added at 70 µL per mg of biomass. This mixture was subjected to ultra-sonication for 15 min, at 20 kHz for 50 cycles in an ice bath. The solution then was centrifuged at 4,000 rpm for 15 min at room temperature to pellet the cellular debris. The supernatant was decanted and transferred to a fresh centrifuge tube, before the solvent was evaporated under a nitrogen stream. To this dried extract, 1 mL of mobile phase containing methanol, ethyl acetate and water (88:10:2, v/v/v) was added and filtered through 0.45 µm nylon filters (Chromacol, Thermo Scientific, Schwerte, Germany) and stored at -20 °C. Carotenoids were identified and quantified by Reverse-phase HPLC (RP-HPLC) analysis.

6.2.5 HPLC analysis of carotenoids

This method is previously described in Chapter-4 (Section 4.2.8).

6.3 Results and Discussion

6.3.1 Strain identification and phylogenetic tree

The two isolates, DT3 and DT4, were identified by the microscopic observation of cell division and 18s rRNA sequencing (Chapter 3, section 3.2.7 and 3.2.8) as a Schizochytrium sp. and a Thraustochytrium sp., respectively. A phylogenetic tree showing the strong homology of these organisms to other available thraustochytrid sequences is presented in Figure 6.1. The 18s rRNA sequences of DT3 and DT4 were deposited in the GenBank database and have been assigned the accession numbers of KF682125 and KF682126, respectively. Using Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990), the sequences of DT3 and DT4 were found to be closely related to Schizochytrium sp. AMCQS1-9 (GenBank accession number JX993843.1) and Thraustochytrium aff. striatum (GenBank accession number HQ228973.1), respectively. The sequences obtained from BLAST were analysed with MEGA 5.1 software (Tamura et al. 2011) to generate a phylogenetic tree. The confidence of branching is shown by the bootstrap values above 50.
Figure 6.1 Molecular phylogenetic tree showing a close relationship between the isolates and other selected sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches.

6.3.2 Cell dry weight determination of both isolates

The cell dry weights of both strains grown under differing carbon concentrations were determined (Figure 6.1 and Figure 6.2). Figure 6.1 has been described in detail previously in Chapter 4 (Figure 4.4). The maximum cell dry weight was obtained in the glycerol medium with both strains, at 16.31 g L\(^{-1}\) for DT3 and 3.1 g L\(^{-1}\) for DT4. The highest cell dry weight in glucose was 4.31 g L\(^{-1}\) for DT3 and 2.45 g L\(^{-1}\) DT4. Higher carbon concentrations (above 8 \%\) might have inhibited the biomass growth in *Schizochytrium* sp. DT3, resulting into a decrease in cell dry weight at 10\% carbon concentration (Figure 6.1). This is in agreement with previous studies where higher glucose and glycerol concentrations inhibited biomass growth (Burja et al. 2006; Huang et al. 2012). In contrast, the cell dry weight of *Thraustochytrium* sp. DT4 increased gradually with increasing carbon concentration (Figure 6.2). Different responses towards higher carbon concentrations may be attributed to the specific thraustochytrid strain. Scott et al. (2011) reported an increase in biomass when glycerol was used in the fermentation medium instead of glucose. Similar findings were observed in our previous study using a variety thraustochytrid strains (Chapter 4).
Figure 6.2 Cell dry weight of *Schizochytrium* sp. DT3 at various carbon concentrations.

Figure 6.3 Cell dry weight of *Thraustochytrium* sp. DT4 at various carbon concentrations.
6.3.3 Standard curve preparation for carotenoid analysis

All standards were prepared in the concentration range of 1-5 µg mL\(^{-1}\) and stored at -20 °C for further use. The calibration curves were prepared using the above mentioned HPLC program (Refer Chapter-4, section 4.2.8). All the carotenoids in the samples were calculated from these standard curves (Figure 6.4). The slope, intercept and the R\(^2\) values are given in Table 6.1.

![Figure 6.4 Calibration curves for each carotenoid standard.](image-url)
Table 6.1 Carotenoid standards.

<table>
<thead>
<tr>
<th>Carotenoid standards</th>
<th>Slope</th>
<th>Intercept</th>
<th>R²</th>
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<tr>
<td>Astaxanthin</td>
<td>114.85</td>
<td>61.63</td>
<td>0.9972</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td>11.148</td>
<td>8.0899</td>
<td>0.9916</td>
</tr>
<tr>
<td>Canthaxanthin</td>
<td>63.566</td>
<td>33.877</td>
<td>0.9902</td>
</tr>
<tr>
<td>β-cryptoxanthin</td>
<td>19.586</td>
<td>16.811</td>
<td>0.9953</td>
</tr>
<tr>
<td>Echinenone</td>
<td>32.647</td>
<td>18.2</td>
<td>0.9861</td>
</tr>
<tr>
<td>β-carotene</td>
<td>12.717</td>
<td>11.814</td>
<td>0.9906</td>
</tr>
</tbody>
</table>

### 6.3.4 Carotenoid content of two thraustochytrid strains grown with glucose or glycerol as the media carbon source

The total carotenoid content in DT3 was greatest when grown in glucose, with the highest carotenoid content recorded in 4 % glucose at 417.5 µg g⁻¹. For DT4 carotenoid content was highest when grown in glycerol, with a maximum carotenoid content of 402.3 µg g⁻¹ in 2 % glycerol, with 4 % glycerol giving a similar value (Figure 6.5 and Figure 6.6). This is similar to the total carotenoid level of about 450 µg g⁻¹ for *Thraustochytrium* sp. CHN-1 when grown in glucose medium (Carmona et al. 2003). It has been previously described in chapter 4 that glycerol can enhance carotenoid levels in some thraustochytrids in comparison to those obtained from organisms grown on glucose, which is consistent with our results with the DT4 strain. Another recent study showed that glycerol enhanced the carotenoid content in *Chlorella* sp. (Singh et al. 2013). However, one common finding with both of our thraustochytrid strains was the similar pattern in carotenoids produced when the strains were grown in a glycerol medium. Both strains showed the highest carotenoid production at low levels of glycerol (2 and 4 %). These levels gradually decreased as the concentration of glycerol increased, which is consistent with results reported by Saenge et al (2011); who observed higher biomass growth but not carotenoid content. Another recent study that used several red yeast strains reported similar observations when cultivated on glycerol (Petrik et al. 2013). The key precursor for the biomass production and carotenoids synthesis is acetyl-CoA (Lee and Schmidt-Dammert 2002). Thus, it may be assumed that the biomass and the production of carotenoids...
compete for the acetyl-CoA derived from the glycerol in the medium (Cutzu et al. 2013). This would suggest that more acetyl-CoA has been converted into biomass, resulting in higher biomass content while not increasing carotenoid content. In general, an increase in sugar concentration increases thraustochytrid growth and total carotenoid production. Glycerol at high concentration inhibited total carotenoids unlike glucose. A gradual increase in total carotenoid content in both strains was observed with the increase in glucose concentration. This means that glucose was more easily converted into carotenoids than glycerol. Strategies such as adding glucose after few days of fermentation in glycerol could result in high yields of both biomass and carotenoid content.

Figure 6.5 Total carotenoids in *Schizochytrium* sp. DT3 at various carbon concentrations.
Figure 6.6 Total carotenoids in *Thraustochytrium* sp. DT4 at various carbon concentrations.

The primary purpose for using glycerol in growth media is to decrease the cost of fermentation, as glycerol is cheaper than glucose. Glucose alone has been accounted for 80% of the total production cost for lipids (Li et al. 2007). In addition, glycerol has recently been used as promising substrate for secondary metabolites production (Abad and Turon 2012). Glycerol has been used with *Phaffia rhodozyma* (astaxanthin), *Sporobolomyces ruberrimus* (astaxanthin), *Blakeslea trispora* (β-carotene) and *Rhodotorula glutinis* (mainly β-carotene) (Kusdiyantini et al. 1998; Mantzouridou et al. 2008; Saenge et al. 2011; Valduga et al. 2009). Other low cost substrates such as distillery waste streams (Yamasaki et al. 2006), brewery by-products (Quilodran et al. 2010) and waste syrups (Iwasaka et al. 2013) have been used in the fermentation of thraustochytrids to produce carotenoids. Other microorganisms, such as red yeasts, have utilised radish brine (Malisorn and Sunthornsuk 2008), agricultural wastes (Aksu and Eren 2005), carob pulp syrup and sugarcane molasses (Freitas et al. 2014) for carotenoids production, although these carbon sources have not been tested with thraustochytrids. The current production of natural carotenoids is based on plant materials. Fermentation-based carotenoid production can be made economically feasible by using low cost substrates such as biodiesel glycerol (Tinoi et al. 2005). However, the use of crude glycerol from the
biodiesel industry requires certain technical difficulties to be overcome before it can be utilised on a larger scale (Yang et al. 2012).

The major carotenoids in both strains examined were astaxanthin, zeaxanthin, canthaxanthin, echinenone and β-carotene. All carotenoids were in similar ratios regardless of the carbon source they were grown on. The exceptions were zeaxanthin and β-cryptoxanthin, which were not observed in the DT3 strain when grown in the glucose-containing medium, but were present when grown with glycerol (Figure 6.7 and Figure 6.8).

![Figure 6.7 Carotenoids composition in Schizochytrium sp. DT3 at various glucose concentrations.](image-url)

Figure 6.7 Carotenoids composition in *Schizochytrium* sp. DT3 at various glucose concentrations.
In the DT3 strain the major pigment was β-carotene (258 µg g⁻¹ in glucose and 249 µg g⁻¹ in glycerol). Astaxanthin (63.7 µg g⁻¹) and β-carotene (258 µg g⁻¹) reached maximal levels when grown in medium containing 4 % glucose and decreased at higher levels of glucose (Figure 6.7). Higher concentrations of glucose may inhibit the formation of carotenoids (Yamane et al. 1997). The detection of high levels of β-carotene in DT3 indicates that β-carotene is poorly converted to other xanthophylls in this strain. The conversion of β-carotene is oxygen dependent and its poor conversion may be due to the limited oxygen available in the shake flask cultures (Aki et al. 2003). *Schizochytrium* sp. HX-308 has been reported to produce 95 µg g⁻¹ of β-carotene in fed batch cultivation using a glucose medium (Ren et al. 2014).

Canthaxanthin and echinenone increased slightly with the increasing glucose concentration. Astaxanthin content was similar to our previously reported level of 29.7 µg g⁻¹ for *Schizochytrium* S31 in chapter 4. Ultra sonication was found to be an efficient method for the disruption of thraustochytrid cells and the release of membrane-bound carotenoids (Singh et al. 2013). Burja et al. (2006) showed lower levels of astaxanthin production (1.53 µg g⁻¹) and β-carotene production (20.2 µg g⁻¹) in *Thraustochytrium* sp. ONC-T18 than found in our results for the DT3 strain. High levels of astaxanthin and β-carotene have been reported in other pigmented microorganisms such as *Haematococcus pluvalis* (Lorenz and Cysewski 2000), *Phaffia rhodozyma* (Schmidt et al. 2011), *Dunaliella salina* and *Chlorella* sp.
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(Guedes et al. 2011; Singh et al. 2013). Thraustochytrids are usually grown heterotrophically. The advantages of using heterotrophic culture over autotrophic growth are primarily the ease of fermentation and the high cell densities that can be obtained. However, higher amounts of astaxanthin have been reported when thraustochytrid cultures (mutant *Schizochytrium* sp. and *Thraustochytrium* sp.) are grown in the presence of a light source (Chatmdurong et al. 2007; Yamaoka et al. 2004). This indicates that autotrophic or mixotrophic growth of thraustochytrids may be more useful than heterotrophic culture for increasing carotenoid production.

DT3 grown in glycerol exhibited higher astaxanthin content (70.1 µg g⁻¹) than when grown in glucose, but levels decreased as the carbon concentration increased (Figure 6.8). β-carotene content increased with higher carbon source levels in both glycerol and glucose media, while other carotenoids decreased or remained unchanged. β-cryptoxanthin was present in DT3 when grown in 2 % and 4 % glycerol but not at higher levels or when grown in glucose (Figure 6.7).

In the DT4 strain carotenoid levels were essentially unchanged with varying levels of glucose or glycerol (Figure 6.9). The astaxanthin content was higher in the DT4 strain than in DT3 and thus can be promoted as a potential astaxanthin producer comparable or higher than other strains studied (Burja et al. 2006; Carmona et al. 2003). In addition, glycerol led to higher astaxanthin levels in the DT4 strain than was observed for glucose (Figure 6.10). Astaxanthin has several advantages over other carotenoids that include increased stability, high antioxidant capacity and high tinctorial properties (Dufosse et al. 2005). For these reasons the production of astaxanthin has commercial potential and is also required as a supplement in aquaculture feed (Lorenz and Cysewski 2000). Thus, it would be worthwhile optimising astaxanthin production in the DT4 strain. Increasing the glycerol concentration increased the cell dry weight but did not improve the level of individual carotenoids in either strain. The higher levels of carotenoids observed at low glycerol concentration is in agreement with previous studies (Cutzu et al. 2013; Saenge et al. 2011). Zeaxanthin content (121.8 µg g⁻¹) was also relatively high in the DT4 strain (Figure 6.10) and was at higher levels than reported for *Thraustochytrium* sp. ONC-T18 (Burja et al. 2006). However, it was not greater than the reported values of *Chlorella* sp. which is known for its zeaxanthin accumulation (Inbaraj et al. 2006; Singh et al. 2013). Zeaxanthin naturally occurs in the retina and may aid in the prevention of age-related macular degeneration (Beatty et al. 1999; Mares-Perlman et
Glucose and glycerol both resulted in β-carotene accumulation in the DT3 strain, whereas, glycerol resulted primarily in astaxanthin and zeaxanthin production in the DT4 strain. Carotenoids are often produced under stress related conditions as they act as lipid soluble membrane antioxidants (Burja et al. 2006; Campenni et al. 2013). The ability of thraustochytrids to adapt and grow on variety of carbon sources including industrial by-products could be of interest for potential biotechnological applications.

Figure 6.9 Carotenoids composition in *Thraustochytrium* sp. DT4 at various glucose concentrations.
6.3.5 Overall carotenoid yield in two thraustochytrid isolates grown on glucose and glycerol fermentation media

The overall carotenoid yield was directly proportional to the biomass yield. The total carotenoid yield was higher when the DT3 strain was grown on glycerol when compared to glucose. This was primarily due to the higher biomass levels (Figure 6.11 and Figure 6.12). With glycerol as the carbon source the maximum carotenoid production in DT3 was 3407.9 µg L⁻¹, whereas the maximum production with glucose was 1515.6 µg L⁻¹ (Figure 6.11). Lower levels of carotenoid were observed in the DT4 strain, compared to the DT3 strain, with 757.3 µg L⁻¹ and 921.5 µg L⁻¹ in glucose and glycerol, respectively (Figure 6.12). Carotenoid yield has been reported at high levels in carotenogenic yeasts (Petrik et al. 2013). Carotenoid formation in the microorganisms is dependent on the strain type, culture medium and cultivation conditions (Sandmann et al. 1999). Higher glycerol concentrations (4 and 6 %) suppressed carotenoid production in both strains, whereas a gradual increase was observed in the glucose medium. Higher glucose concentrations did not impact carotenoid production in Schizochytrium sp. KH105 (Aki et al. 2003). We have previously reported that higher glycerol levels inhibited lipid production in some thraustochytrids (Chapter 4). High levels of glycerol can increase viscosity that
might inhibit secondary metabolite production (Scott et al. 2011). DT3 produced more biomass than DT4, resulting in overall higher carotenoid production levels in DT3.

Figure 6.11 Total carotenoids yield in *Schizochytrium* sp. DT3 at various carbon concentrations.

Figure 6.12 Total carotenoids yield in *Thraustochytrium* sp. DT4 at various carbon concentrations.
The carotenoid profile of DT3 and DT4 showed that individual carotenoid levels gradually increased with increasing in carbon concentration (Figure 6.13, Figure 6.14, Figure 6.15 and Figure 6.16). The highest level of each carotenoid produced in DT3 and DT4 grown on glycerol were astaxanthin (1012.5 and 281.7 µg L\(^{-1}\)), zeaxanthin (1016.5 and 276.5 µg L\(^{-1}\)), canthaxanthin (709.8 and 131.7 µg L\(^{-1}\)), echinenone (801.2 and 135.7 µg L\(^{-1}\)) and β-carotene (3382.9 and 203.7 µg L\(^{-1}\)), respectively. This is similar to the carotenoid yield in *Thraustochytrium* sp. ONC-T18 (Burja et al. 2006). *Schizochytrium* sp. KH105 was reported to accumulate 5-6 fold more astaxanthin than observed in our study (Aki et al. 2003). Strain KH105 has also been reported to accumulate 1.1 mg L\(^{-1}\) astaxanthin when grown on waste syrup (Iwasaka et al. 2013). β-Carotene accumulation for DT3 in 10 % glucose was comparable to that observed for strain KH105. The majority of carotenoids are chemically synthesized and currently only astaxanthin from microalgal sources has been in commercial production. Strain development strategies using DT3 strain might exceed the current production of β-carotene and astaxanthin making it a potential candidate for mass production.

![Figure 6.13 Carotenoids yield in Schizochytrium sp. DT3 at various glucose concentrations.](image-url)
Figure 6.14 Carotenoids yield in *Schizochytrium* sp. DT3 at various glycerol concentrations.

Figure 6.15 Carotenoids yield in *Thraustochytrium* sp. DT4 at various glucose concentrations.
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6.4 Conclusion

Two new strains of thraustochytrid were isolated from mangrove areas in Victoria near Barwon waters. They were identified as *Schizochytrium* sp. DT3 and *Thraustochytrium* sp. DT4 on the basis of microscopic examination of cell division pattern and 18s rRNA sequencing. The appearance of orange colouration on agar plates was due to the production of pigments by the isolates. Both strains were able to utilise glucose and glycerol media to produce a number of carotenoids. The main carotenoid species in both strains were astaxanthin, zeaxanthin, canthaxanthin, echinenone and β-carotene. Further experiments investigating nitrogen depletion and optimisation of the carotenoid extraction procedures may improve their production, making them potentially useful pigment producers. Low cost substrates such as agricultural residues or industrial wastes may also be used to minimise the production costs.

Figure 6.16 Carotenoids yield in *Thraustochytrium* sp. DT4 at various glycerol concentrations.
Chapter 7: Summary and future aspects
The biodiversity of marine ecosystems is vast and represents a considerably underutilised resource for the isolation of novel bioactive compounds. The potential for the discovery of pharmacologically active compounds, that may be beneficial to human health, has gained attention in recent decades. Marine environments provide stringent conditions which help shape microorganisms with highly specialised and unique biochemical compositions. Obtaining a better understanding of the physiological conditions of marine microbial communities will enable us to better exploit these organisms for the production of important bioactive compounds. Recent developments in marine ecology, genomics, metabolomics, molecular biology, drug development and improvements in fermentation technologies are important for gaining an improved understanding of the usefulness of the biodiversity present in the marine environment.

The main objective of this study was to explore the Victorian local marine environment for oleaginous microorganisms that have potential in nutraceutical applications and biodiesel production. For nutraceutical applications organisms producing long chain polyunsaturated fatty acids, such as DHA, were of interest. An aim of this research was to isolate potential microbes as an alternative source to fish oil of omega-3 fatty acids and explore their ability to grow on low cost carbon sources, particularly glycerol. Thus several growth media and their effect on lipids and DHA production were investigated. In addition, the ability of the isolated microorganisms to produce commercially important carotenoids was investigated.

Chapter 2 describes the isolation of oleaginous yeasts and their growth on media containing glucose to produce omega-3 fatty acids. Marine water, degraded leaves and sediments samples were collected and screened using different isolation techniques. A number of red pigmented microbes were isolated, including four isolates labelled as AMCQ1D, AMCQ8A, AMCQ10C and AMCQ12C. All isolates showed a red colour on agar plates, except AMCQ1D. Scanning electron microscopy indicated that the isolates were *Rhodotorula* species. Two of the isolates then were identified based on 18S rRNA sequencing and assigned as *Rhodotorula* sp., AMCQ8A and *Rhodotorula* sp. AMCQ10C.

AMCQ8A produced the highest cell dry weight of the four isolates studied. FTIR-micro-spectroscopy was performed with the four isolates to determine the biochemical composition of cells. A prominent unsaturated fatty acid peak was
observed in the spectrum of AMCQ8A, but not in other isolates. Moreover, AMCQ8A showed a higher lipid to amide ratio, demonstrating the accumulation of lipids. Gas chromatography (GC) analysis also showed the presence of more lipids in AMCQ8A, confirming the FTIR results. More than 90% of the fatty acids were found to be palmitic acid, stearic acid, oleic acid and linoleic acid. The omega-3 fatty acid, α-linolenic acid (ALA) was present at a higher level than in other isolates. Based on the FTIR and GC analysis, AMCQ8A was determined to be the best lipid producer and was thus selected for further evaluation and culture optimisation. Culture optimisation led to improvements in oleic acid, linoleic acid and ALA content. Different fermentation strategies including changing the C:N ratio could further enhance the lipid production of this isolate.

For the work described in Chapter 3 the aim was to isolate marine microbes that produced omega-3 fatty acids, particularly DHA. A second sample collection was undertaken to obtain omega-3 fatty acid rich thraustochytrids. Different isolation methods were used, including pollen baiting and direct plating techniques, which led to the successful isolation of 13 thraustochytrid strains. The difference between the isolates obtained from pollen-baiting and direct plating techniques was investigated. Repeated subculturing was performed and 11 isolates were subsequently analysed for the production omega-3 fatty acids. The baiting isolates showed DHA accumulation of 11-41% (of TFA), whereas plating isolates resulted in a narrower range of 19-25% (of TFA). The main fatty acids present in the isolates were palmitic acid, stearic acid, EPA, DPA-n6 and DHA. Three of the isolates (AMCQS5-3, AMCQS5-4 and AMCQS5-5) were found to produce DHA at high levels (>39% of TFA), while two isolates (AMCQS1-9 and AMCQS1-10) produced significant amounts of saturated fatty acids. These organisms warrant further investigation for use in applications such as omega-3 and biofuel production, respectively. Some of the isolates also produced carotenoids. Seven isolates were selected on the basis of gas chromatography analysis and identified by microscopic examination and 18s rRNA sequencing. The isolates were closely related to Thraustochytrium sp., Schizochytrium sp. and Ulkenia sp. On the basis of its high DHA content (% TFA), Thraustochytrium sp. AMCQS5-5 was selected for optimisation of growth on a variety of carbon sources.
In Chapter 4, the *Thraustochytrium* sp. AMCQS5-5 was grown on glycerol and glucose and analysed for its ability to convert these carbon sources into lipid biomass. *Schizochytrium* sp. DT3 (isolated from Mangrove site, Barwon waters, Victoria) was also grown in glycerol-containing medium. Both strains showed improved biomass growth, lipids and DHA production with glycerol, compared with that obtained using glucose. However, high glycerol concentrations (> 6 %) inhibited DHA production in both strains. Both strains, *Thraustochytrium* sp. AMCQS5-5 and *Schizochytrium* sp. DT3, were grown using potentially low cost sugars hydrolysate from pre-treated raw wood biomass of hemp (*Cannabis sativa*). Both strains efficiently converted the sugars hydrolysate into lipids and produced a biomass level comparable to that from glucose as the carbon source. Further, the coloured isolates were examined for carotenoid production using solvent extraction. Thraustochytrid strains grown in glycerol medium produced more carotenoids than when grown in glucose medium. Hence, the potential of these strains can be exploited to utilise low cost-substrates for both omega-3 fatty acids and carotenoid production.

In Chapter 5 we investigated the ability of Tween 80 and sodium thiosulphate to improve lipid productivity in the isolated *Thraustochytrium* sp. AMCQS5-5. Tween 80 and sodium thiosulphate improved biomass growth resulting in higher cell dry weight. Although, both additives reduced DHA levels as a percentage of TFA, overall DHA accumulation in mg g⁻¹ was enhanced due to an increase in TFA levels with these additives.

In Chapter 6, two coloured strains, *Schizochytrium* sp. DT3 and *Thraustochytrium* sp. DT4, were grown in glycerol and glucose media for carotenoid production. Solvent extraction coupled with ultra-sonication was performed to disrupt the cells and improved carotenoid extraction yields. Glycerol as the carbon source resulted in increased carotenoid production at low concentration, but substrate inhibition was observed at higher concentrations. Overall, total carotenoids levels were higher in *Schizochytrium* sp. DT3 than in *Thraustochytrium* sp. DT4. If carotenoid levels can be increased further then these could be a value added co-product that could decrease the production costs of DHA.

To the best of our knowledge, this is the first study that explored the Victorian local marine environment for lipid producing marine microorganisms. These oleaginous microbes have the potential to compete with fish oil as a commercial source of...
omega-3 fatty acids, overcoming the limitation of fish stocks for oil production. Currently, the cost of fermentation of DHA and EPA is significantly higher than obtaining these fatty acids from fish oil. However, fermentation enables better control of EPA and DHA ratios, is a renewable source, and results in extremely low contaminant levels. The cost of fermentation can be decreased using low cost carbon sources such as glycerol or waste carbon sources, resulting in further decreases in omega-3 production costs.

Potential coproduction of lipids and other metabolites such as carotenoids, polysaccharides, enzymes and sterols is possible with thraustochytrids and makes these organisms interesting as both omega-3 producers and biofuel producers. The application of metabolic engineering techniques to improve the yields of DHA and other compounds in wild-type strain is in an early stage of development. In fact, there is no full genomic sequence of a thraustochytrid yet published. Applying metabolic engineering approaches to these organisms could further improve oil, DHA and carotenoid yields in the future. The global market for omega-3 oils incorporated infant formulas, food supplements and medicinal benefits is still growing. Thus, the identification of currently undiscovered strains, the development of improved fermentation techniques and metabolic engineering approaches are key areas of future research for thraustochytrids.
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