New Australian thraustochytrids: A Renewable Source of Biofuels, Omega-3 Oils and other Bioproducts

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Declaration

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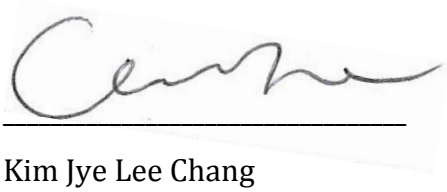

Kim Jye Lee Chang
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Abbreviations

The following abbreviations have been used in this thesis:

15:0	pentadecanoic acid
16:0	palmitic acid
17:0	heptadecanoic acid
18S rRNA	18S ribosomal RNA gene
AA	arachidonic acid (20:4ω6)
ANACC	Australian National Algae Culture Collection
AQIS	Australian Quarantine and Inspection Service
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
DCW	Dry cell weight
DHA	docosahexaenoic acid (22:6ω3)
DMOX	4,4-dimethyloxazoline
DPA-3	docosapentaenoic acid (ω3)
DPA-6	docosapentaenoic acid (ω6)
EPA	eicosapentaenoic acid (20:5ω3)
EPS	exopolysaccharides
ERoEI	energy returned on energy invested
FAME	fatty acid methyl ester
FAS	fatty acid synthase
FID	flame ionization detector
GC	gas chromatography
GC-MS	gas chromatography- mass spectrometry
Glu	glucose
Gly	glycerol
HC	hydrocarbon
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
HRD	hydroprocessed renewable biodiesel
LCA	Life-cycle assessment

LC-PUFA	long chain (≥C_{20}) polyunsaturated fatty acid
MeOH	methanol
MUFA	monounsaturated fatty acid/s
Nutr	nutrients
OC-FA	odd-chain fatty acids
PCR	polymerase chain reactions
PKS	polyketide synthase
PUFA	polyunsaturated fatty acid/s
SD	standard deviation
SFA	saturated fatty acid
SPI	septum-programmable injector
TAG	triacylglycerols
TC	thraustochytrids collection
TFA	total fatty acid/s
Tr	trace
X:YωZ	This was adopted for the naming of fatty acids, where X refers to the number of carbon atoms in the molecule, Y refers to the number of double bonds in the molecule, and Z indicates the carbon position of the first double bond from the terminal methyl end (CH3) of the molecule. The latter is generally referred to as omega Z (e.g. ωZ) or n-Z (e.g. n minus Z).

Publications

The following peer-reviewed publications have been either wholly or partially derived from work associated with this thesis:

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Kim Jye Lee Chang, Geoff Dumsday, Peter D. Nichols, Graeme A. Dunstan, Susan I. Blackburn and Anthony Koutoulis, **High cell density cultivation of a novel *Aurantiochytrium* sp. strain TC 20 in a fed-batch system using glycerol to produce feedstock for biodiesel and omega-3 oils,** Applied Microbiology and Biotechnology (2013) 97:6907–6918

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Kim Jye Lee Chang, Lucas Rye, Tim Grant, Graeme A. Dunstan, Susan I. Blackburn, Peter D. Nichols and Anthony Koutoulis, **Life-cycle assessment: Heterotrophic cultivation of thraustochytrids for biodiesel production,** manuscript in preparation.

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Paper 2, Biodiscovery of new Australian thraustochytrids for production of biodiesel and long-chain omega-3 oils: *Located in chapter 2. Candidate was the primary author, author 3 and author 4 contributed to the lipid identification, author 6 assisted with DNA extraction and sequencing software, and author 7 contributed to the pigment isolation and analysis. Authors 1, 2, 3, and 4 assisted with refinement and presentation.*

Paper 3, Comparison of thraustochytrids *Aurantiochytrium* sp., *Schizochytrium* sp., *Thraustochytrium* sp. and *Ulkenia* sp. for production of biodiesel, long-chain omega-3 oils and exopolysaccharides: *Located in chapter 4. Candidate was the primary author, author 3 and author 4 contributed to the lipid identification, and with author 8 contributed to the EPS isolation and characterisation. Authors 1, 2, 3, and 4 assisted with refinement and presentation.*

Paper 4, High cell density cultivation of a novel *Aurantiochytrium* sp. strain TC 20 in a fed-batch system using glycerol to produce feedstock for biodiesel and omega-3 oils: *Located in chapter 5. Candidate was the primary author, author 3 and author 4 contributed to the lipid identification, and with author 9 contributed to the experimental design and Bioreactor operation. Authors 1, 2, 3, and 4 assisted with refinement and presentation.*

Paper 5, Life-cycle assessment: Heterotrophic cultivation of thraustochytrids for biodiesel production: *Located in chapter 6. Candidate was the primary author, author 10 and author 11 contributed to the idea, LCA software operation and development. Authors 1, 2, 3, and 4 assisted with refinement and presentation.*

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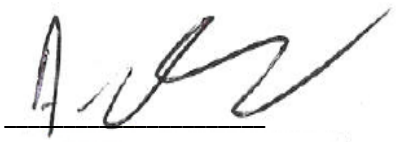
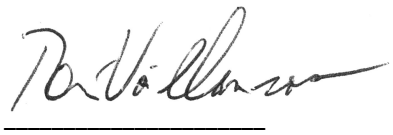
Signed:  

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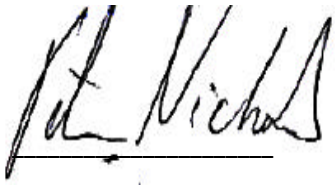

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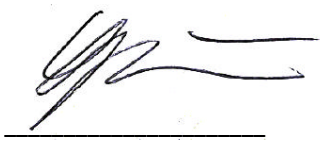

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Abstract

The potential of biofuel production from microalgae is of intense interest globally owing to growing concern with rising crude oil prices and future availability. In addition to producing lipids for potential biofuel application, thraustochytrids are capable of forming other high-value bioproducts, such as proteins, enzymes, omega-3 polyunsaturated fatty acids (PUFA), carotenoid pigments and exopolysaccharides (EPS). The co-production of high-value bioproducts during biofuel production is desirable when it adds greater value to the production process and improves process economics.

Thirty-six new thraustochytrids have been isolated from the southeast coast of Tasmania and far north Queensland. They were separated into eight chemotaxonomic groups (A – H) based on fatty acid and sterol composition, with the groups clustered closely with four different genera (*Aurantiochytrium*, *Schizochytrium*, *Thraustochytrium* and *Ulkenia*) based on 18S rDNA molecular identification. In an initial screening study, some strains produced > 60 % docosahexaenoic acid (DHA) under unoptimized culture conditions. *Aurantiochytrium* sp. strains (groups G and H) contained 15:0 (pentadecanoic acid) at between 20 – 30 % of the total fatty acids (TFA) and 16:0 (palmitic acid) in the range of 7 – 15 % TFA, suggesting these strains could be potential candidates for biodiesel production. β,β-Carotene, canthaxanthin and astaxanthin were identified in pigmented strains. Part of the process to scale up is to select the best performing strain based on growth and biochemical characteristics. In the subsequent trials, eight thraustochytrid strains from the different chemotaxonomic groups (A – H) were compared in 1 L scale baffled shake flasks for the synthesis of EPS, in addition to biomass yield and fatty acid profiles. The crude chemical characterization of the EPS, which were released into the culture media by these strains, was performed as an initial step in

determining the potential for biotechnological application of these biomaterials. *Aurantiochytrium* sp. strain TC 20 had the highest biomass production (18.5 g/L) and oil yield (7.5 g/L) after 9 days of growth in 4 % w/v glucose basal media at 20 °C, with 0.18 g/L EPS extracted from the supernatant. The maximum yield of EPS was observed in *Schizochytrium* sp. strain TC 02 (0.3 g/L). High biomass producing strains that also had high lipid and high EPS yield may be better candidates for commercial production of biofuels and other bioproducts. The next phase was to optimize biomass in 2 L bioreactors. The growth of *Aurantiochytrium* sp. TC 20 was also investigated using glycerol as a carbon source. Glycerol is becoming increasingly available, because it is a by-product of biofuel production from vegetable oil and animal fats. Fortification of the feed with additional nutrients improved the biomass yield from 56 g/L (34 % total fatty acids) to 71 g/L (52 % total fatty acids, cell dry weight) at 69 h.

A life-cycle assessment, from the upstream biomass production to the direct emission of biodiesel combustion, was applied to assess the energy balance and the potential environmental impacts of this heterotrophic microalgal-derived biodiesel. The scenario analysis of a virtual production facility, modeled on experimental yield data, demonstrated that cultivation of heterotrophic microalgae for the production of biodiesel is comparable in terms of greenhouse gas emissions and energy usage to production of petroleum diesel. The LCA identified that improvements in cultivation conditions, in particular the bioreactor energy inputs and microalgae yield, will be critical in developing a sustainable production system. This study demonstrates the potential of heterotrophic cultivation of newly isolated endemic thraustochytrids to provide Australia's transportation fleet with a secure, environmentally sustainable alternative fuel feedstock, and co-production of high value bioproducts that can provide additional revenue to benefit the economics of biofuel production.

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Chapter 1 Introduction: Microalgae - A renewable source of biofuels, omega-3 oils and other co-products

Background

Microalgae are a large and diverse group of aquatic plants found in both freshwater and marine environments. Important components of many ecosystems, they constitute a major food source for organisms at the base of the food web, and are responsible for over half of the global primary productivity (Van den Hoek et al. 1995). The process in nature that forms petroleum from sedimentary organic matter (including microalgae) is thought to take millions of years and occurs through processes involving underground heat and pressure (Walters 2006). Phototrophic microalgae utilize solar energy to combine water with carbon dioxide (CO_2) to create biomass, which contains lipids (oils), carbohydrates, proteins and other biochemical constituents. Alternatively, heterotrophic microalgae can grow on organic substances such as sugars and organic acids as the sole source of carbon and energy (Spolaore et al. 2006).

Microalgal biomass has been utilized as a feedstock for direct extraction of oil to produce biofuels. Several different types of renewable biofuels derived from microalgae have been studied extensively. These include biohydrogen (Esper et al. 2006; Kapdan and Kargi 2006; Levin et al. 2004), methane (Spolaore et al. 2006), hydrocarbons from the green alga *Botryococcus braunii* (Casadevall et al. 1985; Banerjee et al. 2002; Qin 2005), and lipid-based biodiesel (Schenk et al. 2008; Griffiths and Harrison 2009).

The potential of biofuel production from microalgae is of intense interest internationally with growing concerns on the peak oil crisis and is the subject of extensive exploration (Greenwell et al. 2009; Chisti 2007; Brennan and Owende 2010; Donohue and Cogdell 2006; Qiang et al. 2008; Huntley and Redalje 2007; Schenk et al. 2008).

Cultivation of microalgae

Microalgae have been historically cultivated as a source of food supplements in human nutrition e.g., *Arthrospira* (*Spirulina*) in Asia, Central America and Africa (Lee 1997). Due to their inherent biochemical properties, microalgae have also been developed for various applications including aquaculture and agrifeeds, nutraceuticals and high value biochemicals, as well as wastewater treatment (Spolaore et al. 2006).

Despite the significant potential of commercial microalgae cultivation, several challenges have hindered the development of the technology to large-scale commercial viability that would allow economic sustainability for commodity production. The high capital cost of the existing technologies and the need to design a high yield production system matching specific microalgal species or strains have generally made commercial production economically unfeasible (Benemann 2008).

Open versus closed cultivation systems

Under natural growth conditions, phototrophic algae absorb sunlight and CO_2 from the air and nutrients in their aquatic habitats. Algae can be cultivated photoautotrophically, where they use light as a sole energy source that is converted to chemical energy through photosynthesis. The use of natural sun light and CO_2 in an open pond system replicates the optimum natural growth conditions (Brennan and Owende 2010). The U.S. Department of Energy's

Aquatic Species Program (ASP) demonstrated that open ponds are the optimal economic design and used them for its experiments (Sheehan et al. 1998b). Most industrial large-scale production is done in "open ponds" and uses natural sunlight (Ogbonna and Tanaka 1998; Benemann 2008). In addition, controlled artificial light sources are used in a closed cultivation systems known as photobioreactors (PBRs). In PBRs microalgae can sustain high growth rates with high irradiance (i.e. high energy input) (Richmond 2004).

Open pond systems are less efficient than closed photobioreactors in terms of biomass productivity. This difference can be attributed to several factors, including evaporation losses, temperature fluctuation in the growth media, CO_2 deficiencies, inefficient mixing, and light limitation (Chisti 2007; Brennan and Owende 2010). A comparison of open pond and different types of bioreactor systems is given in Table 1-1.

Alternatively, heterotrophic microalgae grow in stainless steel fermenters on organic substances (e.g. sugars, organic acids) as the only carbon and energy source. Some microalgae (such as *Chlorella vulgaris*) can grow under heterotrophic, autotrophic, as well as mixotrophic conditions, which is a combination of heterotrophic and autotrophic growth (Liang et al. 2009). Several studies reveal that heterotrophic cultivation has the potential for higher production of biomass than autotrophic cultivation and accumulation of high lipid content in cells (Brennan and Owende 2010; Liang et al. 2009). This high production can be pinpointed to several causes, including simpler scale-up settings (independent of light, less land required), a high degree of growth control, minimal contamination, low capital cost (per unit biomass produced per time) and higher cell densities than are achievable in photoautotrophic systems (Brennan and Owende 2010).

Table 1-1 Advantages and limitations of open ponds and photobioreactors (adapted from Brennan and Owende, 2010)

System	Advantages	Limitations
Raceway pond	Relatively cheap	Poor biomass productivity
	Easy to clean	Large area of land required
	Utilises non-agricultural land	Limited to a few strains of algae
	Low energy inputs	Poor mixing, light & CO_2 utilisation
	Easy maintenance	Cultures are easily contaminated
Tubular photobioreactor	Large illumination surface area	Some degree of wall growth
	Suitable for outdoor cultures	Fouling
	Relatively cheap	Requires large land space
	Good biomass productivities	Gradients of pH, dissolved oxygen & CO_2 along the tubes
Flat plate photobioreactor	High biomass productivities	Difficult scale-up
	Easy to sterilise	Difficult temperature control
	Low oxygen build-up	Small degree of hydrodynamic stress
	Readily tempered	Some degree of wall growth
	Good light path	
	Large illumination surface area	
	Suitable for outdoor cultures	
Column photobioreactor	Compact	Small illumination area
	High mass transfer	Expensive compared to open ponds
	Low energy consumption	Shear stress
	Good mixing & low shear stress	Sophisticated construction
	Easy to sterilise	
	Reduced photoinhibition & photo-oxidation	

Energy balance - inputs, outputs - is very important when considering energy products such as biofuels. Key factors such as strain selection, photosynthetic efficiency (for autotrophic cultivation), the utilisation of sugars (for heterotrophic cultivation), productivity for lipids and ease of harvesting, determine the energy balance of the production system, as well as the economic viability of production (Brennan and Owende 2010). Clarens et al. (2010) showed that algae-derived biofuel production is energy intensive and requires six times the energy of growing crops with greater net greenhouse gas emissions than for other biofuel sources. However, this study was compromised by the assessment of life-cycle impact as this was based on computer models of decades-old data and out-dated production and refining technologies.

Potential of microalgal oil for transport fuels

The use of microalgae as alternative sources of energy has been explored since the 1950s and the energy shocks in the 1970s prompted widespread interest in microalgal biofuels, such as liquid transport fuels as well as hydrogen production by microalgae and methane production from waste water treatment (Benemann 2000; Levin et al. 2004; Esper et al. 2006). These studies previously demonstrated the promise of the technology, but the commercial success of large-scale production has been elusive.

From 1978-1996 the US Department of Energy funded a program known as the Aquatic Species Program (ASP) to develop biodiesel from algae. The program focused on the production of biodiesel from high lipid-content microalgae grown in ponds, utilizing waste CO_2 from coal-fired power plants (Sheehan et al. 1998b). However, the program was discontinued because of federal budget cutbacks and low oil prices, making production of algae for biofuels an uneconomic endeavour. The estimated cost of algal oil production was in the range US$ 40 – $ 60 per barrel compared to US$ 20 per barrel for crude oil in

1995 (Pienkos and Darzins 2009). Sun et al. (2011) demonstrated that algal oil production costs US$ 10.87 –13.32 per gallon i.e. to produce US$ 456.54 - 559.44 per barrel, while the current crude oil price is US$ 97 per barrel. Since the mid 1990s a number of reviews have examined the potential of microalgae as a biofuel source. Until the present time, various studies have again not yet achieved any major advances in terms of productivity and therefore cost (Chisti 2007; Sheehan et al. 1998b; Benemann 2008).

Some biotechnology companies are now claiming that they can produce up to 6000 gallons of fuel per acre per year (Mascarelli 2009a). Solazyme, Inc. (South San Francisco, USA) has reported using heterotrophic microalgae to produce more than 10,000 gallons of oil at a quality that meets existing fuel standards. The company signed an US$ 8.5 million deal to produce commercial quantities of algal fuel for the US Navy (Mascarelli 2009b). Solazyme's fuel feedstock has already been demonstrated and approved as a commercial aviation fuel blend (Goossens 2011).

Industry giants such as Dow Chemical Co., ExxonMobil Corp., British Petroleum (BP) P.L.C, and Chevron Corp. have recently invested in companies seeking to develop renewable liquid fuels from microalgae (see Table 1-2). The U.S Department of Energy has allocated nearly US$ 80 million for advanced biofuels research and fuelling infrastructure (US DOE 2010b).

Table 1-2 Major investments during 2008 - 2009 in biotech companies to develop renewable liquid fuels from microalgae in USA (summarised from Mascarelli, 2009b)

Biotech company	Investor	Goals
Algenol Biofuels (Bonita Springs, Florida) <www.algenolbiofuels.com>	US$ 25 million grant from the US Department of Energy applied by Dow Chemical Company	Ethanol production from algae
Martek Biosciences (Columbia, Maryland), now DSM <http://www.martek.com>	US$ 10 million from BP	Algal biofuel
Sapphire Energy (San Diego, California) <www.sapphireenergy.com>	US$100 million from Gates's Cascade Investments and the Rockefeller family's venture-capital firm Venrock.	Genetic engineering to improve algal traits, including improved protection from predators and low cost harvestability. Genetically manipulate the algae to produce oils that are nearly identical to crude oil
Solazyme (South San Francisco, California) <www.solazyme.com>	US$ 8.5 million from the US Navy. Undisclosed amount from Chevron Corp	Produce commercial quantities of algal fuel
Synthetic Genomics (La Jolla, California) <www.syntheticgenomics.com>	US$ 600 million over 5 to 6 years from ExxonMobil Corp.	Produce liquid transportation fuels from algae

The interest in and demand for a renewable source of transport fuels from microalgae is now booming globally due to the increasing concerns about rising crude oil prices, global warming and energy security (Chisti 2007; Gavrilescu and Chisti 2005). Other factors driving the trends include government incentives and the opportunity to reduce greenhouse gas emissions, as well as the rise of new regional business enterprises. In Australia, there are a numbered of initiatives underway. The Moree Plains Shire Council in New South Wales signed a memorandum of understanding (MoU) with Cubic QED to develop an algae-based biodiesel plant. The company projected a total of 2.4 tonnes of microalgal biomass per day that will produce 1200 litres of biodiesel a day, with the dried by-products fed to livestock as a high nutrient supplement (Cubic 2009).

In South Australia, a new AU$ 5 million Algae Biofuels Facility has been established at the aquatic science laboratories of the South Australian Research and Development Institute (SARDI). The new facility was funded through the National Collaborative Research and Infrastructure Strategy (NCRIS). One of its major clients was the South-Australian based Algal Fuels Consortium ™ comprising SARDI Aquatic Sciences, Flinders Partners, Flinders University, CSIRO Energy Transformed Flagship and Sancon Resources Recovery (Homer 2010).

MBD Energy has partnered with James Cook University (JCU) to develop a new algal biofuel research and development facility at the university's Townsville campus. The plant aims to develop algal carbon capture and storage technology in plastic sausage-shaped photobioreactors (Solar Environmental Tube System). The company claimed that the 5000 square-metre research plant has the capacity to produce 14,000 litres of oil and 25,000 kilograms of algal feed for livestock from every 100 tonnes of carbon consumed (MBD Energy 2010).

Benefits of algal derived biofuel

Renewable transport fuels, such as bioethanol from carbohydrates (sugar), and biodiesel from lipids (oils), have been commercially produced from specific crops such as soybean, palm oil, sugarcane, corn, rapeseed, flax and jatropha (Schenk et al. 2008; Chisti 2007). However, these agrofuels (biofuels that are produced from specific food crops rather than from waste processes) have been criticized for their devastating environmental impacts, especially deforestation for the creation of monoculture plantations. In addition, they have been held accountable for worsening food crises in developing and third world countries since the use of food crops for agrofuel production decreases food supply production (Rosegrant 2008).

The advantages of using microalgae for biofuel production is that algae can be cultivated in saline or brackish water on non-arable land that is not favourable for terrestrial plant growth. Moreover, microalgae have the capacity to produce considerable oil that could exceed 60 % by weight of dry biomass in some species (Metting 1996). They can grow very rapidly with a doubling time of 3.5 h during exponential growth (Chisti 2007). As well, microalgae perform 10 – 50 times more efficient photosynthesis than terrestrial plants (Huntley and Redalje 2007). Unlike terrestrial plants that have limited harvest times, microalgae can be harvested for much of the year. Their advantages extend to a potential annual oil productivity that is higher than those for conventional crop based biodiesel. Whereas rapeseed typically yields 1190 L/ ha of biodiesel, microalgae could generate at least 12,000 L/ ha of biodiesel (Schenk et al. 2008; Sheehan et al. 1998b).

Another interesting and key aspect about photosynthetic microalgae is that they can even exploit waste CO_2 resources, such as exhaust fumes from coal power plants, and fix CO_2 from the atmosphere. The energy efficiency of microalgae has prompted interest in their use for greenhouse gas (GHG) reductions by capturing CO_2 and power plant flue gases for GHG abatement (Hsueh et al. 2007). It is estimated that replacing just 10 % of Australia's mineral diesel with microalgae-derived biodiesel would bring about a reduction of nearly four million tonnes of carbon dioxide emissions from fossil fuels (Rann 2009). This can be in conjunction with wastewater treatments or co-production of high value co-products (Harmelen and Oonk 2006).

In another venture, JCU and MBD Energy will collaborate to construct test facilities at the Tarong Energy Power Station in Queensland, and two coal-fired power stations at Loy Yang Power in Victoria and Eraring Energy in New South Wales. The trial aims to use microalgae to capture carbon dioxide greenhouse gases from the power stations' emission chimneys and the resultant microalgal biomass used for oil and algal meal production. These products could be used to replace fish oil and fish meal, both of which are under increasing and unsustainable global demand (MBD Energy 2010). The Western Australian government recently agreed to fund a AU$ 10 million pipeline that will carry seawater to a commercial facility in Karratha invested by Aurora Algae, Inc., which is developing a commercial facility estimated at AU$ 86 million that will produce biodiesel, omega-3 fatty acids for health supplements, and protein-rich biomass for aquaculture and animal feed (Lees 2013). As a result, microalgae are presently attracting intense interest in industry for their promising and significant potential to displace some of the huge amount of global petroleum diesel usage.

Nevertheless, microalgae derived biodiesel is presently considered an expensive venture due to the high investment cost and intensive facility maintenance required. The photosynthetic efficiencies of cultivation systems need to be improved to support high cell density growth (i.e. low biomass presently occurs due to the limiting factors of light penetration, gas exchange, nutrient mixing) in order to create enough fuel to be competitive with petroleum diesel on a large scale (Brennan and Owende 2010). Furthermore, the high costs of the harvesting and extraction systems used for algal biomass obtained from small cell size species have impeded the development of algal biofuel technology for commercial production. Other major challenges remaining are algal strain selection and optimization of biomass and oil production for an effective and efficient production system (Chisti 2008).

Biodiesel potential of algae

Biodiesel is the methyl esters of fatty acids (fatty acid methyl ester [FAME]), and is produced by transesterification of triacylglycerols (TAG) obtained from plant seeds with high natural oil content (e.g. rapeseed oil, soybean oil, palm oil, and sunflower seed) or animal fats (Chisti 2007). The interest in microalgae for biodiesel production is due to the high TAG content of some species, which are the best substrate to produce biodiesel. The total lipid content in microalgae may vary from 1– 85 % (Table 1-3) of dry weight, with values higher than 40 % being typically achieved under nutrient limitation (Chisti 2007).

Table 1-3 Oil content of selected microalgae (data from Chisti, 2007)

Class	Species	Oil content (% dry wt)
Bacillariophyceae	*Cylindrotheca* sp.	16–37
Bacillariophyceae	*Nitzschia* sp.	45–47
Bacillariophyceae	*Phaeodactylum tricornutum*	20–30
Chlorophyceae	*Botryococcus braunii*	25–75
Chlorophyceae	*Chlorella* sp.	28–32
Chlorophyceae	*Dunaliella primolecta*	23
Chlorophyceae	*Nannochloris* sp.	20–35
Chlorophyceae	*Neochloris oleoabundans*	35–54
Dinophyceae	*Crypthecodinium cohnii*	20
Eustigmatophyceae	*Nannochloropsis* sp.	31–68
Eustigmatophyceae	*Monallanthus salina*	> 20
Haptophyceae	*Isochrysis* sp.	25–33
Labyrinthulomycetes	*Schizochytrium* sp.	50–77
Prasinophyceae	*Tetraselmis suecica*	15–23

Temperature, irradiance and nutrient availability have been shown to affect both lipid composition and lipid content in many algae (Harwood and Guschina 2009; Qiang et al. 2008). In general, high irradiances stimulate TAG accumulation (Roessler, 1990), while under low irradiances, mainly polar lipids (phospholipids and glycolipids), structurally and functionally associated with cell membranes, are synthesized (Qiang et al. 2008).

The types of fatty acids present in microalgal TAG will affect the biodiesel quality. The quality of biodiesel is quantified using cetane number, cold-flow properties, oxidative stability, and iodine value, which are determined by the structure of the FAME (Chisti 2007). In turn, the properties of FAME are determined by the characteristics of the fatty acids. Fatty acids with fewer double bonds (the degree of unsaturation) and shorter chain length (number of carbons < C_{20}) are more desirable for biodiesel production due to their oxidative and thermal stability. Oxidation results in the formation of undesirable products, such as alcohols that reduce the flash point of biodiesel, aldehydes that cause rancidity, and short-chain fatty acids that are corrosive to engine components (Knothe 2007; Monyem et al. 2000). Furthermore, saturated fatty acids typically have higher solidification temperatures and therefore cannot be used at lower temperatures. The large amount of PUFA with four or more double bonds in some microalgal oils limits the number of microalgal species that can be used (Rodolfi et al. 2009).

The cetane number, which is derived from a standardised test used to measure the ignition quality of diesel fuel, is the key indicator of the quality of diesel fuel. Cetane number increases with increasing length of fatty acid carbon chains and more saturation (fewer double bonds) (Gerpen 2005). The current standard for diesel sold in Australia requires a minimum cetane number of 46, while European diesel has a minimum cetane number of 51. Cetane number affects

combustion irregularity as it relates to the ignition delay time of a fuel upon injection into the combustion chamber. An adequate cetane number for a specified engine associates with improving ignitions, fuel combustion, reduced noise and white smoke that contains carbon monoxide (CO), nitrogen oxide (NO_x), and hydrocarbon (HC) emissions (Wallington et al. 2006).

Strain selection for production of biodiesel

Strain selection should involve screening a wide range of natural isolates for traits including high growth, high lipid productivity, broad environmental tolerances, and co-production of high value-added by products. Strain selection also requires coupling with high biomass production technologies. Genetic and metabolic engineering are likely to have an impact on the performance of algal strains for biofuel production (Dunahay et al. 1996). However, the development of microalgae in Australia for biodiesel necessitates matching algal strains to climate and, preferably using endemic strains to protect Australia's biodiversity and to adhere to Australian Quarantine and Inspection Service (AQIS) regulations (Blackburn et al. 2005; Blackburn and Volkman 2012).

The US Department of Energy's ASP showed that after screening, isolation, and characterisation, the 300 species considered most suitable out of 3000 strains of oil-producing organisms in the collections were mostly green algae and diatoms (Sheehan et al. 1998b).

Potential of microalgae for omega-3 oils and co-products

It is generally acknowledged that dietary consumption of omega-3 long-chain polyunsaturated fatty acid (LC-PUFA), in particular DHA, has many benefits in human health (Tapiero et al. 2002). Recent studies have shown that the consumption of omega-3 LC-PUFA helps prevent the risk of cardiovascular diseases, neural disorders, arthritis, asthma and skin diseases in humans (Takahata et al. 1998; Horrocks and Yeo 1999; He et al. 2004; Horrocks and Farooqui 2004; Kris-Etherton et al. 2004; Balk et al. 2006; Mozaffarian and Rimm 2006; Lin et al. 2010). The main source of the omega-3 LC-PUFA to date has been from fish oil which was originally a by-product of the fish meal industry (Armenta and Valentine 2013). However the production of fish meal and oil (ie. including for use in aquafeeds and fish oil capsules) is dependent on wild fisheries and there is a growing concern about the health of ocean fish stocks, the ecological effects of industrial fishing and also the levels of pollutants in some oils (Pauly et al. 2002). The average Australian and many other western diets have been recognized as being deficient in omega-3 LC-PUFA (Meyer et al. 2003; Danaei et al. 2009), highlighting the importance of and need for increased availability and consumption of the health-benefitting omega-3 LC-PUFA. Furthermore, the growing worldwide population will place further pressure on the fish oil supply. Microalgae such as members of the genera *Thraustochytrium, Nannochloropsis*, *Attheya, Pseudonitzschia* and *Rhodomonas* possess the ability to produce a number of omega-3 LC-PUFA, especially DHA and EPA.

Already a small player in the supply chain for these valuable oils, microalgae and other microbial sources of oil could be an increasing contributor to future renewable sources of omega-3 LC-PUFA rich oil for the nutraceutical industry.

In addition to their growth and harvest for oils (for biofuels and omega-3 LC-PUFA), microalgae can produce a wide range of potential bioproducts (e.g.

carotenoid pigments, industrial enzymes, and exopolysaccharides (EPS) and processing byproducts - such as algal meal - may be of use in animal and fish feeds, adding greater value to the production process pipeline with improved economics (Ratledge and Cohen 2008; Huntley and Redalje 2007; Li et al. 2008; Stephens et al. 2010; Wijffels and Barbosa 2010). The carotenoid pigments such as astaxanthin, which is a valuable red carotenoid pigment, is used as a nutraceutical as well as for cosmetics due to its high antioxidant activities (Lorenz and Cysewski 2000). It is also a supplement for pigmentation in aquaculture (Boussiba 2000; Johnson and An 1991). The co-production of both biodiesel and value-added bioproducts presents a way to compensate the expensive technology needed for production and extraction of microalgal oils.

However, there are a few microalgal species cultured globally for health food supplements. Large-scale phototrophic commercial cultivation of *Chlorella vulgaris* commenced during the 1960s by Japanese companies such as Chlorella Industry (Minato, Tokyo) and Nichon Chlorella (Kunitchi, Tokyo) (Lee 1997; Borowitzka 1999; Spolaore et al. 2006). In the USA, *Spirulina* (*Arthrospira*) plants were established by Cyanotech (Hawaii, USA) and Earthrise Farms (California, USA). In South Australia and Western Australia, the green alga *Dunaliella salina*, a source of β-carotene, is commercially produced in hypersaline outdoor opens ponds by BASF (Baden Aniline and Soda Factory, previously Betatene Pty Ltd). This operation is the largest producer of natural β-carotene globally (> 80 %) for dietary supplements, food colourings, and aquaculture feed (Curtain 2000).

Martek Biosciences (Columbia and Boulder, USA; the latter operation formerly known as OmegaTech), that is now part of the Royal DSM (Dutch State Mines), are producing microalgae heterotrophically (using glucose and yeast extract) in conventional fermentors for the production of long-chain (LC, $\geq C_{20}$) omega-3 fatty acids (Barclay et al. 1994a). Martek uses the diatom *Nitzschia alba* (high in

eicosapentaenoic acid, 20:5ω3 [EPA]) and the heterotrophic dinoflagellate *Crypthecodinium cohnii* (high in docosahexaenoic acid, 22:6ω3 [DHA]) for their production of LC omega-3 oils. Martek also cultures thraustochytrids heterotrophically to produce LC omega-3 fatty acids such as EPA and DHA (Raghukumar 2008). More recently, companies such as Cyanotech (Hawaii, USA), Mera Pharmaceuticals (Hawaii, USA) have used *Haematococcus pluvialis* to produce the carotenoid pigment astaxanthin.

Rationale for this study

The potential of biofuel production from microalgae has been of increasing interest due to growing concerns with rising crude oil prices, future availability and the overuse of fossil fuels. In addition to utilizing the lipids for biofuel production, microalgae are capable of producing other high-value bioproducts, such as proteins, enzymes, PUFA, carotenoid pigments and EPS. The formation of high-value co-products during biofuel production is desirable when it adds greater value to the production process and improved process economics (Li et al. 2008; Stephens et al. 2010; Wijffels and Barbosa 2010). Due to their fast growth rate and high lipid content, thraustochytrids have potential for producing both a feedstock for omega-3 LC-PUFA rich oils as well as the less unsaturated fatty acids suitable for biodiesel. Despite the widespread interest in thraustochytrids, limited data is available for endemic Australian strains due to national quarantine restrictions. The availability of thraustochytrid strains from culture collections and information for optimal growth conditions will be required for any future commercial production of this sought after group.

Objectives of this study

The overall objective of this project is to investigate microalgal strains, especially thraustochytrids, from Australian waters for their capacity to co-produce biofuels, omega-3 oils and other co-products as well as biomass for food, feed and fuel applications. Developing microalgae for biomass and oil production requires significant consideration on whether different oils for different applications can be economically obtained from one type of microalgae.

This following approach was used to address this objective:

While the Australia National Algae Culture Collection (ANACC) is a very valuable resource of microalgal biodiversity, it was necessary to undertake targeted biodiscovery for high oil producing thraustochytrids from hypersaline and other extreme environments. This study was focused on the biodiscovery process that included sampling, isolation and characterising new thraustochytrid strains from the southeast coast of Tasmania and far north Queensland (Chapter 2).

The fatty acid and sterol profiles were used to characterise the newly isolated thraustochytrid strains into eight chemotaxonomic groups (A – H). Chemotaxonomic profiles were compared with the corresponding 18S rRNA gene sequence data to examine the potential to be useful tools in the interpretation of phylogenetic relationships. The screening trial led to the identification of a number of unusual odd-chain (OC) –PUFA: 19:4ω5, 21:4ω5, 21:5ω5 and 21:4ω7, in thraustochytrid strains of chemotaxonomic groups A, B and C (Chapter 3).

Thraustochytrids not only possess the ability to produce a number of ω3 LC-PUFA (DHA and EPA), but also have demonstrated their potential for other co-products such as EPS. In the subsequent trials, eight thraustochytrid strains from the different chemotaxonomic groups (A – H) were compared in 1 L scale baffled shake flasks for the synthesis of EPS, in addition to determination of biomass yield and fatty acid profiles (Chapter 4). The growth rate, biomass and lipid productivities of these strains were examined with two different glucose concentrations, 2 % w/v or 4 % w/v in batch cultures conditions.

An interesting potential of culturing thraustochytrids is to use glycerol as a carbon source since glycerol is byproduct of biodiesel production especially from plant and animal oils, and its excess production is problematic. The growth of *Aurantiochytrium* sp. TC 20 was investigated using glycerol as a carbon source and the biomass was optimized in 2 L bioreactors (Chapter 5).

A preliminary life-cycle assessment was performed to evaluate the energy balance and the potential environmental impacts of this heterotrophic microalgal-derived biodiesel (Chapter 6). The use of a glycerol stream as a carbon source for a heterotrophic algal production system not only could potentially reduce the greenhouse gas emissions, but also substantially reduce the cost of commercial production of algal-derived biodiesel. The development of a hypothetical biodiesel commercial production scenario was developed and later populated using information from the literature as well as results from engineering correlations. Cultivation data is based on experimental data from our 2 L lab scale fed-batch bioreactors (Chapter 5).

Chapter 2 Biodiscovery of new Australian thraustochytrids for production of biodiesel and long-chain omega-3 oils

Abstract

Heterotrophic growth of thraustochytrids has potential in co-producing a feedstock for biodiesel and long-chain (LC, ≥C_{20}) omega-3 oils. Biodiscovery of thraustochytrids from Tasmania (temperate) and Queensland (tropical), Australia, covered a biogeographic range of habitats including fresh, brackish and marine waters. Thirty six thraustochytrid strains were isolated and separated into eight chemotaxonomic groups (A- H) based on fatty acid (FA) and sterol composition which clustered closely with four different genera obtained by 18S rDNA molecular identification. Differences in the relative proportions (% FA) of long-chain C_{20}, C_{22}, omega-3 and omega-6 polyunsaturated fatty acids (PUFA), including docosahexaenoic acid (DHA), docosapentaenoic acid, arachidonic acid, eicosapentaenoic acid (EPA) and saturated FA, as well as the presence of odd-chain PUFA (OC-PUFA) were the major factors influencing the separation of these groups. OC-PUFA were detected in temperate strains of groups A, B and C (*Schizochytrium* and *Thraustochytrium*). Group D (*Ulkenia*) had high omega-3 LC-PUFA (53 % total fatty acid [TFA]) and EPA up to 11.2 % TFA. Strains from groups E and F (*Aurantiochytrium*) contained DHA levels of 50-61 % TFA after 7 days of growth in basal medium at 20 °C. Group G and H (*Aurantiochytrium*) strains had high levels of 15:0 (20-30 % TFA) and the sum of saturated FA was in the range 32-51 %. β,β-Carotene, canthaxanthin and astaxanthin were identified in selected strains. Phylogenetic and chemotaxonomic groupings demonstrated similar patterns for the majority of strains. Our results demonstrate the potential of these newly isolated Australian thraustochytrids for the production of biodiesel in addition to omega-3 LC-PUFA rich oils.

Introduction

The interest in and demand for a renewable source of transport fuels from alternative feedstock such as microalgae is now expanding globally due to increasing concerns about the peak oil crisis, rising crude oil prices, global warming and energy security (Chisti 2007; Gavrilescu and Chisti 2005). Microalgal strain selection and optimization of growth and oil production remain key issues. Development of microalgae in Australia for biodiesel requires matching algal strains to climatic conditions and, in particular, using endemic strains to protect Australia's unique biodiversity.

Thraustochytrids are heterotrophic protists found ubiquitously in the marine environment (Barclay et al. 1994b). It is considered that thraustochytrids play an important role in the marine ecosystem: they can be bacterivores, detritivores and parasites (Maas et al. 1999; Mo et al. 2002; Raghukumar 2002). Thraustochytrids were originally considered to be closely related to primitive fungi and protozoans (Ellenbogen and Aaronson 1969), however, recent molecular phylogenetic studies have resulted in their classification into the class Labyrinthulomycota and phylum Heterokonta within the kingdom Chromista. This phylum also includes the chromophyte algae such as brown algae and diatoms (Porter 1990; Cavalier-Smith et al. 1994; Leander et al. 2004).

Thraustochytrids have received increasing attention in recent years owing to their capacity to produce high amounts of omega-3 long chain ($\geq C_{20}$) polyunsaturated fatty acids (omega-3 LC-PUFA), including docosahexaenoic acid (DHA, 22:6ω3) and eicosapentaenoic acid (EPA, 20:5ω3) and, for a limited number of strains, the omega-6 LC-PUFA arachidonic acid (AA, 20:4ω6) (Iida et al. 1996; Jiang et al. 2004; Jain et al. 2007; Lewis et al. 1999).

Many studies have shown that the consumption of omega-3 LC-PUFA, in particular DHA, helps prevent the risk of cardiovascular diseases, neural disorders, arthritis, asthma and skin diseases in humans (Takahata et al. 1998; Horrocks and Yeo 1999; Horrocks and Farooqui 2004; Kris-Etherton et al. 2004). DHA is also essential for neural and retinal development during fetal life and infancy (Ratledge 2004; Forsyth and Carlson 2001).

The current major global source of omega-3 LC-PUFA is fish oil, which is sourced from wild fisheries. However, there is a growing concern about the health of ocean fish stocks, the ecological effects of industrial fishing and also the levels of pollutants in some fish oils (Pauly et al. 2002). Furthermore, the growing worldwide population and the expanding aquaculture industry are now placing pressure on both fisheries and fish oil supply. Thraustochytrids are an alternate renewable source of omega-3 LC-PUFA rich oil for the feeds, food, nutraceutical and other industries.

Due to their fast growth rate and high lipid content, thraustochytrids have potential for producing both a feedstock for omega-3 LC-PUFA rich oils as well as the shorter chain fatty acids suitable for biodiesel (Johnson and Wen 2009). Despite the widespread interest in thraustochytrids, the availability of strains from culture collections is currently limited (Rosa et al. 2010a).

The objective of this study was to investigate new thraustochytrid strains recently isolated from Australian waters for their potential in co-producing a feedstock for biofuels, omega-3 LC-PUFA rich oils and other co-products, as well as to provide biomass for food, feed and fuel applications. This study provides a perspective on the biodiscovery of Australian thraustochytrids from the southeast coast of Tasmania (temperate) and north of Queensland (tropical).

The specific aspects of biodiscovery relevant to thraustochytrids include isolation from environmental samples, identification, and screening of thraustochytrids for their biotechnological potential, and as a source of carotenoid pigments, biofuels and omega-3 LC-PUFA. In addition, the profiling of the fatty acids and sterols, and the 18S rRNA gene sequence data from these new Australian thraustochytrid strains has enabled their taxonomic grouping based on both molecular and chemical data.

Materials and methods

Sampling location, isolation and growth of culture organisms

Samples were collected from 42 sites along three different transects in two locations: temperate Tasmania and tropical Queensland (Table 2-1). All samples were collected from moist sediments, which were periodically covered by seawater or freshwater and included shells, leaf litter, seaweeds or small stones from the same site. GPS locations and the salinity of the water collected with each sample was also recorded.

Samples were collected in sterile 50 mL centrifuge tubes (Iwaki, Japan) from estuarine environments, and were stored at <10 °C and subsequent isolation steps were performed within 24 h. Samples were initially diluted by ten-fold serial dilution and plated on solid culture medium and subsequently incubated at 20°C. Isolation medium was prepared by dissolving 1 % (w/v) glucose, 0.2 % (w/v) peptone, 0.2 % (w/v) yeast extract, 0.5 % (w/v) monosodium glutamate, 0.2 % (w/v) corn steep liquor and 1 % (w/v) agar (Sigma-Aldrich, St. Louis, MO, USA) in 1 L of filter-sterilised natural seawater (diluted to 20- 50 % (v/v) sea water depending on sample salinity at collection, Table 2-1), which was treated using a filtration series incorporating three 12" Millipore® cartridge filters (5 µm prefilter, activated charcoal filter for organics removal and a Durapore™ 0.45 µm

filter) and finally a Millipak-40™ 0.22 µm disc filter. This medium was adapted from Honda et al. (1998) and Rosa et al. (2010). The mixture was autoclaved at 110 °C for 20 min, cooled to about 50 °C and 1 mL of antibiotic solution was aseptically added. The antibiotic solution was prepared by dissolving penicillin (60 mg) and streptomycin (250 mg) in 100 mL of distilled water filter sterilized with 0.2 µm filter (Raghukumar 1992).

The inoculated agar plates were examined daily by light microscopy (Zeiss Axioplan Compound Microscope, NY, USA) and thraustochytrid-like colonies were aseptically transferred to new agar plates and incubated at 20 °C. Morphological characteristics of thraustochytrids include the presence of ectoplasmic net elements and zoospores with heterokont flagella. Furthermore, the vegetative stages of most thraustochytrids consist of clusters of single cells that are spherical to ovoid in shape measuring 5-20 µm in diameter (Porter 1990; Honda et al. 1998; Raghukumar 2002; Leander et al. 2004). After 2-7 days incubation at 20 °C, all axenic colonies growing on the agar plates were streaked onto a different plate consisting of the same agar medium without antibiotics to check for purity. A maintenance protocol involving cryo-preservation of strains in liquid nitrogen was adopted from Cox et al. (2009).

Thraustochytrids were characterized based on morphological examination using light microscopy (Zeiss Axioplan Compound Microscope, NY, USA) and molecular identification (18S rRNA gene sequences). These strains are held in the Australian National Algae Culture Collection (http://www.csiro.au/ANACC). Cultures were maintained on a basal medium consisting of: 1 L natural seawater, 0.5 % (w/v) of bacteriological peptone, 0.2 % (w/v) glucose and 0.2 % (w/v) yeast extract (Sigma-Aldrich, St. Louis, MO, USA).

Table 2-1 Sample collection sites and habitat in this study

Habitat within section of transect	Distance from origin along transect (km)[1]	Number of sites	Salinity range of samples
Temperate Tasmania			
Sandy beach	0- 0.5	3	34.6- 35
River mouth and estuary	0.6- 1.0	6	33.3- 34.4
Mud flats	1.1- 1.2	3	26.2- 36.8
Salt marsh	0.9- 1.1	3	27.9- 36.5
River, billabong, dam (freshwater)	1.2- 1.4	5	0.1- 7
Tropical Queensland transect A[2]			
Mud flats adjacent to mangrove forest	0- 0.1	2	30.2- 33.2
Seaward edge of mangrove forest	0.09- 0.1	3	33.2- 34.4
Within the mangrove forest	0.07- 0.1	2	34.4- 40.4
Landward edge of mangrove forest	0.13- 0.3	3	34.4- 40.4
Tropical Queensland transect B[2]			
Seaward edge of mangrove forest	0- 0.14	3	33
Within the mangrove forest	0.06- 0.29	6	34- 40
Landward edge of mangrove forest	0.33- 0.55	3	30- 31

[1]Distance from origin refers to the distance between the sampling sites from the first sampling point. Overlap of distance between habitat was due to the side of transect sampled. [2]Tropical transects A and B were 2 km apart.

Cultures were grown in sterile 50 mL polystyrene cell culture flasks containing a cap fitted with a 0.2 μm filter (Greiner Bio-One, Germany) and were shaken at 100 rpm (20 °C) to facilitate culture mixing for 7 days. To harvest biomass the culture broth was filtered through pre-weighed 47 mm diameter Whatman® GF/C glass fiber filters (Sigma-Aldrich, St. Louis, MO, USA). After rinsing with 3.15 % (w/v) ammonium formate, the filters were folded and wrapped in aluminum foil and placed directly into liquid nitrogen (-196 °C). Prior to analysis the samples were freeze-dried overnight and weighed to determine dry weight.

Preparation and analysis of fatty acid methyl esters (FAME) and sterols

Freeze-dried samples were directly transesterified with methanol: chloroform: HCl (l0:1:1 v/v/v) to produce FAME and free sterols. BSTFA (N,O-Bis(trimethylsilyl)trifluoroacetamide) (Altech, Deerfield, IL, USA) was added to convert any hydroxyl-containing lipids (such as sterols) to their OTMSi-ether lipids, as described in Lee Chang et al. (2011). Gas chromatographic analysis was carried out on a Varian 3800 gas chromatograph equipped with a HP5 ultra 2 capillary column (50 m × 0.32 mm i.d.; 0.17 μm film thickness), flame ionization detector (FID) and septum-programmable injector (SPI). Samples were injected at 45 °C and thereafter the oven temperature was raised to 140 °C at 30 °C/ min and finally to 310 °C at 3 °C/ min where it was held for 10 min. To quantify co-eluting fatty acids, samples were run on a Varian 3800 gas chromatograph fitted with an SGE BPX-70 capillary column (50 m × 0.32 mm i.d.; 0.25 μm film thickness). Samples were injected at 45 ˚C; after 1 min the oven temperature was raised by 30 ˚C/ min to 120 ˚C and then by 3˚C/ min to 255 ˚C where it was held for 20 min. Peak identifications were based on comparison of retention time data with data obtained for authentic and laboratory standards. Peaks were quantified with Galaxie chromatography software (Palo Alto, CA, USA).

GC-MS analysis of FAME was performed to confirm component identifications and was carried out on a Finnigan GCQ Plus™ GC-MS ion-trap fitted with an on-column injector set at 45 °C. Double bond locations were determined by EI GC–MS analysis of the DMOX derivatives of the free FA, as described in Lee Chang et al. (2011). Samples were injected using an AS2000 auto sampler onto a retention gap attached to a non polar HP-5 Ultra 2 bonded-phase column (50 m x 0.32 mm i.d. x 0.17 µm film thickness). The initial temperature of 45 °C was held for 1 min, followed by temperature programming at 30 °C/ min to 140 °C then at 3 °C/ min to 310 °C where it was held for 12 min. Helium was used as the carrier gas. Mass spectrometer operating conditions were: electron impact energy 70 eV; emission current 250 µamp, transfer line 310 °C; source temperature 240 °C; scan rate 0.8 scan/ sec and mass range 40-650 Da. Mass spectra were acquired and processed with Thermo Scientific Xcalibur™ software (Waltham, MA, USA).

Extraction, amplification and sequencing of 18S ribosomal RNA genes

Biomass for DNA extraction was obtained after 7 days of growth in liquid culture medium. DNA was extracted from 100 µL samples using the FastDNA SPIN for Soil Kit (MP Biomedicals, Solon, OH, USA). The method generally followed the manufacturer's instructions, with the exception that, following the bead-beating step, the aqueous fraction was extracted using one volume of phenol:chloroform (Fluka, Castle Hill, NSW, Australia) and then one volume of phenol:chloroform:isoamylalcohol (Fluka), and separated using phase lock gel tubes (5 Prime, Hamburg, Germany) before combining with binding matrix and continuing as per the manufacturers instructions. DNA extractions were subsequently quantified using a Nano-Drop spectrophotometer (Nanorop, Wilmington, DE, USA).

The 18S rRNA gene was amplified from DNA extractions using the NSF-370F (AGGGYTCGAYYCCGGAGA), NSR-1787 (CYGCAGGTTCACCTACRG) Lab-1017R (GACTACGATGGTATCTAATCATCTTCG) primer sets (Siboni et al. 2010). Polymerase chain reactions (PCRs) comprised 1 U of AmpliTaq gold polymerase (Applied Biosystems, Carlsbad), 10mM dNTPs and 12.5 pmol of each primer in a total volume of 25 mL. PCR was performed using an Eppendorf thermal cycler (Eppendorf, Hamburg, Germany) with 30 cycles of 1 min denaturation at 95 °C, 1 min of annealing at 45 °C and 1 min extension at 72 °C, followed by a single extension step of 10 min at 72 °C. PCR products were visualised using 1.5 % agarose gel electrophoresis to ensure successful amplification of products the correct size. PCR products were purified using the Ampure (Agencourt, USA) and then Sanger sequenced using the F primer at Macrogen Korea.

Pigment extraction and analysis

Three mL of culture was filtered through a 47 mm diameter Whatman® GF/C glass fiber filter (Sigma-Aldrich, St. Louis, MO, USA) and stored in liquid nitrogen until analysis. To extract pigments, filters were ground in a glass mortar on ice with 100 % acetone (3 mL) and the extract was quantitatively transferred into 10 mL centrifuge tubes. Samples were vortexed for about 30 s and sonicated in an ice-water bath for 15 min in the dark. Samples were kept in the dark at 4°C for approximately 15 h. Extracts were sonicated in an ice-water bath for 15 min and centrifuged to remove the filter paper and transferred to a 10 mL volumetric flask. Water (1 mL) was added to the sample extract and the volumetric flask was made up to 10 mL with 100 % acetone (final extract mixture of 9:1 acetone:water [v:v]).

The final extract was filtered through a 0.2 µm membrane filter (Whatman®, anatope) prior to analysis by high performance liquid chromatography (HPLC), comprising a 2695XE separations module with column heater and refrigerated autosampler and a 2996 photo-diode array detector (Waters cooperation, Milford, MA, USA). Cultures were analysed using a C_8 column and binary gradient system with an elevated column temperature following a modified version of the (Van Heukelem and Thomas 2001) method. The separated pigments were detected at 436 nm and identified against standard spectra using Empower™ software (Waters cooperation, Milford, MA, USA). Concentrations of chlorophyll a, chlorophyll b, β,β-carotene filters (Sigma-Aldrich, St. Louis, MO, USA) and other pigments in sample chromatograms were determined from standards (DHI, Hørsholm, Denmark).

Statistical analysis

Multivariate statistical analysis of profiles was performed using Primer 6 (PRIMER-E Ltd, Plymouth, UK). Fatty acid and sterol profiles were compared among strains using clustering based on a Bray Curtis similarity matrix of chemotaxonomic profiles of individual strains. Phylogenetic analysis of 18S rRNA genes was performed using the ARB software package (Ludwig et al. 2004). Sequences from this study and closest reference sequences were imported into the Silva (Release 105) ARB database (Pruesse et al. 2007), automatically aligned and then corrected manually. Phylogenetic trees of sequences >400 bp were calculated using the Neighbour-joining method (Saitou and Nei 1987) using Felsenstein correction, 500 bootstraps. Shorter sequences were added to the tree using the parsimony method, without changing the overall tree topology. Sequences from this study were deposited in the GenBank database (accession numbers JN675242-JN675277).

Comparison of the similarity of the structure of similarity between chemotaxonomic and 18S rRNA gene similarity matrices was conducted using the RELATE function (Spearman correlation, 9999 permutations) in Primer 6 (PRIMER-E Ltd, Plymouth, UK), whereby the null hypothesis is that there is no similarity between the assemblage patterns for the 18S rRNA gene sequence and chemotaxonomic groupings.

Results

Biodiscovery

Of the five habitats along the temperate sampling transect (Table 2-1), one strain (TC 10) was isolated from the sandy beach sediments. The sample included various seaweeds and the site was adjacent to a freshwater storm water drain. All the other 17 temperate strains were isolated from the mudflat and adjacent saltmarsh (equidistant along the transect). Of the four habitat groups along the tropical sampling transects (Table 2-1), one strain (TC 20) was isolated from the seaward edge of one transect of the mangrove forest. This sample included mud and various dead mangrove leaves. All the other 17 tropical strains were isolated from the landward edge of both mangrove forest transects. No strains were isolated from sampling sites with salinities less than 26, even though initial isolations had been performed with isolating media of salinities similar to those of the sampling sites (Table 2-1).

Fatty acid and sterol profiles

Fatty acid and sterol profiles of 36 newly isolated temperate and tropical thraustochytrids strains are shown in Table 2-2. Based on their profiles, the thraustochytrid strains were separated into eight groups (A to H) using 80 % similarity of the cluster analysis (Fig. 2-1).

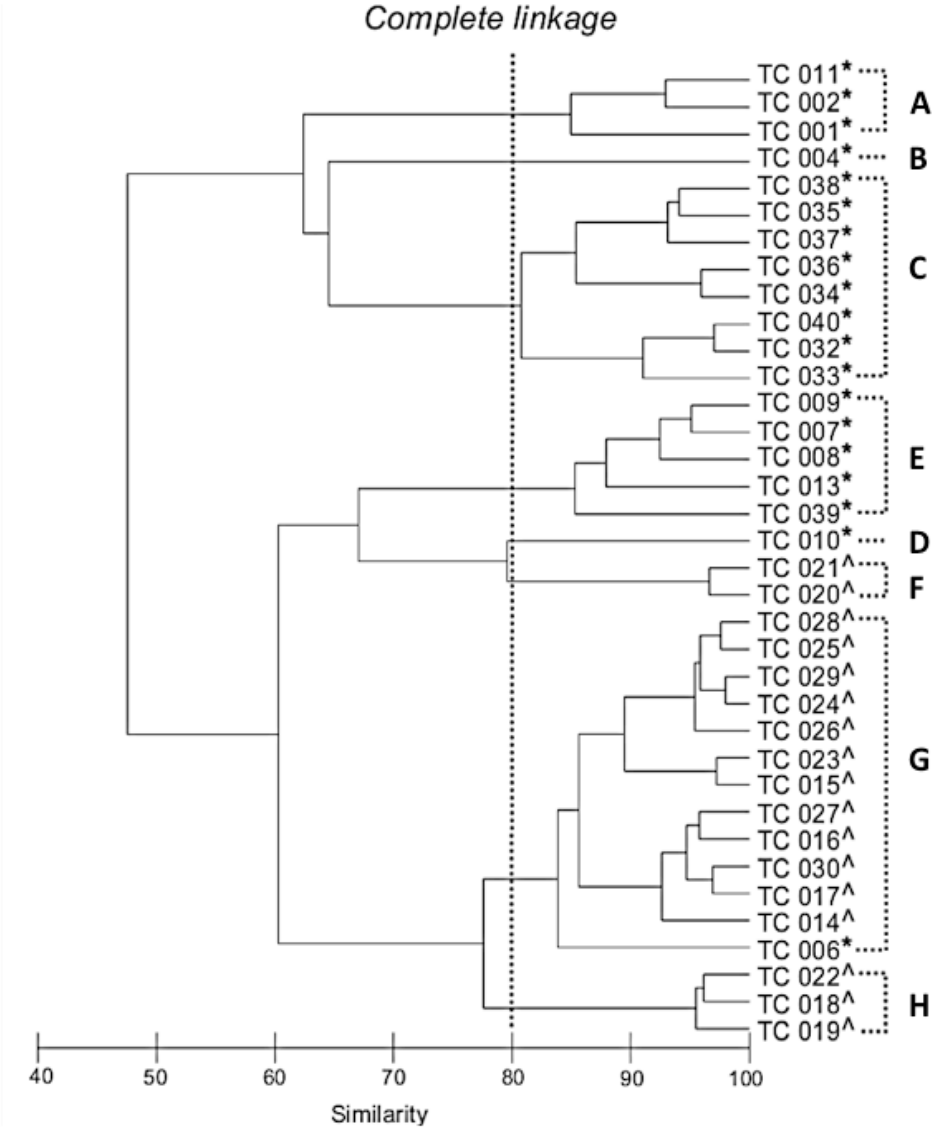


Figure 2-1 Chemotaxonomic grouping of 36 strains of temperate (*) and tropical (^) new Australian thraustochytrids based on fatty acid and sterol profiles formed by using the complete linkage function to compare Bray-Curtis similarity matrices (Spearman correlation, 9999 permutations) in Primer 6 (PRIMER-E Ltd, Plymouth, UK)

Group A strains (*Schizochytrium* spp.) contained high levels of omega-3 LC-PUFA (29 % TFA) and omega-6 LC-PUFA (34 % TFA), with OC-PUFA present and brassicasterol as a major sterol; Group B (*Thraustochytrium* sp.) had high C_{22} PUFA with 30 % of DHA and 13 % docosapentaenoic acid (DPA-6), with OC-PUFA also present and cholesterol as the major sterol; Group C (mostly *Thraustochytrium* spp.) contained high omega-3 LC-PUFA (47 % TFA), relatively low DPA-6 (0.8-1.8 % TFA), with OC-PUFA and cholesterol as the major sterol; Group D (*Ulkenia* sp.) had high omega-3 LC-PUFA (53 % TFA), 16:0 (24 % TFA) and no OC-PUFA with cholesterol, stigmasterol and brassicasterol as major sterols; Group E (*Aurantiochytrium* spp.) contained 60 % of total fatty acids as DHA, no OC-PUFA with cholesterol and stigmasterol as major sterols; Group F (*Aurantiochytrium* spp.) had high DHA (51 % TFA) and 16:0 (23 % TFA), no OC-PUFA; Group G (*Aurantiochytrium* spp.) contained high DHA (43 % TFA) and 15:0 (23 % TFA), no OC-PUFA; Group H (*Aurantiochytrium* spp.) had high 15:0 (30 % TFA) and saturated fatty acids (52 % TFA) and no OC-PUFA.

Strains from groups E, G and H (*Aurantiochytrium* sp.) all shared cholesterol and stigmasterol as their major sterols (Table 2-2). Figure 2-2 summarizes the percentage of LC-PUFA in all strains to highlight the potential of production of omega-3 LC-PUFA from these strains for potential use in nutraceuticals and other applications. The percentages of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) in the 36 strains are shown in Figure 2-3 to highlight the potential of production of these fatty acids from these strains for potential use in biodiesel.

Table 2-2 Fatty acid (as % of total FA) and sterol composition (g/ 100 g dry weight) for thraustochytrid strains from southeast Tasmania and north Queensland

TC	01*	02*	11*	04*	32*	33*	34*	35*	36*	37*	38*	40*	10*	07*	08*	09*	39*	13*	20^	21^	06*	14^	15^	16^	17^	23^	24^	25^	26^	27^	28^	30^	29^	18^	19^	22^
CS Number [a]	979	980	981	982	983	984	985	986	987	988	989	990	991	992	993	994	995	996	997	998	999	1000	1001	1002	1003	1004	1005	1006	1007	1008	1009	1011	1010	1012	1013	1014
Group	A	A	A	B	C	C	C	C	C	C	C	C	D	E	E	E	E	E	F	F	G	G	G	G	G	G	G	G	G	G	G	G	G	H	H	H
Salinity [b]	26	34	26	33	31	31	31	31	31	31	31	31	33	30	30	30	31	30	33	31	30	34	35	35	33	31	37	37	37	33	40	30	40	40	40	31
12:0	-	-	-	-	0.4	0.2	0.5	0.3	0.4	0.3	0.4	0.4	-	-	-	-	-	-	Tr	0.1	-	0.2	0.2	0.2	0.1	0.2	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.2	0.2
14:0	1.7	1.9	1.9	0.2	2.7	2.8	2.9	2.8	2.6	2.7	2.9	2.5	1.1	0.3	0.7	0.4	0.6	0.2	1.9	2.1	2.6	4.1	7.0	4.3	2.7	7.2	4.2	4.3	5.7	3.9	3.8	2.7	4.4	4.9	7.0	6.3
15:0	7.1	5.7	6.5	5.7	13.7	16.3	7.1	8.5	6.7	11.1	9.7	13.4	5.9	5.9	6.7	6.7	8.2	2.4	6.2	8.0	19.6	29.2	20.6	24.3	25.9	21.4	20.9	20.3	22.3	27.0	21.8	25.8	20.8	30.6	31.9	28.5
16:0	13.3	17.1	15.0	9.6	16.9	17.5	16.1	19.4	17.2	19.6	18.2	15.2	23.6	8.4	7.3	9.6	6.9	11.0	22.3	22.7	6.7	8.0	14.5	8.7	8.0	15.5	10.7	10.7	10.3	6.8	10.3	7.4	12.1	11.3	11.4	12.4
17:0	2.2	2.3	2.4	6.4	2.3	2.3	1.6	1.7	1.8	2.3	1.9	2.3	1.8	1.8	2.2	1.9	3.1	1.2	1.5	1.9	2.7	3.4	2.6	2.8	3.8	2.7	2.9	2.8	2.6	3.0	3.3	3.7	3.2	3.4	3.2	3.5
18:0	0.8	1.8	1.4	0.4	0.8	0.8	2.3	0.9	2.4	0.9	1.4	0.8	0.5	0.5	0.5	0.2	0.4	0.4	0.2	0.3	0.1	0.6	0.1	0.2	0.2	0.3	0.8	0.8	1.0	0.5	0.9	0.8	0.7	0.1	0.1	0.3
16:1ω9	0.3	0.3	0.3	0.2	0.3	0.4	0.4	0.2	0.3	0.2	0.3	1.3	0.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16:1ω7	0.4	0.6	0.5	0.1	0.5	0.8	0.4	0.3	0.3	0.4	0.5	0.5	0.9	0.1	0.2	0.2	0.3	0.2	0.1	0.2	0.3	0.2	0.1	0.3	0.1	0.3	0.2	0.2	0.3	0.2	0.2	0.1	0.2	0.1	0.3	0.4
17:1ω8	3.2	3.2	3.4	0.9	3.7	5.0	1.2	1.9	1.6	2.7	2.0	3.9	-	-	0.2	-	0.2	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17:1ω6	0.2	0.2	0.2	-	0.2	0.3	0.1	0.1	0.1	0.1	0.1	0.2		0.1	1.1	0.4	1.9	-	-	-	1.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18:1ω9	2.1	5.4	3.8	0.3	2.4	3.3	5.7	1.6	6.2	1.9	2.6	2.7	1.0	-	-	-	-	-	0.2	0.2	-	0.4	Tr	Tr	Tr	-	0.4	0.4	0.4	0.4	0.3	0.3	0.4	Tr	-	-
18:1ω7	0.6	1.0	0.8	2.0	1.1	1.4	1.3	1.2	1.0	0.9	1.3	1.1	1.6	0.4	1.4	1.1	2.3	0.6	-	-	0.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18:2ω6	1.9	3.1	2.5	0.3	2.1	2.2	1.8	2.1	2.5	2.3	2.4	2.1	0.5	-	0.2	-	-	-	0.1	0.1	0.2	-	Tr	Tr	Tr	0.1	-	-	-	-	-	-	-	-	0.1	0.2
18:3ω6	0.6	0.7	0.7	-	0.7	0.7	0.2	0.6	0.3	0.8	0.5	0.6	0.4	-	0.1	-	-	-	0.4	0.2	0.1	0.2	0.1	0.1	Tr	0.1	0.1	0.1	0.4	0.1	0.1	0.1	0.1	0.1	0.1	0.1
18:4ω3	-	-	-	0.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.4	0.5		0.1	0.1	0.1	0.1	Tr	0.1	0.1	0.2	0.1	0.1	0.2	0.1	0.1	0.1	0.1
19:1ω10	0.5	0.6	0.6	2.2	0.5	0.4	0.1	0.3	0.2	0.4	0.3	0.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19:1ω9	0.5	0.4	0.4	-	0.5	0.6	0.2	0.2	0.3	0.3	0.3	0.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19:2ω7	0.5	0.3	0.4	1.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19:3ω8	0.2	0.1	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19:3ω5	0.3	0.3	0.3	-	0.1	0.1	Tr	Tr	Tr	0.1	0.1	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19:4ω5	2.8	1.5	1.9	1.6	1.1	1.2	0.6	0.8	0.4	1.0	0.8	1.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 2-2 (continued)

TC	01*	02*	11*	04*	32*	33*	34*	35*	36*	37*	38*	40*	10*	07*	08*	09*	39*	13*	20^	21^	06*	14^	15^	16^	17^	23^	24^	25^	26^	27^	28^	30^	29^	18^	19^	22^
CS Number [a]	979	980	981	982	983	984	985	986	987	988	989	990	991	992	993	994	995	996	997	998	999	1000	1001	1002	1003	1004	1005	1006	1007	1008	1009	1011	1010	1012	1013	1014
Group	A	A	A	B	C	C	C	C	C	C	C	C	D	E	E	E	E	E	F	F	G	G	G	G	G	G	G	G	G	G	G	G	G	H	H	H
20:3ω6, DGLA	1.8	2.5	2.3	0.1	0.7	0.7	0.8	0.7	0.7	0.8	0.7	0.8	0.1	0.1	0.1	0.1	0.1	-	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
20:4ω6, AA	11.3	12.3	12.2	1.8	1.0	1.0	1.6	1.0	1.2	1.2	1.3	1.1	2.3	1.3	1.6	1.1	0.4	0.9	1.2	1.2	0.8	0.5	0.5	0.7	0.7	0.5	0.7	0.7	0.7	0.5	0.6	1.0	0.6	0.5	0.5	0.5
20:4ω3	0.4	0.3	0.3	0.1	0.9	0.4	1.0	0.7	0.8	0.8	0.5	0.9	0.1	0.4	0.4	0.3	0.4	0.3	0.6	0.6	0.3	0.3	0.4	0.4	0.4	0.3	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.1	0.3	Tr
20:5ω3, EPA	6.9	4.5	5.8	9.2	9.9	8.3	8.0	10.2	8.6	10.6	8.7	9.6	11.2	7.8	5.7	8.5	5.1	6.4	1.9	1.4	2.9	2.1	2.5	2.5	2.2	2.0	2.6	2.7	2.6	2.3	2.6	3.0	2.2	1.7	1.9	2.0
21:4ω7	2.9	1.8	2.0	7.2	0.6	0.7	0.5	0.4	0.4	0.4	0.7	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21:4ω5	0.2	0.2	0.2	-	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21:5ω5	0.3	0.1	0.1	1.9	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21:6ω2	0.5	0.4	0.4	0.6	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22:3ω6	0.2	0.2	0.7	0.5	0.2	0.2	0.3	0.4	0.4	0.3	0.3	0.2	0.4	-	0.4	0.3	0.3	1.1	-	-	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22:4ω6	1.1	1.4	1.2	0.4	0.2	0.4	0.2	0.2	0.2	0.2	0.3	0.2	0.7	0.7	0.5	0.7	0.2	0.4	Tr	Tr	0.3	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr
22:4ω3	Tr	0.2	0.1	0.5	0.1	0.1	0.2	0.2	0.2	0.2	0.2	0.1	0.8	0.3	0.1	0.3	0.2	0.4	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22:5ω3	1.2	0.8	0.9	0.5	1.0	0.9	1.7	1.2	1.5	1.2	1.2	1.0	0.9	4.0	2.7	4.3	3.2	1.8	1.2	0.9	1.5	1.4	1.4	1.5	1.8	1.1	1.5	0.1	0.1	2.0	0.1	0.1	1.6	1.2	1.2	1.2
22:5ω6, DPA-6	7.4	10.0	8.4	12.8	0.8	0.9	1.8	0.6	1.2	0.7	1.7	0.9	5.9	10.8	12.1	9.0	4.7	11.9	9.2	9.3	12.7	7.9	8.0	9.4	9.6	7.7	9.3	9.3	9.4	8.8	9.7	10.4	9.2	8.4	6.8	7.7
22:6ω3, DHA	25.7	18.5	21.1	29.5	32.9	28.5	40.1	40.0	38.9	34.1	37.2	33.2	37.5	56.5	55.3	54.0	60.8	60.4	51.7	49.4	46.3	40.4	41.2	44.0	43.7	39.6	44.4	46.4	42.4	43.1	45.3	43.3	43.3	37.0	34.3	36.1
23:5ω6	0.2	0.1	0.1	0.8	0.4	0.4	0.1	0.1	0.1	0.1	0.4	0.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SUM SFA	25.4	29.1	27.4	22.5	37.2	40.3	30.8	33.9	31.4	37.4	34.9	35.0	32.9	17.1	17.4	18.9	19.4	15.2	32.3	35.2	31.9	45.5	45.1	40.4	40.7	47.4	39.8	39.1	42.0	41.4	40.2	40.4	41.3	50.5	54.0	51.1
SUM MUFA	7.4	11.1	9.5	4.0	9.0	12.1	9.6	6.1	10.1	7.0	7.4	10.4	4.0	1.1	3.2	1.9	4.9	1.3	0.5	0.6	2.6	0.6	0.1	0.3	0.2	0.3	0.7	0.6	0.8	0.7	0.5	0.5	0.6	0.2	0.3	0.5
SUM PUFA	67.2	59.8	63.0	73.4	53.8	47.6	59.6	60.0	58.4	55.6	57.7	54.6	63.1	81.9	79.4	79.2	75.7	83.5	67.3	64.3	65.5	53.8	54.8	59.2	59.0	52.2	59.5	60.3	57.2	57.9	59.3	59.1	58.1	49.4	45.8	48.4
SUM others [c]	0.8	0.7	0.9	1.2	0.7	0.7	0.5	0.7	0.7	0.7	0.7	0.8	0.3	0.1	0.1	0.5	0.4	-	0.4	0.4	Tr	0.2	0.2	0.1	0.1	0.1	0.1	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1
TOTAL ω3	34.5	24.4	28.5	40.5	44.8	38.3	51.1	52.4	50.0	46.8	47.8	44.9	52.6	69.1	64.3	67.6	69.8	69.3	56.2	53.0	51.2	44.4	45.7	48.6	48.3	43.2	49.1	49.8	45.7	48.0	48.5	47.0	47.7	40.1	37.8	39.5
TOTAL ω6	31.9	34.9	33.9	30.3	8.8	9.2	8.4	7.4	8.2	8.5	9.7	9.5	10.5	12.8	15.0	11.4	5.8	14.2	11.0	11.2	14.3	9.2	9.0	10.6	10.7	8.9	10.3	10.5	11.4	9.9	10.7	12.0	10.3	9.2	7.9	8.8
FAME (g/ 100 g DW)	6.7	9.1	5.2	4.5	8.0	2.9	7.2	9.2	8.9	9.8	7.8	8.8	8.8	6.4	3.1	4.7	5.7	1.7	7.8	7.7	7.3	10.1	6.5	9.1	4.0	7.1	50.4	18.8	6.0	9.7	8.0	2.8	17.6	7.8	13.0	7.7
DW (mg/ mL)	1.5	1.7	1.4	1.1	0.7	0.7	0.9	0.7	0.7	0.6	0.8	0.7	0.3	0.5	1.9	0.6	1.3	1.3	1.0	1.3	0.8	0.9	1.2	1.1	1.7	0.8	0.2	0.5	0.8	1.4	0.9	2.0	0.6	1.0	0.4	0.6

Table 2-2 (continued)

TC	01*	02*	11*	04*	32*	33*	34*	35*	36*	37*	38*	40*	10*	07*	08*	09*	39*	13*	20^	21^	06*	14^	15^	16^	17^	23^	24^	25^	26^	27^	28^	30^	29^	18^	19^	22^
CS Number [a]	979	980	981	982	983	984	985	986	987	988	989	990	991	992	993	994	995	996	997	998	999	1000	1001	1002	1003	1004	1005	1006	1007	1008	1009	1010	1011	1012	1013	1014
Group	A	A	A	B	C	C	C	C	C	C	C	C	D	E	E	E	E	E	F	F	G	G	G	G	G	G	G	G	G	G	G	G	G	H	H	H
Sterols (g/ 100 g DW)																																				
Cholesterol	0.1	0.1	0.1	1.0	0.5	0.2	0.5	0.6	0.6	0.5	0.6	0.6	0.9	0.7	0.3	0.7	0.6	0.3	0.2	0.3	0.5	0.6	0.4	0.5	0.2	0.4	3.1	1.3	0.4	0.5	0.6	1.2	0.2	0.4	0.7	0.4
Desmosterol	-	-	Tr	-	-	-	-	-	-	-	-	-	-	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr
Brassicasterol	0.5	0.5	0.3	Tr	-	-	-	-	-	-	-	-	0.3	0.2	0.1	0.1	-	Tr	Tr	Tr	0.1	0.1	0.1	0.1	Tr	0.1	0.5	0.2	0.1	0.1	0.1	0.2	Tr	0.1	0.1	0.1
Lathosterol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Tr	0.1		0.1	Tr	0.1	Tr	Tr	0.3	0.1	Tr	0.1	0.1	0.1	Tr	Tr	0.1	Tr
Stigmasterol	-	-	-	Tr	-	-	-	-	-	-	-	-	0.4	0.4	0.2	0.4	0.3	0.2	0.1	0.1	0.2	0.2	0.2	0.2	0.1	0.1	1.0	0.4	0.2	0.2	0.2	0.4	0.1	0.1	0.3	0.2
Unknown 4	-	-	-	Tr	-	-	-	-	-	-	-	-	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	Tr	0.1	0.5	0.2	0.1	0.1	0.1	0.1	Tr	0.1	0.1	0.1
Unknown 7	-	-	Tr	Tr	-	-	-	-	-	-	-	-	Tr	Tr	Tr	Tr	Tr	Tr	Tr	0.1	Tr	Tr	Tr	Tr	Tr	Tr	0.2	0.1	Tr	0.1	Tr	0.1	Tr	Tr	Tr	Tr
Total sterols	0.7	0.6	0.4	1.1	0.5	0.2	0.5	0.6	0.6	0.5	0.6	0.6	1.7	1.8	0.6	1.3	1.0	0.8	0.4	0.7	1.0	1.1	0.8	1.0	0.4	0.8	5.8	2.4	0.8	1.1	1.1	2.2	0.3	0.8	1.4	0.8

[a] CS Number is assigned for strains held in Australian National Algae Culture Collection (ANACC) hereafter strains will be referenced by their sample code –TC

[b] Salinity refers to salinity of sample at collection

[c] Other FA refer to: 19:0, 20:0, 21:0, 14:1, 15:1, 20:1ω9, 20:1, 22:1ω9, 16:3ω3, 16:2ω3, 17:2, 17:3, 18:2, 18:3ω3, 20:2ω6, 20:3ω3, 20:1ω9, 22:2ω6

Tr denotes Trace < 0.01; '-' denotes not detected; '*' denotes strains were isolated from temperate Tasmania; '^' denotes strains were isolated from tropical Queensland

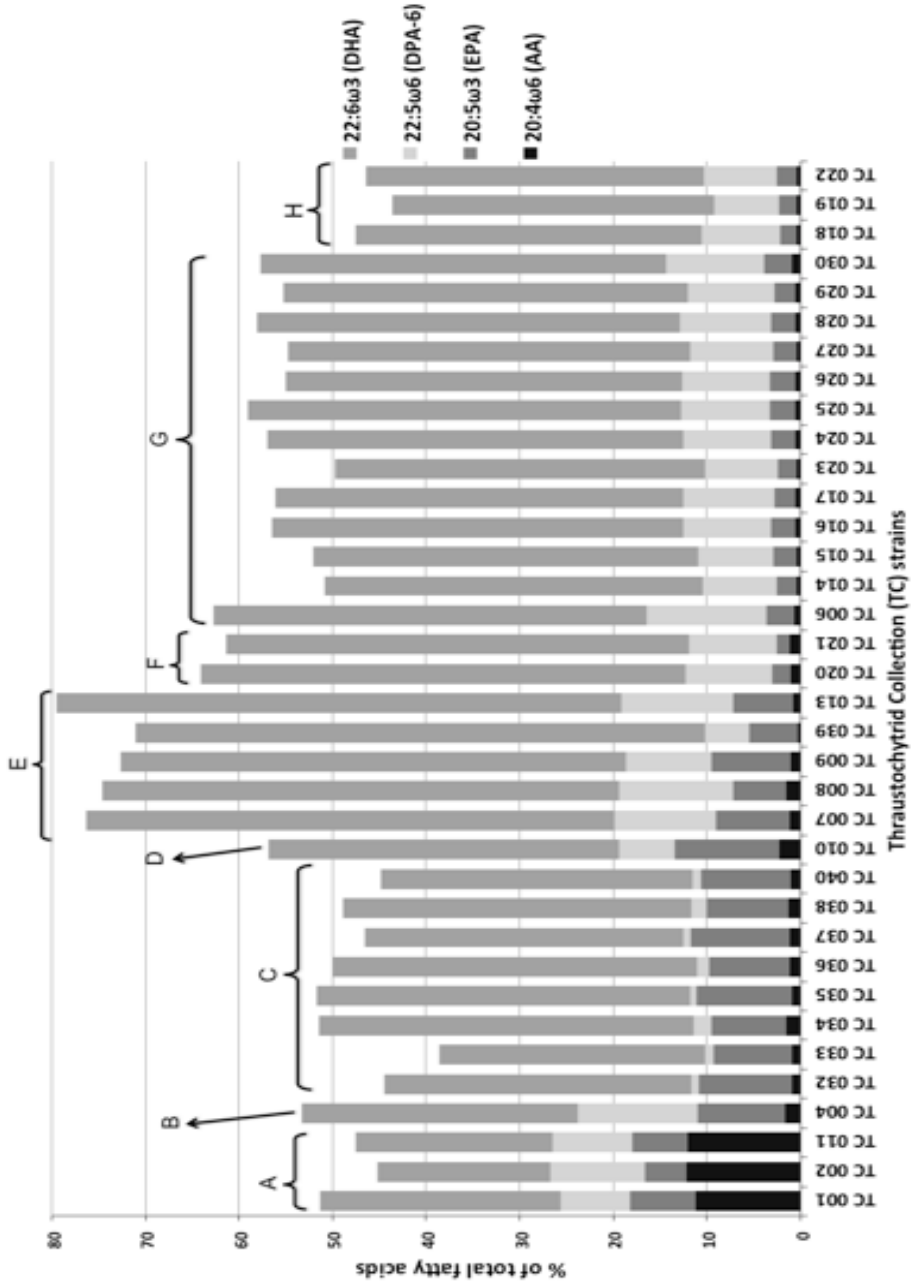


Figure 2-2 Percentage of long chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA) in 36 new Australian thraustochytrid strains. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; AA, arachidonic acid; DPA-6, docosapentaenoic acid. Group E (5 strains) had up to 60 % of total fatty acids as DHA

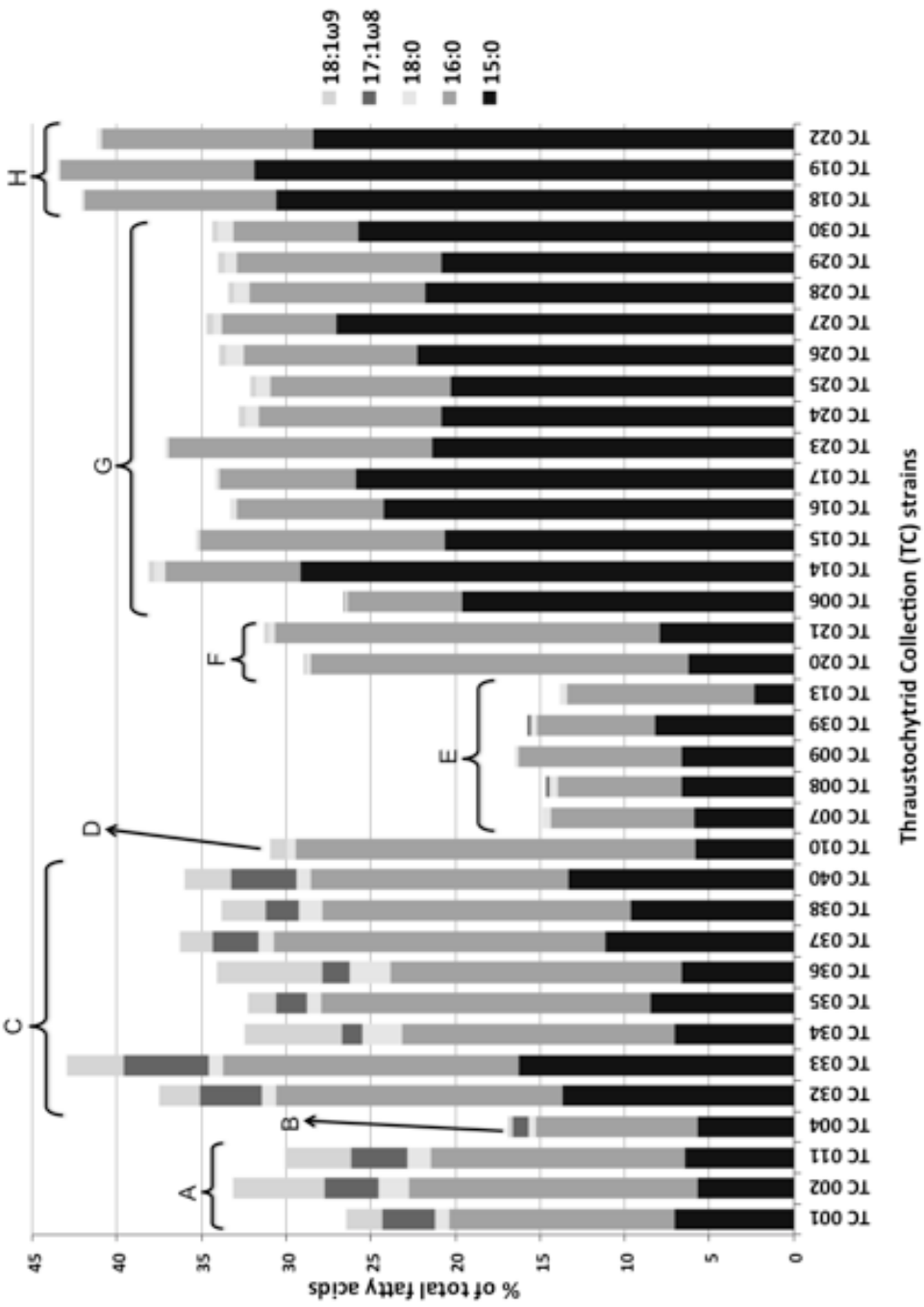


Figure 2-3 Percentage of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) in 36 new Australian thraustochytrid strains. Group C (8 strains) contained the highest relative levels of MUFA and is the most suited to biodiesel production

18S rRNA gene sequencing

A phylogenetic tree of 18S rRNA was generated based on sequences of the 36 Australian strains in the present study and 18S rRNA sequence data from thraustochytrid strains available in the published literature (Honda et al. 1999; Huang et al. 2003; Yang et al. 2010; Yokoyama et al. 2007; Yokoyama and Honda 2007) with *Thraustochytrium pachydemum* included as the outgroup (Fig. 2-4). Based on previous studies, the 36 Australian strains group to the following genera in two distinct clusters: (i) *Aurantiochytrium* and *Schizochytrium*; and (ii) *Thraustochytrium* and *Ulkenia*. The first cluster was comprised of two distinctive clades (Fig. 2-4). The first clade contained 19 strains from chemotaxonomic groups F, G, H and 3 of the 4 strains from group E and were grouped together with several *Aurantiochytrium* spp. strains (Fig. 2-4). The second clade had three strains of chemotaxonomic group A together with *Schizochytrium sp*. SEK 345. The second cluster was also comprised of two distinctive clades (Fig. 2-4). The first clade contained 8 strains from chemotaxonomic group C and 1 of the 4 strains from group E and were grouped together with *Thraustochytrium kinnei* (Fig. 2-1). The second clade had three strains of chemotaxonomic group A together with *Schizochytrium sp*. SEK 345. In the second clade, strain TC 04 of chemotaxonomic group B was closest to *Thraustochytrium striatum*, whereas TC 10 (chemotaxonomic group D) clustered closely with *Ulkenia visurgensis* (Fig. 2-4). These two chemotaxonomic groups (B and D) were further apart from all the other Australian strains. Interestingly, the fact that of the four strains of chemotaxonomic group E, one clustered with Group G, one with Group C and the other two clustered between Groups F, G and H (Fig 2-4.) This suggests that Group E was a polyphyletic grouping of strains with similar chemical compositions.

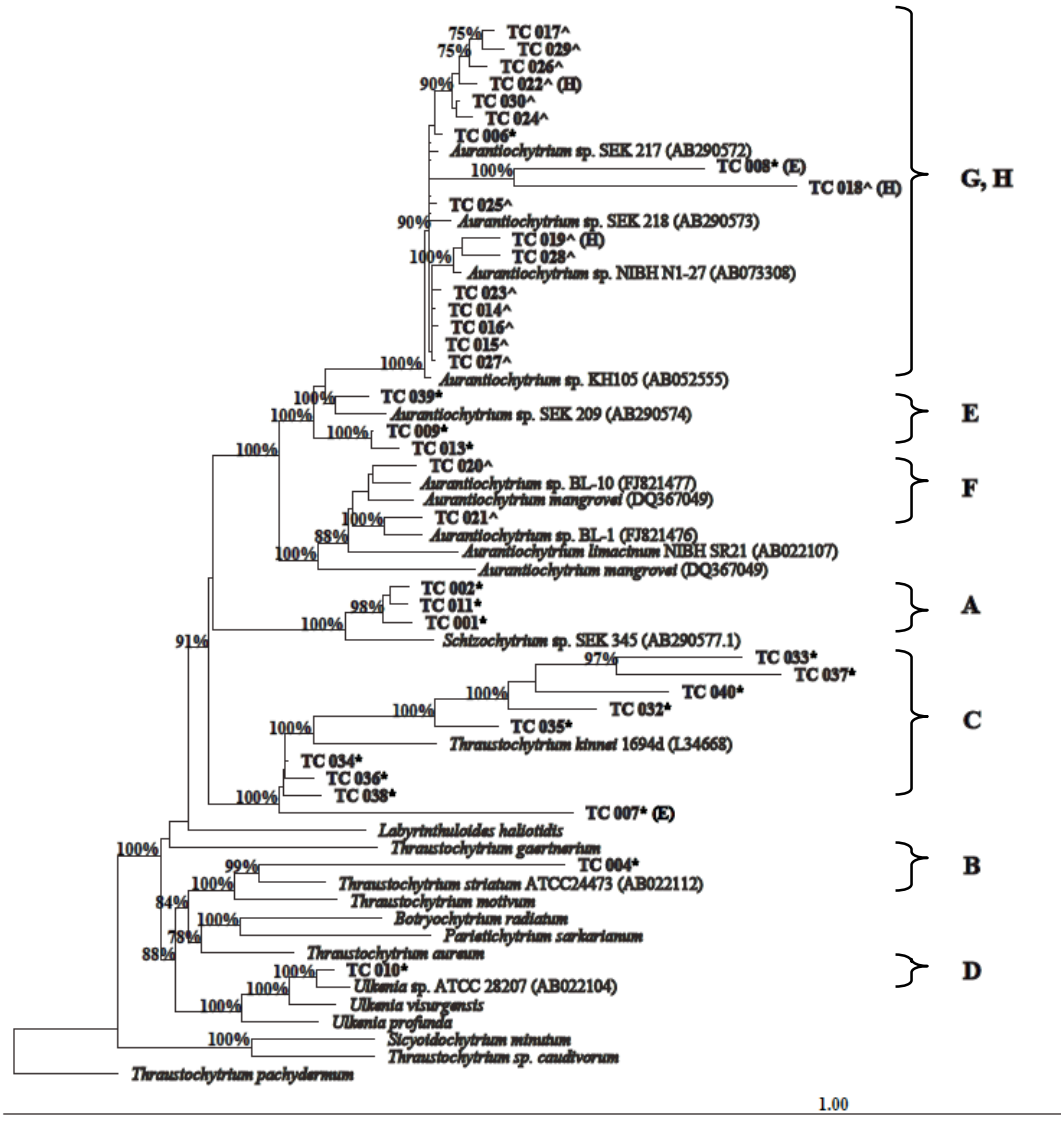


Figure 2-4 Phylogeny of thraustochytrid partial 18S rRNA gene sequences from DNA extracts of the 36 new Australian strains (* temperate strains, ^ tropical strains) and reference sequences from the database. Parentheses indicate sequences matched to chemotaxonomic groups. Bootstrap values > 75 % are shown at the nodes. Two strains which were chemotaxonomically grouped as E, clustered more closely with other groups. These were TC 08 (clustered with groups G and H) and TC 07 (clustered with group C). Group G strains were clustered with group H strains. Most strains in groups G and H were from tropical north Queensland, except TC 06 and TC 08

In the present study, there was a correlation between the groups formed using 18S rRNA sequences data and the chemotaxonomic groups based on fatty acid profiles of 7-day old cultures grown in basal medium ($P < 0.0001$, $R = 0.596$).

Pigment profiles

Pigments observed via HPLC obtained from representative strains selected from each of the 8 chemotaxonomic groups were identified based on retention times and absorption spectra of pigment standards of β,β-carotene, canthaxanthin and astaxanthin (Table 2-3). β,β-Carotene, canthaxanthin and astaxanthin were detected in orange and cream colored strains. Pigments found in other colored strains were not detected in white colored strains (Table 2-3).

Table 2-3 Pigment composition and content (μg/L) of selected strains from each taxonomic group (based on fatty acid and sterol profiles)

Strains	Group	Colour	Genus	β,β-Carotene (μg/L)	Canthaxanthin (μg/L)	Astaxanthin (μg/L)	Pigment yield (μg/g DW)
TC 02	A	Cream	*Schizochytrium*	56.7	-	-	33.4
TC 04	B	Orange	*Thraustochytrium*	-	-	112.3	102.3
TC 33	C	White	*Thraustochytrium*	-	-	-	-
TC 10	D	Cream	*Ulkenia*	-	-	23.4	71.7
TC 08	E	Cream	*Aurantiochytrium*	25.7	28.9	-	28.2
TC 20	F	White	*Aurantiochytrium*	-	-	-	-
TC 06	G	Orange	*Aurantiochytrium*	16.8	27.7	-	53.9
TC 30	G	Cream	*Aurantiochytrium*	11.7	-	-	5.7
TC 18	H	Cream	*Aurantiochytrium*	15.7	-	-	15.9

'-' denotes not detected

Discussion

Sampling location and isolation

The selection of habitats covered a diversity of freshwater, brackish, and marine environments (Table 2-1). Thraustochytrids were isolated from silt, mud and organic rich sediments in both temperate and tropical environments. It was also observed that thraustochytrids were isolated only from the landward and seaward edges of the tropical mangrove forest. This observation is consistent with the results obtained from the temperate environment sampled where no thraustochytrids were isolated from open water and the low tide zone using this isolation technique. Mangroves are adapted to survive in muddy sediments along shorelines of beaches and estuaries. Even though temperate Tasmania has many areas with muddy sediments, it is the only Australian state not to support mangroves due to the cool climate. With the exception of one strain (collected in sediment near a stormwater outlet in a temperate transect), all thraustochytrids in this study were isolated from muddy sediments of mudflat and saltmarsh. While it has been reported that thraustochytrids are frequently collected from mangrove forests (Honda et al. 1998; Raghukumar 1988; Fan et al. 2007; Leańo et al. 2003), our experience is that they were abundant in fine muddy sediment of intertidal areas containing organic rich material of both marine and terrestrial origin that support the growth of thraustochytrids (Raghukumar 2002). Although the cell population of thraustochytrids might be higher at the edge of the sample zone compared to those of open water zone and tidal areas, other contaminants such as bacteria and fungi outgrew thraustochytrids before an axenic culture could be isolated.

Thraustochytrids were only isolated from brackish and marine environments. No thraustochytrids were isolated from fresh water samples. This can be explained by the fact that while thraustochytrids are halophilic and show a large

degree of salinity tolerance, they have an obligate requirement for Na^+ ions (Raghukumar 2008). They show optimal growth at salinities above 15 at 20-30 °C (Leańo et al. 2003). Yokochi et al. (1998) reported that *Schizochytrium limacinum* SR21 (ATCC MYA-1381), which has been renamed *Aurantiochytrium limacinum* SR21 (Yokoyama and Honda 2007), could grow in a medium without salt, with the highest dry cell weight at 50 % seawater and could be maintained at up to 200 % seawater. In contrast, growth of *Thraustochytrium aureum* ATCC 34304 was completely inhibited at 0 % and 200 % seawater, with the highest dry cell weight obtained in 50 % seawater (Iida et al. 1996).

It is acknowledged that the composition of the culture media can select for different Labyrithulomycetes species or strains that can grow on that substrate and the culture conditions employed (Rosa et al. 2010a; Raghukumar 2002). Raghukumar (2002) indicated that culture techniques are likely to reveal only a portion of the species present and therefore underestimate the actual natural population. Rosa et al. (2010) demonstrated a modified method of pollen baiting and direct plating on agar medium for a more efficient isolation of thraustochytrid strains from both cold and temperate environments in southern Argentina. In addition, media selection for thraustochytrid culturing is vital including setting the desired strain selection parameters that matches a production system. For example, optimal growth of *Aurantiochytrium limacinum* SR21 in half-strength seawater not only lowers production cost, but also minimizes corrosive effects on conventional fermenters by decreasing sodium chloride concentration (Honda et al. 1998; Fan and Chen 2007).

Groupings based on 18S rRNA gene sequence, fatty acid and sterol profiles

Phylogenetic and chemotaxonomic grouping of the 36 new Australian thraustochytrid strains illustrated similar relationships for the majority of strains in the present study. Table 2-4 summarizes the relationships derived from 18S rRNA gene sequence data, chemotaxonomic characteristics based on fatty acid and sterol profiles, as well as pigment profiles of 7-day-old cultures grown in basal medium.

Fatty acids profiles have been previously used to characterize microorganisms (Huang et al. 2003; Yang et al. 2010; Yokoyama and Honda 2007), where separation of taxa was largely based on the presence or absence of specific fatty acids. Huang et al. (2003) suggested that thraustochytrids can be divided into five groups based on their PUFA profiles. These groups were categorized by the presence or absence of dihomo-γ-linolenic acid (20:3ω6, DGLA), as well as the relative amounts of AA, EPA, DPA-6 and DHA in the total fatty acids. In the present study, the level of DGLA detected was 0.1- 2.2 % TFA in all thraustochytrid strains from genera *Aurantiochytrium*, *Schizochytrium*, *Thraustochytrium* and *Ulkenia* (Table 2-2). However, it should be noted that the relatively low glucose concentration (0.2 % w/v) used may have influenced the fatty acid compositions resulting in higher amounts of DGLA than was detected in the Huang et al. (2003) study.

Our group A had a similar fatty acid profile as one of the categories proposed by Huang et al. (2003) characterized by 6.3- 13.6 % AA in addition to production of DHA, DPA-6 and EPA. In their study this group was clustered closely with *S. aggregatum* ATCC 28209 in the phylogenetic tree, which is consistent with the present study, as our group A also clustered closely with *S. aggregatum* (Table 2-4, Fig. 2-4).

Table 2-4 Summary of chemotaxonomic characteristics and 18s rRNA sequence data of thraustochytrid strains examined in the present study

Genus	*Schizochytrium*	*Thraustochytrium*		*Ulkenia*	*Aurantiochytrium*			
Group	A	B	C	D	E	F	G	H
Temperate	+	+	+	+	+	-	+	-
Tropical	-	-	-	-	-	+	+	+
Fatty acids[1]								
OC-PUFA	+	+	+	-	-	-	-	-
22:6ω3, DHA	21.8	29.5	35.6	37.5	57.4	50.6	43.3	35.8
22:5ω6, DPA-6	8.6	12.8	1.1	5.9	9.7	9.2	9.3	7.6
20:4ω6, AA	11.9	1.8	1.2	2.3	1.1	1.2	0.6	0.5
20:5ω3, EPA	5.7	9.2	9.2	11.2	6.7	1.7	2.5	1.9
15:0	6.4	5.7	10.8	5.9	6.0	7.1	23.1	30.3
16:0	15.1	9.6	17.5	23.6	8.7	22.5	10	11.7
Pigments								
β,β-Carotene	+	-	-	-	+	-	+	+
Canthaxanthin	-	-	-	-	+	-	+	-
Astaxanthin	-	+	-	+	-	-	-	-
Sterols								
Cholesterol	Tr	+	+	+	+	+	+	+
Stigmasterol	-	-	-	+	+	Tr	+	+
Brassicasterol	+	-	-	+	Tr	Tr	Tr	Tr

[1]Mean of % TFA within strains in the group. Groups B and D - one single strain; '+' denotes presence and '-'; denotes absence, 'Tr' denotes trace < 0.01 of total fatty acids or sterols

Yang et al. (2010) demonstrated that thraustochytrid strains with higher DHA production were found within the strains that have closer phylogenic relationships. The authors suggested strains can be categorized based on: (i) their FA profiles that were defined by a high unsaturated fatty acid content, (ii) the presence of 20:3ω6 and 20:4ω6, and (iii) the relative amount of 22:5ω6 and 22:5ω3 (Yang et al. 2010). Huang et al. (2003) indicated that the different PUFA profiles probably resulted from different biosynthetic pathways, such as by the conventional desaturation and elongation pathway and/or the polyketide synthase (PKS) pathway. The PKS pathway does not require multiple desaturase and elongase enzymes, but instead employs a PKS gene cluster for the synthesis of LC-PUFA (Metz et al. 2001). It has been suggested that thraustochytrids might utilize both pathways for PUFA biosynthesis depending on substrate availability (Lippmeier et al. 2009).

While the amounts and proportions of the various fatty acids can vary with culture conditions, generally the presence/absence of specific fatty acids is genetically determined due to the enzymes present. These comparisons highlight the potential difficulties in trying to categorize thraustochytrids by their PUFA profiles alone (Huang et al. 2003). Fatty acid and sterol data were analysed by Bray-Curtis similarity analysis for grouping the strains chemotaxonomically, rather than PUFA profiles alone. The relative level of DHA was one of the influential factors in grouping thraustochytrids analyzed as 7-day-old broth cultures.

The occurrence of OC-PUFA was observed only in strains of chemotaxonomic groups A, B and C, thus further distinguishing these three groups from the other chemotaxonomic groups. These strains belonged to the genera *Schizochytrium* and *Thraustochytrium* that were isolated from the temperate environment. These OC-PUFA have the potential to be a signature marker for different groups

of thraustochytrids (Lee Chang et al. 2011). Future research with these strains will examine the use of higher glucose concentrations (and other carbon sources) in order to determine the effect of organic substrate on the FA profiles.

Our study has shown that there was strong correlation between the groups defined using 18S rRNA gene sequence data and those obtained from fatty acid and sterol profiles of 7-day old cultures grown in basal medium. The largest grouping from the 18S rRNA gene sequences all belonged to the genus *Aurantiochytrium* that combined chemotaxonomic groups G and H, except for one strain (TC 08) that belonged to chemotaxonomic group E. The differences between fatty acid profiles of groups G, H and E of genus *Aurantiochytrium* mainly resulted from the relative levels of EPA and 15:0 (Table 2-4). One strain (TC 07) from group E clustered more closely with group C of genus *Thraustochytrium* (Fig. 2-4), while the occurrence of OC-PUFA and lower proportion of DHA in group C were the factors contributing to their chemotaxonomic separation (Fig. 2-1). The rest of chemotaxonomic group E clustered closely based on the 18S rRNA gene phylogeny (Fig. 2-4). Other strains from chemotaxonomic groups A, C and F grouped closely based on 18S rRNA gene sequences, indicating the relative specificity of the fatty acid profiles of these strains. Comparison of thraustochytrid chemotaxonomic profiles with their 18S rRNA gene sequence data have demonstrated that such chemotaxonomic methods have the potential to be useful tools in the interpretation of thraustochytrid phylogenetic relationships.

Biodiversity

Strains from chemotaxonomic groups F, G and H of genus *Aurantiochytrium* were isolated from a tropical mangrove environment in far north Queensland, except for TC 06 which was isolated from the temperate environment.

This finding is consistent with available literature in that chemotaxonomic group F strains clustered closely with subtropical *Aurantiochytrium mangrovei* (*Schizochytrium mangrovei*), which is a thraustochytrid found to be associated with mangrove leaves and detritus from subtropical mangrove habitats (Jiang et al. 2004). Five tropical strains were clustered closely to each other (TC 14, 15, 16, 23 and 27) in the genus *Aurantiochytrium*, suggesting that they may belong to the same species. However, the five strains were isolated from separate sample sites and on both transects through the tropical mangrove forest, suggesting widespread occurrence in the environmental sampled.

In contrast to all the tropical strains (groups F, G and H) being representatives of the one named genus, *Aurantiochytrium*, the temperate strains (groups A, B, C, D and E) clustered closely to either of the four genera identified in the study, namely *Schizochytrium, Thraustochytrium, Ulkenia* and *Aurantiochytrium* (Table 2-4). It is generally held that the tropical habitats support a more diverse flora and fauna. However in this case, even though the mangrove forest supported a cline of mangroves species along the transects, the muddy sediment below appeared relatively homogeneous. It was only where this cline was interrupted (at the extreme ends of the transects) that thraustochytrids were isolated. The relatively higher habitat diversity of the temperate sampling transect (saltmarsh/mudflat) may have resulted in a greater variety of thraustochytrids being isolated/present.

Bowles et al. (1999) observed that the mean percentage of DHA (and PUFA in general) was higher in cold temperate thraustochytrids. We observed that the percentage of DHA, ranging from 19-61 % (mean 39.1 %) of the total FA of cold temperate strains (groups A, B, C, D and E) was slightly lower compared to those of the tropical strains ranging from 34-52 % (mean 43 %).

However, the temperate water strains contained a higher relative level of total PUFA (mean SUM 66 %) compared to the level observed for the tropical strains (mean SUM 57 %) (Table 2-1). Other strains of chemotaxonomic groups A, B, C, D, and E were isolated from the temperate environment. Three out of the five groups (A, B and C which clustered closely with *Schizochytrium* sp. and *Thraustochytrium* sp.) from the temperate environment had OC-PUFA present in the FA profiles. None of the isolated tropical strains contained OC-PUFA when grown under the conditions investigated in this study.

Omega-3 oil production

It is generally acknowledged that dietary consumption of omega-3 LC-PUFA, in particular DHA, has many benefits in human health (Tapiero et al. 2002). From a nutritional point of view, the total amount of omega-3 LC-PUFA in diets is important, and it is considered beneficial to obtain high ratios of omega-3/ omega-6 PUFA and DHA/EPA (Takahata et al. 1998). While chemotaxonomic groups A and B strains (*Schizochytrium* spp. and *Thraustochytrium* sp.) had less DHA and a lower omega-3/ omega-6 PUFA ratio than the other strains (*Ulkenia* sp. and *Aurantiochytrium* spp.), all strains would be well suited to provide these requirements. Chemotaxonomic group F strains closely clustered to the highly productive and high DHA-containing *Aurantiochytrium limacinum* SR21 (Yokochi et al. 1998) based on the 18S rRNA sequence data. This association suggests thraustochytrid strains from group F may have similar potential to be used as sustainable sources of omega-3 LC-PUFA for nutraceuticals and other applications as has been the case for *Aurantiochytrium limacinum* SR21 (Yokochi et al. 1998).

Through strain selection and manipulation of culture conditions to optimize chemical composition, thraustochytrid oils can be further tailored with specific qualities such as high DHA, low DPA-6, high omega-3 PUFA, and high omega-3/ omega-6 ratios. The relative amounts of omega-3 LC-PUFA also would be enhanced after removal of the biodiesel fraction. If there was a difference between the predominance of particular fatty acids in the different lipid classes (as occurs in fish oils) (Guil-Guerrero et al. 2007), the omega-3 LC-PUFA rich polar lipids and less unsaturated fatty acid rich neutral lipids (for biodiesel) can be separated by an inexpensive liquid/liquid partitioning as has been used for *Aurantiochytrium limacinum* SR21(Yamamura and Shimomura 1997). However, analysis of *Schizochytrium* sp. F26-b lipid classes has shown the fatty acid compositions to be quite similar (Abe et al. 2006), suggesting a simple winterization may be more appropriate. This involves the separation of omega-3 LC-PUFA and biodiesel fractions with temperature due to differential melting points of fatty acids resulting from differences in chain length and degree of unsaturation (Guil-Guerrero et al. 2007).

Biodiesel production

Group G strains had high levels of 13:0 and 15:0 saturated OC-FA. High levels of saturated and monounsaturated FA is more desirable for biodiesel production due to the increased oxidative stability of biodiesel as well as the thermal stability of biodiesel (Monyem et al. 2000; Knothe 2007). Strains from groups G and H contained 15:0 at between 20-30 % of the total FA. Group C (8 strains) was also suited to biodiesel production, with high SFA (mean 35 % TFA) and MUFA (mean 9 % TFA).

Johnson and Wen (2009) demonstrated the potential of producing biodiesel from *A. limacinum* SR21 using direct transesterification and the scaled-up production resulted in about 200 mL of liquid fuel from 400 g of biomass that was grown in a medium containing 90 g/L crude glycerol and 10 g/L corn steep solid dissolved in artificial seawater. The main aim of the present study was one of biodiscovery of novel strains for further development, and therefore the low FA yields observed were not obtained from cultures with optimised growth, fatty acid content or composition.

Ideally, high biomass producing strains will have higher yields and therefore be better candidates for biodiesel production. There has been little work in Australia to date examining thraustochytrids with regards to biodiesel production. Future research will involve the use of different growth parameters in order to optimise the biomass and oil yield and measurement of the biodiesel quantity and quality from these thraustochytrid derived FAME.

Further studies will also be required to explore methods to extract LC-PUFA for nutraceuticals and at the same time extract SFA and MUFA for biodiesel production. In order to achieve cost-effective extraction of thraustochytrids to co-produce both omega-3 LC-PUFA and biodiesel, future research will include targeting strains having both saturated and preferably monounsaturated fatty acids and LC-PUFA as major constituents. Although these FA groups may be in different in lipid classes, under high oil producing growth conditions, they may be associated with the neutral lipid classes, with further chemical processing required to separate these different fractions as noted above. In addition, the use of lipases can be used to digest specific LC-PUFA from the lipid classes that can then be harvested as free fatty acids.

Sterols

Sterols are an important component of cell membrane lipids derived from oxygenation of squalene (Lewis et al. 2001; Volkman et al. 1998). Squalene is a natural antioxidant and it has been suggested that it could effectively reduce the incidence of coronary heart disease and cancer (Smith 2000). Lewis et al. (2001) demonstrated that changes to cultures conditions, such as the level of dissolved oxygen, incubation temperature, and culture age, can influence squalene and sterol production in thraustochytrids. Several studies demonstrated that thraustochytrids have the potential to be a microbial source of squalene for the food and cosmetic industries (Fan et al. 2010; Lewis et al. 2001). Only trace amounts of squalene were detected in the present study (data not shown). Fan et al. (2010) proposed that this might be due to rapid conversion of squalene to other components during growth, as squalene is an intermediate in the sterol metabolic pathway. Importantly, the low glucose concentration (0.2 % w/v) basal medium used for the screening purposes of this study may have influenced squalene content. Chemotaxonomic groups E, G and H (*Aurantiochytrium* spp.) strains all shared cholesterol and stigmasterol as their major sterols, and this is consistent with 18S rRNA data where they were clustered closely in the phylogenetic tree. These differences between groups and the consistency with the clustering shown by the phylogeny, suggests that like the fatty acids, the sterols may be robust chemotaxonomic biomarkers (Volkman et al. 1998).

Pigments

Thraustochytrids can produce pigments such as astaxanthin when grown heterotrophically on organic substrates (Carmona et al. 2003). Astaxanthin is a valuable red carotenoid pigment that can be used as a nutraceutical in food and feeds as well as being considered as a bioactive compound for use in cosmetics due to its high antioxidant activities (Lorenz and Cysewski 2000).

It is also a dietary supplement for enhancing pigmentation in aquaculture species (Johnson and An 1991; Boussiba 2000). The astaxanthin content in thraustochytrids was relatively low compared to *Haematococcus* sp. (Boussiba and Vonshak 1991). In the present study, nine strains, one from each of seven different chemotaxonomic groups (A – E, H) and two strains from group G, were characterized for pigment (Table 2-3). No pigments were detected in the white coloured colonies. The orange and cream coloured strains contained β,β-carotene or astaxanthin, at up to 57 μg/ g and 100 μg/ g of dry weight, respectively.

The co-production of both biodiesel and other high value co-products presents a way to compensate for the expensive technology needed for the production and extraction of thraustochytrid oils. To achieve commercial interest, the yield of pigment would need to be much higher than was found in this preliminary study, and as in the case of increasing biomass and fatty acid yield and tailoring fatty acid composition for biodiesel or omega-3 LC-PUFA, pigment production could be manipulated using culture conditions. Interestingly, non-pigmented strains of *Aurantiochytrium* sp. were found to produce higher biomass and more of the omega-3 LC-PUFA than the pigmented strains which contained higher levels of saturated fatty acids (Fan et al. 2009).

Further studies will be carried out with these newly isolated Australian thraustochytrid strains, including growth at temperatures above 20°C and using higher concentrations of glucose and other carbon sources to maximize production of biomass and products. The content of pigments and fatty acids and other components will need to be determined under different growth conditions as part of a wider strain selection and optimization strategy.

Chapter 3 Odd-chain polyunsaturated fatty acids in thraustochytrids

Abstract

A series of unusual odd-chain fatty acids (OC-FA) were identified in two thraustochytrid strains, TC 01 and TC 04, isolated from waters off the south east coast of Tasmania, Australia. FA compositions were determined by capillary GC and GC–MS, with confirmation of polyunsaturated fatty acids (PUFA) structure performed by analysis of 4,4-dimethyloxazoline derivatives. PUFA constituted 68–74 % of the total FA, with the essential PUFA; eicosapentaenoic acid (20:5ω3, EPA), arachidonic acid (20:4ω6, AA) and docosahexaenoic acid (22:6ω3, DHA), accounting for 42–44 % of the total FA. High proportions of the saturated OC-FA 15:0 (7.1 % in TC 01) and 17:0 (6.2 % in TC 04) were detected. The OC-FA 17:1ω8 was present at 2.8 % in TC 01. Of particular interest, the C_{21} PUFA 21:5ω5 and 21:4ω7 were detected at 3.5 % and 4.1 %, respectively, in TC 04. A proposed biosynthesis pathway for these OC-PUFA is presented. It is possible that the unsaturated OC-PUFA found previously in a number of marine animals were derived from dietary thraustochytrids and they could be useful biomarkers in environmental and food web studies.

Introduction

Thraustochytrids are heterotrophic protists, commonly found in marine and estuarine environments (Porter and Lingle 1992; Raghukumar 2002). Thraustochytrids are considered to play an important role as decomposers (ie. detritivores) in the marine ecosystems (Sharma et al. 1994; Raghukumar 2002). Thraustochytrids are ubiquitous on dead or decaying plant material, such as seaweed and submerged leaves, and are capable of producing extracellular enzymes that chemically alter and mineralise detritus. In addition, they can be pathogenic parasites on animals such as juvenile abalone (*Haliotis kamtschatkana*) and hard-shell clam (*Mercenaria mercenaria)* (Whyte et al. 1994; Maas et al. 1999; Mo et al. 2002; Bower 1987). Some thraustochytrids appear to have mutualistic associations with reef-building corals such as *Favia* sp. (Siboni et al. 2010) and *Fungia granulosa* (Harel et al. 2008).

Thraustochytrids were considered to be closely related to primitive fungi and protozoans (Ellenbogen and Aaronson 1969). More recently, molecular phylogenetic studies have resulted in their classification into the class Labyrinthulomycota and phylum Heterokonta in the kingdom Chromista, which also includes the chromophyte algae such as brown algae and diatoms (Porter 1990; Cavalier-Smith et al. 1994; Leander et al. 2004). They are characterised by the capacity to produce high amounts of omega-3 long chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA), including docosahexaenoic acid (22:6ω3, DHA) and eicosapentaenoic acid (20:5ω3, EPA) and the omega-6 LC-PUFA arachidonic acid (20:4ω6, AA) (Iida et al. 1996; Jiang et al. 2004; Jain et al. 2007).

Thraustochytrids can be grown heterotrophically in fermenters with organic substances (e.g. sugars, organic acids) as the only carbon and energy source (Raghukumar 2008; Vazhappilly and Chen 1998; Barclay et al. 1994b). Several studies have revealed that heterotrophic cultivation of microalgae results in high production of biomass and accumulation of high lipid content in cells (Liang et al. 2009; Miao and Wu 2006). The advantages of heterotrophic production in fermenters over photoautotrophic systems (open ponds and closed photobioreactors) include simpler scale-up (independent of light, less land required), higher degree of growth control, minimal contamination, lower capital cost and higher cell densities (Brennan and Owende 2010).

Due to their fast growth rate and high lipid content, thraustochytrids have potential for producing a feedstock for biofuels and omega-3 oils for nutraceuticals, as well as animal feeds. We are examining the potential of Australian thraustochytrids for use as potential source organisms for biodiesel, omega-3 oils and other co-products. As part of this research, we isolated and examined the lipid profiles of 36 thraustochytrid strains from both temperate and tropical Australian waters. Here we report on the unusual occurrence of OC-FA, including OC-PUFA, in two thraustochytrid strains.

Materials and methods

Culture maintenance

Thraustochytrids were isolated from benthic and water samples from the south east coast of Tasmania and far north of Queensland of Australia, and identified both by morphological examination using light microscopy and molecular identification (18S rDNA sequences, Chapter 2). The strains *Schizochytrium* TC 01 and *Thraustochytrium* TC 04 were isolated from southeast Tasmania and were maintained on a basal medium that consists of: 1 l natural seawater, 0.5 % (w/v) of bacteriological peptone, 0.2 % (w/v) glucose and 0.2 % (w/v) yeast extract (Sigma-Aldrich, St. Louis, MO, USA). Cultures were grown in sterile 50 ml polystyrene cell culture flasks including a cap fitted with a 0.2 μm filter (Greiner Bio-One, Germany) that were shaken at 100 rpm (20 °C) to facilitate culture mixing for 7 days. To harvest biomass the culture broth was filtered through pre-weighed 4.5 cm diameter Whatman® GF/C glass fiber filters (Sigma-Aldrich, St. Louis, MO, USA). After rinsing with 3.15 % (w/v) ammonium formate, the filters were folded and wrapped in aluminum foil and placed directly into liquid nitrogen (-196 °C). Prior to analysis the samples were freeze-dried overnight, and weighed to determine dry weight.

Lipid extraction

Freeze-dried filters with biomass were transferred to glass test tubes fitted with screw caps, then directly transesterified with methanol:chloroform:HCl (l0: 1 :1 v/v/v) under nitrogen and heated for 2 h at 80 °C with vigorous shaking every l5 min. After cooling to room temperature, 2.5 ml of Milli-Q® water (Millipore, Milford, MA, USA) and 1.8 ml of hexane:chloroform (4:1, v/v) was added to the tube. The tube was shaken and centrifuged at 2000 rpm for 5 min to break phase. The upper phase containing FAME was removed and the lower phase extracted a further two times. Following removal of solvent with nitrogen gas, 80 µl of BSTFA (N,O-Bis(trimethylsilul)trifluoroacetamide) (Alltech, Deerfield, IL, USA) was added and the vial was heated for an hour at 60 ºC to convert any hydroxyl-containing lipids (such as sterols) to their OTMSi-ether lipids. After removal of excess BSTFA with nitrogen gas, 1 ml of chloroform containing a known concentration of methyltricosanoate was added to each tube as an internal injection standard and the samples were analyzed by GC.

The naming of FAME has adopted the convention: X:YωZ, where X refers to the number of carbon atoms in the molecule, Y refers to the number of double bonds in the molecule, and Z indicates the carbon position of the first double bond from the methyl end (CH_3) of the molecule. The latter is generally referred to as Omega Z (e.g. ωZ) or altematively n-Z (e.g. n minus Z). The alternative delta or Δ convention indicates the carbon position of a double bond from the carboxylic acid (COO-) of the FAME.

Preparation of 4,4-dimethyloxazoline (DMOX) derivatives

The DMOX method was adapted from (Christie 1998). FAME were first saponified and, after extraction and removal of solvent, the resulting FFA were converted to their acid chlorides using oxalyl chloride (0.5 ml) at ambient temperature overnight. After addition of Milli-Q® water, the acid chloride

products were extracted (2 × 2 ml; hexane/chloroform 4:1, v/v). After removal of the solvent with nitrogen gas, the products were reacted with 2-amino-2-methylpropanol in dichloromethane (0.5 ml of a 10 mg/ ml solution, stored over molecular sieve). After 1 h at ambient temperature, the solvent was removed under a stream of nitrogen, trifluoracetic acid anhydride (1 ml) was added, and the mixture was placed in an oven at 40 °C for 1 h. The excess reagent was again removed under a stream of nitrogen and, after addition of Milli-Q® water (2 ml), the DMOX products were extracted (2 × 2 ml) as before and redissolved in DCM prior to GC and GC-MS analysis.

GC and GC-MS analysis

GC analysis of FAME was performed using an Agilent Technologies 7890A GC (Palo Alto, CA, USA) equipped with a non-polar Equity-1 ™ fused silica capillary column (15 m x 0.1 mm i.d., 0.1 mm film thickness), an FID, a split/splitless injector and an Agilent Technologies 7683 Series auto sampler and injector with Helium as the carrier gas. Samples were injected in splitless mode at an oven temperature of 120 °C and after injection the oven temperature was raised to 270 °C at 10 °C/ min and finally to 310 °C at 5 °C/ min. Peaks were quantified with Agilent Technologies ChemStation software (Palo Alto, CA, USA).

GC-MS analysis of the FAME and DMOX derivatives was performed to confirm component identifications and was carried out on a Finnigan GCQ Plus™ GC-MS ion-trap fitted with an on-column injector set at 45 °C. Samples were injected using an AS2000 auto sampler onto a retention gap attached to a non polar HP-5 Ultra 2 bonded-phase column (50 m x 0.32 mm i.d. x 0.17 µm film thickness). The initial temperature of 45 °C was held for 1 min, followed by temperature programming at 30 °C/ min to 140 °C then at 3 °C/ min to 310 °C where it was held for 12 min. Helium was used as the carrier gas. Mass spectrometer operating conditions were: electron impact energy 70 eV; emission current 250

μamp, transfer line 310 °C; source temperature 240 °C; scan rate 0.8 scan/ sec and mass range 40-650 Da. Mass spectra were acquired and processed with Thermo Scientific Xcalibur™ software (Waltham, MA, USA).

Results

Of the 36 thraustochytrid strains analyzed, 19 strains had high proportions of saturated OC-FA; 12 strains had long-chain (≥C_{21}) OC-PUFA. The highest proportions of OC-FA were detected in two strains, *Schizochytrium* TC 01 and *Thraustochytrium* TC 04. The FA profiles of TC 01 and TC 04 were dominated by PUFA (68–70 % of total FA), saturated fatty acids (SFA, 22–25.4 %) and monounsaturated fatty acids (MUFA, 3.8–6.5 %), with OC-FA at 21.2–27 % (Table 3-1). The sum of SFA and MUFA were higher in TC 01 compared to TC 04, and the sum of PUFA and of OC-FA were higher in TC 04 compared to TC 01.

The main PUFA were DHA, docosapentaenoic acid (DPA-6, 22:5ω6), AA and EPA for both strains (Table 3-1). These four components accounted for more than 50 % of the total FA. The AA/EPA ratio and the ω6/ω3 ratio were higher in TC 01 compared to TC 04. The total level of C_{20} PUFA was higher in TC 01 (20.7 %) compared to TC 04 (11.8 %). Additionally, in TC 04 DHA and DPA-6 were detected at 31.1 % and 13.4 %, respectively. Of particular interest, a series of OC-FA were detected, and in decreasing order of abundance for TC 01 were: 15:0 (7.1 %), 17:1ω8 (2.8 %), 19:4ω5 (2.8 %), 17:0 (2.2 %), 21:5ω5 (1.6 %), 21:4ω7 (1.4 %), 19:1ω10 (0.7 %), 17:2ω5 (0.6 %) and 21:4ω5 (0.5 %). For TC 04 OC-FA were: 17:0 (6.2 %), 15:0 (5.2 %), 21:4ω7 (4.1 %), 21:5ω5 (3.5 %), 19:1ω10 (2.2 %), 19:3ω5 (1.3 %), 21:6ω2 (1.1 %), 17:1ω8 (0.8 %) and 21:4ω5 (0.6 %) (Table 3-1). The long-chain (≥C_{21}) OC-PUFA were found at higher levels in TC 04 (9.3 %) compared to TC 01 (3.7 %), whereas the shorter chain (<C_{21}) OC-PUFA were more abundant in TC 01 (8.1 %) compared to TC 04 (5.4 %) (Table 3-1).

Table 3-1 Fatty acid (FA) composition (%) of two thraustochytrid strains (TC 01 and TC 04) isolated from the south east coast of Tasmania (n = 2)

Fatty acid	TC 01 (mean ± SD)	TC 04 (mean ± SD)
14:0	1.8 ± 0.0	0.2 ± 0.0
15:0	7.1 ± 0.0	5.2 ± 0.6
16:1ω10	0.3 ± 0.0	0.2 ± 0.0
16:1ω7	0.5 ± 0.1	0.1 ± 0.1
16:0	13.2 ± 0.1	9.3 ± 0.5
17:2ω5	0.6 ± 0.2	0.3 ± 0.1
17:1ω8	2.8 ± 0.5	0.8 ± 0.2
17:1ω7	0.1 ± 0.2	0.1 ± 0.2
17:0	2.2 ± 0.0	6.2 ± 0.2
18:3ω6	0.6 ± 0.0	0.1 ± 0.1
18:3ω3	Trace[b]	0.1 ± 0.1
18:2ω6	1.9 ± 0.0	0.4 ± 0.1
18:1ω9	2.2 ± 0.2	0.3 ± 0.0
18:1ω7	0.6 ± 0.1	1.2 ± 1.1
18:0	0.8 ± 0.1	0.4 ± 0.1
19:4ω5	2.8 ± 0.0	0.1 ±0.1
19:3ω8	0.2 ± 0.0	0.1 ± 0.1
19:3ω5	0.4 ± 0.1	1.3 ± 0.3
19:2ω7	0.4 ± 0.1	0.3 ± 0.4
19:1ω10	0.7 ± 0.2	2.2 ± 0.1
19:1ω8	0.1 ± 0.1	0.2 ± 0.2
19:0	0.2 ± 0.0	0.2 ± 0.1
20:4ω6, AA	11.3 ± 0.0	1.8 ± 0.0
20:5ω3, EPA	6.8 ± 0.2	9.5 ± 0.4
20:3ω6	1.8 ± 0.0	0.2 ± 0.0
20:4ω3	0.4 ± 0.0	0.1 ± 0.0
20:2ω6	0.3 ± 0.0	0.1 ± 0.2
20:0	0.1 ± 0.0	0.1 ± 0.1
21:5ω5	1.6 ± 0.1	3.5 ± 0.6
21:4ω7	1.4 ± 0.0	4.1 ± 0.0
21:4ω5	0.5 ± 0.0	0.6 ± 0.1
21:6ω2	0.2 ± 0.2	1.1 ±1.6
22:5ω6, DPA-6	6.3 ±1.6	13.4 ± 0.8
22:6ω3, DHA	26.4 ± 0.9	31.1 ± 2.3
22:4ω6	1.2 ± 0.2	0.6 ± 0.3
22:5ω3, DPA-3	1.2 ± 0.0	0.6 ± 0.1
22:4ω3	0.1 ± 0.2	0.4 ± 0.2
22:0	0.1 ± 0.1	0.4 ± 0.5
Others[a]	0.4 ± 0.3	0.3 ± 0.1
SUM SFA	25.4 ± 0.0	21.9 ± 0.9
SUM MUFA	6.5 ±1.3	3.8 ± 0.2
SUM PUFA	68.1 ±1.2	74.2 ±1.1
SUM OC-FA	21.2 ± 0.5	27.0 ± 0.6

[a]Others refer to FA < 0.1%: 16:1ω5, 18:4ω3, 18:1ω6, 19:2ω5, 20:3ω3, 20:1ω5, 21:0, 21:3ω7 and 23:5ω5; [b]Trace denotes <0.05% of total FA

The electron impact (EI) mass spectra (MS) of odd-chain fatty acid methyl esters (FAME) gave the characteristic diagnostic ions for SFA (*m/z* 74,87), MUFA (*m/z* 55, 74), PUFA (dienes *m/z* 67, 81 and for 3 or more double bonds *m/z* 79, 91) and typical fragmentation patterns with small but discernible M^+ peaks. Double bond locations were determined by EI GC–MS analysis of DMOX derivatives of FA. An interval of 12 Da, instead of the regular 14 Da observed between the peaks of adjacent ion clusters at n and n − 1 carbon atoms in the FAME indicates a double bond between carbons n and n + 1 in the molecule. An intense fragment ion at *m/z* 152 indicates a double bond at C-4, while intense fragment ions at *m/z* 153 (often accompanied by a high 152) indicates a double bond at C-5 (Spitzer 1996; Christie 2009; Saito 2007; Zhang et al. 1988).

The major C_{21} OC-PUFA in TC 01 was identified as 21:5ω5 (4,7,10,13,16) based on the MS (Fig. 3-1A). A molecular ion of *m/z* 369 was present, with an M − 1 ion at *m/z* 368 also present, and the fragmentations observed were consistent with this structure for a DMOX derivative of a FA with a base peak of *m/z* 113. An elevated *m/z* 152 (indicating Δ4 unsaturation) and the characteristic 12 Da gaps occurred at m/z 166/178 (Δ7), 206/218 (Δ10), 246/258 (Δ13) and 286/298 (Δ16) (Table 3-2).

In TC 04 the dominant C_{21} OC-PUFA was 21:4ω7 (5,8,11,14) and the MS showed a molecular ion of m/z 371, with an M − 1 ion at *m/z* 370 also present, base peak of *m/z* 113, elevated *m/z* 153 (indicating Δ5 unsaturation), and the characteristic 12 Da gaps at m/z 180/192 (Δ8), 220/232 (Δ11) and 260/272 (Δ14) (Fig. 3-1B).

Table 3-2 Characteristic and diagnostic fragment ions from DMOX derivatives of selected fatty acids from thraustochytrids TC 01 and TC 04

Fatty acid	Molecular ion	Diagnostic ions*
17:2ω5 (9,12)	319	196/208; 236/248
18:1ω6 (12)	335	238/250
19:4ω5 (5,8,11,14)	342 (M-1)	152/153; 180/192; 220/232; 260/272
19:3ω8 (5,8,11)	344 (M-1)	152/153; 180/192; 220/232
19:3ω5 (8,11,14)	345	182/194; 222/234; 262/274
19:2ω7 (9,12)	347	196/208; 236/248
19:2ω5 (11,14)	347	224/236; 264/276
20:5ω3 (5,8,11,14,17)	354 (M-1)	152/153; 180/192; 220/232; 260/272; 300/312
21:5ω5 (4,7,10,13,16)	368 (M-1)	152; 166/178; 206/218; 246/258; 286/298
21:4ω7 (5,8,11,14)	370 (M-1)	153; 180/192; 220/232; 260/272
21:4ω5 (7,10,13,16)	370(M-1)	168/180; 208/220; 248/260; 288/300
21:3ω7 (8,11,14)	373	182/194; 222/234; 262/274
21:6ω2 (4,7,10,13,16,19)	366 (M-1)	152; 166/178; 206/218; 246/258; 286/298; 326/338
22:6ω3 (4,7,10,13,16,19)	380 (M-1)	152, 166/178; 206/218; 246/258; 286/298; 326/338

* DMOX FAME derivatives also had intense ions at m/z 98, 113, 126

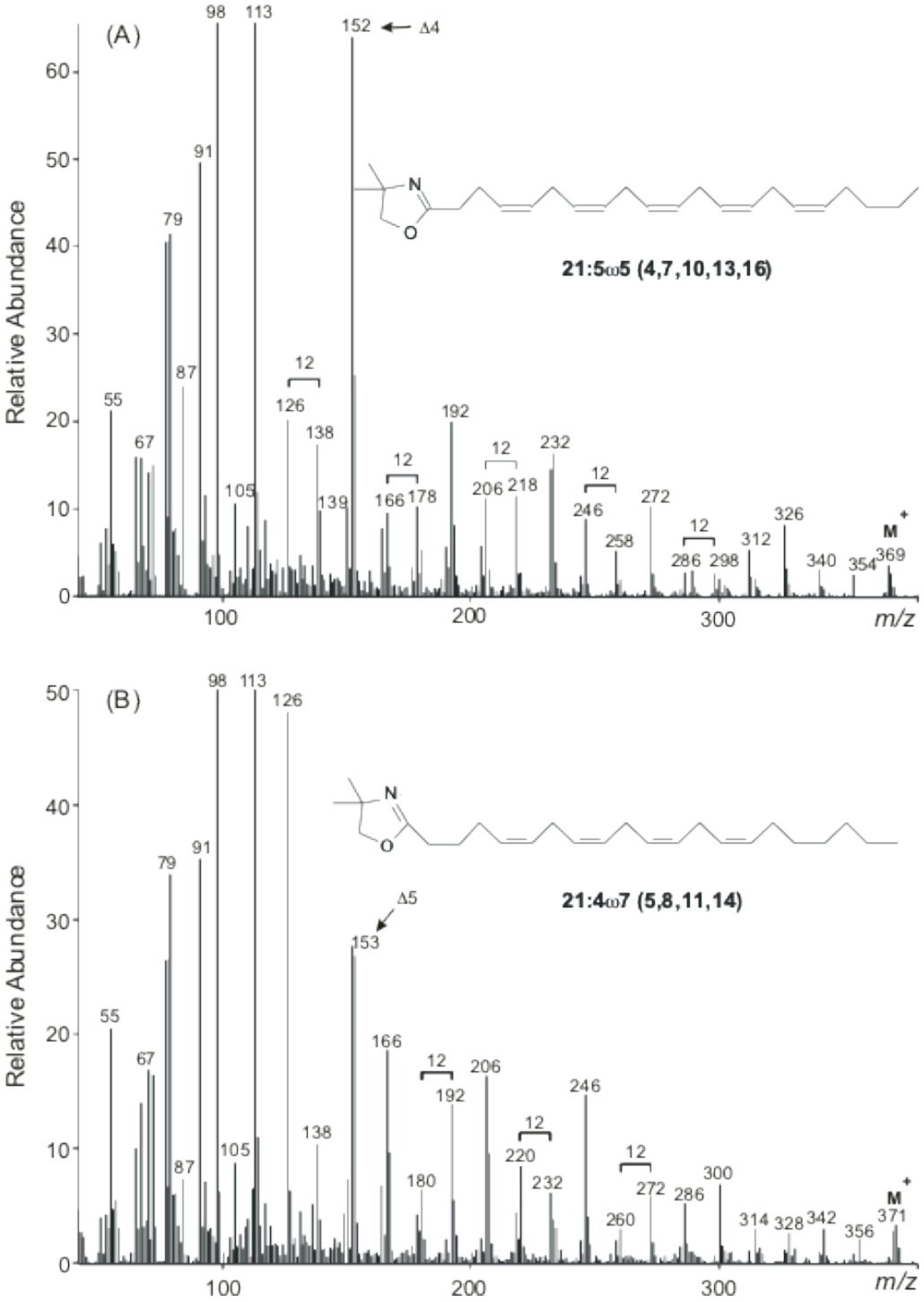


Figure 3-1 Mass spectra of DMOX derivatives of the two major C_{21} PUFA: (**A**) 21:5ω5 (4,7,10,13,16) and (**B**) 21:4ω7 (5,8,11,14). Ions used to identify double bond positions at Δ4 and Δ5 are 152 and 153, respectively. Other double bond positions are located where a gap of 12 amu is flanked by two intense fragment ions of 40 amu difference

Discussion

The FA profiles of thraustochytrid strains TC 01 and TC 04 were similar to those of other reported thraustochytrids (Burja et al. 2006; Raghukumar 2008), with high proportions of omega-3 LC-PUFA including DHA and EPA as the main components and accounting for 33–41 % of the total FA. However, the profiles of these strains were different from other reports due to the relatively high proportions of saturated, monounsaturated and polyunsaturated OC-FA (Fig. 3-2). There has been one report of high proportions of saturated OC-FA (15:0 at 34 %) in a thraustochytrid strain with the proportions of saturated OC-FA being able to be manipulated with culture conditions (Wang et al. 2000). Unsaturated OC-FA were not reported in that study.

Saturated OC-FA are major fatty acids in certain groups of bacteria, but are typically minor (<1 %) components in lower plants or animals (Rezanka and Sigler 2009). Odd-chain MUFA and PUFA of the families ω9 and ω3 FA have been identified in some invertebrates (Rezanka and Sigler 2009). For example, 21:5ω3 occurred in a marine sponge; 23:1ω9 and 21:5ω3 in a freshwater amphipod (*Gammarus*), crabs (*Rhithropanopeus harrisii*), crayfish (*Astacus leptodactylus eichwaldi*) and bivalves (*Mytilaster lineatus*). These FA were proposed to most likely accumulate from the diet (Rezanka and Sigler 2009). The unsaturated methylene interrupted OC-FA identified in thraustochytrids in our study were predominantly ω7 and ω5 FA with smaller amounts of ω8 FA (Table 3-1). Interestingly, 17:2ω5, 19:4ω5 and 21:4ω5 have also been detected in the bivalve *Megangulus zyonoensis* (Kawashima and Ohnishi 2004).

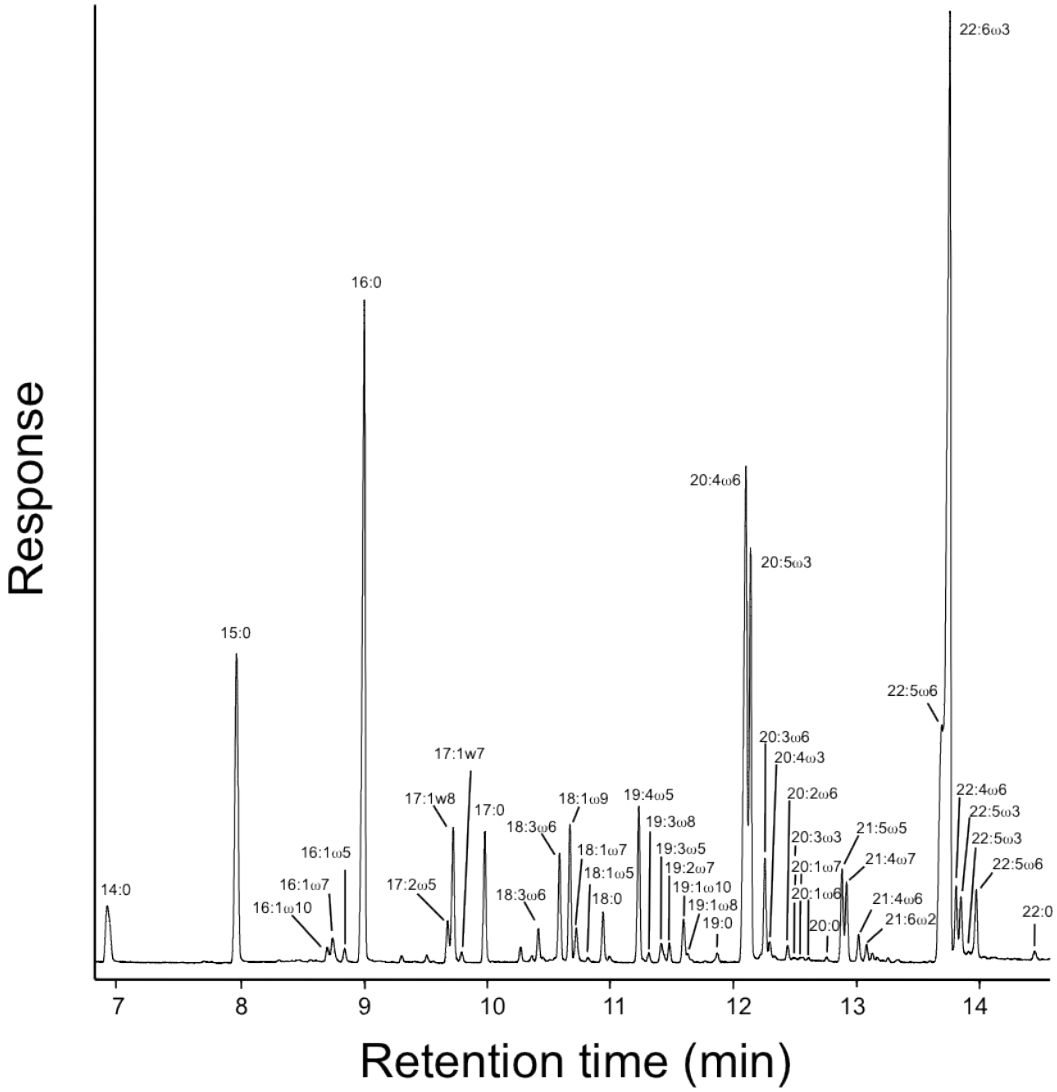


Figure 3-2 Gas chromatogram of the total FA (as FAME) of thraustochytrid TC01, showing unusual odd-chain polyunsaturated fatty acids

Furthermore, 19:4ω5, 21:4ω7, 21:4ω5 and 21:5ω2 have been identified in the amphipod *Pontoporeia femorata* (Paradis and Ackman 1976). This species of amphipod is known to be a detritivore feeding on decaying organic matter in marine sediments (Lopez and Elmgren 1989), and it is possible that the presence of unsaturated OC-FA in these marine animals was derived from dietary thraustochytrids. In a broader context these unusual OC-PUFA could be useful biomarkers in benthic food chain studies. The novel OC-PUFA 21:5ω5 has been detected in rat liver cells in a study by Nakano et al. (2000). The study demonstrated the biosynthesis of highly desaturated PUFA in animal cells using exogenous C_{19} PUFA for the formation of C_{21} PUFA. The authors suggested the OC-PUFA were metabolized through the same route that EPA is biosynthesized to DHA.

To our knowledge, 19:4ω5, 21:4ω5, 21:5ω5 and 21:4ω7 have not been reported in thraustochytrids. Based on detection of the OC-PUFA in the two thraustochytrid strains, we propose a pathway from saturated 17:0 and 19:0 leading to the synthesis of 19:4ω5 and also the C_{21} PUFA. Propionate has been suggested as a precursor for these long-chain OC-FA (Paradis and Ackman 1976; Ackman 1965). The necessary desaturation and elongation step are included in the pathway (Fig. 3-3). This pathway requires the same enzymatic reactions for elongation and desaturation as those in the conventional aerobic PUFA biosynthesis pathway.

Zhang et al. (2006) demonstrated a mutant of the fungus *Mortierella alpina*, which is defective in its Δ12 desaturase activity, but exhibited enhanced activities of Δ5 and Δ6 desaturases and produced up to 80 % of OC-FA when grown on n-alkane. Zhang et al. (2006) proposed a biosynthetic pathway analogous to the biosynthetic pathway for the n-9 series. It involved: 17:0 ➔ 17:1ω8 ➔ 17:2ω8 ➔ 19:2ω8 ➔ 19:3ω8. This is consistent with our proposed pathway, although 17:2ω8 and 19:2ω8 were not detected in our two strains. In another study by Shimizu et al. (1991), *Mortierella alpina* showed high production of OC-PUFA with a proposed biosynthetic route to 19:4ω5. The authors of this study suggested an analogous pathway to the n-6 route to arachidonic acid as follows: 17:0 ➔ 17:1ω8 ➔ 17:2ω5 ➔ 17:3ω5 ➔ 19:3ω5 ➔ 19:4ω5. As 17:3ω5 was not detected in our thraustochytrid strains, the absence of these intermediates might be due to their low abundance and therefore below the detection limit, possibly due to being very efficiently converted to other fatty acids. Alternatively, the presence of a Δ8 desaturase would result in 19:1ω8 and 19:2ω5 being the precursors for the long-chain ω8 and ω5 OC-PUFA, respectively, as suggested in Figure 3-3.

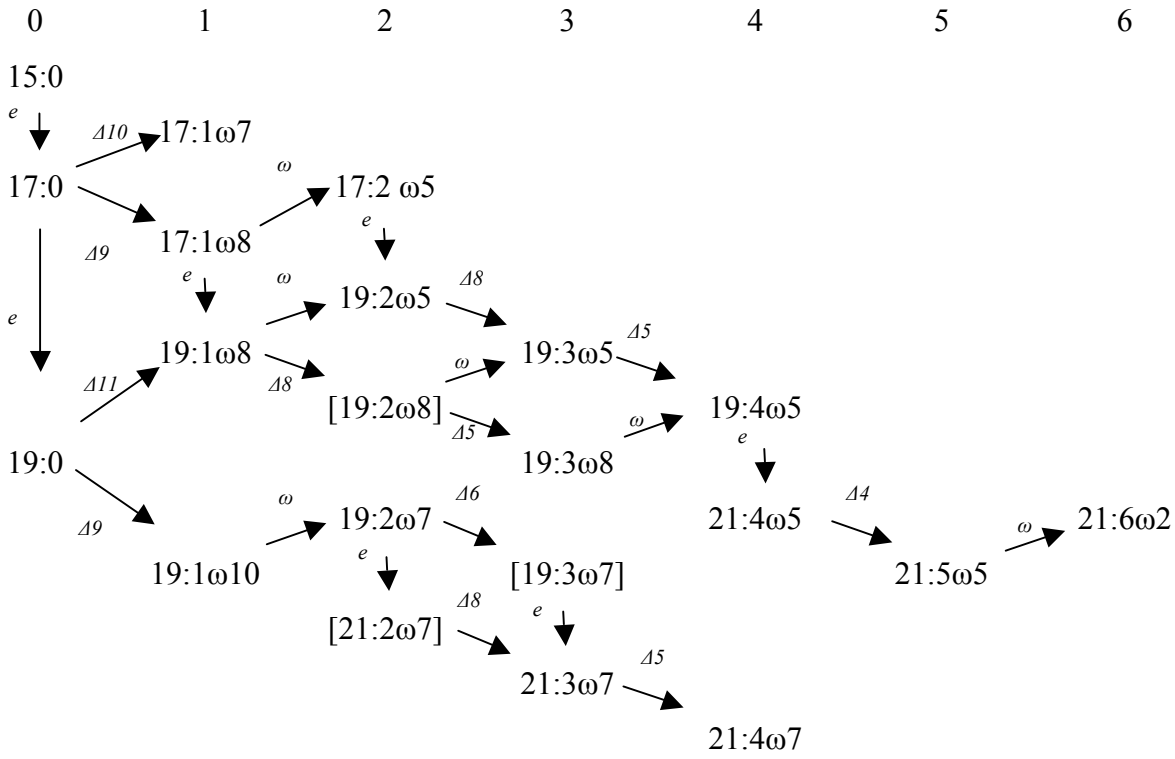


Figure 3-3 Proposed OC-FA biosynthesis pathway in thraustochytrids TC 01 and TC 04. Top row indicates number of double bonds; vertical arrows indicate elongase (e). Excluding the initial Δ9, Δ10 or Δ11 desaturation, upward angled arrows indicate methylene interrupted desaturation towards the omega (ω, methyl) side of an existing double bond (as occurs in organisms which can produce PUFA de novo, including most photosynthetic and some heterotrophic organisms), downward angled arrows indicate methylene interrupted desaturation towards the delta (Δ, carboxyl) side of an existing double bond, FA in brackets were not identified in our samples, but indicate likely pathway intermediates

An alternative pathway for LC-PUFA ($\geq C_{20}$) biosynthesis was described by Metz et al. (2001). This pathway does not require multiple desaturase and elongase enzymes, but instead employs a polyketide synthase (PKS) gene cluster for the synthesis of LC-PUFA (Metz et al. 2001). The high level of DPA-6 in TC 04 could also indicate the involvement of the PKS-like pathway, although the low level (0.1 %) of ALA in TC 04 also indicates the use of the conventional PUFA biosynthesis pathway. Furthermore, Lippmeier et al. (2009) suggested these thraustochytrids might utilize both pathways for PUFA biosynthesis depending on substrate availability. Further studies involving supplementation with radio labeled precursors would help to understand the PUFA biosynthetic pathway in these thraustochytrids.

Given the potential of thraustochytrids for co-producing a feedstock for biodiesel and omega-3 oils in heterotrophic cultivation conditions, the identification of OC-FA provides additional insight into the biology of thraustochytrids, biosynthesis of fatty acids, and their potential roles in benthic and pelagic food webs. Future research is required on the possible bioactivity of these OC-FA to determine their suitability for novel applications and commercialization.

Chapter 4 Comparison of thraustochytrids *Aurantiochytrium* sp., *Schizochytrium* sp., *Thraustochytrium* sp. and *Ulkenia* sp. for production of biodiesel, long-chain omega-3 oils and exopolysaccharides

Abstract

Heterotrophic growth of thraustochytrids has potential in co-producing biodiesel for transportation, as well as producing a feedstock for omega-3 long-chain (≥C20) polyunsaturated fatty acids (LC-PUFA), especially docosahexaenoic acid (DHA) for use in nutraceuticals. In this study, we compared eight new endemic Australian thraustochytrid strains from the genera *Aurantiochytrium*, *Schizochytrium*, *Thraustochytrium* and *Ulkenia* for the synthesis of exopolysaccharide (EPS), in addition to biodiesel and LC-PUFA. *Aurantiochytrium* sp. strains readily utilized glucose for biomass production and increasing glucose from 2 %w/v to 4 %w/v of the culture medium resulted in increased biomass yield by an average factor of 1.7. *Ulkenia* sp. strain TC 10 and *Thraustochytrium* sp. strain TC 33 did not utilize glucose, while *Schizochytrium* sp. strain TC 02 utilized less than half the glucose available by day 14, and *Thraustochytrium* sp. strain TC 04 utilized glucose at 4 %w/v but not 2 %w/v of the culture suggesting a threshold requirement between these values. Across all strains increasing glucose from 2% w/v to 4% w/v of the culture medium resulted in increased total FAME content by an average factor of 1.9. Despite an increasing literature demonstrating the capacity of thraustochytrids for DHA synthesis, the production of EPS from these organisms is not well documented. A broad range of EPS yields were observed. The maximum yield of EPS was observed for *Schizochytrium* sp. strain TC 02 (299 mg/L). High biomass producing strains that also have high lipid and high EPS yield, may be better candidates for commercial production of biofuels and other co-products.

Introduction

Exploration for microalgae-derived biofuel has increased due to growing concern with rising crude oil prices, and the future availability and overuse of fossil fuels. In addition to utilizing the lipids for biofuel production, microalgae are capable of producing other high-value bioproducts, such as proteins, enzymes, polyunsaturated fatty acids (PUFA), carotenoid pigments and exopolysaccharides (EPS). The formation of high-value co-products during biofuel production is desirable when it adds greater value to the production process and improved process economics (Li et al. 2008; Stephens et al. 2010; Wijffels and Barbosa 2010). In a previous screening study, we demonstrated that recently isolated endemic Australian thraustochytrid strains were promising candidates for heterotrophic production of biodiesel, omega-3 long chain ($\geq C_{20}$) polyunsaturated fatty acids (omega-3 LC-PUFA) and carotenoid pigments (Lee Chang et al. 2012). Eight chemotaxonomic groups (A- H) were identified based on fatty acid and sterol composition, with the groups clustered closely with four different genera obtained by 18S rDNA molecular identification. *Aurantiochytrium* sp. strains (group G and H) contained 15:0 (pentadecanoic acid) at between 20 – 30 % of the total fatty acids (TFA) and 16:0 (palmitic acid) in the range of 7 – 15 % TFA. Furthermore, the *Thraustochytrium* sp. strains (Group C) were also suited to biodiesel production, with high levels of saturated fatty acids (SFA, mean 35 % TFA; 15:0, 7– 16 % TFA and 16:0, 15– 20 % TFA) and monounsaturated fatty acids (MUFA, mean 9 % TFA). These shorter chain fatty acids with high levels of SFA and MUFA are more desirable for biodiesel production due to the increased oxidative and thermal stability of biodiesel (Knothe 2007, 2008; Monyem et al. 2000).

Thraustochytrids are heterotrophic protists commonly found in the marine environment and play an important role in marine ecosystems; they can be bacterivores, detritivores or parasites (Lee Chang et al. 2012; Maas et al. 1999; Raghukumar 2002). Unlike phototrophic algae, thraustochytrids are usually grown heterotrophically for mass production in stainless steel fermenters using complex organic substances, including by-products from other processes (e.g. sugars, organic acids) as a sole carbon and energy source. Some strains of thraustochytrids are known to produce high levels of omega-3 LC-PUFA, including docosahexaenoic acid (DHA, 22:6ω3) and eicosapentaenoic acid (EPA, 20:5ω3), which are fatty acids essential for human health (Jain et al. 2007). A range of studies have shown that consumption of omega-3 LC-PUFA helps prevent the risk of cardiovascular diseases, neural disorders, arthritis, asthma and skin diseases in humans (Horrocks and Farooqui 2004; Horrocks and Yeo 1999; Kris-Etherton et al. 2004; Takahata et al. 1998). DHA is also essential for neural and retinal development during fetal life and infancy (Forsyth and Carlson 2001; Ratledge 2004). While the capacity of thraustochytrids to produce high amounts of omega-3 LC-PUFA has been well documented (Iida et al. 1996; Jain et al. 2007; Jiang et al. 2004; Lewis et al. 1999), to our knowledge, there have been only limited reports examining the production of EPS by thraustochytrids (Jain et al. 2005; Raghukumar et al. 2000).

Bacteria and microalgae living in aquatic ecosystems commonly secrete extracellular polymeric substances. These can be formed as capsular material that closely surrounds the microbial cell or they may be released into the surrounding environment as dispersed slime with no obvious association to any one particular cell (Decho 1990; Sutherland 1982; Wotton 2004). A large proportion (40 – 95 %) of this polymeric material is EPS and it may also include proteins, nucleic acids and lipids (Flemming and Wingender 2001).

EPS form a highly hydrated matrix (Flemming et al. 1999) and provides a layer of protection to cells against toxic compounds (Bitton and Freihofer 1977; Jeanthon and Prieur 1990) or digestion by other organisms (Caron 1987). Recent studies have shown that microbial EPS improves the bioavailability of trace nutrients, including iron, to phytoplankton in iron-limited regions of the world's ocean (Hassler et al. 2011). Jain et al. (2005) demonstrated that EPS play an important role in preventing cellular desiccation in thraustochytrids.

There has been a growing interest in the isolation and identification of new microbial polysaccharides with biotechnological potential. Polysaccharides produced by microorganisms have a range of uses as gelling, flocculent and thickening agents in the food and beverage, oil, cosmetic, paper, paint, adhesive and textile industries (Sandford 1984; Sutherland 1972). Clinical applications include pharmaceuticals, cancer therapy, drug delivery, promotion of bone healing and wound dressings for patients with burns, chronic ulcers or extensive tissue loss (Guezennec 2002; Labare et al. 1989; Sutherland 1998; Weiner 1997; Zanchetta et al. 2003). As high energy compounds, EPS are also suitable for fermentation into bio-ethanol as a biofuel (González López et al. 2009). Alternatively, EPS may be a high-value co-product formed in low amounts during the microbial biofuel production (Donot et al. 2012).

This study examines newly isolated Australian thraustochytrid strains from eight different chemotaxonomic groups, which have been detailed previously (Lee Chang et al. 2012). Although a number of studies now exist for a range of thraustochytrids, limited data is available for endemic Australian strains due to national quarantine restrictions. The availability of thraustochytrid strains from culture collections and information for optimal growth conditions will be required for any future commercial production of this sought after group.

The eight strains were cultured in baffled shake flasks to compare the biomass yield and fatty acid profiles. This study also details the isolation of EPS material released into the culture media by these strains, and describes the crude chemical characterization of the EPS as an initial step in determining the potential for biotechnological application of these biomaterials.

Materials and methods

Microorganisms and culture condition

Thraustochytrid strains (see Table 4-1) used in this study are part of the Australian National Algae Culture Collection (http://www.csiro.au/ANACC). Strain isolation information, medium preparation and culturing conditions have been reported previously (Lee Chang et al., 2012).

Table 4-1 List of thraustochytrid strains and their chemotaxonomic and molecular grouping, based on Lee Chang et al. (2012)

TC	Group	CS Number*	Genus	Genebank accession	Colour
02	A	CS-980	*Schizochytrium*	JN675267	Off white
04	B	CS-982	*Thraustochytrium*	JN675271	Orange
09	E	CS-994	*Aurantiochytrium*	JN675249	White
10	D	CS-991	*Ulkenia*	JN675268	Cream
18	H	CS-1012	*Aurantiochytrium*	JN675255	Cream
20	F	CS-997	*Aurantiochytrium*	JN675250	White
30	G	CS-1011	*Aurantiochytrium*	JN675265	Pale orange
33	C	CS-984	*Thraustochytrium*	JN675251	White

*CS Number is assigned for strains held in Australian National Algae Culture Collection (ANACC) and hereafter strains will be referenced by their sample code –TC

The culture medium consisted of (g/L): sea salts (20), glucose (2), bacteriological peptone (2) and yeast extract (2) (Sigma-Aldrich, St. Louis, MO, USA). Filter sterilized (0.2 μm) metal solution (1 mL/L) and vitamin solution (1 mL/L) were added after autoclaving. The metal solution contained (mg/L): $MgSO_4.7H_2O$ (200), KH_2PO_4 (200), $NaHCO_3$ (100), $MnCl_2.4H_2O$ (9), $Fe_3Cl_3.6H_2O$ (3), $ZnSO_4.7H_2O$ (1), $CoSO_4.5H_2O$ (0.3) and $CuSO_4.5H_2O$ (0.2). The vitamin mixture contained (mg/L): pyridoxine hydrochloride (0.2), thiamine (0.1), pantothenic acid (0.1), aminobenzoic acid (0.1), riboflavin (0.1), nicotinamide (0.1), biotin (0.04), folic acid (0.04) and vitamin B12 (0.002).

For the primary seed cultures, strains were revived from cryogenic storage by rapidly thawing the cryovials in a 50 °C water bath until the contents melted (Cox et al. 2009). The thawed cultures (1 mL) were slowly pipetted into 10 mL of culture medium in sterile 50 mL polystyrene cell culture flasks fitted with 0.2 μm filter caps (Greiner Bio-One, Germany). The flasks were incubated at 20 °C in the dark for 7 days without agitation (Cox et al. 2009).

For the secondary seed cultures, after 7 days, 10 mL of the primary seed cultures were aseptically added to 1 L baffled flasks containing 200 mL of culture medium supplemented with 20 g/L of glucose. The purity of the secondary seed cultures was checked using light microscopy (Zeiss Axioplan Compound Microscope, NY, USA) and streak plating onto solid medium. The flasks were incubated in a shaking incubator at 20 °C and 200 rpm and cultures were sub-sampled at 3 days after being acclimated in the high glucose growth media. The secondary seed cultures (50 mL) were used to inoculate the flask culture experiment.

A series of flask culture experiments were performed to examine the influence of initial glucose concentrations on biomass and lipid production by different strains. This study examined 32 flasks (8 strains, n = 2 replicate flasks, in 2 glucose concentration treatments) containing 1 L of culture medium with the two different glucose concentrations, 20 g/L or 40 g/L in 2 L baffled flask. All flasks were incubated in a shaking incubator at 20 °C and 200 rpm. Cultures were sampled at different time intervals from 0 to 21 days. The supernatant of sampled culture broths were used for glucose and pH measurements. Supernatants from samples of the 7-day-old cultures (2 % w/v glucose) were used to harvest EPS and details of the methodology are described below.

Biomass harvest and analysis of fatty acid methyl esters (FAME)

To harvest biomass, a 10 mL sub-sample was aseptically removed from the flasks, transferred into a 15 mL tube and centrifuged at 3400 x *g* at 20 °C for 10 min. The supernatant was removed, and the pellet was resuspended and washed with 10 mL of sterile 3.15 % (w/v) ammonium formate solution. Cells were again pelleted by centrifugation at 3400 x *g* at 20 °C for 10 min. The pellets were freeze-dried overnight and weighed to determine dry cell weight (DCW). Residual glucose in the culture supernatants was measured using a GlucCell® Glucose Monitoring System (Cesco Bioproducts, GA, USA).

Freeze-dried samples were directly transesterified with methanol: chloroform: HCl (l0:1:1 v/v/v) to convert fatty acids from the complex lipids into fatty acid methyl esters (FAME), as described previously (Lee Chang et al. 2011). Because most of the oil in thraustochytrids is comprised of triacylglycerol containing fatty acids (85-95 %), total FAME was used as a surrogate for total lipid. Individual fatty acids are expressed as a percentage of the total fatty acids (TFA).

Gas chromatography (GC) to quantify the fatty acids was performed on an Agilent Technologies 7890A GC (Palo Alto, CA, USA) equipped with a non-polar Equity-1™ fused silica capillary column (15 m x 0.1 mm i.d., 0.1 mm film thickness), flame ionization detector (FID) and split/splitless injector. Samples were injected in splitless mode at an oven temperature of 120 °C and after injection, the oven temperature was increased to 270 °C at 10 °C/ min and then to 310 °C at 5 °C/ min. Peaks were quantified with Agilent Technologies ChemStation software (Palo Alto, CA, USA).

GC-mass spectrometry (GC-MS) analysis of FAME was performed to confirm component identifications and was carried out on a Finnigan GCQ Plus™ GC-MS ion-trap fitted with an on-column injector set at 45 °C. Samples were injected using an AS2000 auto sampler onto a retention gap attached to a non polar HP-5 Ultra 2 bonded-phase column (50 m x 0.32 mm i.d. x 0.17 µm film thickness). The initial temperature of 45 °C was held for 1 min, followed by temperature programming at 30 °C/ min to 140 °C then at 3 °C/ min to 310 °C where it was held for 12 min. Helium was used as the carrier gas. Mass spectrometer operating conditions were: electron impact energy 70 eV; emission current 250 µamp, transfer line 310 °C; source temperature 240 °C; scan rate 0.8 scan/ sec and mass range 40-650 Da. Mass spectra were acquired and processed with Thermo Scientific Xcalibur™ software (Waltham, MA, USA).

Harvesting and characterisation of polysaccharides

Cultures were centrifuged to remove cellular material at 10, 000 x *g* and 20 °C for 2 h (Sorvall, K.I. Scientific Pty. Ltd., North Ryde, NSW, Australia). Biomass yield was determined as described above. Supernatants were decanted. Sodium azide (0.4 g/L, Ajax Chemical Ltd., Melbourne, VIC, Australia) was added to supernatants to prevent bacterial growth before pressure filtration through a muffled (450 °C, 18 h) glass fibre filter (GF/F, Whatman International Ltd,

Maidstone, UK) and then through a cellulose nitrate filter (0.45 μm, Sartorius Australia, Pty. Ltd., East Oakleigh, VIC, Australia). The filtrates were then subjected to ultrafiltration to remove small proteins and media components and to concentrate the EPS solution (100,000 Dalton MWCO, Pellicon XL, Millipore Corp, Billerica, MA, USA).

The concentrated retentates were frozen, freeze-dried and weighed. For the crude chemical characterization, solutions of EPS (1 mg/mL) were prepared in Milli-Q™ water and used for subsequent colorimetric assays. Uronic acid content of the EPS was determined by the meta-hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen 1973; Filisetti-Cozzi and Carpita 1991), with D-glucuronic acid as a standard (Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia).

Protein content was determined by the bicinchoninic acid (BCA) protein assay (Smith et al. 1985) with serum albumin as the standard (Sigma-Aldrich Pty. Ltd.). The total neutral carbohydrate content was determined by the orcinol-sulfuric acid method modified by Rimington (1931), using D-glucose (Sigma-Aldrich Pty. Ltd.) as a standard.

Results and Discussion

Effect of glucose on biomass yield by different thraustochytrid strains

The biomass production of different strains, from the genera *Aurantiochytrium*, *Schizochytrium*, *Ulkenia* and *Thraustochytrium*, including eight different chemotaxonomic groups, is shown in Figures 4-1a, 4-2a, and 4-3a, respectively. The glucose assay was performed to determine whether the amount of glucose in the media was the limiting factor in the growth of different thraustochytrid strains (Figs. 4-1b, 4-2b, and 4-3b). The glucose consumption patterns in *Schizochytrium*, *Ulkenia* and *Thraustochytrium* spp. strains were very different to those observed in *Aurantiochytrium* sp. strains.

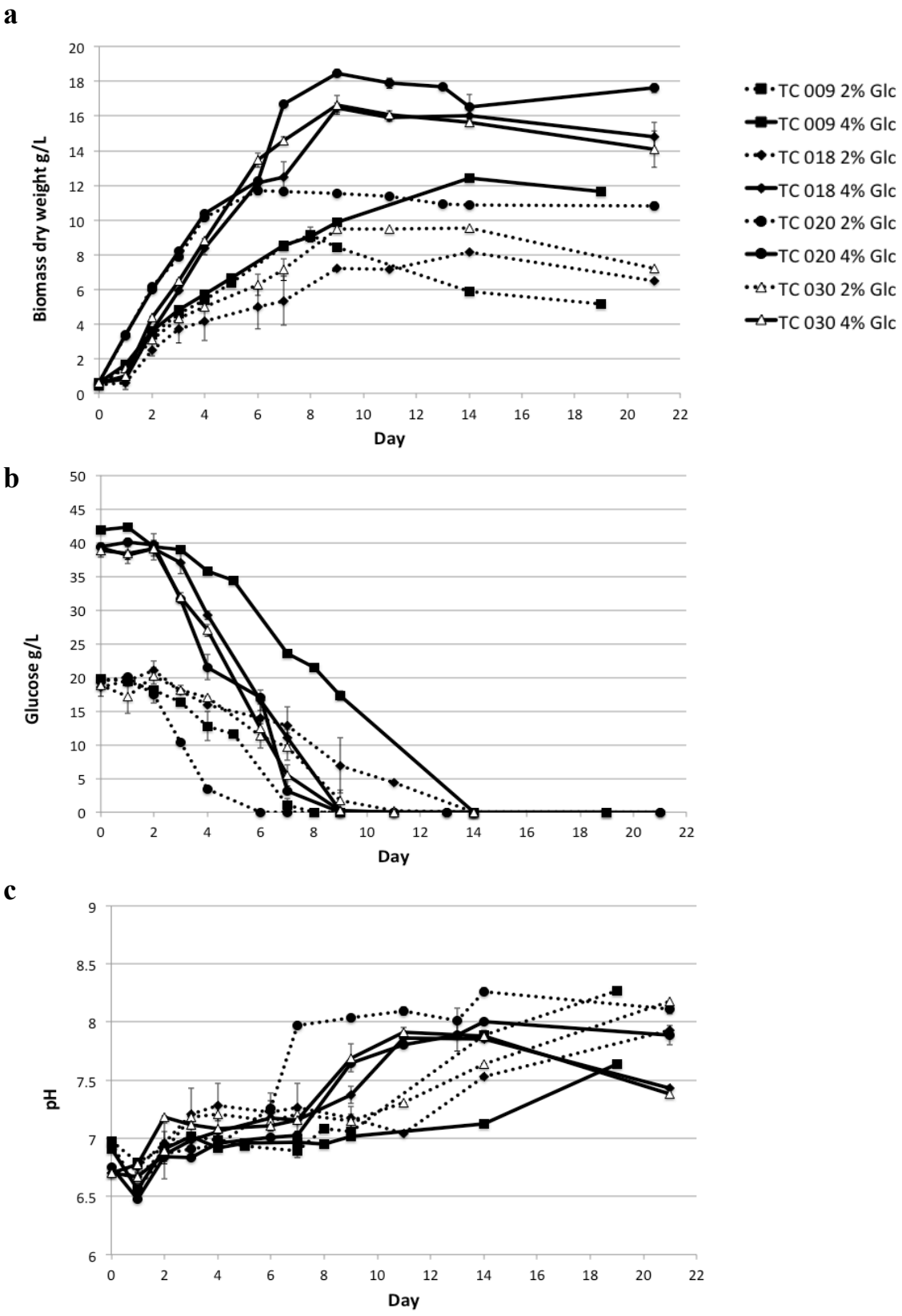


Figure 4-1 *Aurantiochytrium* sp. strains TC 09 (Group E), 18 (Group H), 20 (Group F), and 30 (Group G) in 2 % w/v and 4 % w/v glucose. (**a**) biomass yield g/L, (**b**) glucose concentration (g/L), and (**c**) pH change in culture media

The *Aurantiochytrium* sp. strains TC 09, 18, 20 and 30 had biomass yields in the range of 7.2 – 18.5 g/L at day 9, in the 2 % w/v and 4 % w/v glucose culture media (Fig. 4-1a). The *Aurantiochytrium* sp. strains TC 20 achieved relatively high biomass in both 2 % w/v (11.5 g/L) and 4 % w/v (18.5 g/L) media indicating that this strain grew well under these conditions. It was determined that by day 6 *Aurantiochytrium* sp. strain TC 20 had consumed all the glucose in the 2 % w/v glucose medium (Fig. 4-1b). This level of consumption did not occur in the 4 % w/v glucose media until day 9 (Fig. 4-1b).

The depletion of glucose in both media resulted in a reduction in biomass therefore at the end of the arithmetic growth of strain TC 20. Figures 4-1a and 4-1b demonstrate that there was a good relationship between the amount of the glucose in the media and growth for the *Aurantiochytrium* sp. strains TC 09, 18, 20 and 30. The biomass production in all *Aurantiochytrium* sp. strains steadily increased as the glucose concentration declined. The biomass production of *Aurantiochytrium* sp. strain TC 09 was considerably lower (8.4 g/L in 2 % w/v glucose and 9.9 g/L in 4 % w/v glucose at day 9) than that of TC 20.

As the glucose assay is performed from a very small amount, < 3 μL, of diluted sample, there may be variability between samples. The use of this glucose assay should be considered as an initial indicator of growth and for understanding the pattern of glucose consumption of these strains. Furthermore, the standard errors for biomass yield of *Aurantiochytrium* sp. strain TC 18 at days 3, 4, 6 and 7 were relatively large (Fig. 4-1a), possibly due to clumping of the cell biomass that caused the cells to disperse unevenly.

The effect of an additional 2 % w/v glucose in the culture medium was to increase the biomass yield by an average factor of 1.7. The growth profile of *Aurantiochytrium* sp. strains in this study was similar to those of other *Aurantiochytrium sp.* strains reported. Our strain clustered closely with the highly productive and high DHA-containing *Aurantiochytrium limacinum* SR21 (formerly *Schizochytrium limacinum* SR21) based on 18S rRNA sequence data (Lee Chang et al. 2012).

Yokochi et al. (1998) demonstrated the level of biomass and DHA production of *A. limacinum* SR21 in response to the composition of the growth medium. Similar yields of biomass (> 15 g/L) and DHA content (> 500 mg/L) were reported when glucose, fructose or glycerol (3 % w/v) was used as a carbon source, with low biomass yield (> 5 g/L) occurring when starch was used as a carbon source (Yokochi et al. 1998). The authors also reported that the total fatty acid content of strain SR21 increased as the concentration of the nitrogen source decreased. Higher biomass (35 g/L) and DHA (4.2 g/L) yields were obtained by increasing the carbon (9 % w/v) concentration (Yokochi et al. 1998). Growth of *Aurantiochytrium* sp. strain OUC-88 (which was a UV-induced mutant of *A. limacinum* SR21) was optimized with different carbon sources (3 % w/v of glucose, fructose, starch, potato powder or glycerol) and nitrogen sources (yeast extract, peptone, tryptone or soybean cake hydolysate) (Zhu et al. 2008). Zhu et al. (2008) also reported that potato powder yielded the highest biomass (14 g/L), while maximum DHA yield (1.78 g/L) was achieved in the glucose-containing medium.

The biomass production profile of *Schizochytrium* sp. strain TC 02 and *Ulkenia* sp. strain TC 10 in the 2 % w/v and 4 % w/v glucose culture media is shown in Figure 4-2a. The glucose concentrations of *Ulkenia* sp. strain TC 10 in the 2 % w/v and 4 % w/v glucose culture media remained unchanged by day 14 suggesting this strain did not utilize glucose as a growth substrate (Fig. 4-2b). Therefore the decline in biomass production is not explained by the availability of glucose. Quilodrán et al. (2010) reported that a native Chilean *Ulkenia* sp. (Strain AS4-A1) grew best in (2 % w/v) starch (biomass yield of 4.9 g/L, DHA 54 mg/g, 39 % TFA at day 3) when compared to other carbon sources such as glucose and maltose. In the current study, the biomass of *Schizochytrium* sp. strain TC 02 gradually increased as the glucose concentration declined (Fig. 4-2b). On day 14, the glucose concentration of the 4 % w/v medium declined to 2.5 % w/v and 1.22 % w/v remained in the 2 % w/v medium (Fig. 4-2b). The biomass yield of TC 02 in 4 % w/v glucose medium (9.7g/L) was markedly higher than that (1.4 g/L) in the in 2 % w/v glucose medium (Fig. 4-2a). Similar trends were observed in the case of *Thraustochytrium* sp. strain TC 04. A biomass of 13.3 g/L was produced by day 21 in the 4 % w/v glucose medium (Fig. 4-3a). The growth of TC 04 in 2 % w/v was markedly lower than in the 4 % w/v medium (1.6 g/L biomass at day 21). This may be explained by the acclimatization to glucose, which may have occurred in the 4 % w/v medium, but not in the case of 2 % w/v glucose medium. *Thraustochytrium* sp. strain TC 33, had a biomass yield in the range of 1.9 – 2.1 g/L at day 7, in the 2 % w/v and 4 % w/v glucose culture media (Fig. 4-3a). The overall growth with TC 33 was poor and the glucose remained unchanged until day 14 (Fig. 4-3b).

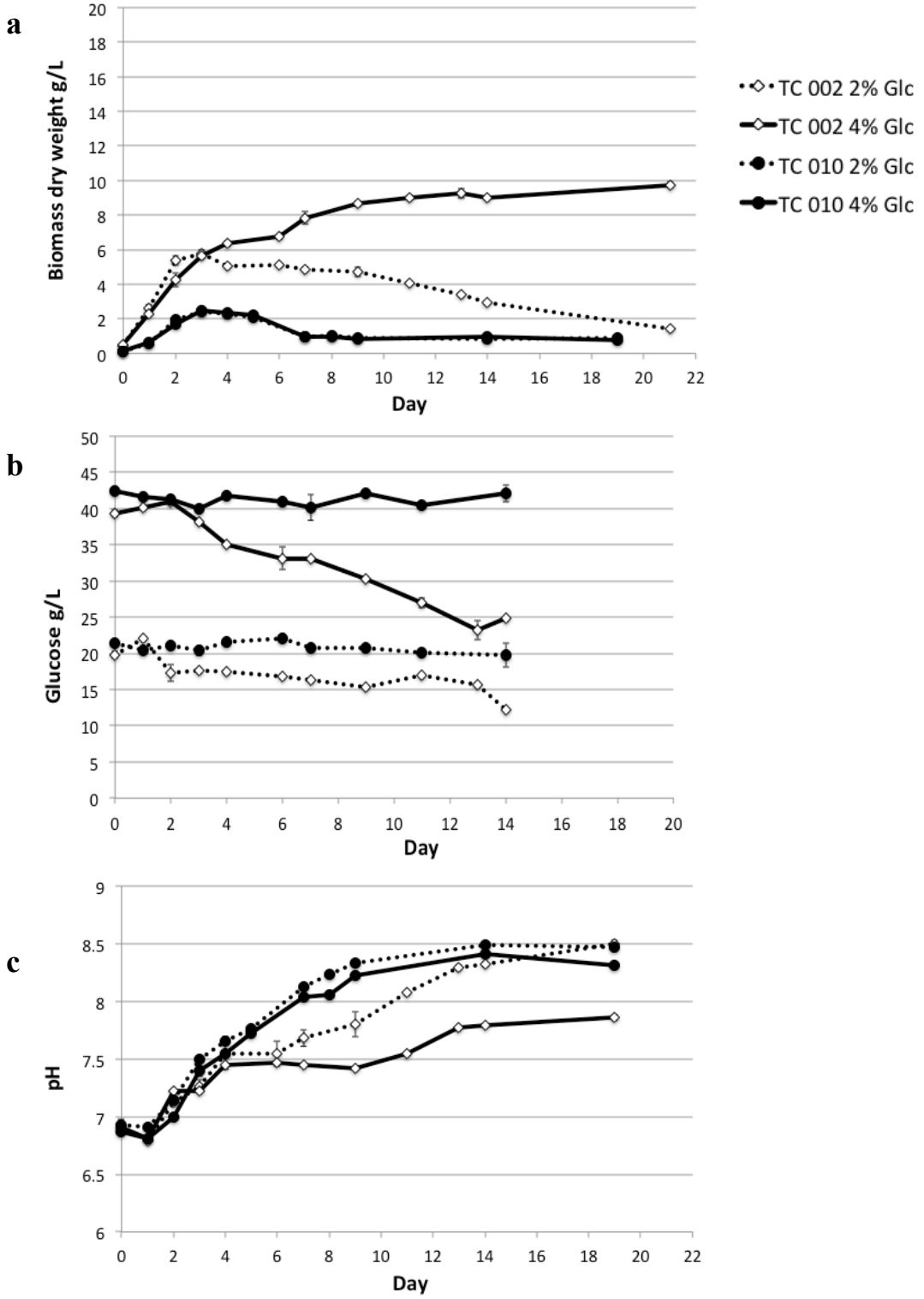


Figure 4-2 *Schizochytrium* sp. strain TC 02 (Group A) and *Ulkenia* sp. strain TC 10 (Group D) in 2 % w/v and 4 % w/v glucose. (**a**) biomass yield g/L, (**b**) glucose concentration (g/L), and (**c**) pH change in culture media

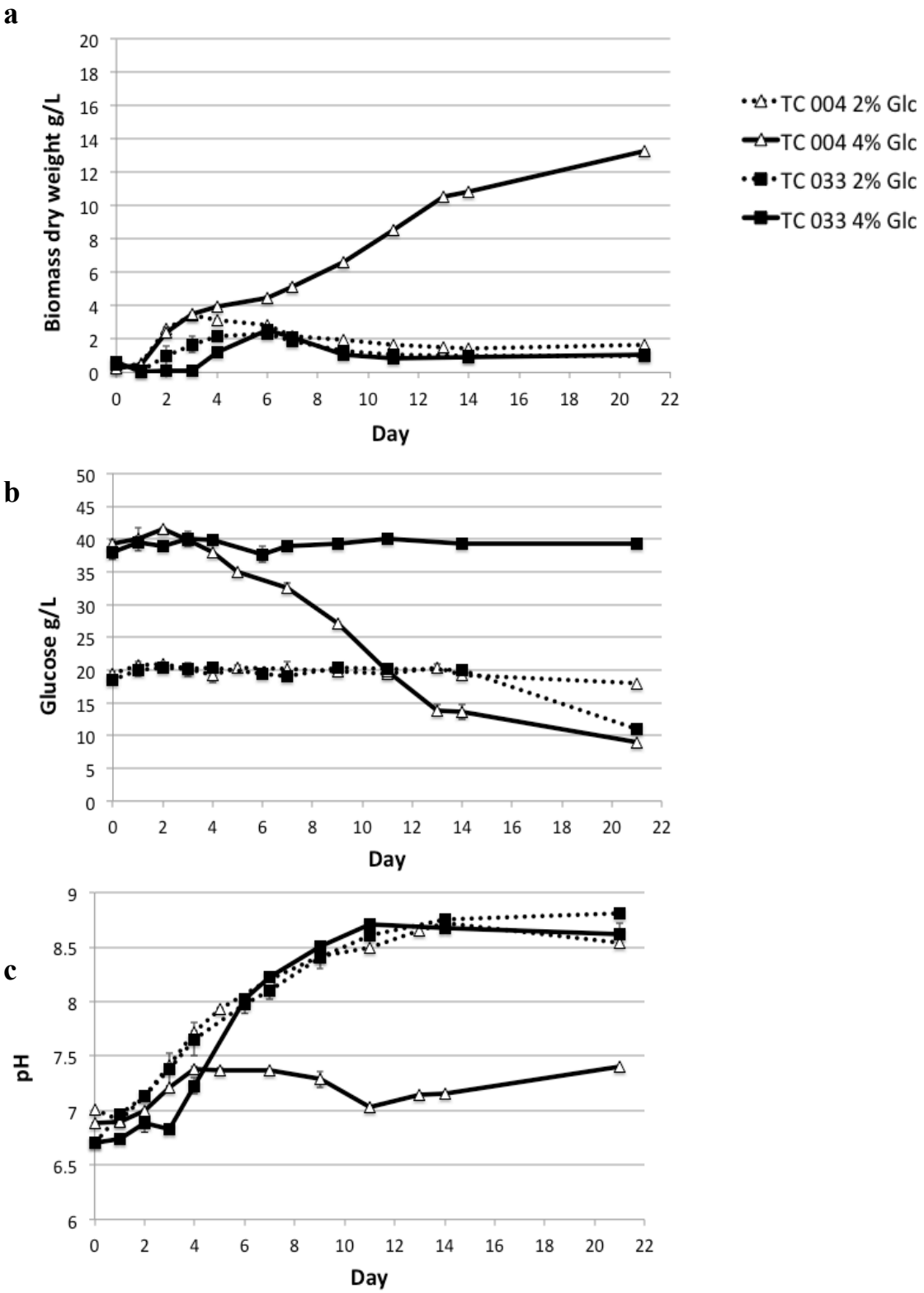


Figure 4-3 *Thraustochytrium* sp. strains TC 04 (Group B) and TC 33 (Group C) in 2 % w/v and 4 % w/v glucose. (**a**) biomass yield g/L, (**b**) glucose concentration (g/L), and (**c**) the pH change in culture media

Vazhappilly and Chen (1998) reported that poor biomass yield was observed in *Schizochytrium aggregatum* ATCC 28209 (0.5 g/L) and *Thraustochytrium aureum* ATCC 28211 (0.1 g/L) in glucose (0.5 % w/v) medium after 23 days of cultivation. The authors also demonstrated that these strains could utilize acetate (0.1 % w/v) as the sole carbon and energy source with moderate growth observed (Vazhappilly and Chen 1998). The glucose assimilating profiles in our *Schizochytrium* sp. strain TC 02 and *Thraustochytrium* sp. strain TC 33 were very similar to those reported by Vazhappilly and Chen (1998). In cultures of strains TC 02 and TC 33, slow growth was observed and a longer cultivation time (20–21 days) was required to produce substantial amounts of biomass (Figs 2a and 3a). In contrast, Singh and Ward (1996) reported that the biomass and DHA production of *Thraustochytrium roseum* ATCC28210 in media containing starch (2.5 % w/v) as a carbon source, was 10.4 g/L biomass and 1011 mg/L DHA in 5 days.

It is often acknowledged that a high carbon to nitrogen ratio in the culture medium is important for microbial growth and lipid production, and that lipid accumulation in oleaginous microorganisms is often triggered by nitrogen depletion with an excess of carbon source (Bowles et al. 1999; Hall and Ratledge 1977; Ratledge and Wynn 2002; Burja et al. 2006). As the cell grows, it quickly depletes the nitrogen source, but it continues to assimilate the excess carbon source for lipid synthesis (Ratledge and Wynn 2002). Glucose is the most common carbon source that is used to cultivate thraustochytrids.

Different carbon sources such as fructose, glycerol, maltose, starch, sucrose, etc (Bahnweg 1979b; Bajpai et al. 1991b; Singh and Ward 1996; Yokochi et al. 1998; Burja et al. 2006), and different nitrogen sources such as yeast extract, peptone, sodium glutamate, ammonium sulfate, etc (Yang et al. 2010; Bahnweg 1979a; Yokochi et al. 1998) have been examined for different thraustochytrids strains. Bahnweg (1979a, b) showed that different thraustochytrid strains have different preferences for growth substrates. It is important to identify the best substrate for a strain before adjusting the carbon to nitrogen ratio to optimize biomass and lipid production. This is probably why several studies have reported that carbon to nitrogen ratio variations only affected lipid accumulation in some thraustochytrid strains. Lewis (2001) reported that there was no clear correlation between the initial carbon: nitrogen ratio in the medium (glucose and sodium glutamate) on either biomass or lipid production by thraustochytrid strain ACEM 6063.

Correlation of growth and pH change

It is thought that the pH of the culture medium is a good indication of the growth of thraustochytrids. The pH of the culture media of the different thraustochytrid strains is shown in Figures 4-1c, 4-2c, and 4-3c. The pH of the culture media increased over time in actively growing strains. This indicated that the nitrogen source was being utilized resulting in the formation of alkaline by-products from de-amination of amino acids in the complex medium. Once the nitrogen sources were exhausted, the excess carbon source became the sole energy source available. The thraustochytrid strains that were able to assimilate the glucose as a carbon source continued to grow until the glucose was depleted as described above. However, the carbon source utilization releases acidic end products (Wu et al. 2005).

Wu et al. (2005) reported that *Schizochytrium* sp. released metabolic acids such as malic acid, citric acid, pyruvic acid and fumaric acid during growth. Optimal growth was observed at pH 7.

Our observations are consistent with the findings of Wu et al. (2005) in that the strains with good growth (biomass yield > 8 g/L), and which could utilize glucose, were able to maintain the pH at the range of 7.5 to 8.2, while in those that could not utilize glucose, higher pH values (ranging 8.2 - 8.8) were observed (Figs 4-2c and 4-3c). This trend was more apparent in the case of *Aurantiochytrium* sp. strains (Fig. 4-1c) compared to the *Schizochytrium*, *Ulkenia* and *Thraustochytrium* spp. strains (Figs 4-2c and 4-3c). The exception to this is those strains that have been acclimatized to glucose, such as *Thraustochytrium* sp. strain TC 04 (in the 4 % w/v glucose medium), which were able to maintain the pH at 7.4. The pH in these cultures did not increase greatly across the length of the trial (Fig. 4-3c). In addition, *Schizochytrium* sp. strain TC 02 in 4 w/v glucose (pH 7.8 at day 19) showed that the pH increased more slowly than that of 2 % w/v glucose (pH 8.5 at day 19) (Fig. 4-2c)

Biomass and fatty acid profiles

The total FAME content of the eight strains was measured on days 4, 9 and 14 (Fig. 4-4a). Overall, the FAME content of the four *Aurantiochytrium* sp. strains, TC 09, 18, 20, and 30 was relatively high (in the range of 42 – 404 mg/g), compared to those in *Schizochytrium* sp. strain TC 02, *Ulkenia* sp. strain TC 10 and *Thraustochytrium* sp. strains TC 04 and 33 (range of 14 – 162 mg/g) (Fig. 4-4a). The lower FAME yield in these strains could be the result of their poorer biomass (Figs 4-4a and 4-4b). In general, the total FAME content (mg/g) of each strain generally follows the same pattern as the biomass yield (Figs 4-4a and 4-4b). This relationship indicates that thraustochytrid cells accumulate lipid during cell growth.

Across all strains increasing glucose from 2 % w/v to 4 % w/v of the culture medium resulted in increased total FAME content by an average factor of 1.9, even though for some of these strains there was not a measurable amount of glucose utilized (Fig. 4-2 and 4-3). The fatty acid profiles for the eight thraustochytrid strains in 4 % w/v glucose at day 9 are shown in Table 4-2. The percentage of DHA (22:6ω3) and 16:0 of the eight strains in two different culture media (2 % w/v and 4 % w/v glucose) are shown in Figures 4-5a and 4-5b respectively. Overall, there was no consistant difference in the percentages of DHA and 16:0 between the different levels of glucose for each strain (Figs 4-5a and 4-5b). The effects of media composition on the biomass and fatty acid profiles of thraustochytrids have already been demonstrated (Bajpai et al. 1991a; Bajpai et al. 1991b; Li and Ward 1994; Singh and Ward 1996; Yokochi et al. 1998).

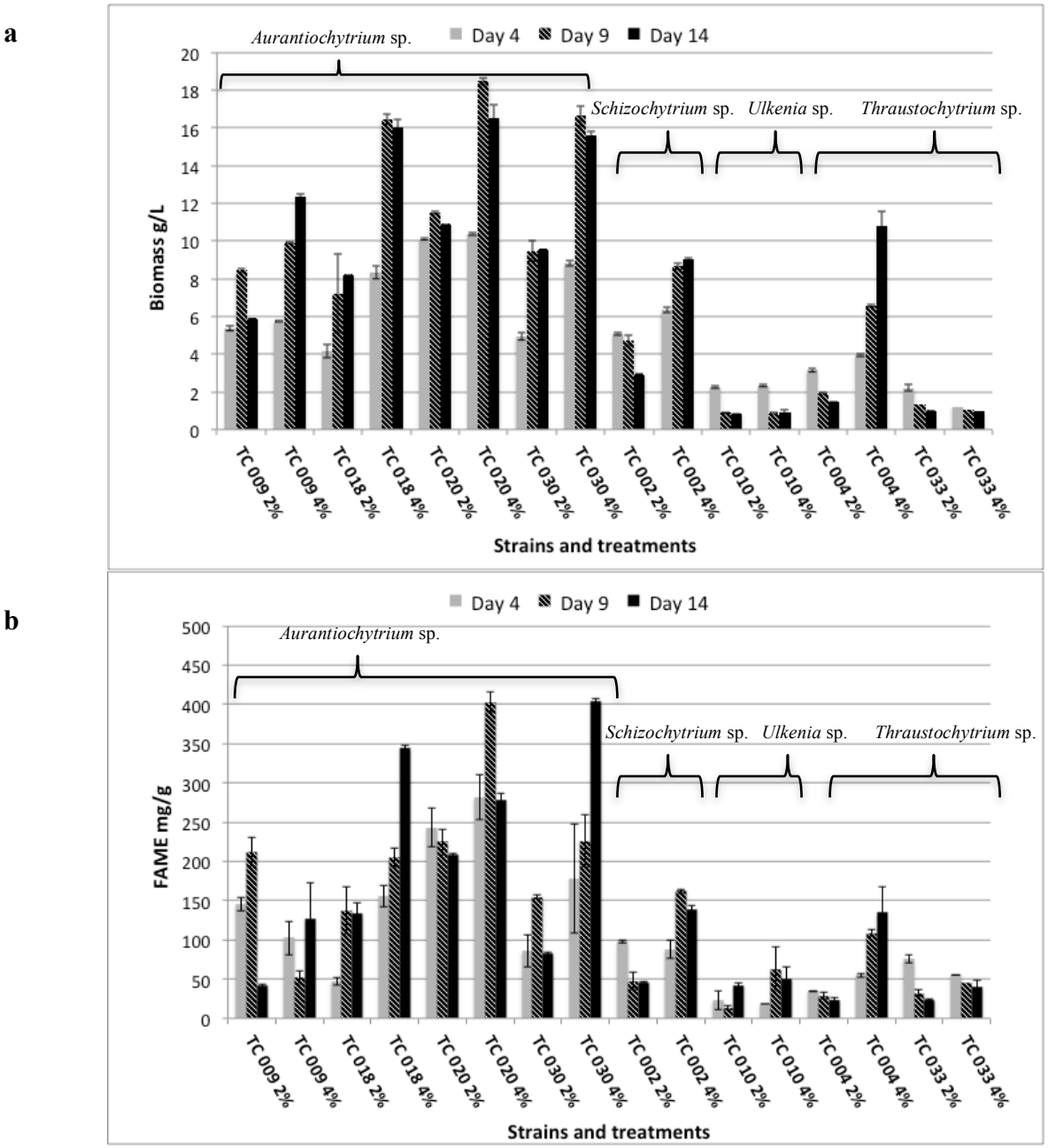


Figure 4-4 The total content of (**a**) fatty acid methyl esters, FAME mg/g, and (**b**) biomass g/L, of eight different thraustochytrid strains in 2 % w/v and 4 % w/v glucose

Table 4-2 Fatty acid composition (as percentage of total fatty acids) for eight thraustochytrid strains grown in 4 % w/v glucose in shake flask culture at day 9

Genus	*Aurantiochytrium*				*Schizo-chytrium*	*Ulkenia*	*Thraustochytrium*	
Group	E	F	G	H	A	D	B	C
TC	09	20	30	18	02	10	04	33
14:0	9.5 ± 0.0	2.9 ± 1.4	29.8 ± 0.1	29.2 ± 0.2	2.7 ± 0.3	0.1 ± 0.1	1.1 ± 0.1	1.6 ± 0.4
15:0	2.4 ± 0.0	1.9 ± 0.0	1.6 ± 0.0	2.1 ± 0.0	-	0.1 ± 0.0	-	-
16:0	41 ± 0.0	39.8 ± 0.2	33.5 ± 0.1	35.9 ± 0.0	18.1 ± 0.0	27.6 ± 0.0	34.8 ± 0.0	16.8 ± 0.0
18:3ω6	Tr	0.3 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	1.2 ± 0.0	-	0.3 ± 0.0	Tr
18:2ω6	-	0.1 ± 0.2	0.1 ± 0.0	0.1 ± 0.0	7.9 ± 0.0	1.7 ± 0.0	1.5 ± 0.0	0.2 ± 0.0
18:3ω3	-	0.1 ± 0.2	-	-	-	8.8 ± 0.0	Tr	-
18:1ω9	-	0.5 ± 0.2	-	-	38.4 ± 0.0	10.5 ± 0.0	6.6 ± 0.0	0.2 ± 0.0
18:0	0.7 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.7 ± 0.0	4.8 ± 0.0	4.4 ± 0.0	19.9 ± 0.0	0.2 ± 0.0
20:4ω6	0.4 ± 0.0	1.2 ± 0.0	0.9 ± 0.0	1 ± 0.0	6.6 ± 0.0	6.1± 0.0	7 ± 0.0	0.3 ± 0.0
20:5ω3	0.9 ± 0.0	0.5 ± 0.0	0.9 ± 0.0	1.1 ± 0.0	1 ± 0.0	5.0 ± 0.0	0.9 ± 0.0	7.5 ± 0.0
20:3ω6	0.2 ± 0.0	0.4 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	3.3 ± 0.0	0.5 ± 0.0	0.3 ± 0.0	0.4 ± 0.0
20:4ω3	0.4 ± 0.2	0.3 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.2 ± 0.1	0.2 ± 0.5	0.1 ± 0.1	0.6 ± 0.0
20:2ω6	Tr	Tr	-	-	0.5 ± 0.0	0.6 ± 0.5	0.4 ± 0.0	-
20:1ω11	Tr	Tr	0.1 ± 0.0	0.1 ± 0.0	-	0.9 ± 0.0	-	-
20:1ω9	Tr	-	-	-	0.2 ± 0.0	-	0.1 ± 0.0	-
20:1ω7	-	-	-	-	-	0.2 ± 0.6	-	-
20:0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	Tr
22:5ω6	9.6 ± 0.0	2.5 ± 0.0	5.7 ± 0.0	5.1 ± 0.0	5.9 ± 0.0	10.1 ± 0.0	18.2 ± 0.0	0.5 ± 0.0
22:6ω3	32.1 ± 0.0	46.7 ± 0.0	23.5 ± 0.0	22.2 ± 0.0	4.2 ± 0.0	13.7 ± 0.1	2.7 ± 0.0	69 ± 0.0
22:4ω6	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	1.6 ± 0.0	3.9 ± 0.0	4.8 ± 0.0	-
22:5ω3	1.4 ± 0.6	0.4 ± 0.5	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.2	2.4 ± 1.5	0.2 ± 0.6	2 ± 0.1
Others [a]	1.1 ± 0.2	1.6 ± 0.2	2.1 ± 0.0	1.3 ± 0.0	2.7 ± 0.1	3.3 ± 0.5	0.8 ± 0.2	0.7 ± 0.0
Total FAME mg/g	52 ± 7.9	403 ± 13	226 ± 33	205 ± 12	162 ± 2.6	63 ± 27.4	109 ± 4.5	46 ± 2.0

[a] Others refer to: 16:1ω5, 16:1ω7, 16:1ω9, 17:0, 18:1ω5, 18:1ω7, 18:4ω3, 19:0, 22:0, C_{20} PUFA, 20:1ω5, C_{22}PUFA, 22:1ω7, 22:1ω9, 22:1ω11; 'Tr' denotes Trace < 0.01; '-' denotes not detected

The characteristics of chemotaxonomic groupings of strains based on their fatty acids profile (in 2 % w/v and 4 % w/v glucose media) remained very similar compared to the screening data previously reported for these strains (in basal medium containing 0.2 % w/v glucose), though different culture media and culture conditions (different flasks sizes and culture volumes) were used (Lee Chang et al. 2012; Lee Chang et al. 2011). None of the odd-chain LC-PUFA previously detected in the low nutrient containing screening study were detected in the current study with 2 % w/v and 4 % w/v glucose. The percentage of DHA ranged between 20 – 49 % TFA for *Aurantiochytrium* sp. strains. The *Aurantiochytrium* sp. strains TC 09 (Group E) and TC 20 (Group F) contained DHA levels of 32 – 47 % TFA, and 16:0 ranged from 40 – 41 % TFA after 9 days of growth in 4 % w/v glucose medium (Table 4-2). While the *Aurantiochytrium* sp. strains TC 30 (Group G) and TC 18 (Group H) were previously reported to be high in the SFA 15:0 (23 – 30 % TFA) (Lee Chang et al. 2012), the SFA 14:0 was observed in the range of 29 – 30 % TFA in this study (Table 4-2).

The Group A *Schizochytrium* sp. strain TC 02 was characterized as high in omega-6 PUFA such as arachidonic acid (AA, 22:6ω3, 6.6 % TFA), dihomo-γ-linolenic acid (DGLA, 20:3ω6, 3.3 % TFA), and docosapentaenoic acid (DPA6, 22:5ω6, 5.9 % TFA) (Table 4-2). High levels of 18:1ω9 were observed in Groups A (*Schizochytrium* sp. strain TC 02, 38.4 % TFA), C (*Thraustochytrium* sp. strain TC 04, 6.6 % TFA) and D (*Ulkenia* sp. strain TC 10, 10.5 % TFA) compared to other groups (ranged from 0.1- 0.5 % TFA) (Table 4-2). This is similar to previously reported levels of 18:1ω9 in Groups A, C and D, which were higher (in the range 1– 6 % TFA) compared to the other groups (range 0.1 – 0.3 % TFA) (Lee Chang et al. 2012).

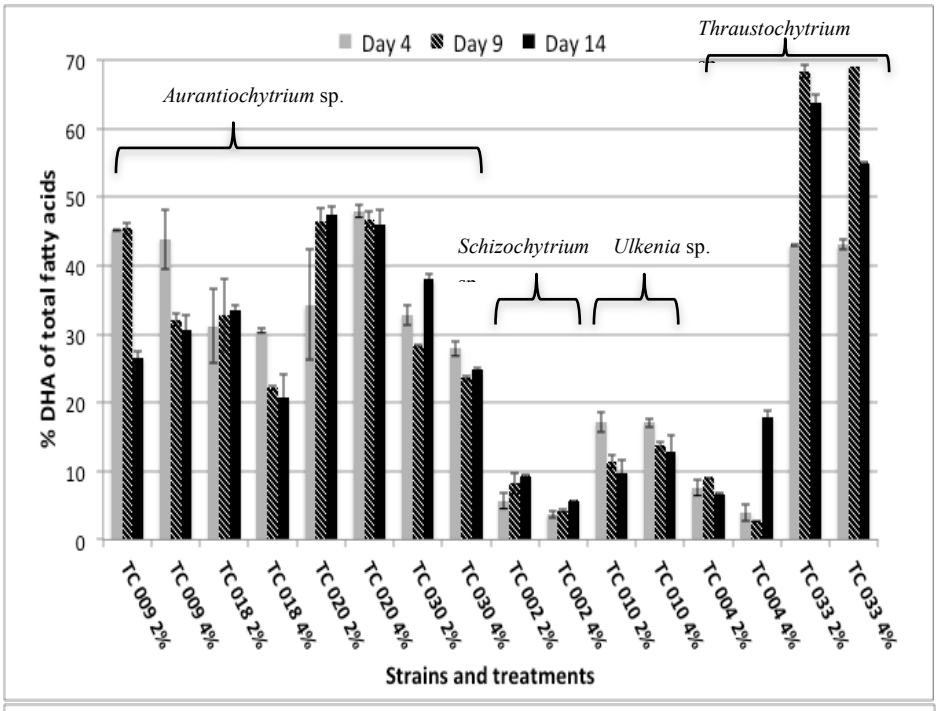
Ulkenia sp. strain TC 10 (Group D) had high levels of omega-3 PUFA (53 % TFA), 16:0 (24 % TFA) as reported previously (Lee Chang et al. 2012); this is similar to

the current study in which high levels of 16:0 (27.6 % TFA) and omega-3 PUFA such as 18:3ω3 (8.8 % TFA), EPA (5 % TFA), and DPA6 (10 % TFA) were observed.

Thraustochytrium sp. strain TC 04 (Group B) had high levels of C_{22} PUFA with 13 % DPA6 reported in Lee Chang et al. (2012), and in the current study similarly high levels of DPA6 (18.2 %) was also observed in this group (Table 4-2). While *Thraustochytrium* sp. strain TC 33 (Group C) previously was found to contain high omega-3 LC-PUFA (47 % TFA) with relatively low DPA6 (0.8 – 1.8 % TFA) (Lee Chang et al. 2012), again the pattern is similar to the current study with 69 % DHA and 0.5 % DPA6 observed (Table 4-2). Low biomass (0.9 – 1.3 g/L) and high DHA (68 – 69% TFA) was observed in *Thraustochytrium* sp. strain TC 33 in both media at day 9 (Fig. 4-5a).

High levels of biomass yield will be ideal for economical and environmentally sustainable biodiesel production because of the associated high lipid content and productivity. Furthermore, quick growth and high biomass producing strains will have higher lipid productivity and therefore are better candidates for biodiesel production. *Aurantiochytrium* sp. strain TC 20 (Group F) had the highest biomass (18.5 g/L) and lipid content (403 mg/g) in 4 % w/v glucose at day 9 (Figs 4-4a and 4-4b). The high level of 16:0 (39.8 % TFA) and DHA (46.7 % TFA) found in TC 20 suggests that this strain could potentially be used for co-production of a feedstock for biodiesel and omega-3 LC-PUFA.

a



b

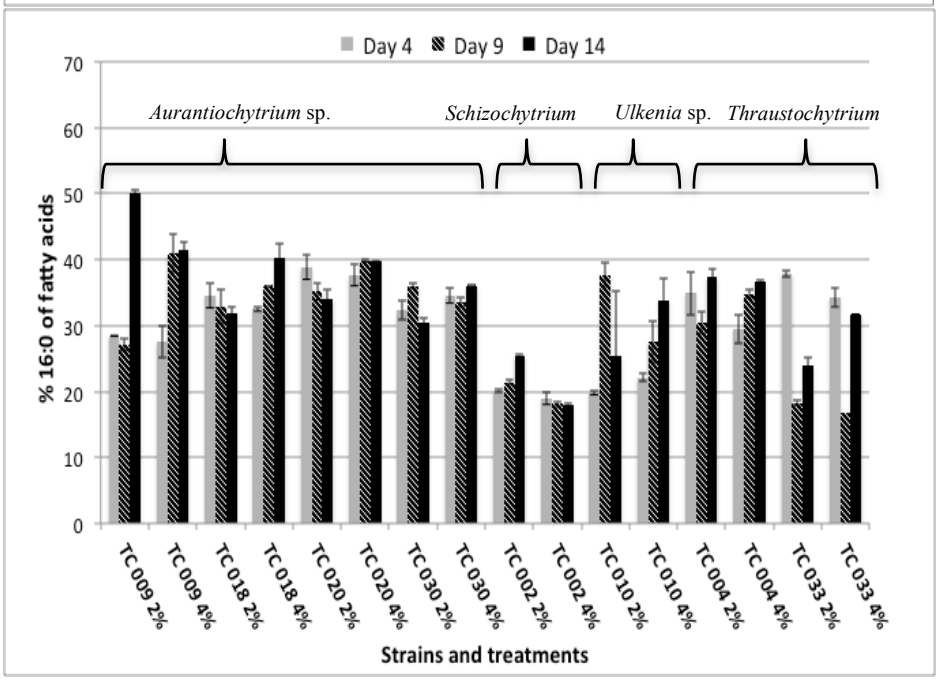


Figure 4-5 The percentage of fatty acid methyl esters, FAME (**a**) docosahexaenoic acid, DHA, and (**b**) palmitic acid, 16:0, of eight different thraustochytrid strains in 2 % w/v and 4 % w/v glucose

Yield and crude chemical characterization of exopolysaccharides

EPS yield data are presented in Table 4-3. *Schizochytrium* sp. strain TC 02 produced the highest yield of EPS on a culture volume basis (299 mg/L). As discussed above, this strain did not consume significant amounts of glucose during growth (Fig. 4-2b). Many marine microbes are capable of producing EPS on sea water alone (Decho 1990) and during carbon limitation since many species make use of non-sugar carbon sources for EPS production (Sutherland 1997). Further increases in EPS yield may be possible by providing alternative carbon sources for growth.

Aurantiochytrium sp. strain TC 30 had one of highest EPS yields (283 mg/L) as well as one of the highest biomass yields observed in our experiment (Fig. 4-1a). In contrast, *Thraustochytrium* sp. strain TC 33 yielded the lowest amount of EPS on a culture volume basis (55 mg/L). It is interesting to note that the low EPS yield observed in *Thraustochytrium* sp. strain TC 33 was coupled with the poorest growth and lowest biomass yields over the course of the experiment (Fig. 4-3a). It appears that for several of the thraustochytrid strains examined in this study, EPS production may be related to biomass production under these yet to be optimized culture conditions.

Many factors can affect EPS yield. These can include nutrients such as the carbon or nitrogen source (Decho 1990; Kucuk and Merih 2009), carbon to nitrogen ratio (Williams and Wimpenny 1978) or physical factors such as temperature (Mancuso Nichols et al. 2005), pH or the amount of agitation (Kucuk and Merih 2009). Marine microbes grown in laboratory cultures produced EPS in response to limitation of nutrients such as nitrogen, phosphorus, sulfur and potassium (Sutherland 1982).

Table 4-3 EPS yield at day 7 for eight thraustochytrid strains grown in shake flask cultures with 2 % w/v glucose

Thraustochytrid strains	EPS yield based on culture volume (mg/L)	EPS yield based on cell dry weight (mg/g)
TC 09	129	15
TC 18	248	46
TC 20	179	15
TC 30	283	40
TC 02	299	61
TC 10	92	94
TC 04	257	119
TC 33	55	29

Most bacteria release the largest quantity of EPS during stationary growth phase in laboratory culture (Decho 1990; Manca et al. 1996). In the limited data available for thraustochytrids, Jain et al. (2005) reported the EPS production of *Schizochytrium* sp. strains CW1 and SC-1 was observed at all growth stages and the highest EPS production occurred during stationary phase. These authors found EPS yields for four thraustochytrids grown for 7 days in growth media with 2% w/v glucose ranged from 300 mg/L for *Thraustochytrium aggregatum* Ulken strain A6T to 1100 mg/L for the *Schizochytrium* sp. strain CW1 (Jain et al. 2005). In the current study, biomass data indicate that at day 7, when EPS were harvested, the cultures varied in their stages of growth (Figs 4-1a, 4-2a and 4-3a).

The lowest EPS yield was produced by *Thraustochytrium* sp. strain TC 33, which after 7 days appears to have been in early stationary phase based on biomass measurements (Fig. 4-3a). *Schizochytrium* sp. strain TC 02 and *Thraustochytrium* strain TC 04, which yielded the most EPS on a culture volume basis (Table 4-3), appear to have been in mid-stationary phase of growth based on the biomass measurements (Figs 4-2a and 4-3a, respectively). Further studies that monitor EPS through the life of the cultures of these thraustochytrid strains will enable pinpointing timing of maximum yields for individual strains.

The crude chemical composition of purified EPS from thraustochytrid cultures is presented in Figure 4-6. All EPS samples included protein, neutral sugars and uronic acids in varying amounts. The total amount of EPS represented by protein, neutral sugars and uronic acids ranged from 73 % for *Aurantiochytrium* sp. strain TC 20 to 94 % for *Aurantiochytrium* sp. strain TC 30 (Fig. 4-6). The unaccounted material present in this EPS may be lipid, nucleic acid or some other as yet unidentified components. In these EPS samples, protein accounted for approximately 21 – 41 % of the composition of EPS produced by these strains. EPS produced by *Aurantiochytrium* sp. strain TC 30 included the greatest abundance of neutral sugars (66 %), while EPS produced by *Thraustochytrium* sp. strain TC 04 contained the least (approximately 38 %, Fig. 4-6). Low content of uronic acid was observed in the EPS of *Aurantiochytrium* sp. strains TC 09, 18, 20 and 30 at approximately 1 % of the total weight of EPS, compared to that of *Schizochytrium* sp. strain TC 02, *Ulkenia* sp. strains TC 10*, Thraustochytrium* sp. strains TC 04 and 33 (which ranged from 2 – 5 % of the total weight of EPS) (Fig. 4-6).

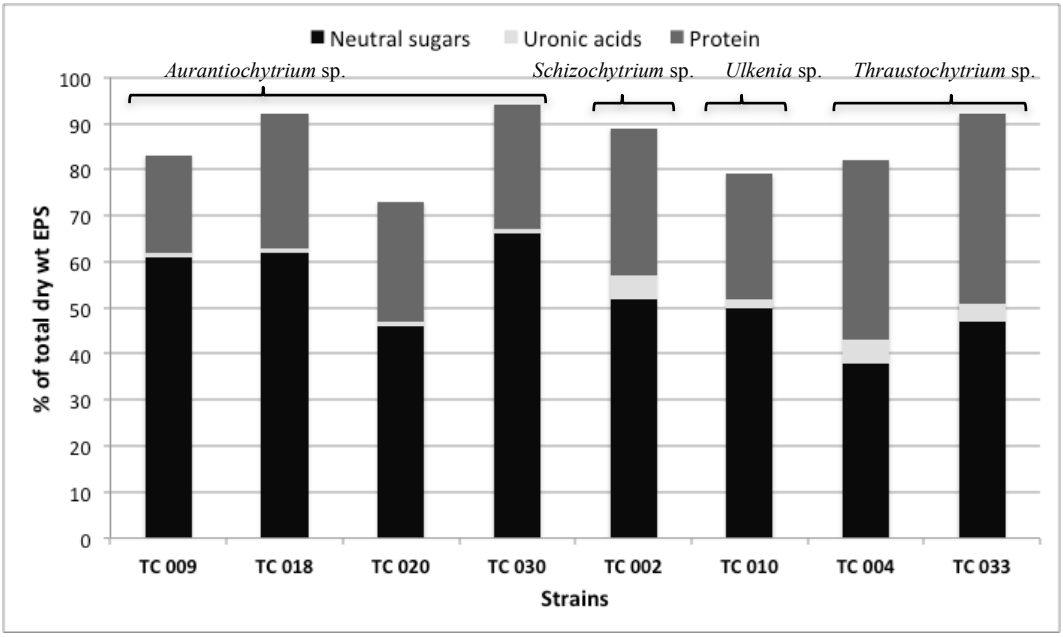


Figure 4-6 Crude chemical compositions of purified EPS produced by thraustochytrid strains grown in liquid shake flask cultures. Abundances of neutral sugars, uronic acids and protein are reported as percentage of total EPS

The proportion of uronic acids in the EPS is representative of the carboxyl functional groups present. These contribute to the anionic character of the polysaccharide at pH higher than 3 (pKa 3.2). At neutral pH, these acidic groups would be negatively charged and the polymer would be chemically 'sticky.' Other functional groups such as sulfate, hydroxyl and aminyl groups may also be present in these polysaccharides. The interaction of the carboxyl groups with hydroxyl groups, as well as with other functional groups, affects the overall tertiary structure of the polysaccharide and its interaction with its environment (Rendleman 1978).

Further chemical and structural elucidation is required for the samples of EPS examined in the current study to better assess their biotechnological potential. The search for novel EPS with desirable properties for use in commercial applications requires knowledge of the chemical composition and structure of the polymers. The chemical composition of EPS impact on the tertiary structure and over-all physicochemical characteristic of the polymer in the surrounding aqueous environment (Decho 1990). The structure of the polysaccharide determines its function in the environment and ultimately defines potential commercial applications that may occur in the future.

Conclusion

The co-production of both biodiesel and other high value co-products such as LC omega-3 oils and EPS presents a way to compensate for the cost of the technology needed for the production and extraction of thraustochytrid oils. This study demonstrated that glucose is a major factor in the biomass production of *Aurantiochytrium* sp. strains and also indicates that *Aurantiochytrium* sp. strains can utilize glucose as a carbon source better than *Schizochytrium*, *Ulkenia* and *Thraustochytrium* spp. strains. A high biomass producing strain such as *Aurantiochytrium* sp. strain TC 20 (Group F), with 18.5 g/L at day 9 in 4 % w/v glucose, has high lipid (403 mg/g of total FAME, 39.8 % of 16:0, 46.7 % of DHA) and EPS (179 mg/L at day 7 in 2 %w/v glucose medium) yields, and therefore is a better candidate for commercial production of biofuels and other co-products. To achieve commercial interest, the yield of biomass would need to be much higher than was found in this study. As is the case for increasing biomass and fatty acid yield and tailoring fatty acid composition for biodiesel or omega-3 LC-PUFA, EPS production could be manipulated using culture conditions.

Chapter 5 High cell density cultivation of a novel *Aurantiochytrium* sp. strain TC 20 in a fed-batch system using glycerol to produce feedstock for biodiesel and omega-3 oils

A recently isolated Australian *Aurantiochytrium* sp. strain TC 20 was investigated using small-scale (2 L) bioreactors for the potential of co-producing biodiesel and high value omega-3 long-chain polyunsaturated fatty acids. Higher initial glucose concentration (100 g/L compared to 40 g/L) did not result in markedly different biomass (48 g/L) or fatty acid (12 – 14 g/L) yields by 69 h. This comparison suggests factors other than carbon source were limiting biomass production. The effect of both glucose and glycerol as carbon sources for *Aurantiochytrium* sp. strain TC 20 was evaluated in a fed-batch process. Both glucose and glycerol resulted in similar biomass yields (57 g/L and 56 g/L, respectively) by 69 h. The agro-industrial waste from biodiesel production - glycerol - is a suitable carbon source for *Aurantiochytrium* sp. strain TC 20. Approximately half the fatty acids from *Aurantiochytrium* sp. strain TC 20 are suitable for development of sustainable, low emission sources of transportation fuels and bioproducts. To further improve biomass and oil production, fortification of the feed with additional nutrients (nitrogen sources, trace metals and vitamins) improved the biomass yield from 56 g/L (34 % total fatty acids) to 71 g/L (52 % total fatty acids, cell dry weight) at 69 h; These yields are to our knowledge around 70% of the biomass yields achieved by other researchers using glycerol. However, our yields were achieved in less than half the time, and were markedly greater than the yields achieved using other industrial wastes. The fast growth and suitable fatty acid profile of this newly isolated *Aurantiochytrium* sp. strain TC 20 highlights the potential of co-producing the drop-in biodiesel and high value omega-3 oils.

Introduction

The potential of biofuel production from microalgae has been of increasing interest due to growing concerns with rising crude oil prices, future availability and the overuse of fossil fuels. Many single cell organisms, including microalgae, bacteria, fungi, and yeasts, have been shown to accumulate useful oils under certain conditions (Chisti 2008; Ratledge 2004). Microalgae have received much attention because of their reported capacity to accumulate large amounts of intracellular lipids with concentrations exceeding 60 % of dry weight in some species (Metting 1996; Chisti 2007). Furthermore, microalgae have an added advantage in that many strains produce a wide range of potential bioproducts (e.g. carotenoid pigments, industrial enzymes, polyunsaturated fatty acids (PUFA), and exopolysaccharides and their byproducts - such as algal meal - may be of use in animal and fish feeds, adding greater value to the production process pipeline with improved economics (Huntley and Redalje 2007; US DOE 2010a; Li et al. 2008; Stephens et al. 2010; Wijffels and Barbosa 2010).

One group of microalgae - thraustochytrids - are heterotrophic protists found ubiquitously in the marine environment playing an important role in marine ecosystems; they can be bacterivores, detritivores or parasites (Maas et al. 1999; Raghukumar 2002). Unlike phototrophic algae, thraustochytrids are usually grown heterotrophically for mass production in stainless steel fermentors using complex organic substances, including by-products from other processes (e.g. sugars, organic acids) as a sole carbon and energy source.

In an initial isolation and screening study, we demonstrated that recently isolated endemic Australian thraustochytrid strains were promising candidates for production of biodiesel, omega-3 long-chain (LC, $\geq C_{20}$) PUFA (LC-PUFA) and carotenoid pigments (Lee Chang et al. 2012). We reported that *Aurantiochytrium* sp. thraustochytrids (Groups G and H as identified by chemo and molecular

taxonomy) contained pentadecanoic acid (15:0) at between 20-30 % of the total fatty acids (TFA); these strains, along with the *Thraustochytrium* sp. strains (Group C), were deemed to be suited for biodiesel production, as they contained high levels of saturated fatty acids (SFA, mean 35 % TFA) and monounsaturated fatty acids (MUFA, mean 9 % TFA). In a follow-up study, a number of thraustochytrids were grown in shake-flask cultures to identify high lipid producing strains (Lee Chang et al., manuscript submitted).

Dietary consumption of omega-3 LC-PUFA, in particular EPA and DHA, helps reduce the risk of cardiovascular diseases, neural disorders, arthritis, asthma and skin diseases in humans (Danaei et al. 2009; Mozaffarian and Rimm 2006). DHA is also essential for neural and retinal development during fetal life and infancy (Forsyth and Carlson 2001; Ratledge 2004). The average Australian and many other western diets have been recognized as being deficient in omega-3 LC-PUFA (Meyer et al. 2003; Danaei et al. 2009), highlighting the importance of increased availability and consumption of omega-3 fatty acids. Currently, most omega-3 LC-PUFA is extracted from fish oil sourced from wild fisheries. However, growing concerns exist over the health of ocean fish stocks, the ecological effects of industrial fishing, and high levels of pollutants in some fish oils (Pauly et al. 2002). As a result, alternative sources of omega-3 LC-PUFA, including microbial production systems, are being sought as replacements for fish oil (William et al. 2005; Kralovec et al. 2012). Thraustochytrids are known to produce high amounts (> 30 % of total fatty acids) of omega-3 LC-PUFA, including docosahexaenoic acid (DHA, 22:6ω3) and eicosapentaenoic acid (EPA, 20:5ω3) (Jain et al. 2007; Lewis et al. 1999). For example, *Schizochytrium* sp. (thraustochytrid) and *Crypthecodinium cohnii* (dinoflagellate) have been used to produce DHA-containing oil by Martek Biosciences Corporation that is now part of the Royal DSM (Dutch State Mines) (Ratledge 2012).

The degree of saturation of the fatty acids is important with respect to the suitability of the microbial-derived oil for biodiesel production. Fatty acids with fewer double bonds (the degree of unsaturation) are more stable to oxidation compared to the PUFA. Oxidation results in the formation of undesirable products, such as alcohols that reduce the flash point of biodiesel, aldehydes that cause rancidity, and short-chain fatty acids that are corrosive to engine components (Knothe 2007; Monyem et al. 2000). Furthermore, saturated fatty acids typically have higher solidification temperatures and therefore cannot be used at lower temperatures. Therefore high levels of saturated and monounsaturated FA are more desirable for biodiesel production due to the increased oxidative stability as well as the thermal stability of biodiesel. Due to their high lipid productivity, thraustochytrids have the potential for production of both omega-3 LC-PUFA rich lipids as well as the shorter chain fatty acids suitable for biodiesel (Johnson and Wen 2009).

Agro-industrial wastes such as spent yeast from brewery, empty palm fruit bunches, and coconut water, have been very recently explored for producing high value omega-3 oils including DHA using *Aurantiochytrium* sp. strains (Ryu et al. 2012; Kim et al. 2012; Hong et al. 2012; Liang et al. 2010). The biomass and DHA yields under varied cultivation conditions and systems using different *Aurantiochytrium* sp. strains are summarized in Table 5-1. Both pure and crude glycerol (a raw product of the transesterification process used for biodiesel industry) have been examined to be used as a carbon source for cultivation of thraustochytrids (Abad and Turon 2012).

Table 5-1 Summary of studies using different agro-industrial wastes and carbon sources to obtain DHA from *Aurantiochytrium* sp. strains

Carbon source	Treatment	Agitation speed, temperature	Strain	DCW g/L	DHA g/L	Time h	Reference
Palm empty fruit bunches	5 L bioreactor	50 rpm, 28°C	*Aurantiochytrium* sp. KRS101	34.4	5.4	36	(Hong et al. 2012)
Spent yeast (glycerol supplement)	Fed-batch, 500 ml baffled flask	170 rpm, 28 °C	*Aurantiochytrium* sp. KRS101	31.8	4.2	130	(Ryu et al. 2012)
Sweet sorghum juice 50 % v/v	250 ml Erlenmeyer flask	170 rpm, 20°C	*Aurantiochytrium limacinum* SR21[a] (ATCC MYA-1381)	9.4	2.4	120	(Liang et al. 2010)
Coconut water 33 %v/v	250 ml Erlenmeyer flask	200 rpm, 25°C	*Aurantiochytrium mangrovei* Sk-02[b]	28.6	6	96	(Unagul et al. 2007)
Glucose 4 % w/v	Fed-batch, 500 ml baffled flask	200 rpm, 28°C	*Aurantiochytrium* sp. KRS101	22.1	3.1	168	(Kim et al. 2012)
Glucose 10 % w/v	3.5 L bioreactor	300-500 rpm, 28°C	*Aurantiochytrium limacinum* SR21[a] (ATCC MYA-1381)	52	9.8	72	(Rosa et al. 2010b)
Crude glycerol 10 % w/v	250 ml Erlenmeyer flasks	170 rpm, 20°C	*Aurantiochytrium limacinum* SR21[a] (ATCC MYA-1381)	22.1	5	168	(Pyle 2008)
Glycerol kept above 2 % v/v	Fed excess C, limit N, 1.8 L bioreactor	1300 rpm, 28°C	*Aurantiochytrium* sp. T66 (ATCC PRA-276)	100	15.6	168	(Jakobsen et al. 2008)
Glycerol 4% w/v (Total fed 17.2 % w/v)	Fed-batch, 2 L bioreactor	300-1150 rpm, 20°C	*Aurantiochytrium* sp. TC 20 (CS-997)	70	14.3	69	This study

Formerly [a]*Schizochytrium limacinum* SR21, [b]*Schizochytrium mangrovei* Sk-02

The rise in biodiesel production has resulted in a surplus of low cost glycerol internationally. Therefore the use of a crude glycerol stream as a feedstock could substantially reduce the cost of commercial production of biodiesel using a heterotrophic algal production system. However, glycerol utilization is limited to certain species and strains; not all heterotrophic algae can utilize glycerol as a carbon source (Hong et al. 2011). In this study, we investigate the potential of glycerol as a carbon source for the cultivation of a recently isolated Australian *Aurantiochytrium* sp. strain (Lee Chang et al. 2012). This *Aurantiochytrium* sp. strain TC 20 is closely clustered to the highly productive and high DHA-containing *Aurantiochytrium limacinum* SR21 (Yokochi et al. 1998) based on the 18S rRNA sequence data (Lee Chang et al. 2012). This association suggests that this strain has similar potential to be used as a sustainable source of omega-3 LC-PUFA for nutraceuticals and other applications as already established using *Aurantiochytrium limacinum* SR21 (Yokochi et al. 1998). We compare our results for an Australian *Aurantiochytrium* sp. strain TC 20 with those recently reported for other *Aurantiochytrium* sp. strains grown on other agro-industrial wastes. We also demonstrate the high cell density cultivation of *Aurantiochytrium* sp. TC 20 using a fed-batch production process and examine the effect of fortification of the feed with additional nutrients including vitamins and minerals to further increase biomass yield.

Materials and methods

Microorganism and culture conditions

Aurantiochytrium sp. strain TC 20 (CS number: CS-997, GenBank accession number: JN675250) isolated previously (Lee Chang et al. 2012) was used in the present study. The strain is held in the Australian National Algae Culture Collection (http://www.csiro.au/ANACC). Medium preparation was performed as described previously (Lee Chang et al. 2012).

The culture medium consisted of (g/L): sea salts (20), glucose (2), bacteriological peptone (2), yeast extract (2) (Sigma-Aldrich, St. Louis, MO, USA), metal solution (1 mL/L) and vitamin solution (1 mL/L) that were filter sterilized (0.2 μm) and added after autoclaving. The metal solution contained (mg/L): $MgSO_4.7H_20$ (200), KH_2PO_4 (200), $NaHCO_3$ (100), $MnCl_2.4H_2O$ (9), $Fe_3Cl_3.6H_2O$ (3), $ZnSO_4.7H_2O$ (1), $CoSO_4.5H_2O$ (0.3) and $CuSO_4.5H_2O$ (0.2). The vitamin mixture contained (mg/L): pyridoxine hydrochloride (0.2), thiamine (0.1), pantothenic acid (0.1), aminobenzoic acid (0.1), riboflavin (0.1), nicotinamide (0.1), biotin (0.04), folic acid (0.04) and vitamin B12 (0.002).

For the primary seed culture, strains were revived from cryogenic storage by rapidly thawing the cryovials in a 50 °C water bath until the content melted (Cox et al. 2009). The thawed culture (1 mL) was slowly pipetted into 10 mL of culture medium in a sterile 50 mL polystyrene cell culture flask fitted with a 0.2 μm filter cap (Greiner Bio-One, Germany). The flasks were incubated at 20 °C in the dark for 7 days without agitation (Cox et al. 2009).

For the secondary seed culture, after 7 days, 10 mL of the primary seed culture was aseptically pipetted into a 1 L Erlenmeyer flask containing 200 mL of culture medium supplemented with 20 g/L of glucose.

The flask was incubated in a shaking incubator at 20 °C and 200 rpm and cultures were sub-sampled at 3 days after being acclimated in the high glucose growth media. The secondary seed culture (80 mL) was used to inoculate both bioreactor experiments. The purity of the secondary seed culture was checked using light microscopy (Zeiss Axioplan Compound Microscope, NY, USA) and streak plating onto solid medium.

Two fed-batch experiments were performed using Sartorius Biostat B bioreactors (Sartorius Stedim, Australia) fitted with 2 L vessels (Fig. 5-1) that contained 1.6 L of medium supplemented with a carbon source. For the first experiment comprising two treatments (n = 2 replicate fermentors per treatment), the medium was initially supplemented with 40 g/L or 100 g/L glucose with the addition of 400 mL of 660 g/L glucose at glucose exhaustion occurring at 29 h and 45.6 h respectively (with a feed rate of 30 mL/h).

Figure 5-1 Two litres stirred tank bioreactors connected to a Biostat B (Sartorius Stedim, Australia) control system

For the second experiment, two of the three treatments contained medium initially supplemented with 40 g/L glucose (Glu) or glycerol (Gly), with the addition at 26 h of 400 mL of 660 g/L glucose or glycerol respectively (with feed rate of 18 mL/h) before the carbon source was exhausted. For the third treatment, the medium was also initially supplemented with glycerol (Gly/ Nutr) at 40 g/L with the addition at 26 h of 400 mL of 660 g/L glycerol; in contrast to the other two treatments, 100 mL of concentrated nutrients was also added at 26 h (20 g yeast extract, 4 mL trace metal mix, 4 mL vitamin mix, 1.23 g $MgSO_4.7H_2O$ and 4 g MSG) at the same feed rate as the other two treatments.

Each Sartorius Biostat B bioreactor was fitted with sensors for temperature, pH and optical density. Optical density was important for real-time assessment of cell growth, however, thraustochytrids tend to clump together at different stages of the life cycle resulting in variable observations and the data are not presented. In comparison, biomass showed more consistency among replicates and was used to quantitate growth. For all fed-batch fermentations, the temperature was maintained at 20°C and the culture was maintained at pH 7.0 via the automatic addition of ammonia solution (10 % w/v) or phosphoric acid (10 % v/v). Dissolved oxygen was controlled at 30 % of saturation by varying the stirrer speed (300 to 1150 rpm) and air flow rate (0.3 to 1.5 L/min). Morphological examination of cells using light microscopy revealed the cells were healthy and resistant to shear stress caused by the impeller (up to 1000 rpm) in the bioreactor.

Biomass harvest and analysis of fatty acid methyl esters (FAME)

To harvest biomass, a 10 mL sub-sample was aseptically removed from the bioreactors, transferred into a 15 mL tube and centrifuged at 4000 rpm at 20°C for 10 min. The supernatant was removed, and the pellet was resuspended and washed with 10 mL of sterile 3.15 % (w/v) ammonium formate solution. Cells were again pelleted by centrifugation at 4000 rpm at 20 °C for 10 min. The pellets were freeze-dried overnight and weighed to determine dry cell weight (DCW). Residual glucose in the culture supernatants was measured using a YSI glucose analyser (Yellow Springs Instruments, Ohio, USA).

Freeze-dried samples were directly transesterified with methanol: chloroform: HCl (l0:1:1 v/v/v) to convert fatty acids from the complex lipids into fatty acid methyl esters (FAME), as described by Lee Chang et al. (2011). Because most of the oil in thraustochytrids is comprised of triacylglycerol containing fatty acids (85-95 %), total FAME was used as a surrogate for total lipid. Individual fatty acids are expressed as a percentage of the total fatty acids (TFA). Gas chromatography was used to quantitate the fatty acids and was performed on an Agilent Technologies 7890A GC (Palo Alto, CA, USA) equipped with a non-polar Equity-1™ fused silica capillary column (15 m x 0.1 mm i.d., 0.1 mm film thickness), a flame ionization detector (FID) and split/splitless injector. Samples were injected in splitless mode at an oven temperature of 120 °C and after injection the oven temperature was increased to 270 °C at 10°C/ min and then to 310 °C at 5 °C/ min. Lipid classes were identified according to standard mixtures 18-5A that was obtained from Nu-Chek-Prep, Inc. (Elysian, MN, USA). Peaks were quantified with Agilent Technologies ChemStation software (Palo Alto, CA, USA).

GC-MS analysis of FAME was performed to confirm component identifications and was carried out on a Finnigan GCQ Plus™ GC-MS ion-trap fitted with an on-column injector set at 45 °C. Samples were injected using an AS2000 auto

sampler onto a retention gap attached to a non polar HP-5 Ultra 2 bonded-phase column (50 m x 0.32 mm i.d. x 0.17 μm film thickness). The initial temperature of 45 °C was held for 1 min, followed by temperature programming at 30 °C/ min to 140 °C then at 3 °C/min to 310°C where it was held for 12 min. Helium was used as the carrier gas. Mass spectrometer operating conditions were: electron impact energy 70 eV; emission current 250 μamp, transfer line 310 °C; source temperature 240 °C; scan rate 0.8 scan/ sec and mass range 40-650 Da. Mass spectra were acquired and processed with Thermo Scientific Xcalibur™ software (Waltham, MA, USA).

Lipid class composition was determined by analysing a portion of the total lipid extract using thin layer chromatography – flame ionisation detection (Iatroscan Mk V TH-10, Iatron Laboratories, Japan). Lipid extracts (1 μL) were applied onto duplicate chroma rods using disposable micro-pipettes. Rods were developed using a hexane/ diethyl ether/ acetic acid (60:17:0.2 v/v/v) solvent system in a sealed glass tank containing filter paper. After development, the rods were dried at 60°C for 10 min and analysed. Peak areas were quantified using SIC-480II Iatroscan™ Integrating Software v.7.0-E (System Instruments Co., Mitsubishi Chemical Medicine Corp., Japan).

Results

Effect of initial glucose levels (40 g/L and 100 g/L) on cell growth and lipid yield

In the experiment 1, changes in glucose concentration and biomass with fermentation time for *Aurantiochytrium* sp. strain TC 20 are shown in Figures 5-2 and 5-3. When the medium had an initial glucose content of 40 g/L, biomass decreased once glucose became depleted at 27 h (Fig. 5-2). At 29 h, a dose of glucose (660 g/L) was fed into the bioreactor (Fig. 5-2). Cultures grown using the higher initial glucose (100 g/L) had a final biomass yield of 47.7 g/L, similar to the 48.3 g/L for the 40 g/L glucose treatment at 69 h of fermentation (Figs. 5-2 and 5-3).

Biomass in the fermentation of strain TC 20 increased in a linear relationship with glucose consumption. Both biomass and ammonia consumption increased as glucose was fed into the bioreactor (Fig. 5-2). The higher initial glucose level delayed exhaustion of glucose from 27 h to 45 h and resulted in a higher biomass than for the fermentor containing the lower initial glucose concentration (24.6 g/L biomass at 27 h for initial 40 g/L glucose (Fig. 5-2) and 38.8 g/L biomass at 45 h for the initial 100 g/L glucose treatment (Fig. 5-3)).

Although both treatments yielded relatively similar biomass at 69 h, the total FAME content was improved with the higher (100 g/L) glucose treatment (Fig. 5-3). The total FAME content at 49 h and 69 h was 8 g/L and 12 g/L (25 % of dry weight), respectively, in the 40 g/L glucose treatment (Fig. 5-2). In the 100 g/L glucose treatment, total FAME content was 16 g/L (40 % of dry weight) at 49 h and then dropped to 14 g/L (29 % of dry cell weight) at 69 h (Fig. 5-3).

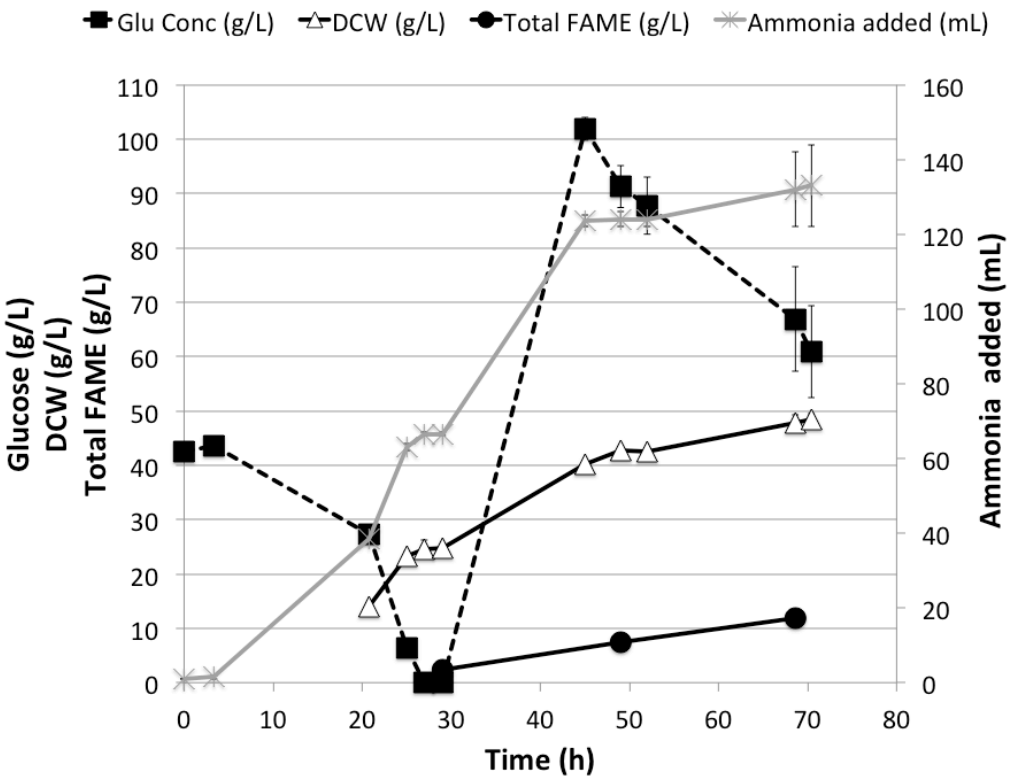


Figure 5-2 Fed-batch cultivation of *Aurantiochytrium* sp. strain TC 20 in a 2 L bioreactor with 1.6 L of medium. The initial medium contained 40 g/L glucose. A dose of glucose (660 g/L) was fed into the bioreactor at 29 h. Data given are the mean of two replicate bioreactors under the same conditions. Abbreviations: Glu, glucose; DCW, dry cell weight

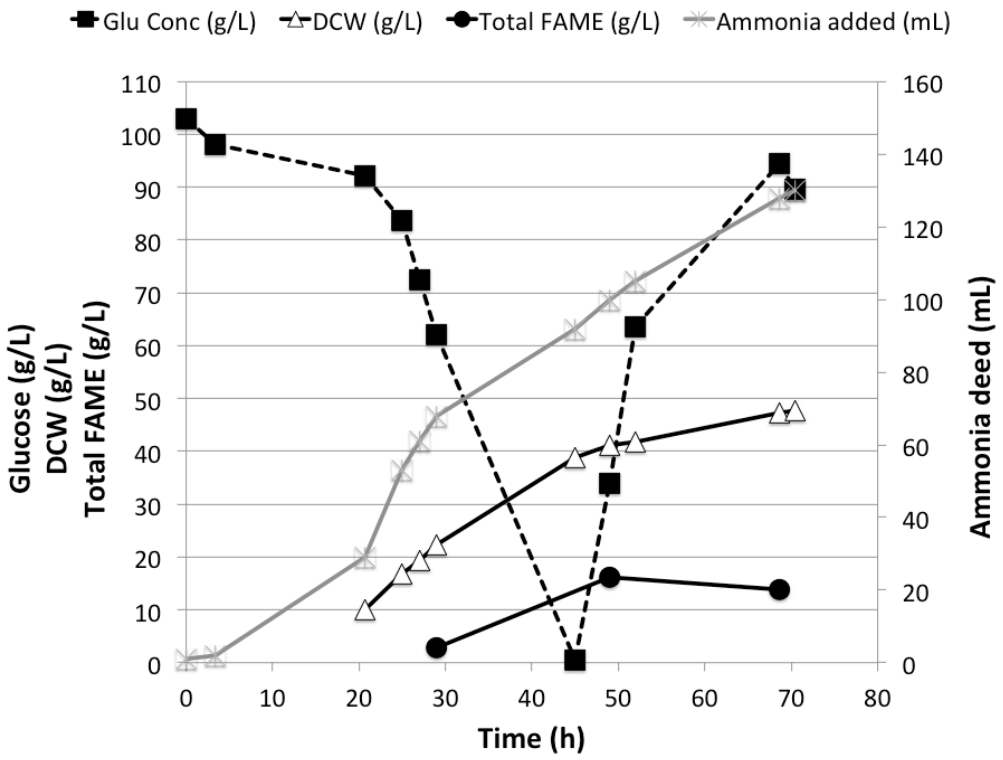


Figure 5-3 Fed-batch cultivation of *Aurantiochytrium* sp. strain TC 20 in a 2 L bioreactor with 1.6 L of medium. The initial medium contained 100 g/L glucose. Seed culture was inoculated at 5% (v/v). A dose of glucose (660 g/L) was fed into the bioreactor at 45.6 h. Data given are the mean of two replicate bioreactors under the same conditions. Abbreviations: Glu, glucose; DCW, dry cell weight

Evaluation of glycerol as carbon source

In the experiment 2, both glucose (Glu, Fig. 5-4) and glycerol (Gly, Fig. 5-4) were tested, with an initial concentration of 40 g/L, as the carbon source for the cultivation of our *Aurantiochytrium* sp. Strain TC 20. The glucose consumption pattern was similar to experiment 1 (Fig. 5-2), where in the first 20 h glucose was consumed at about 0.5 g/L/h. When the culture actively grew in arithmetic fashion, glucose was consumed at a rate of 5 g/L/h (a 10-fold increase). Glucose (660 g/L, Glu, Fig. 5-4) or glycerol (Gly, Fig. 5-4) was fed to the fermentors at 18 mL/h, from 25.4 h (commencing before the initial carbon source was exhausted). The DHA yield of 145.9 mg/g DCW (43.2 % TFA) in the glucose treatment was lower than achieved (159.7 mg/g DCW, 48.4 % TFA) when glycerol was used as carbon source (Table 5-2). While comparing the effects of glucose or glycerol as a carbon source, we observed that the additional feeding of the carbon source before it was completely exhausted improved biomass and FAME yield (Fig. 5-4), compared to the Experiment 1 fermentations (Figs. 5-2 and 5-3) under the same growth conditions. The biomass yield improved from 50 g/L (Figs. 5-2 and 5-3) to 55 g/L (Fig. 5-4) at 69 h of fermentation. The FAME content of the strain in the experiment 2 fermentations with additional carbon used (Fig. 5-4) ranged between 18-19 g/L and was up to 2-fold higher than observed with the experiment 1 fermentations (Figs. 5-2 and 5-3).

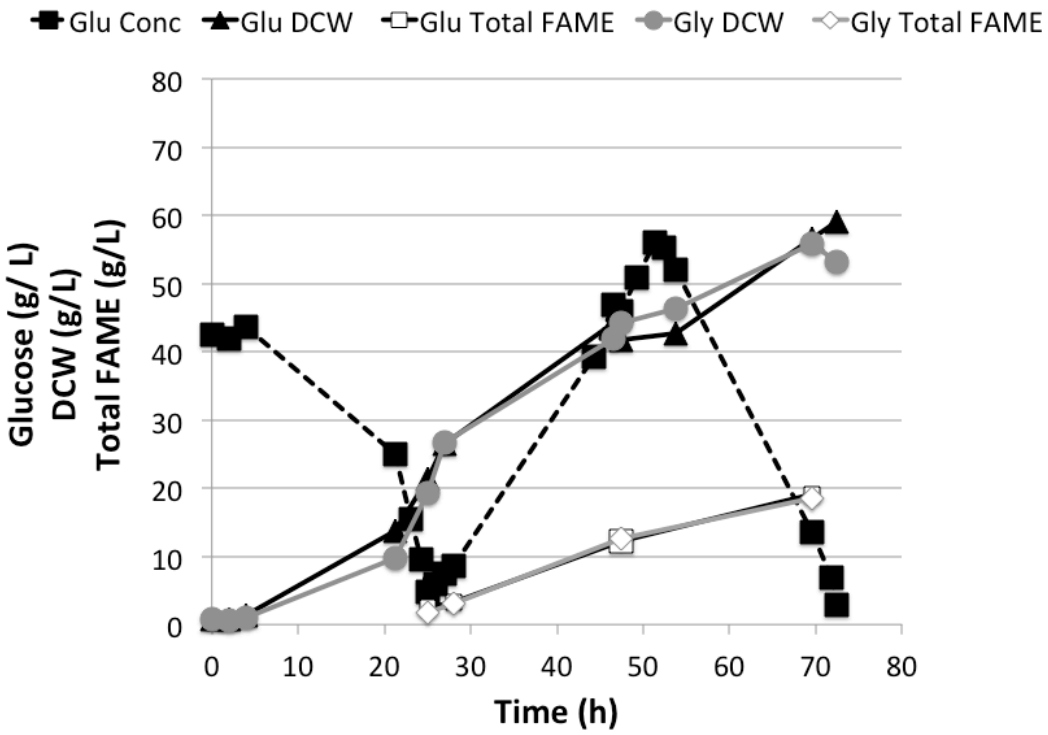


Figure 5-4 Fed-batch cultivation of *Aurantiochytrium* sp. strain TC 20 in a 2 L bioreactor with 1.6 L of medium. The initial medium contained 40 g/L glucose (Glu) or glycerol (Gly). An additional 400 mL 660 g/L of glucose (Glu) or glycerol (Gly) was fed into the bioreactor at 25.4 h. Abbreviations: DCW, dry cell weight

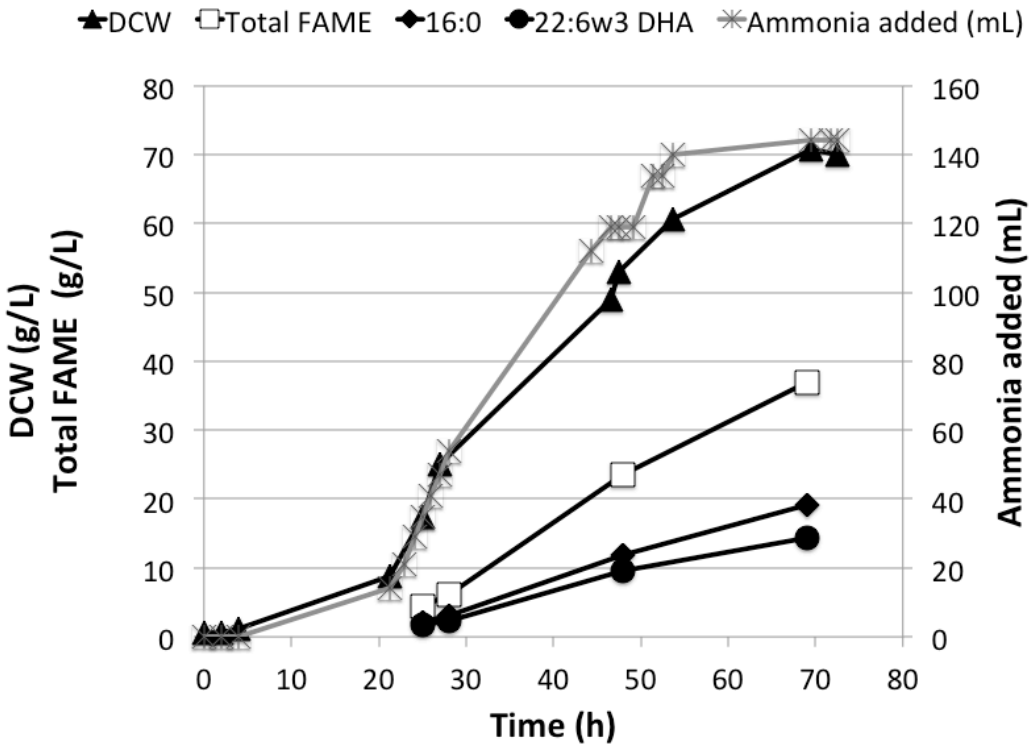


Figure 5-5 Effect of addition of nutrients (Gly/ Nutr) in a fed-batch cultivation of *Aurantiochytrium* sp. strain TC 20 in a 2 L bioreactor with 1.6 L of medium. The initial medium contained 40 g/L glycerol. At 25.4 h, an additional feed of 400 mL 660 g/L glycerol + 100 mL of concentrated nutrients (yeast extract, trace metal mix, vitamin mix, $MgSO_4.7H_2O$ and MSG) was added at 18 mL/h (total volume of fermentor = 2 L). Abbreviation: DCW, dry cell weight

Glycerol and additional nutrients in fed-batch cultivation

In experiment 2 the effect of fed-batch glycerol with additional nutrients (yeast extract, trace metal mix, vitamin mix, $MgSO_4.7H_2O$ and MSG) was also investigated (Gly/ Nutr, Fig. 5-5). This combined fed-batch glycerol and extra nutrients (Gly/ Nutr) cultivation from 25.4 h showed markedly increased biomass and FAME yield compared with the experiment 1 fermentations, achieving a FAME content of 52.2 % DCW and biomass of 70.8 g/L DCW at 69 h (Table 5-2 and Fig. 5-5). Fatty acid profiles of the *Aurantiochytrium* sp. strain TC 20 in the three different fermentation treatments of experiment 2 (Figs. 5-4 and 5-5) were similar (Table 5-2) and a representative gas chromatogram is shown in Fig. 5-6. High levels of SFA, mainly 16:0, ranged from 45 to 52 % of the TFA and PUFA also ranged from 45 to 52 % TFA; PUFA was mostly DHA with lower levels of docosapentaenoic acid (DPA-6, 0.9- 4.2 %), (Table 5-2).

The relative proportion of DHA (38.9 % TFA) in the glycerol added nutrient fed-batch system (Gly/ Nutr) was lower than occurred for the glycerol (48.4 % TFA) and glucose (43.2 % TFA) treatments (Table 5-2). However, the absolute amount of DHA and hexadecanoic acid (16:0) produced per volume of medium was higher (14.3 g/L and 19.1 g/L respectively for the Gly/ Nutr treatment) compared to when only the carbon source (glycerol or glucose) was fed-batch (8.2-8.9 g/L and 8.3-9.6 g/L respectively) (Fig. 5-6a and 5-6b). The relative proportion of DPA-6 was less than that in a previous screening study where this strain was reported to have 9.3 % DPA-6 (as % TFA) in flask culture (Chapter 4). In the present study, 0.9 % DPA-6 was detected in the glucose and glycerol treatments and 4.2 % DPA-6 was recorded in the glycerol added nutrient (Gly/Nutr) fed-batch fermentation (Table 5-2). The relative percentage of 16:0 (51.7 % TFA) in the Gly/ Nutr system was slightly higher than observed for the glucose (Glu) and glycerol (Gly) treatments (50.3 % and 45.2 %, respectively).

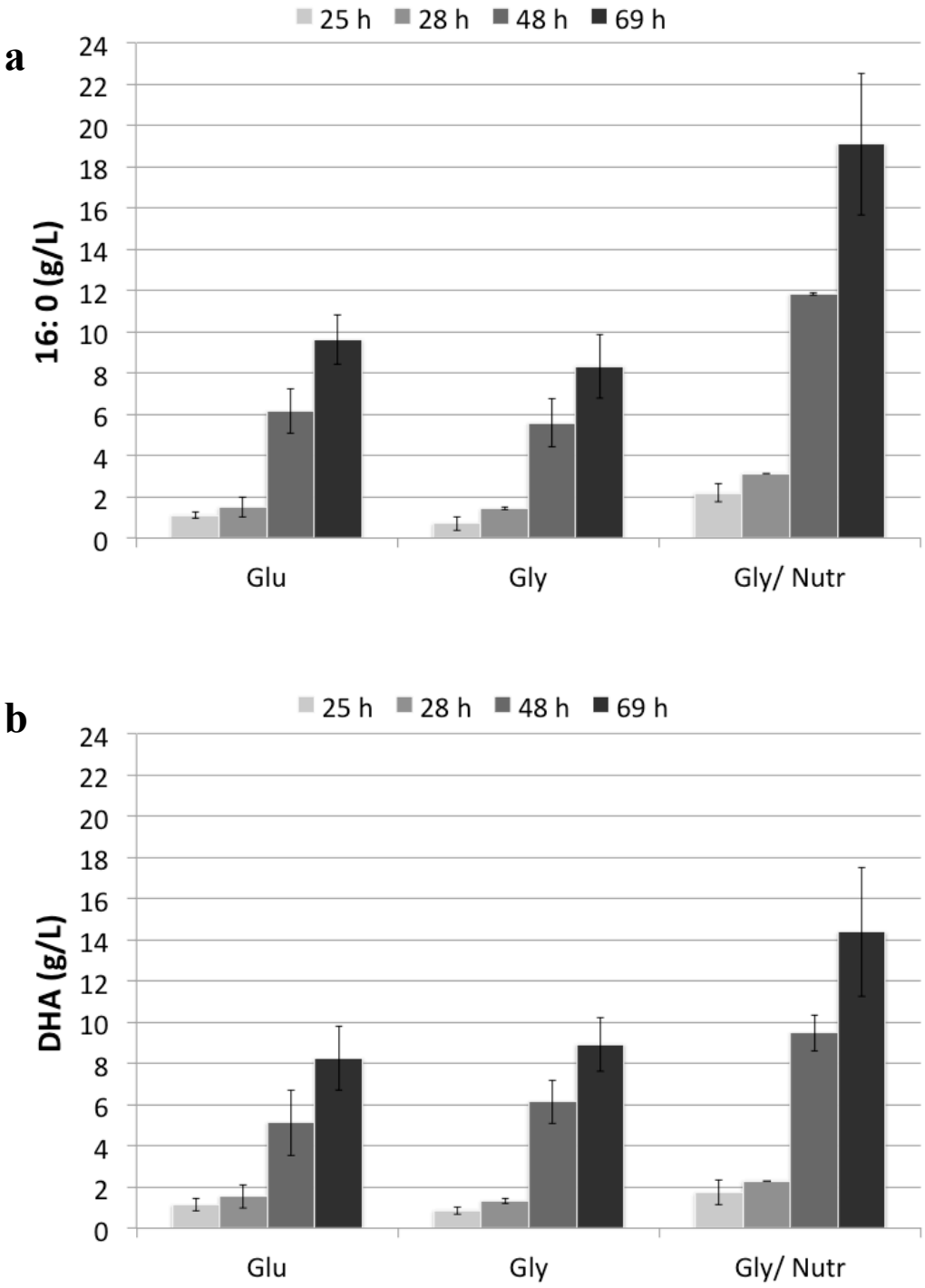


Figure 5-6 Yield of (**a**) saturated fatty acid, 16:0, and (**b**) docosahexaenoic acid, DHA, in different treatments of the *Aurantiochytrium* sp. strain TC 20. All 3 treatment media were initially with 40 g/L of glucose (Glu) or glycerol (Gly). An additional 400 mL of 660 g/L glucose or glycerol was fed at 25.4 h. Bioreactor Gly/ Nutr had an additional 100 mL of concentrated nutrients (yeast extract, trace metal mix, vitamin, $MgSO_4.7H_2O$ and MSG) added at 25.4 h

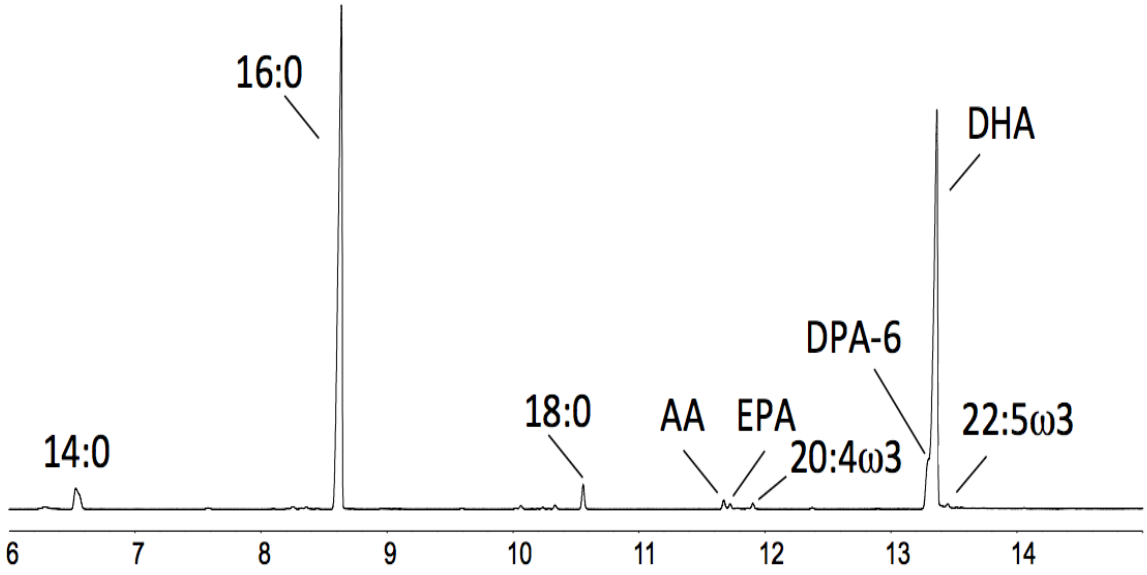


Figure 5-7 Partial gas chromatogram of the total FA (as FAME) of the thraustochytrid *Aurantiochytrium* sp. strain TC 20 at 69 h of fermentation with glycerol as the carbon source (Gly/ Nutr). The shoulder on the front of DHA is DPA-6. Abbreviations: AA, 20:4ω6; EPA, 20:5ω3; DPA-6, 22:5ω6; DHA, 22:6ω3

Discussion

Effect of initial glucose levels on biomass and fatty acid production

In the first experiment, higher initial glucose treatment (100 g/L compared to 40 g/L) did not result in markedly different biomass (about 48 g/L) or fatty acid (12-14 g/L) yields by 69 h. The exhaustion of glucose at 27 h appears to be critical, as the limited carbon source caused cell growth to decrease; however, an additional dose of glucose enabled biomass production to resume (Fig. 5-2). Furthermore, the trend of the glucose consumption rate at 25 h and in the following period from 30 h to 45 h, where an absence of data points existed (occurring overnight), suggested the glucose concentration in the medium of the fermentor with 100 g/L of initial glucose was very likely to have been consumed earlier than the 45 h sampling point (Fig. 5-3). Therefore it is likely that glucose starvation had occurred between 35 h – 40 h, and again this glucose depletion will have caused cell growth to decrease. This suggests other factors were limiting biomass production at higher initial glucose treatment. These observations are consistent with the results obtained from Hong et al. (2011) where the authors compared the growth of *Aurantiochytrium* sp. KRS101 in different initial glucose concentrations and found DCW increased as glucose concentration increased up to 60 g/L; a further increase in glucose concentration had no effect on DCW (Hong et al. 2011). Interestingly both of our treatments had high amounts of glucose remaining at the conclusion of the experiment (60 g/L and 90 g/L; Fig. 5-2 and Fig. 5-3), suggesting the reduced growth rates by 69 h were due to a non-carbon nutrient.

Higher initial glucose (100 g/L) did not result in a direct increase in the rate of biomass production, but rather the isolate actively produced biomass for a longer period than with a lower initial glucose concentration (i.e. the glucose ran out later with growth being prolonged) (Fig. 5-3). In addition, Kim at al. (2012) demonstrated that carbon (glucose) consumption was linearly associated with increased biomass regardless of growth stage, while the uptake of nitrogen (yeast extract) was during the initial stage of cultivation of *Aurantiochytrium* sp. KRS101.

The increased ammonia consumption and biomass demonstrated that the availability of the carbon source in the fermentation medium markedly impacted growth of this *Aurantiochytrium* sp. strain. A similar trend was evident when the medium had a higher initial glucose content (100 g/L) (Fig. 5-3). It is often acknowledged that a high carbon to nitrogen ratio in the culture medium is important for microbial growth and lipid production. The lipid accumulation is often triggered by nitrogen depletion with an excess of carbon source (Bowles et al. 1999; Yokochi et al. 1998; Hall and Ratledge 1977; Ratledge and Wynn 2002). Lewis (2001) reported there was no clear correlation between the initial carbon: nitrogen ratio in the medium (glucose and sodium glutamate) on either biomass or lipid production by Thraustochytrid strain ACEM 6063. Ganuza et al. (2008) demonstrated a high biomass yield (63 g/L at 49 h of fermentation) of *Schizochytrium* sp. strain G13/2S grown in an unlimited nitrogen supply using an ammonium/ pH-auxostat fed-batch system with glucose (150 g/L) as the carbon source. The authors suggested this pH-auxostat strategy could be used to achieve high biomass production for the early stage of cultivation, while lipid accumulation can be triggered by nitrogen depletion at the final stages for maximum lipid accumulation (Ganuza et al. 2008).

Comparison of glucose and glycerol as carbon source

In the second experiment, both glucose and glycerol were examined as carbon sources in a fed-batch process. Even though the seed culture of the *Aurantiochytrium* sp. TC 20 strain was not acclimated in glycerol, the biomass and total FAME content were very similar for both the glucose and glycerol treatments, 55.6 g/L DCW with 33.8 % total FAME and 55.9 g/L with 33 % total FAME, respectively, at 69 h of fermentation (Table 5-2, Fig. 5-4).

The finding of higher DHA yield in the glycerol treatment (159.7 mg/g DCW, 48.4 % TFA) compared to the glucose treatment (145.9 mg/g DCW 43.2 % TFA) is consistent with Yokochi et al. (1998), where the authors demonstrated that DHA production of *Aurantiochytrium limacinum* SR21 was enhanced when glycerol was used as the carbon source. Yokochi et al. (1998) showed that the lipid content and biomass yields were very similar when glucose or glycerol was used as carbon sources. Hong et al. (2011) investigated various carbon sources including arabinose, fructose, lactose, maltose, sucrose, xylose, glycerol, and crude glycerol with the *Aurantiochytrium* sp. KRS101 and obtained the maximum lipid content (45 % DCW) and highest biomass (10 g/L DCW) when glucose was used. These differences suggest that responses to various carbon sources are strain specific. Furthermore, Scott et al. (2011) reported *Thraustochytrium* sp. ONC-T18 yielded higher biomass (31.7 g/L) in crude glycerol, which is derived from biodiesel production without purification, than for all other carbon treatments (pure glycerol, glucose/glycerol and glucose) (<23 g/L) after 5 days of fermentation. The authors proposed that high amounts of ions (i.e. potassium, phosphorus and sodium) in the crude glycerol supported stronger growth relative to the other media where glucose was the carbon source (Scott et al. 2011).

Glycerol and additional nutrients in fed-batch cultivation

Jakobsen et al. (2008) reported up to 100 g/L biomass for *Aurantiochytrium* sp. strain T66 was achieved in a feed-batch cultivation using an excess of carbon source (glycerol) with nitrogen limitation, and a constant high level of dissolved O_2 (20 % of saturation). The fast growth and high biomass together with high lipid content of *Aurantiochytrium* sp. strain TC 20 is comparable to or greater than values recently reported for a number of thraustochytrid strains (Table 5-1). Whilst the culture conditions differed, of particular interest, our study has achieved around 70 % of the biomass yield in less than half of the time reported in Jakobsen et al. (2008) using glycerol. Our yields were also markedly higher than achieved using other industrial wastes (Table 5-1). This comparison highlights that there is still scope for further fine-turning our growth conditions as we did not examine the effect of nitrogen starvation.

The high yield of DHA (14.3 g/L) and 16:0 (19.1 g/L) in the Gly/ Nutr treatment collectively highlight the potential for markedly enhanced production of these major fatty acids from strain TC 20. The decrease in the level of DPA-6 under the current fermentation conditions could be due to better O_2 supply in the fermentor compared to that of flask culture, with the O_2-independent polyketide synthase (PKS) pathway being more active in the flask culture. In the fermentor, O_2 was monitored and the conventional fatty acid synthase (FAS) pathway may be more actively involved. The higher palmitic acid (16:0) observed in the fermentation culture compared to that of the flask culture (Lee Chang et al. 2012) might also be explained by enhancement of the FAS pathway due to the unlimited O_2 supply. Our findings are consistent with those reported by Jakobsen et al (2008) in that O_2 limitation hindered the O_2-dependent desaturase(s) resulting in production of MUFA, while the O_2-independent polyunsaturated fatty acid synthase was favored for LC-PUFA production.

High levels of SFA (51.7 % TFA) are suitable for biodiesel production due to the increased oxidative stability and thermal stability required (Knothe 2007; Monyem et al. 2000). Johnson and Wen (2009) demonstrated the potential of producing biodiesel from *A. limacinum* SR21 using direct transesterification and the scaled-up production resulted in about 200 mL of liquid fuel from 400 g of biomass that was grown in a medium containing 90 g/L of crude glycerol and 10 g/L corn steep solid.

pH regulation of *Aurantiochytrium* sp. TC 20 in fermentation

The high growth rate of strain TC 20 during fermentation was accompanied by high ammonia consumption (Fig. 5-2). It is possible that pH control is a critical parameter for the growth of this strain. The pH decrease occurred at the same time that glucose was being utilized. The pH decreased due to the carbon source utilization and resultant acidic end product release, and the pH control facility of the fermentor then added ammonia to balance the pH at 7. As a result, ammonia was added to the fermentation and the rising ammonia consumption was a further indication of cell growth (Fig. 5-2). Wu et al. (2005) reported that *Schizochytrium* sp. released metabolic acids such as malic acid, citric acid, pyruvic acid and fumaric acid during growth with optimal growth at pH 7. As discussed previously, Ganuza et al. (2008) demonstrated the use of ammonia to maintain pH and as a nitrogen source and this further suggests the importance of supporting healthy cell growth under optimal pH condition.

A small amount (9 - 10 mL) of phosphoric acid (10 % v/v) was added to the fermentation by the pH control facility of the fermentor over the course of the experiment to maintain the pH at 7 (data not shown). It is possible that the observed pH increase was due to alkaline by-products formation resulting from de-amination of the amino acids in the complex medium. Such a situation would indicate that this *Aurantiochytrium* sp. strain TC 20 was co-metabolising the

glucose and amino acids from the peptone and yeast extract. The total amounts of phosphoric acid (10 % v/v) added to fermentations were similar in both the glucose treatment (10 mL) and glycerol treatment (9 mL). However, with the Gly/ Nutr treatment, where a total of 22 mL phosphoric acid was added to the fermentation, better growth was observed and therefore more alkaline by-products were produced further supporting the suggestion of co-metabolism of glucose and amino acids.

Biodiesel and long-chain omega-3 fatty acid production

It is often acknowledged that lipid accumulation in photosynthetic microalgal cells is triggered by limitation of the nitrogen source (Ratledge and Wynn 2002). However, LC-PUFA synthesis takes place in cells undergoing balanced growth rather than nutrient limitation (Ratledge and Wynn 2002). This is consistent with our observations that, with an unlimited supply of a nitrogen source in the fermentation (as ammonia that was automatically added to maintain the culture at pH 7), the total lipid content increased as the biomass increased.

There has been little research in Australia to date examining thraustochytrids with regards to biodiesel production. The main aim of the present study was to commence the optimization of biomass yield for recently isolated strains. Future research will continue in this context and will also involve optimization of the fatty acid yield and measurement of the biodiesel yield and quality from these thraustochytrid derived FAME. High biomass producing growth conditions had higher fatty acid and DHA yields and therefore would be a good cultivation strategy for potential commercial production. The fatty acid profiles in our different trials showed markedly similar results (Table 5-2), which demonstrates that *Aurantiochytrium* sp. strain TC 20 is generally robust and, under the growth conditions employed, not overly influenced by changes in feed supplements.

Table 5-2 Fatty acid profiles (as either % of total FA or mg/g biomass) of *Aurantiochytrium* sp. strain TC 20 (CS number: CS-980, GenBank accession numbers: JN675267) at 69 h of fermentation in different fed-batch treatments of experiment 2: (i) glucose (Glu); (ii) glycerol (Gly) and (iii) glycerol and nutrients (Gly/ Nutr)

	Carbon source		
FA	Glu	Gly	Gly/ Nutr
Percentage composition			
14:0	1.1	0.9	1.5
15:0	0.1	0.1	0.2
16:0	50.3	45.2	51.7
18:0	1.4	1.3	1.0
20:4ω6 AA	0.6	0.6	0.5
20:5ω3 EPA	0.4	0.4	0.4
20:4ω3	0.4	0.4	0.4
22:5ω6 DPA-6	0.9	0.9	4.2
22:6ω3 DHA	43.2	48.4	38.9
22:4ω6	0.4	0.5	0.4
Other FA[a]	0.4	0.4	0
SUM SFA	53.0	47.6	54.4
SUM MUFA	0.5	0.7	0.5
SUM PUFA	46.3	51.7	45.0
ω3/ω6	21.6	24.8	7.7
Total FAME (% of DCW)	33.8	33.0	52.2
Biomass (DCW g/L)	56.62	55.9	70.81
mg FAME / g DCW			
Total FAME (mg/g)	337.6	330.2	521.7
16:0	169.8	149.3	269.9
20:4ω6 AA	2.2	1.9	2.9
20:5ω3 EPA	1.2	1.3	1.9
22:5ω6 DPA-6	3.1	2.9	22.1
22:6ω3 DHA	145.9	159.7	203.1

[a]Others: 20:3ω6, 22:5ω3 DPA (3), C_{22}PUFA, 22:1ω11c and 22:0

Table 5-3 Lipid class composition (as % of total lipids) of *Aurantiochytrium* sp. strain TC 20 (Gly/ Nutr) at 69 h of fermentation with glycerol as the carbon source. Composition determined using thin layer chromatography – flame ionisation detection

Lipid class	% Composition	Standard error (n = 2)
Hydrocarbon	1	0.3
Triacylglycerol (TAG)	93	11.1
Free fatty acid	1	0.1
Diacylglycerol (DAG)	1	0.1
Phospholipid (PL)	3	0.8

The simple fatty acid profile of this strain, having both saturated fatty acids (45 – 52 % 16:0) and omega-3 LC-PUFA (39 – 48 % 22:6ω3 DHA) as major constituents (Table 5-2), makes it potentially a very good candidate to co-produce both omega-3 LC-PUFA and biodiesel. The relative amounts of omega-3 LC-PUFA would be enhanced after removal of the biodiesel fraction. Furthermore, the lipid class profile of *Aurantiochytrium* sp. strain TC 20 contained 93 % triacylglycerol and 3 % phospholipid (Table 5-3), and a simple winterization could be used for the separation of omega-3 LC-PUFA and biodiesel fractions due to the differential melting points of these fatty acids resulting from differences in chain length and degree of unsaturation (Guil-Guerrero et al. 2007).

In conclusion, the *Aurantiochytrium* sp. strain TC 20 isolated from Australian waters can utilize glycerol as a carbon source for the potential co-production of a feedstock for biofuels and omega-3 LC-PUFA, in particular DHA, rich lipids. The biomass yields are comparable to those obtained with glucose, with an improved DHA yield achieved. Fed-batch cultivation with additional carbon source and nutrients achieved enhanced biomass and lipid yield relative to additional carbon source alone. Future research will be required to further enhance biomass and oil yields and to explore methods to separate the high value LC-PUFA (for nutraceuticals) from the SFA and MUFA for biodiesel production. The role of individual nutrients will be examined in chemically defined media to better identify the essential ingredients required for further increasing and optimizing biomass and oil yield of *Aurantiochytrium* sp. strain TC 20.

Chapter 6 Life-cycle assessment: Heterotrophic cultivation of thraustochytrids for biodiesel production

Abstract

This study provides a life cycle assessment of the energy balance and the potential environmental impacts of heterotrophic microalgae derived biodiesel estimated from the upstream biomass production to the downstream emissions from biodiesel combustion. Heterotrophic microalgae can be cultivated using a waste product from biodiesel production such as glycerol as a carbon source. The oils within the algal biomass can then be converted to biodiesel using transesterification or hydroprocessing techniques. This may provide a solution to the limited availability of biomass feedstock for production of bio-refined transportation fuels. The life cycle assessment of a virtual production facility, modeled on experimental yield data, has demonstrated that cultivation of heterotrophic microalgae for the production of biodiesel is comparable, in terms of greenhouse gas emissions and energy usage (90 g CO2e/MJ), to fossil diesel (85 g CO2e/MJ). The life cycle assessment identified that improvement in cultivation conditions, in particular the bioreactor energy inputs and microalgae yield, will be critical in developing a sustainable production system. Our research shows the potential of heterotrophic microalgae to provide Australia's transportation fleet with a secure, environmentally sustainable alternative fuel.

Introduction

The use of microalgae as alternative sources of energy has been explored since the 1950s and the energy shocks in the 1970s prompted widespread interest in microalgal biofuels, such as hydrogen production by microalgae and methane production from waste water treatment (Benemann 2000; Levin et al. 2004; Esper et al. 2006). From 1978 – 1996 the U.S. Department of Energy funded a program known as the Aquatic Species Program to develop biodiesel from algae. The program focused on the production of biodiesel from high lipid-content microalgae grown in ponds, utilizing waste CO_2 from coal-fired power plants (Sheehan et al. 1998b). However, the program was discontinued due to federal budget cutbacks and decreasing oil prices. These studies demonstrated the promise of the technology, but the commercial success of large-scale production has been elusive. The renewed interest and demand in microalgae-derived biodiesel is now increasing globally due to growing concern with depletion of fossil fuels and anthropogenic climate change. These two factors are driving development of an economical and environmentally sustainable, low greenhouse gas emission transportation fuel.

In addition to producing lipids for conversion to biodiesel, microalgae are capable of producing high-value bioproducts, such as carotenoid pigments, industrial enzymes, omega-3 polyunsaturated fatty acids (PUFA), and exopolysaccharides (EPS). Other low value byproducts – such as algal meal – may be of use in animal and fish feeds or for fermentation as energy and nutrient sources, adding greater value to the production process pipeline with improved process economics (Huntley and Redalje 2007; US DOE 2010a; Li et al. 2008; Stephens et al. 2010; Wijffels and Barbosa 2010).

Numerous research and start-up companies have shown that heterotrophic cultivation could result in higher production of biomass and accumulation of high oil content in cells compared to that achieved using photoautotrophic cultivation (Brennan and Owende 2010; Liang et al. 2009). The majority of photoautotrophic systems for production of biofuels are depending on photosynthesis of phototrophic microalgae in outdoor cultivation using CO_2 and sunlight as carbon and energy sources respectively. However, phototrophic cultivation provides low biomass dry weight yield per litre of cultivation medium, with values of 25 $g/m^2/d$ frequently cited (Davis et al. 2012). This means of cultivation, with low cell concentrations achieved, significantly increases processing cost (i.e. harvesting, dewatering and oil extraction) and thus represents a significant economic barrier if the system is designed to produce only low-value commodity oil.

Alternatively, some microalgae can grow heterotrophically in stainless steel fermenters on organic substances (e.g. sugars, organic acids) as the only carbon and energy source. Yan et al. (2011) demonstrated that heterotrophic cultivation of the green alga *Chlorella protothecoides* has the potential to provide significantly higher biomass yields, and established an equivalent bio-oil yield of 8.3 ml per cultivation litre per day. Solazyme, Inc. (South San Francisco, USA) has reported using heterotrophic microalgae to produce more than 10,000 gallons of oil at a quality that meets existing fuel standards (Peterka 2013). The company have successfully partnered with the US Navy to produce commercial quantities of algal fuel. Solazyme's fuel feedstock has already been demonstrated and approved as a commercial aviation fuel blend. In addition, Solazyme is producing oil for a range of higher value applications such as – chemicals, nutritional, cosmetic and personal care products.

Furthermore, the limited biomass feedstock availability is expected to restrict industry uptake of bio-refined transportation fuel (Rye et al. 2010). Heterotrophic cultivation of another microalgal group – the thraustochytrids - using waste product from biodiesel production such as glycerol as a carbon source can be used for biomass production. The oils in this biomass feedstock can then be converted to biodiesel using transesterification to produce fatty acid methyl esters (FAME) or hydroprocessing to produce hydrocarbons from the oils, and may provide a potential solution.

Thraustochytrids are heterotrophic protists found ubiquitously in the marine environment and play an important role in marine ecosystems; they can be bacterivores, detritivores or parasites (Maas et al. 1999; Raghukumar 2002). However molecular phylogenetic studies have resulted in their classification into the class Labyrinthulomycota and phylum Heterokonta within the kingdom Chromista. This phylum also includes the chromophyte algae such as brown algae and diatoms (Cavalier-Smith et al. 1994; Leander et al. 2004; Porter 1990). They are known to produce high amounts (> 30% of total fatty acids) of omega-3 LC-PUFA (long-chain polyunsaturated fatty acids), including docosahexaenoic acid (DHA, 22:6w3) and eicosapentaenoic acid (EPA, 20:5w3) (Jain et al. 2007; Lewis et al. 1999). Numerous studies have shown dietary consumption of omega-3 LC-PUFA, in particular EPA and DHA, helps reduce the risk of cardiovascular diseases, neural disorders, arthritis, asthma and skin diseases in humans (Loef and Walach 2013; Smith et al. 2011; Danaei et al. 2009; Mozaffarian and Rimm 2006). DHA is also essential for neural and retinal development during fetal life and infancy (Forsyth and Carlson 2001; Ratledge 2004).

In a previous study, we demonstrated that recently isolated endemic Australian thraustochytrid strains were promising candidates for production of biodiesel, omega-3 long-chain ($\geq C_{20}$) PUFA (LC-PUFA), and bio-products such as carotenoid pigments, phytosterols, exopolysaccharides and odd-chain length fatty acids (Lee Chang et al. 2013; Lee Chang et al. 2012; Lee Chang et al. 2011). The fatty acid profile of the species examined contained high levels of saturated fatty acid (SFA), mainly palmitic acid (16:0, up to 52 % TFA), and LC-PUFA was mostly docosahexaenoic acid (DHA, up to 39 % TFA) with low levels of docosapentaenoic acid (DPA-6, up to 4.2 %) (Lee Chang et al. 2013). The degree of unsaturation (number of double bonds) of the fatty acids is important with respect to its suitability for biodiesel production. Fatty acids with fewer double bonds are more stable to oxidation compared to the PUFA. Oxidation results in the formation of undesirable products, such as alcohols that reduce the flash point of biodiesel, aldehydes that cause rancidity, and short-chain ($\geq C_{12}$) fatty acids that are corrosive to engine components (Knothe 2007; Monyem et al. 2000). Furthermore, SFA typically have higher melting point and cannot be used at lower temperatures. Therefore high levels of SFA and monounsaturated FA (MUFA) are more desirable for biodiesel production due to the increased oxidative stability as well as the thermal stability of biodiesel. Due to their high oil productivity, thraustochytrids have the potential for production of both omega-3 LC-PUFA rich oils as well as the shorter-chain, less unsaturated fatty acids suitable for biodiesel (Johnson and Wen 2009).

The rise in biodiesel production from vegetable oils and animal fats has resulted in a surplus of low cost glycerol internationally with crude glycerol prices dropping from $0.25/lb in 2004 to $0.025-0.05/lb in 2006 (Johnson and Taconi 2007; Pyle et al. 2008). The use of a glycerol stream as a carbon source for a heterotrophic algal production system not only could potentially reduce the

greenhouse gas emissions, but also substantially reduce the cost of commercial production of algal-derived biodiesel. The key objective of this study was to investigate the potential of heterotrophic cultivation of thraustochytrids using glycerol to produce bio-oil through a preliminary life-cycle assessment (LCA).

Materials and methods

LCA software

Life cycle assessment (LCA) was performed using SimaPro 7, which is an open structure program that can be used for different types of LCAs. The upstream production (heterotrophic cultivation) and use (processing) stages and the downstream end-of-life stage (combustion emissions) can be specified in as much detail as necessary by selecting processes from the database and by building process trees that can be drawn automatically by the program. The results are presented in graphs, varying from a list of substances (inputs and outputs), characterised scores, normalised scores or evaluated scores. The program visual output provides for easy identification of processes that have a high impact to the overall life cycle.

Life cycle assessment

LCA involves taking into account both upstream and downstream emissions. In the context of biodiesel, this includes not only the combustion emissions from vehicles (referred to as downstream emissions), but also those associated with the overall production of the biofuel: extraction, transportation, processing, conversion and distribution (referred to as upstream emissions) (Grant et al. 2008). The lack of algae-to-biofuel processes operating at scale, coupled with the commercial sensitivities of the sector, required the development of a hypothetical production scenario. This scenario was developed and later populated using information from the literature as well as results from

engineering correlations. The culture of heterotrophic thraustochytrids and subsequent processing and conversion into the biodiesel blend stock is shown in Figure 6-1.

The term "well to wheel emissions" is also used to describe the full fuel LCA. The emissions related to vehicle manufacture, maintenance and disposal, and road building are relevant to total transport emissions, but they are not likely to vary significantly with the nature of the fuel used, and thus these factors are excluded from the LCA (Grant et al. 2008). Apart from analysing the well to wheel emissions profile of a particular transport fuel, LCA is often applied to determine the energy returned on energy invested (ERoEI). The 'invested energy', in a transport fuel context, represents the energy inputs required to produce a unit of fuel. For example, in the case of microalgae derived bio-oils this represents the energy consumed during the growth, harvest, extraction and refining stages (MJ input). The 'energy returned' represents the energy output from combusting the produced unit of fuel (MJ out). Therefore, the amount of energy returned per energy input can be calculated ($ERoEI = MJ_{out} / MJ_{in}$). If the energy input is less than the available combustion energy in the refined fuel, the process is an energy source ($ERoEI > 1$). However, if the $ERoEI < 1$, the process is an energy sink. Importantly, energy cannot be created or destroyed, and as such SimaPro7 includes the embedded energy content of limited fossil fuel resources (i.e. crude oil) as energy inputs.

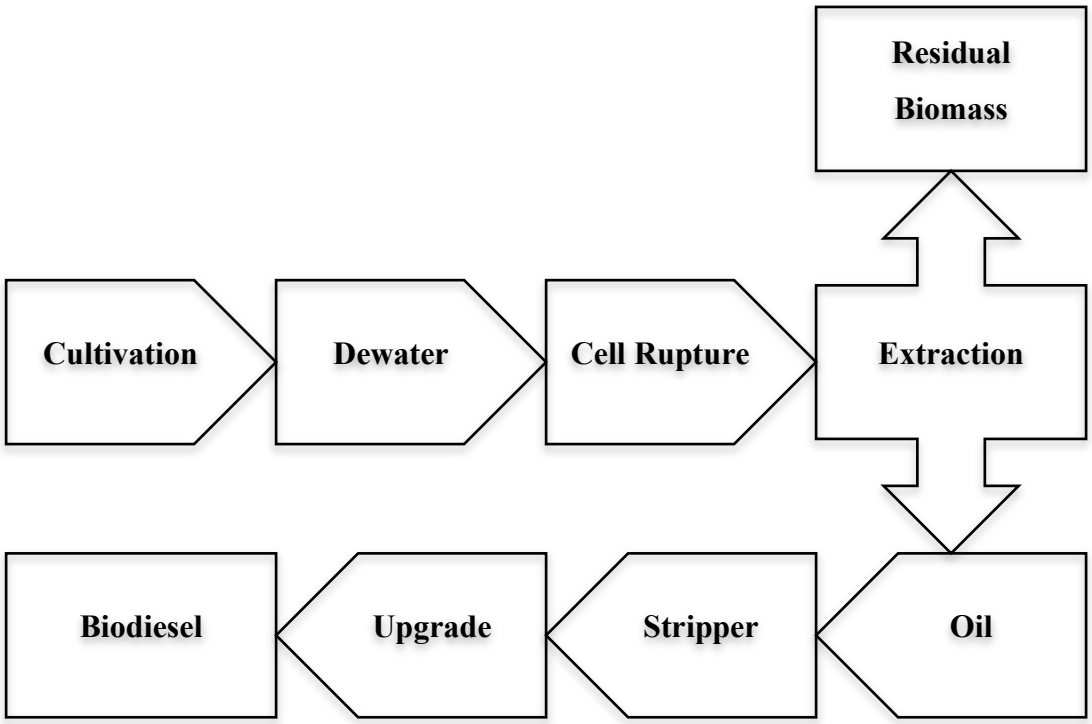


Figure 6-1 Heterotrophic microalgae production process diagram. The upgrade step could be production of biodiesel via either hydroprocessing to produce a hydrocarbon based fuel, or transesterification to produce a fatty acid methyl ester based fuel. The residual biomass could be used elsewhere for example for stock or aquaculture feed or used on-site in an anaerobic digester to produce energy to be fed back into the system

Heterotrophic cultivation

Heterotrophic cultivation of thraustochytrids is assumed to take place using multiple 0.2 ML industrial bioreactors. Cultivation data is based on experimental data from our 2 L lab scale fed-batch bioreactors (Chapter 5). This work reported 71 g/L biomass with FAME content of 52 % (w/w) of dry weight by 69 h using glycerol as a carbon source. Extrapolation of experimental data to the proposed full scale bioreactor was based on maintaining an equivalent aggregator tip speed, and is one of the many scale-up techniques used in industry (Holland and Chapman 1966). Scale-up introduces obvious uncertainty, however, the selection of our experimental yield data (under yet to be be optimized culture conditions) provides for the assessment of a baseline production target. Biomass of thraustochytrids may reach up to 200 g/L (oil content > 50 %) in commercial scale stirred fermentors (100 m^3) (Barclay et al. 2010). For simplicity, only cultivation carbon (e.g. glycerol; assumed to be sourced from Australian biodiesel production from vegetable oil and animal fat) is included in this LCA. The bioreactor operating conditions – scaled up from Lee Chang et al. (2013) (Chapter 4) are shown in Table 6-1. Once the culture reaches sufficient volume (after 69 h), it is transferred for harvesting, oil extraction and subsequent upgrading using transesterification or hydroprocessing techniques (Fig. 6-1).

Table 6-1 Bioreactor inputs (operating conditions) and outputs (microalgal culture) per 0.20 ML reactor batch, used for the LCA (glycerol and outputs scaled up from data in Lee Chang et al., 2013)

Parameter	Value	Unit	Comments
Inputs			
Glycerol	34400	kg	Each unit of algae requires 2.42 units of sugar
Water	0.17	ML	Culture water
Steam	1912	MJ	Bioreactor sterilisation
Heat	3464	MJ	Culture thermal control
Water pump	7	kWh	Bioreactor fill electricity demand
Sparger	836	kWh	Blower electricity demand
Impeller	867	kWh	Electricity demand (equivalent RPM tip speed)
Outputs			
Culture	0.20	ML	Wet microalgae and media

Processing

Harvesting/dewatering is assumed to take place using a centrifuge. This stage removes 30% of the cultivation water. The small size of the algal cells and presence of a cell wall, suggests efficient oil extraction may benefit from the rupture of the microalgal cells prior to oil extraction. This is assumed to be achieved using a high-pressure industrial homogenizer as has been shown to be an effective technique to rupture Nannochloris oculata cell walls (Samarasinghe et al. 2012). Further research is needed to validate whether the cell rupture step may be omitted and which direct solvent extraction method could improve oil extraction from thraustochytrid cells at a commercial scale. In the LCA the lysed cell solution is then mixed with solvent (hexane) in a mixer/settler to extract the oils. A stripper is used to recover the hexane solvent from the oil-solvent solution, producing a crude algal bio-oil, which may subsequently be upgraded into a transportation fuel. The process requirements to produce crude algal bio-oil, as shown in Figure 6-1, are summarised in Table 6-2. For this LCA, the crude algal bio-oil is upgraded into renewable biodiesel fuel using the transesterification or the petrochemical hydroprocessing techniques. The transesterification process reacts the bio-oil with alcohol to produce biodiesel consisting of fatty acid methyl esters (FAME) and glycerol (Figure 6-2). However, due to the high levels of LC-PUFA, not all of the FAME are suitable for direct use as biodiesel. For the purpose of this LCA, the thraustochytrid was assumed to be a low LC-PUFA strain (Fisher et al. 2007). However if a high LC-PUFA strain was used, the separation of the FAME suitable of biodiesel and the LC-PUFA fraction would require an additional processing step such as the relatively mature methods of winterisation and urea complexation etc. (Mendes et al. 2007) but would of course reduce yields of biodiesel. In comparison the hydroprocessing technique removes oxygen, nitrogen and other heteroatoms, producing a

hydrocarbon based hydroprocessed biodiesel. This hydroprocessed biodiesel contains no aromatics and thus the product must be blended with conventional diesel to satisfy certification requirements. Input data for the hydroprocessing requirement in this LCA has been adapted from Stratton et al. (2011). After bio-oil extraction, the residual oil-free biomass stream may be processed further for energy or sugar recovery. In the examples presented the residual oil-free biomass is fed into an anaerobic digester (Oswald and Golueke 1960), where the gases produced are combusted and the energy yielded is fed back into the process to reduce processing costs and help reduce total upstream emissions.

Table 6-2 Process inputs (operational parameters) and outputs (products) per 0.20 ML reactor batch, used for the LCA

Parameter	Value	Unit	Notes
Inputs			
Culture volume	0.20	ML	Wet microalgae and media (from Table 1)
Centrifuge	240	kWh	Dewatering electricity (evodos.eu)
Homogenizer	967	kWh	Cell rupture electricity (niro-soavi.com)
Mixer settler	135	kWh	Oil extraction electricity
Stripper	6941	MJ	Solvent recovery heat (natural gas)
Hexane	1338	kg	Make-up hexane required
Outputs			
Bio-oil	6313	kg	Recovered crude microalgae lipids
Biomass	7177	kg	Recovered dry biomass equivalent after bio-oil extraction

Combustion emissions

To evaluate the total lifecycle footprint of biodiesel derived from thraustochytrids, the combustion emissions must also be considered. Combustion emission data are available in the literature and from engine certification testing (Dobes 1994; Penner et al. 1999; Wahlen et al. 2013). Importantly, although these data are based on conventional fossil fuel, alternative products must be compositionally similar to biodiesel to satisfy the certification requirements. Previous studies have shown variation of gaseous exhaust emissions from microalgal derived biodiesels (Knothe et al. 2006; Wahlen et al. 2013). Thus fossil diesel emission data are used in this LCA, however, a correction is applied to take into account biogenic CO_2.

Under carbon accounting practices, CO_2 emitted during the combustion of a biofuel – entitled biogenic CO_2 – is assumed to be equal to that which was absorbed during photosynthetic growth. Heterotrophic algae utilize organic carbon sources (in this case glycerol) and do not absorb atmospheric CO_2. Nevertheless, the cultivation of plants from which the organic carbon source is obtained, absorbed atmospheric CO_2 to produce it. Therefore the associated combustion emissions are considered biogenic.

Combustion produces gaseous (i.e. carbon dioxide, methane, nitrous oxide, etc) and particulate matter (volatile and non-volatile organics) emissions that must be considered when evaluating the biofuels lifecycle. Total emissions are accounted through a CO_2 equivalent (CO_2e) value based on their global warming potential (GWP) as defined in the Kyoto Protocol. A breakdown of selected exhaust data is shown in Table 6-3; these data are adapted from the CSIRO Simapro database.

The LCA includes electricity sourced from coal, natural gas and related upstream processes (i.e. gas extraction) when calculating the LCA impact. Capital equipment and infrastructure are rarely included when analysing the footprint of the fossil fuel industry, and as such they are excluded from this LCA.

In this study, the LCA impact of refining crude bio-oil to produce diesel is based on energy content allocation, as modeled in the Australasian Unit Process LCI (life cycle inventory) SimaPro7.

Table 6-3 Different greenhouse gases and their global warming potentials (GWP) relative to carbon dioxide (with a 100 year time horizon)

Greenhouse Gas	GWP
Carbon dioxide (CO_2)	1
Methane (CH_4)	21
Nitrous oxide (N_2O)	310

Results and Discussion

Total greenhouse gas impact and energy intensity

Combining the upstream (heterotrophic cultivation and processing stages, see Materials and Method section) and downstream use (combustion emissions) stage provide the total greenhouse gas impact and energy intensity of the heterotrophic microalgae production system (Table 6-4). Emissions data are reported per MJ of product produced (e.g. g CO_2e/MJ) from the batch cultivation system, with the energy intensity representing the ratio of energy output over input (e.g. ERoEI; MJ/MJ). The lifecycle impact of fossil diesel is included for comparison.

Table 6-4. Hydroprocessed renewable diesel (HRD) and Biodiesel emissions (g CO_2e per MJ) and energy returned on energy invested (ERoEI)

Process	Hydroprocessed biodiesel (Glycerol)	Hydroprocessed biodiesel (Molasses)	FAME biodiesel (Glycerol)	Fossil diesel
Upstream (g CO_2e)	71.0	16.5	89.4	15.4
Direct (g CO_2e)	0.53	0.53	0.53	69.7
Lifecycle (g CO_2e)	71.5	17.0	89.9	85.1
Energy in (MJ/kg)	89.3	32.4	87.8	52.0
Energy out (MJ/kg)	43.8	43.8	38.0	43.2
ERoEI (MJ/MJ)	0.49	1.35	0.43	0.83*

* data from Sheehan et al. (1998a)

The results show that production of biodiesel using heterotrophic algae via hydroprocessing (71.5 g CO_2e/MJ) had a superior greenhouse gas footprint for the whole of lifecycle compared with fossil diesel (85.1 g CO_2e/MJ) (Table 6-4). The greenhouse gas footprint of transesterified heterotrophic algal-derived FAME biodiesel (89.9 g CO_2e/MJ) was comparable to fossil diesel. The differences between the algal-derived hydroprocessed and FAME biodiesels were predonminatly due to the different energy densities of the hydrocarbons and FAME of the biodiesels produced (Energy out; Table 6-4).

The elevated upstream emissions from heterotrophic algal-derived hydroprocessed and FAME biodiesels, were due mostly to the upstream impact of the heterotrophic cultivation stage. Specifically these were the glycerol carbon source (61.0 g CO_2e/MJ for hydroprocessed biodiesel and 66.8 g CO_2e/MJ for FAME biodiesel) and the electricity demand of impeller (3.8 g CO_2e/MJ) and air sparging motor (3.6 g CO_2e/MJ) from a mostly fossil based grid (coal). A major impact was also from the hexane used in the solvent extraction during the processing stage (21.8 g CO_2e/MJ for hydroprocessed biodiesel and 24.2 g CO_2e/MJ for FAME biodiesel).

A breakdown of emissions contributing to 3.5% or more of the total impact for FAME biodiesel production is shown in Figure 6-2. The cumulative emissions impact is represented by the thermometer bars shown on each process block. The CO_2e input from the production of glycerol and hexane were quite high (3.79 and 0.41 kg CO_2e/kg of input respectively) and large amounts were used in the production process (33817 and 1338 kg respectively) resulting in the high contributions from these sources (Figure 6-2).

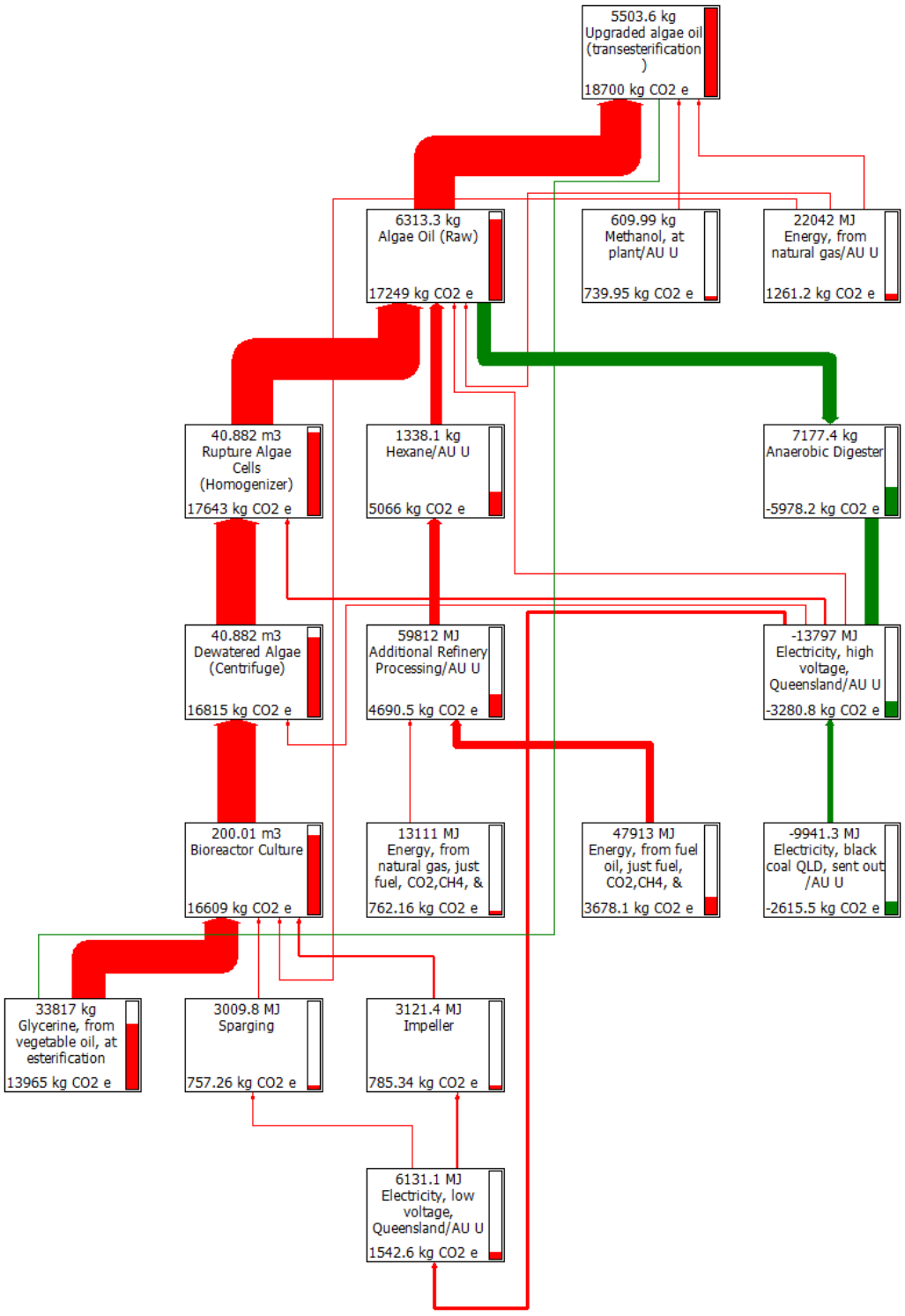


Figure 6-2 LCA of biodiesel derived from heterotrophic microalgae production followed by extraction and transesterification and energy yields resulting from oil-free biomass back to anaerobic digestion

Even though these upstream inputs for the algal derived biodiesel (especially from glycerol) are high, the total CO_2 emitted for the whole of lifecycle is low because the downstream CO_2 emitted is significantly reduced (Table 6-4) due to taking into account the biogenic CO_2 from which the glycerol was obtained, namely the by-product from transesterification in the production of biodiesel from terrestrial plants.

Other carbon sources derived from agro-industrial wastes (such as waste molasses, empty palm fruit bunches, etc) have been explored recently for producing high oil content containing biomass from thraustochytrids (Yan et al. 2011; Hong et al. 2012). However, to date biomass yields have been considerably lower than obtained using glycerol (Lee Chang et al. 2013). For comparison, substituting molasses as the carbon source for the heterotrophic cultivation stage and incorporating this process into the scenario reduces total upstream emissions to 16.5 g CO_2e/MJ (Table 6-4). This is a 77 - 81% reduction of total upstream emissions, and highlights the importance of optimising the culture conditions, including choice of processing technologies and major ingredients, at scale.

The energy return on energy invested (ERoEI) is 0.5 for the production of biodiesel from heterotrophic cultivation of thraustochytrids (Table 6-4). An ERoEI of less than one is an energy sink, as we need twice the energy to produce one unit of energy for use. This is mainly due to costs of running the impeller, centrifugation electricity consumption and also the carbon source with the high process footprint as discussed. The value of ERoEI reported for fossil diesel is 0.8 according to Sheehan et al. (1998a). It is worth noting that when replacing the glycerol with molasses as the carbon source, the ERoEI improved to 1.4.

Identification of other low cost carbon sources such as a lignocellulosic biomass, as well as an appropriate process to convert this feed into carbohydrates suitable for heterotrophic growth, could also be critical in both the economic and sustainable scale-up of this technology.

Use of residual oil-free biomass after extraction of the bio-oil may also improve the process economics through the generation of an additional income stream (e.g. stock feed sales) or reduce operating expenditure through energy or material recovery. In the current study this oil-free biomass was used as an energy source, reducing the energy required by 11.9 MJ/kg. Alternatively, it may be possible to extract the starch (carbohydrates) from the residual microalgae biomass and, using enzymes, convert the biomass into a carbon source for subsequent culture batches.

Conclusion

The LCA, modelled on baseline literature yield data, has demonstrated that cultivation of heterotrophic microalgae for the production of biodiesel stock is economically viable and comparable, in terms of greenhouse gas emissions and energy usage, to fossil diesel. The LCA identified that improvement in cultivation conditions, in particular the bioreactor energy inputs and algae yield, will be critical in developing a sustainable production system. The literature reports that the required yields are close to being demonstrated in the laboratory; however, even if the laboratory yields translate to an industrial scale, the identification of a suitably cheap and readily available sugar or other carbon source, such as glycerol, is critical. The cultivation of high carbon crops such as sugarcane for heterotrophic microalgae requires more land than growing palm oil.

To generate commercial interest in the heterotrophic cultivation of thraustochytrids for bio-oil for use in biodiesel production together with other co-products, there is need for additional research for the optimization of growth conditions. This will need to include industrial scale research, to allow further assessment of the commercial feasibility and to remodel the LCA with a more current glycerol processing footprint. To date, the authors are not aware of any commercial operation producing biodiesel using heterotrophic thraustochytrids at a cost comparable to conventional diesel. Therefore, although our study has focused on limited cultivation stage costs, further research is required to improve the understanding of both the upstream and downstream process economics.

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Chapter 7 Conclusions and future perspectives

Introduction

The overall objective of this project was to investigate microalgal strains, especially thraustochytrids, from Australian waters for their capacity to co-produce biofuels, omega-3 oils and other co-products as well as biomass for food, feed and fuel applications. The approaches used to address this objective included: (i) targeted biodiscovery for high oil producing thraustochytrids from habitats such as fresh, brackish and marine waters; and (ii) to explore the potential of newly isolated endemic Australian thraustochytrid strains for production of microalgal derived biofuel and co-production of other bioproducts by evaluating their growth and lipid profiles.

The study first focused on the biodiscovery process that included sampling, isolation and characterizing new thraustochytrid strains. Secondly, the potential of thraustochytrids for production of biodiesel, omega-3 oils and exopolysaccharides (EPS) was investigated in batch culture conditions. This was conducted by examining the growth rate, biomass, lipid and EPS production of different strains in 1 L scale baffled shake flasks. Following these flask trials, the best performing strain was subsequently optimised and scaled-up by manipulating growth phase and culture conditions in 2 L bioreactors. Finally, a life-cycle analysis was undertaken to understand the energy and economic implications with a view to address whether heterotrophic cultivation of endemic thraustochytrids can provide Australia's transportation fleet with a secure, environmentally sustainable alternative fuel feedstock.

Biodiscovery

A total of 36 thraustochytrid strains were isolated from silt, mud and organic rich sediments in both temperate (southeast coast of Tasmania) and tropical environments (far north Queensland). These strains were categorized using chemical and molecular criteria (Chapter 2). The carotenoid pigments β,β-carotene, canthaxanthin and astaxanthin were detected in orange and cream colored strains. Fatty acid and sterol composition were used to separate the strains into eight chemotaxonomic groups (A – H). Furthermore, phylogenetic analysis of 18S rDNA molecular data showed that the strains clustered closely with four different genera (*Aurantiochytrium*, *Schizochytrium*, *Thraustochytrium* and *Ulkenia*) of known thraustochytrid biodiversity.

There was significant correlation between the phylogenetic and chemotaxonomic groupings of the Australian thraustochytrid strains (based on fatty acid and sterol profiles of 7-day old cultures grown in basal medium, $P < 0.0001$, $R = 0.596$). This finding is in accordance with observations by one previous Australian study where correlation was observed between the groups described by fatty acid and 18S rDNA data (Lewis 2001). Comparison of thraustochytrid chemotaxonomic profiles with their 18S rRNA gene sequence data has demonstrated that such chemotaxonomic methods have the potential to be useful tools in the interpretation of thraustochytrid phylogenetic relationships.

Odd-chain polyunsaturated fatty acids

The structure of unusual odd-chain (OC)-PUFA was observed only in strains of chemotaxonomic groups A, B and C, thus further distinguishing these three groups from the other chemotaxonomic groups (Chapter 3). To my knowledge this is the first report that demonstrates the occurrence of these OC-PUFA, 19:4ω5, 21:4ω5, 21:5ω5 and 21:4ω7, in thraustochytrids.

These OC-PUFA were only detected in un-optimized basal medium (complex medium with low carbon and nitrogen nutrient) that was used in the initial isolation and screening study. The precursor of these long-chain OC-PUFA, propionate, may be derived from amino acids of the complex medium components such as yeast extract, peptone and corn steep liquor. This observation suggests that the biosynthesis of OC-PUFA may be dependent on substrate availability and culture conditions.

Shirasaka et al. (2005) showed that the addition of cyanocobalamin, which is a form of synthetic vitamin B_{12} with cyanide attached to the cobalt atom, decreased the content of shorter OC FA, such as pentadecanoic acid (15:0) and heptadecanoic acid (17:0) in thraustochytrids. The mechanism for upregulation of this was the cobalamin-dependent methylmalonyl-CoA mutase that converts propionic acid to succinic acid, thereby decreasing the availability of the OC precursor. The authors also demonstrated that inhibitors can alter the biosynthetic pathway of fatty acids in *Aurantiochytrium limacinum* SR21 and lead to production of unusual fatty acids such as 20:4ω7 and 22:4ω9 (Shirasaka et al. 2005). Further studies involving supplementation with radio labeled precursors would help to understand the PUFA biosynthetic pathway in thraustochytrids. It is also of interest to investigate the possible bioactivity of these OC-PUFA to determine their suitability for novel applications and commercialization.

One litre batch culture trials

The biomass yield, fatty acid profiles and EPS yields of one representative strain from each of the eight different chemotaxonomic groups (A – H) were evaluated in 1 L baffled shake flasks under batch culture conditions (Chapter 4). These trials initially focused on identifying the suitable carbon substrates for a strain before manipulating the growth conditions and media, such as the carbon to nitrogen ratio; this is vital before undertaking further trials to optimize biomass and lipid production.

The study demonstrated that *Aurantiochytrium* sp. strains could utilize glucose as a carbon source better than *Schizochytrium*, *Ulkenia* and *Thraustochytrium* spp. strains. Other researchers have demonstrated that the *Ulkenia* and *Thraustochytrium* spp. strains prefer starch as a carbon source (Quilodrán et al. 2010). The high biomass producing *Aurantiochytrium* sp. strain, TC 20 of group F, with 18.5 g/L at day 9 in 4 % w/v glucose, had high lipid (403 mg/g of total FAME, 39.8 % of 16:0, 46.7 % of DHA) and EPS (179 mg/L at day 7 in 2 % w/v glucose medium) yields, and therefore is potentially a good candidate for commercial production of biofuels and other bioproducts.

Optimisation in two litre fed-batch fermentation trials in bioreactors

The first part of this study comprised screening thraustochytrids strains in 50 mL polystyrene cell culture flasks containing basal medium. Small culture flasks offered a rapid and simple method for characterising chemotaxonomic relationships, and identifying potentially productive strains and the presence of co-products. However, a drawback of using small culture flasks is the inadequacy of agitation and consequently limited aeration. The 1 L baffled flasks enable more detailed analysis (due to larger sample volume and biomass) and direct comparisons on key strains from different chemotaxonomic groups. Furthermore, larger baffled flasks allow more agitation and improved aeration

with better oxygen supply in culture medium. However, cultivation using bioreactors with optimised culture conditions and full control of growth parameters, such as dissolved oxygen, stirrer speed, pH and air flow rate, is much more productive than flask culture and provides a much more accurate assessment of production potential.

Two litre bioreactors were used to further investigate the growth of *Aurantiochytrium* sp. TC 20 using glycerol as a carbon source. Glycerol is becoming increasingly available, because with the rise in biodiesel production globally; it is a by-product of biodiesel production from vegetable oils and animal fats (Johnson and Taconi 2007). Therefore the use of a glycerol stream as a feedstock is a demonstration of the potential of development of sustainable, low emission sources of transportation fuels and bioproducts

The initial trial with *Aurantiochytrium* sp. TC 20 gave a biomass of 56 g/L (34 % total fatty acids) and this was markedly improved by fortification of the feed with additional nutrients to reach 71 g/L (52 % total fatty acids, cell dry weight) at 69 h.

Life-cycle assessment

A preliminary LCA, modeled on experimental yield data, was performed. The analysis demonstrated that cultivation of heterotrophic microalgae for the production of biodiesel is comparable in terms of greenhouse gas emissions and energy usage to the production of diesel from crude oil. The analysis identified that improvement in cultivation conditions, in particularly the bioreactor energy inputs and algae yield, will be critical in developing a sustainable production system. Further assessment of the commercial feasibility of thraustochytrids derived biodiesel will require larger scale (500 L) cultivation and repetition of the LCA with a more current glycerol processing footprint.

Fatty acid profiles

The relative proportion of the majority of fatty acids of thraustochytrids strains was a stable characteristic. The profiles remained very similar when the strains were grown in different media compared to the screening data for these strains (in basal medium containing 0.2 % w/v glucose), even though different culture media and culture conditions such as different flask sizes and culture volumes were used. *Aurantiochytrium* spp. (Group F) strains had chemotaxonomic characteristics of high DHA (51 % TFA) and 16:0 (23 % TFA), and no OC-PUFA. The simple fatty acid profile of *Aurantiochytrium* sp. strain TC 20 contained 16:0 (22 – 52 % TFA) and DHA (42 – 52 %), while other fatty acids accounted for less than 10 % of TFA. The profile was observed in *Aurantiochytrium* sp. TC 20 grown in different media and conditions throughout the study and is summarized in Table 7-1. The fatty acid profile in 2 L bioreactors trials showed markedly similar results, with 16:0 ranging from 45 – 52 % TFA and DHA from 39 – 48 %, which further demonstrated that the fatty acid profile of this strain is robust and was not influenced greatly by changes of media or culture conditions.

There was a decline in the proportions of fatty acids such as 15:0 (from 6.2 % to 0.1 %), EPA (1.9 % to 0.1 %), DPA-6 (9.2 % to 1 %), and DPA-3 (1.2 % to 0.1 %) from the initial screening trial compared to those observed in the optimization trials with high carbon and nutrients media. This may be explained by the differences in medium constituents that inhibit fatty acid desaturation, thus causing changes in fatty acid profiles, as suggested by Shirasaka et al. (2005). Alternatively these fatty acids may be predominantly membrane bound. In samples with high oil content (e.g. the optimization trials), the membrane bound fatty acids represent a smaller proportion of the total fatty acids.

Table 7-1Fatty acid profiles (as percentage of total fatty acids) of *Aurantiochytrium* sp. strain TC20 (Group F) from three different experiments – (i) Initial screening, (ii) Flask optimization, and (iii) Scale up in bioreactors

Trials	Screening	Optimising	Scaling-up			
Volume	50 mL Flask	1 L Flask	2 L Bioreactor			
Agitation (rpm)	100	200	300 -1150 rpm			
Time	7 d	9 d	69 h			
Medium (carbon source % w/v)	0.2% Glu [a]	4% Glu [b]	10% Glu [c]	4% Glu [d]	4% Gly [e]	4% Gly/ Nutr [f]
14:0	1.9	2.9	1.2	1.1	0.9	1.5
15:0	6.2	1.9	0.1	0.1	0.1	0.2
16:0	22.3	39.8	50.6	50.3	45.2	51.7
18:2ω6	0.1	0.1	0.1	0.1	0.1	Tr
18:1ω9	0.2	0.5	0.2	0.2	0.2	Tr
18:0	0.2	0.5	1.4	1.4	1.3	1.0
20:4ω6, AA	1.2	1.2	0.6	0.6	0.6	0.5
20:5ω3,EPA	1.9	0.5	0.3	0.4	0.4	0.4
20:3ω6, DGLA	0.1	0.4	0.1	0.1	0.1	Tr
20:4ω3	0.6	0.3	0.4	0.4	0.4	0.4
20:0	0.1	0.1	0.1	0.1	0.1	Tr
22:5ω6, DPA-6	9.2	2.5	1.1	0.9	0.9	4.2
22:6ω3, DHA	51.7	46.7	42.4	43.2	48.4	38.9
22:4ω6	Tr	0.2	0.2	0.4	0.5	0.4
22:5ω3, DPA-3	1.2	0.4	0.3	Tr	0.1	Tr
Other FA [g]	3.1	1.9	0.9	0.7	0.7	0.8
Total FAME mg/g	78	403	293	338	330	522
DCW g/L	1	12	48	57	56	71

[a] Basal medium: natural seawater, glucose (0.2 % w/v), peptone (0.5 % w/v) and yeast extract (0.2 % w/v)

[b] Flask culture medium: half-strength sea salts, glucose (2 % w/v), peptone (0.2 % w/v), yeast extract (0.2 % w/v), trace metal mix (0.1 % v/v) and vitamin mix (0.1 % v/v)

[c] Bioreactor culture medium consist the same constituent as the flask culture medium, except fed-batch mode supplemented with initially [c] 10 % w/v glucose (Glu), [d] 4 % w/v Glu, or [e] 4 % w/v Glycerol (Gly) with addition of carbon source at around 26- 29 h

[f] 4 % w/v Gly/ Nutr with the addition of 400 mL 66 % w/v glycerol and 100 mL of concentrated nutrients (20 % w/v yeast extract, 40 % w/v MSG, 4 % v/v trace metal mix, 4 % v/v vitamin mix, and 1.2 % w/v $MgSO_4.7H_2O$) at 26 h

[g] Other FA refers to: 17:0, 19:0, 20:0, 21:0, 14:1, 15:1, 20:1ω9, 20:1, 22:1ω9, 16:1ω7, 16:3ω3, 16:2ω3, 17:2, 17:3, 18:2, 18:3ω3, 18:3ω6, 20:2ω6, 20:3ω3, 20:1ω9, 22:2ω6; Tr denotes Trace < 0.01

In addition, the culture conditions such as limited O_2 supply may favor the O_2-independent polyketide synthase (PKS) over the fatty acid synthase pathway in thraustochytrids (Lippmeier et al. 2009; Ren et al. 2010). The higher level of DPA-6 in flask trials (9.2 % TFA in 500 ml flask and 2.5 % TFA in 1 L flask) compared to that in bioreactors (0.9 – 4.2 % TFA in 2 L bioreactors) may be explained by the O_2-independent PKS pathway being more active in the flask cultures (Table 7-1). The higher level of 16:0 observed in bioreactors (in the range of 45 – 52 %) compared to that in the flasks culture (22 % TFA in 50 mL flasks and 40 % TFA in 1 L flasks) might also be explained by enhancement of the fatty acid synthase pathway due to the unlimited O_2 supply in bioreactors. Similar trends were observed for the other chemotaxonomic groups. This observation highlights the relevance of defining the characteristics of different thraustochytrids based on chemotaxonomic groupings. However, the possible variability of the fatty acid profiles of some thraustochytrid groups, such as the occurrence of OC-PUFA in response to different growth substrate and environmental conditions, will also need to be considered if one is trying to optimize particular fatty acids.

Biofuels, omega-3 oils and co-products

This study provides additional insight into the biology of endemic Australian thraustochytrids, biosynthesis of fatty acids, and their potential roles in benthic and pelagic food webs. This research has demonstrated that heterotrophic cultivation of Australian thraustochytrids is a viable option for biodiesel, omega-3 oils and other co-products. While other studies have identified the attributes of fast growth rate and high lipid content in oleaginous microorganisms (Bowles et al. 1999; Hall and Ratledge 1977; Ratledge and Wynn 2002; Burja et al. 2006), this work sheds light on how endemic Australian thraustochytrids could be effectively scaled-up. Growth characterization (including culture conditions and

lipid profiles) has been performed for high oil producing strains that can utilise an inexpensive by-product as a carbon source, such as glycerol. In addition, a life-cycle analysis was undertaken of the potential environmental impacts in terms of energy balance and greenhouse gases emission of this heterotrophic microalgal-derived biodiesel. Methods for assessing the production of omega-3 PUFA and other high-value bioproducts, such as carotenoid pigments and EPS, which can add greater value to the production process and improved process economics were identified and applied.

Numerous research and start-up companies have shown that heterotrophic cultivation could result in higher production of biomass and accumulation of high lipid content in cells compared to photoautotrophic systems (Brennan and Owende 2010; Liang et al. 2009). Solazyme is among the first companies to pursue heterotrophic cultivation of microalgae for commercial biofuel production, with multiple 128,000 L fermenters and an annual oil production capacity in excess 2 million L (Fiakas 2012). In addition, Yan et al. (2011) demonstrated that heterotrophic cultivation of *Chlorella protothecoides* using waste molasses has the potential to provide significantly higher biomass yields, and reported biomass up to 70.9 g/L with 57.6 % oil content in 178 h of cultivation (Yan et al. 2011). The cultivation of *Aurantiochytrium* sp. TC 20 in glycerol resulted in a biomass of 71 g/L (52 % total fatty acids, cell dry weight) at 69 h, which is 2.5 times higher productivity compared to Yan et al. (2011).

The research makes fundamental contributions in the area of transportation energy by providing a potential solution to the limited availability of biomass feedstock for production of bio-refined fuel. The heterotrophic cultivation of thraustochytrids could be considered as part of the national solution to sustainable fuels while also providing other valuable co-products.

Implications for further research

To generate commercial interest in the heterotrophic cultivation of endemic Australian thraustochytrids for both biofuel and high-value co-products, there is a need for additional research for the optimization of growth conditions, including at industrial scale, to allow further assessment of the productivity and diversification of coproducing high-value bioproducts. Exploring the following areas as future research strategies can facilitate the attainment of these goals:

- Develop a chemically defined medium to identify the key nutrients and energy sources required in the putative biosynthetic pathway of lipids.
- Examine the potential for manipulation of lipid products in thraustochytrids through manipulation of the media and culture conditions *such as temperature, salinity, dissolve oxygen and agitation*.
- Evaluate the optimization and reproducibility of biomass and lipid yield of thraustochytrids in commercial scale fermentation.
- Investigate different solvent and other fractionation methods to separate the high value omega-3 oils from the saturated and monounsaturated FA used for biofuel production.
- Examine the potential bioactivity of the EPS and OC-PUFA derived from heterotrophic growth of thraustochytrids to determine their suitability for novel applications and commercialization.

Bibliography

Abad S, Turon X (2012) Valorization of biodiesel derived glycerol as a carbon source to obtain added-value metabolites: Focus on polyunsaturated fatty acids. Biotechnol Adv 30 (3):733-741

Abe E, Hayashi Y, Hama Y, Hayashi M, Inagaki M, Ito M (2006) A novel phosphatidylcholine which contains pentadecanoic acid at sn-1 and docosahexaenoic acid at sn-2 in *Schizochytrium* sp. F26-b. J Biochem (Tokyo) 140 (2):247-253

Ackman RG (1965) Occurrence of odd-numbered fatty acids in the Mullet *Mugil cephalus*. Nature 208 (5016):1213-1214

Armenta R, Valentine M (2013) Single-cell oils as a source of omega-3 fatty Acids: An overview of recent advances. J Am Oil Chem Soc 90 (2):167-182

Bahnweg G (1979a) Studies on the physiology of thraustochytriales: I. Growth and nitrogen nutrition of *Thraustochytrium* spp., *Schizochytrium* sp., *Japonicum* sp., *Ulkenia* spp. and *Labrynthuloides* spp. Veröff Inst Meeresforsc Bremerh 17 (2):245-268

Bahnweg G (1979b) Studies on the physiology of thraustochytriales: II. Carbon nutrition of *Thraustochytrium* spp., *Schizochytrium* sp., *Japonicum* sp., *Ulkenia* spp. and *Labrynthuloides* spp. Veröff Inst Meeresforsc Bremerh 17 (2):269-273

Bajpai P, Bajpai PK, Ward OP (1991a) Production of docosahexaenoic acid by *Thraustochytrium aureum*. Appl Microbiol Biotechnol 35 (6):706-710

Bajpai PK, Bajpai P, Ward OP (1991b) Optimization of production of docosahexaenoic acid (DHA) by *Thraustochytrium aureum* ATCC 34304. J Am Oil Chem Soc 68 (7):509-514

Balk EM, Lichtenstein AH, Chung M, Kupelnick B, Chew P, Lau J (2006) Effects of omega-3 fatty acids on serum markers of cardiovascular disease risk: A systematic review. Atherosclerosis 189 (1):19-30

Banerjee A, Sharma R, Chisti Y, Banerjee UC (2002) *Botryococcus braunii*: A renewable source of hydrocarbons and other chemicals. Crit Rev Biotechnol 22 (3):245-279

Barclay W, Meager K, Abril J (1994a) Heterotrophic production of long chain omega-3 fatty acids utilizing algae and algae-like microorganisms. J Appl Phycol 6 (2):123-129

Barclay W, Weaver C, Metz J, Hansen J (2010) Development of a docosahexaenoic acid production technology using *Schizochytrium*: historical perspective and update. In: Cohen Z, Ratledge C (eds) Single Cell Oils - Microbial and Algal Oils (2nd Edition). AOCS Press, Champaign, IL, US, pp 75–96

Barclay WR, Meager KM, Abril JR (1994b) Heterotrophic production of long chain omega-3 fatty acids utilizing algae and algae-like microorganisms. J Appl Phycol 6 (2):123-129

Benemann JR (2000) Hydrogen production by microalgae. J Appl Phycol 12 (3):291-300

Benemann JR (2008) Opportunities & challenges in algae biofuels production. <http://www.fao.org/uploads/media/algae_positionpaper.pdf>. Accessed 27 Mar 2010

Bitton G, Freihofer V (1977) Influence of extracellular polysaccharides on the toxicity of copper and cadmium toward *Klebsiella aerogenes*. Microb Ecol 4 (2):119-125

Blackburn SI, Frampton DMF, Jameson ID, Brown MR, Mansour MP, Negri AP, Nichols PD, Parker NS, Robert SS, Bolch CJ, Volkman JK (2005) The CSIRO collection of living microalgae: an Australian perspective on microalgal biodiversity and applications. In: Kasai F, Kaya K, Watanabe MM (eds) Algal culture collections and the environment. Tokai University Press, Hadano-shi, JP, pp 29-63

Blackburn SI, Volkman JK (2012) Microalgae: a renewable source of bioproducts. In: Food and Industrial Bioproducts and Bioprocessing. Wiley-Blackwell, pp 221-241

Blumenkrantz N, Asboe-Hansen G (1973) New method for quantitative determination of uronic acids. Anal Biochem 54 (2):484-489

Borowitzka MA (1999) Commercial production of microalgae: ponds, tanks, tubes and fermenters. J Biotechnol 70 (1-3):313-321

Boussiba S (2000) Carotenogenesis in the green alga *Haematococcus pluvialis*: cellular physiology and stress response. Physiol Plant 108 (2):111-117

Boussiba S, Vonshak A (1991) Astaxanthin accumulation in the green alga *Haematococcus pluvialis*. Plant Cell Physiol 32 (7):1077-1082

Bower SM (1987) *Labyrinthuloides haliotidis* n.sp. (Protozoa: Labyrinthomorpha), a pathogenic parasite of small juvenile abalone in a British Columbia mariculture facility. Can J Zool 65 (8):1996-2007

Bowles RD, Hunt AE, Bremer GB, Duchars MG, Eaton RA (1999) Long-chain n-3 polyunsaturated fatty acid production by members of the marine protistan group the thraustochytrids: screening of isolates and optimisation of docosahexaenoic acid production. J Biotechnol 70 (1-3):193-202

Brennan L, Owende P (2010) Biofuels from microalgae-A review of technologies for production, processing, and extractions of biofuels and co-products. Renew Sust Energ Rev 14 (2):557-577

Burja AM, Radianingtyas H, Windust A, Barrow CJ (2006) Isolation and characterization of polyunsaturated fatty acid producing *Thraustochytrium* species: screening of strains and optimization of omega-3 production. Appl Microbiol Biotechnol 72 (6):1161-1169

Carmona ML, Naganuma T, Yamaoka Y (2003) Identification by HPLC-MS of carotenoids of the *Thraustochytrium* CHN-1 strain isolated from the Seto inland sea, Biosci Biotechnol Biochemvol 67(4):884-888

Caron DA (1987) Grazing of attached bacteria by heterotrophic microflagellates. Microb Ecol 13 (3):203-218

Casadevall E, Dif D, Largeau C, Gudin C, Chaumont D, Desanti O (1985) Studies on batch and continuous cultures of *Botryococcus braunii*: Hydrocarbon production in relation to physiological state, cell ultrastructure, and phosphate nutrition. Biotechnol Bioeng 27 (3):286-295

Cavalier-Smith T, Allsopp MTEP, Chao EE (1994) Thraustochytrids are chromists, not fungi: 18s rRNA signatures of Heterokonta. Phil Trans R Soc B 346 (1318):387-397

Chisti Y (2007) Biodiesel from microalgae. Biotechnol Adv 25 (3):294-306.

Chisti Y (2008) Biodiesel from microalgae beats bioethanol. Trends Biotechnol 26 (3):126-131

Christie WW (1998) Mass spectrometry of fatty acids with methylene-interrupted ene-yne systems. Chem Phys Lipids 94 (1):35-41

Christie WW (2009) The lipid library. Mass Spectrometry of Fatty Acid Derivatives- Dimethyloxazolines (DMOX) of fatty acids. The American Oil Chemists' Society. Available via The American Oil Chemists' Society. <http://lipidlibrary.aocs.org>. Accessed Nov 24 2009

Clarens AF, Resurreccion EP, White MA, Colosi LM (2010) Environmental life cycle comparison of algae to other bioenergy feedstocks. Environ Sci Technol 44 (5):1813-1819

Cox SL, Hulston D, Maas EW (2009) Cryopreservation of marine thraustochytrids (Labyrinthulomycetes). Cryobiology 59 (3):363-365

Cubic QED (2009) Regional carbon neutral energy solution. Cubic, Quod Erat Demonstradum QED. <http://www.cubicqed.com.au/index.html>. Accessed 24 Dec 2011

Curtain C (2000) Plant Biotechnology- The growth of Australia's algal b-carotene industry. Australas Biotechnol 10 (3):19-23

Danaei G, Ding EL, Mozaffarian D, Taylor B, Rehm Jr, Murray CJL, Ezzati M (2009) The preventable causes of death in the United States: Comparative risk assessment of dietary, lifestyle, and metabolic risk factors. PLoS Med 6 (4):e1000058

Decho AW (1990) Microbial exopolymer secretions in ocean environments: their role (s) in food webs and marine processes. Oceanogr Mar Biol Annu Rev 28:73-153

Davis R, Fishman D, Frank E, Wigmosta M, Aden A, Coleman A, Pienkos P, Skaggs R, Venteris E, Wang M (2012) Renewable diesel from algal lipids: an integrated baseline for cost, emissions, and resource potential from a harmonized model. US Department of Energy Biomass Program. Argonne National Laboratory, Argonne, IL; National Renewable Energy Laboratory, Golden, CO; Pacific Northwest National Laboratory, Richland, WA, US

Dobes L (1994) Greenhouse gas emissions in Australian transport in 1900 and 2000, Occasional paper 110, Bureau of Transport and Communications Economics, Canberra, AU <http://www.bitre.gov.au/publications/1995/files/op_110.pdf> Accessed Nov 28 2009

Donohue TJ, Cogdell RJ (2006) Microorganisms and clean energy. Nat Rev Micro 4 (11):800-800

Donot F, Fontana A, Baccou J, Schorr-Galindo S (2012) Microbial exopolysaccharides: Main examples of synthesis, excretion, genetics and extraction. Carbohydr Polym 87 (2):951-962

Dunahay T, Jarvis E, Dais S, Roessler P (1996) Manipulation of microalgal lipid production using genetic engineering. Appl Biochem Biotechnol 57-58 (1):223-231

Ellenbogen BB, Aaronson S (1969) Polyunsaturated fatty acids of aquatic fungi: Possible phylogenetic significance. Comp Biochem Physiol 29:805- 811

Esper B, Badura A, Rögner M (2006) Photosynthesis as a power supply for (bio-) hydrogen production. Trends Plant Sci 11 (11):543-549

Fan K-W, Jiang Y, Ho L-T, Chen F (2009) Differentiation in fatty acid profiles of pigmented and nonpigmented *Aurantiochytrium* isolated from Hong Kong mangroves. J Agric Food Chem 57 (14):6334-6341

Fan KW, Chen F (2007) Production of high-value products by marine microalgae thraustochytrids. In: Shang-Tian Y (ed) Bioprocessing for value-added products from renewable resources. Elsevier, Amsterdam,NL, pp 293-323

Fan KW, Jiang Y, Faan YW, Chen F (2007) Lipid characterization of mangrove thraustochytrid - *Schizochytrium mangrovei*. J Agric Food Chem 55 (8):2906-2910

Fiakas D (2012) Solazyme: microbes in the dark. Alternative Energy Stocks. <http://www.altenergystocks.com/archives/2012/10/solazyme_microbes_in_the_dark_1.html>. Accessed 10 Feb 2013

Filisetti-Cozzi TMCC, Carpita NC (1991) Measurement of uronic acids without interference from neutral sugars. Anal Biochem 197 (1):157-162

Fisher L, Nichols D, Sanderson K (2007) Production of biodiesel. WIPO (: World Intellectual Property Organization.) Patent No. 2008067605. Geneva, CH

Flemming H-C, Wingender J, Moritz R, Borchard W, Mayer C (1999) Physico-Chemical properties of biofilms - a short review. In: Keevil Cw, Dow C, Godfree A, Holt D (eds) Biofilms in the Aquatic Environment. Royal Society of Chemistry, Cambridge, pp 1-12

Flemming HC, Wingender J (2001) Relevance of microbial extracellular polymeric substances (EPSs)- Part I: Structural and ecological aspects. Water Sci Technol 43 (6):1-8

Forsyth JS, Carlson SE (2001) Long-chain polyunsaturated fatty acids in infant nutrition: effects on infant development. Curr Opin Clin Nutr Metab Care 4 (2):123-126

Ganuza E, Anderson A, Ratledge C (2008) High-cell-density cultivation of *Schizochytrium* sp. in an ammonium/pH-auxostat fed-batch system. Biotechnol Lett 30 (9):1559-1564

Gavrilescu M, Chisti Y (2005) Biotechnology--a sustainable alternative for chemical industry. Biotechnol Adv 23 (7-8):471-499

Gerpen JV (2005) Biodiesel processing and production. Fuel Process Technol 86 (10):1097-1107

González López CV, Acién Fernández FG, Fernández Sevilla JM, Sánchez Fernández JF, Cerón García MC, Molina Grima E (2009) Utilization of the cyanobacteria *Anabaena* sp. ATCC 33047 in CO_2 removal processes. Bioresour Technol 100 (23):5904-5910

Goossens E (2011) United agrees to buy algae biofuel from Solazyme for air. Bloomberg. <http://www.bloomberg.com/news/2011-11-07/united-agrees-to-buy-algae-biofuel-from-solazyme-for-air-travel.html >. Accessed 16 Dec 2012

Grant T, Beer T, Campbell PK, Batten D (2008) Life cycle assessment of environmental outcomes and greenhouse gas emissions from biofuels production in Western Australia. Department of Agriculture and Food Government of Western Australia, CSIRO Atmospheric Research, Aspendale, VIC, AU

Greenwell HC, Laurens LML, Shields RJ, Lovitt RW, Flynn KJ (2009) Placing microalgae on the biofuels priority list: a review of the technological challenges. J R Soc Interface 7 (46):703-726

Griffiths M, Harrison S (2009) Lipid productivity as a key characteristic for choosing algal species for biodiesel production. J Appl Phycol 21 (5):493-507

Guezennec J (2002) Deep-sea hydrothermal vents: A new source of innovative bacterial exopolysaccharides of biotechnological interest? J Ind Microbiol Biotechnol 29 (4):204-208

Guil-Guerrero J, López-Martínez J, Rincón-Cervera M, Campra-Madrid P (2007) One-step extraction and concentration of polyunsaturated fatty acids from fish liver. J Am Oil Chem Soc 84 (4):357-361

Hall MJ, Ratledge C (1977) Lipid accumulation in an oleaginous yeast (Candida 107) growing on glucose under various conditions in a one-and two-stage continuous culture. Appl Environ Microbiol 33 (3):577-584

Harel M, Ben-Dov E, Rasoulouniriana D, Siboni N, Kramarsky-Winter E, Loya Y, Barak Ze, Wiesman Z, Kushmaro A (2008) A new Thraustochytrid, strain Fng1, isolated from the surface mucus of the hermatypic coral Fungia granulosa. FEMS Microbiol Ecol 64 (3):378-387

Harmelen Tv, Oonk H (2006) Microalgae biofixation processes: application and potential contributions to greenhouse gas mitigation. Advanced Photosynthetics - Technical report. TNO Built Environment and Geosciences, Apeldoorn, NL

Harwood JL, Guschina IA (2009) The versatility of algae and their lipid metabolism. Biochimie 91 (6):679-684

Hassler CS, Schoemann V, Nichols CM, Butler ECV, Boyd PW (2011) Saccharides enhance iron bioavailability to Southern Ocean phytoplankton. Proc Natl Acad Sci 108 (3):1076-1081

He K, Song Y, Daviglus ML, Liu K, Van Horn L, Dyer AR, Greenland P (2004) Accumulated evidence on fish consumption and coronary heart disease mortality: a meta-analysis of cohort studies. Circulation 109 (22):2705-2711

Holland FA, Chapman FS (1966) Liquid mixing and processing in stirred tanks. Reinhold Pub. Corp., New York, US

Homer A (2010) Microalgae: the next biofuel? Australian Broadcasting Corporation (ABC), South Australia, AU.

<http://www.abc.net.au/rural/sa/content/2009/11/s2742396.htm> Accessed 17 Dec 2012

Honda D, Yokochi T, Nakahara T, Erata M, Higashihara T (1998) Schizochytrium limacinum sp. nov., a new thraustochytrid from a mangrove area in the west Pacific Ocean. Mycol Res 102 (Part 4):439-448

Honda D, Yokochi T, Nakahara T, Raghukumar S, Nakagiri A, Schaumann K, Higashihara T (1999) Molecular phylogeny of labyrinthulids and thraustochytrids based on the sequencing of 18S ribosomal RNA gene. J Eukaryot Microbiol 46 (6):637-647

Hong WK, Rairakhwada D, Seo PS, Park SY, Hur BK, Kim C, Seo JW (2011) Production of lipids containing high levels of docosahexaenoic acid by a newly isolated microalga, *Aurantiochytrium* sp. KRS101. Appl Biochem Biotechnol 164 (8):1468-1480

Hong WK, Yu A, Heo SY, Oh BR, Kim CH, Sohn JH, Yang JW, Kondo A, Seo JW (2012) Production of lipids containing high levels of docosahexaenoic acid from empty palm fruit bunches by *Aurantiochytrium* sp. KRS101. Bioprocess Biosystems Eng DOI: 10.1007/s00449-012-0830-1:1-5

Horrocks LA, Farooqui AA (2004) Docosahexaenoic acid in the diet: its importance in maintenance and restoration of neural membrane function. Prostaglandins Leukot Essent Fatty Acids 70:361-372

Horrocks LA, Yeo YK (1999) Health benefits of docosahexaenoic acid (DHA). Pharmacol Res 40 (3):211-225

Hsueh HT, Chu H, Yu ST (2007) A batch study on the bio-fixation of carbon dioxide in the absorbed solution from a chemical wet scrubber by hot spring and marine algae. Chemosphere 66 (5):878-886

Huang J, Aki T, Yokochi T, Nakahara T, Honda D, Kawamoto S, Shigeta S, Ono K, Suzuki O (2003) Grouping newly isolated docosahexaenoic acid-producing thraustochytrids based on their polyunsaturated fatty acid profiles and comparative analysis of 18S rRNA genes. Mar Biotechnol 5 (450):450 – 457

Huntley M, Redalje D (2007) CO_2 mitigation and renewable oil from photosynthetic microbes: A new appraisal. Mitig Adapt Strat Gl 12 (4):573-608

Iida I, Nakahara T, Yokochi T, Kamisaka Y, Yagi H, Yamaoka M, Suzuki O (1996) Improvement of docosahexaenoic acid production in a culture of *Thraustochytrium aureum* by medium optimization. J Ferment Bioeng 81 (1):76-78

Jain R, Raghukumar S, Sambaiah K, Kumon Y, Nakahara T (2007) Docosahexaenoic acid accumulation in thraustochytrids: search for the rationale. Mar Biol 151 (5):1657-1664

Jain R, Raghukumar S, Tharanathan R, Bhosle NB (2005) Extracellular polysaccharide production by thraustochytrid protists. Mar Biotechnol 7 (3):184-192

Jakobsen A, Aasen I, Josefsen K, Strøm A (2008) Accumulation of docosahexaenoic acid-rich lipid in thraustochytrid *Aurantiochytrium* sp. strain T66: effects of N and P starvation and O_2 limitation. Appl Microbiol Biotechnol 80 (2):297-306

Jeanthon C, Prieur D (1990) Susceptibility to heavy metals and characterization of heterotrophic bacteria isolated from two hydrothermal vent Polychaete Annelids, *Alvinella pompejana* and *Alvinella caudata*. Appl Environ Microbiol 56 (11):3308-3314

Jiang Y, Fan KW, Tsz-YeungWong R, Chen F (2004) Fatty acid composition and squalene content of the marine microalga *Schizochytrium mangrovei*. J Agric Food Chem 52 (5):1196-1200

Johnson DT, Taconi KA (2007) The glycerin glut: Options for the value-added conversion of crude glycerol resulting from biodiesel production. Environ Prog 26 (4):338-348

Johnson EA, An G-H (1991) Astaxanthin from microbial sources. Crit Rev Biotechnol 11 (4):297 - 326

Johnson MB, Wen Z (2009) Production of biodiesel fuel from the microalga *Schizochytrium limacinum* by direct transesterification of algal biomass. Energy Fuels 23 (10):5179-5183

Kapdan IK, Kargi F (2006) Bio-hydrogen production from waste materials. Enzyme Microb Technol 38 (5):569-582

Kawashima H, Ohnishi M (2004) Identification of minor fatty acids and various non-methylene-interrupted diene isomers in mantle, muscle, and viscera of the marine bivalve Megangulus zyonoensis. Lipids 39 (3):265-271

Kim K, Jung Kim E, Ryu B-G, Park S, Choi Y-E, Yang J-W (2012) A novel fed-batch process based on the biology of *Aurantiochytrium* sp. KRS101 for the production of biodiesel and docosahexaenoic acid. Bioresour Technol doi: 10.1016/j.biortech.2012.10.139

Knothe G (2007) Some aspects of biodiesel oxidative stability. Fuel Process Technol 88 (7):669-677

Knothe G (2008) "Designer" Biodiesel: optimizing fatty ester composition to improve fuel properties. Energy Fuels 22 (2):1358-1364

Knothe G, Sharp CA, Ryan III TW (2006) Exhaust emissions of biodiesel, petrodiesel, neat methyl esters, and alkanes in a new technology engine. Energy Fuels 20 (1):403-408

Kralovec JA, Zhang SC, Zhang W, Barrow CJ (2012) A review of the progress in enzymatic concentration and microencapsulation of omega-3 rich oil from fish and microbial sources. Food Chem 131 (2):639-644

Kris-Etherton PM, Hecker KD, Binkoski AE (2004) Polyunsaturated fatty acids and cardiovascular health. Nutr Rev 62 (11):414-426

Kucuk C, Merih K (2009) Extracellular polysaccharide production by *Rhizobium ciceri* from Turkey. Ann Microbiol 59 (1):141-144

Labare MP, Guthrie K, Weiner RM (1989) Polysaccharide exopolymer adhesives from periphytic marine bacteria. J Adhes Sci Technol 3 (1):213-223.

Leander CA, Porter D, Leander BS (2004) Comparative morphology and molecular phylogeny of aplanochytrids (Labyrinthulomycota). Eur J Protistol 40 (4):317-328

Leańo EM, Gapasin RSJ, Polohan B, Vrijmoed LLP (2003) Growth and fatty acid production of thraustochytrids from Panay mangroves, Philippines. Fungal Diversity 12:111-122

Lee Chang KJ, Dumsday G, Nichols PD, Dunstan GA, Blackburn SI, Koutoulis A (2013) High cell density cultivation of a novel *Aurantiochytrium* sp. strain TC 20 in a fed-batch system using glycerol to produce feedstock for biodiesel and omega-3 oils. Appl Microbiol Biotechnol97 (15):6907-6918

Lee Chang KJ, Dunstan GA, Abell G, Clementson L, Blackburn SI, Nichols PD, Koutoulis A (2012) Biodiscovery of new Australian thraustochytrids for production of biodiesel and long-chain omega-3 oils. Appl Microbiol Biotechnol 93 (5):2215-2231

Lee Chang KJ, Mansour MP, Dunstan GA, Blackburn SI, Koutoulis A, Nichols PD (2011) Odd-chain polyunsaturated fatty acids in thraustochytrids. Phytochemistry 72 (11-12):1460-1465

Lee Y-K (1997) Commercial production of microalgae in the Asia-Pacific rim. J Appl Phycol 9 (5):403-411

Lees C (2013) Aurora Algae ready to roll. Yahoo News, The West Australian Opinion. <http://au.news.yahoo.com/thewest/a/-/news/16131164/aurora-algae-ready-to-roll/>. Accessed 11 Mar 2013

Levin DB, Pitt L, Love M (2004) Biohydrogen production: prospects and limitations to practical application. Int J Hydrogen Energ 29 (2):173-185.

Lewis TE (2001) Characterisation and application of Australian thraustochytrids. PhD, University of Tasmania, Hobart, AU

Lewis TE, Nichols PD, McMeekin TA (1999) The biological potential of thraustochytrids. Mar Biotechnol 1:580-587

Lewis TE, Nichols PD, McMeekin TA (2001) Sterol and squalene content of a DHA-producing thraustochytrid: influence of culture age, temperature and dissolved oxygen. Mar Biotechnol 3 (5):439-447

Li Y, Horsman M, Wu N, Lan CQ, Dubois-Calero N (2008) Biofuels from microalgae. Biotechnol Prog 24 (4):815-820

Li Z, Ward OP (1994) Production of docosahexaenoic acid by *Thraustochytrium roseum*. J Ind Microbiol 13 (4):238-241

Liang Y, Sarkany N, Cui Y (2009) Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions. Biotechnol Lett 31 (7):1043-1049

Liang Y, Sarkany N, Cui Y, Yesuf J, Trushenski J, Blackburn JW (2010) Use of sweet sorghum juice for lipid production by *Schizochytrium limacinum* SR21. Bioresour Technol 101 (10):3623-3627

Lin P-Y, Huang S-Y, Su K-P (2010) A meta-analytic review of polyunsaturated fatty acid compositions in patients with depression. Biol Psychiatry 68 (2):140-147

Lippmeier J, Crawford K, Owen C, Rivas A, Metz J, Apt K (2009) Characterization of both polyunsaturated fatty acid biosynthetic pathways in *Schizochytrium* sp. Lipids 44 (7):621-630

Loef M, Walach H (2013) The omega-6/ omega-3 ratio and dementia or cognitive decline: a systematic review on human studies and biological evidence. J nutr gerontol geriatr 32 (1):1-23

Lopez G, Elmgren R (1989) Feeding depths and organic absorption for the deposit-feeding benthic amphipoids *Pontoporeia affinis* and *Pontoporeia femorata*. Limnol Oceanogr 34 (6):982-991

Lorenz RT, Cysewski GR (2000) Commercial potential for *Haematococcus* microalgae as a natural source of astaxanthin. Trends Biotechnol 18 (4):160-167

Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar, Buchner A, Lai T, Steppi S, Jobb G, Förster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, König A, Liss T, Lüßmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A, Schleifer KH (2004) ARB: a software environment for sequence data. Nucleic Acids Res 32 (4):1363-1371

Maas PAY, Kleinschuster SJ, Dykstra MJ, Smolowitz R, Parent J (1999) Molecular characterization of QPX (Quahog Parasite Unknown), a pathogen of *Mercenaria mercenaria*. J Shellfish Res 18 (2):561-567

Manca MC, Lama L, Improta R, Esposito E, Gambacorta A, Nicolaus B (1996) Chemical composition of two exopolysaccharides from *Bacillus thermoantarcticus*. Appl Environ Microbiol 62 (9):3265-3269

Mancuso Nichols C, Bowman JP, Guezennec J (2005) Effects of incubation temperature on growth and production of exopolysaccharides by an Antarctic sea ice bacterium grown in batch culture. Appl Environ Microbiol 71 (7):3519-3523

Mascarelli AL (2009a) Algae: fuel of the future? Environ Sci Technol 43 (19):7160-7161

Mascarelli AL (2009b) Gold rush for algae. Nature News 461:460-461.

MBD Energy (2010) CO_2 to Energy. MBD Energy Limited. <http://www.mbdenergy.com/catalogue/c31>. Accessed 5 March 2010

Mendes A, da Silva TL, Reis A (2007) DHA concentration and purification from the marine heterotrophic microalga *Crypthecodinium cohnii* CCMP 316 by winterization and urea complexation. Food Technol Biotechnol 45 (1):38.

Metting FB (1996) Biodiversity and application of microalgae. J Ind Microbiol Biotechnol 17 (5):477-489

Metz JG, Roessler P, Facciotti D, Levering C, Dittrich F, Lassner M, Valentine R, Lardizabal K, Domergue F, Yamada A, Yazawa K, Knauf V, Browse J (2001) Production of polyunsaturated fatty acids by polyketide synthases in both prokaryotes and eukaryotes. Science 293 (5528):290-293

Meyer B, Mann N, Lewis J, Milligan G, Sinclair A, Howe P (2003) Dietary intakes and food sources of omega-6 and omega-3 polyunsaturated fatty acids. Lipids 38 (4):391-398

Miao X, Wu Q (2006) Biodiesel production from heterotrophic microalgal oil. Bioresour Technol 97 (6):841-846

Mo C, Douek J, Rinkevich B (2002) Development of a PCR strategy for thraustochytrid identification based on 18S rDNA sequence. Mar Biol 140 (5):883-889

Monyem A, Canakci M, Van Gerpen JH (2000) Investigation of biodiesel thermal stability under simulated in-use conditions. Appl Eng Agric 16 (4):373-378

Mozaffarian D, Rimm EB (2006) Fish intake, contaminants, and human health: Evaluating the risks and the benefits. JAMA: J Am Med Assoc 296 (15):1885-1899

Ogbonna JC, Tanaka H (1998) Cyclic autotrophic/heterotrophic cultivation of photosynthetic cells: A method of achieving continuous cell growth under light/dark cycles. Bioresour Technol 65 (1-2):65-72

Oswald WJ, Golueke CG (1960) Biological transformation of solar energy. In: Wayne WU (ed) Advances in applied microbiology, vol 2. Academic Press, pp 223-262

Paradis M, Ackman R (1976) Localization of a marine source of odd chain-length fatty acids. I. The amphipod Pontoporeia femorata (Kröyer). Lipids 11 (12):863-870

Pauly D, Christensen V, Guenette S, Pitcher TJ, Sumaila UR, Walters CJ, Watson R, Zeller D (2002) Towards sustainability in world fisheries. Nature 418 (6898):689-695

Penner JE, Lister D, Griggs DJ, Dokken DJ, McFarland M (1999) Aviation and the Global Atmosphere: A Special Report of the Intergovernmental Panel on Climate Change. University Press, Cambridge, UK

Weekly Diesel prices report (2013) Australian Instutute of Petroluem <www.aip.com.au/pricing/facts/Weekly_Diesel_Prices_Report.htm>. Accessed 18th Jan 2013

Pienkos PT, Darzins A (2009) The promise and challenges of microalgal-derived biofuels. Biofuels, Bioproducts and Biorefining 3 (4):431-440

Porter D (1990) Phylum Labyrinthulomycota. In: Margulis L, Corliss JO, Melkonian M, Chapman DJ (eds) Handbook of Protoctista. Jones and Bartlett, Boston, US, pp 388-398

Porter D, Lingle WL (1992) Endolithic thraustochytrid marine fungi from planted shell fragments. Mycologia 84 (3):289-299

Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, Glöckner FO (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res 35 (21):7188-7196

Pyle DJ (2008) Use of biodiesel-derived crude glycerol for the production of omega-3 polyunsaturated fatty acids by the microalga *Schizochytrium limacinum*. Virginia Polytechnic Institute and State University, US

Pyle DJ, Garcia RA, Wen Z (2008) Producing docosahexaenoic acid (DHA)-Rich algae from biodiesel-derived crude glycerol: Effects of impurities on DHA production and algal biomass composition. J Agric Food Chem 56 (11):3933-3939

Qiang H, Milton S, Eric J, Maria G, Matthew P, Michael S, Al D (2008) Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J 54 (4):621-639

Qin J (2005) Bio - Hydrocarbons from Algae Impacts of temperature, light and salinity on algae growth. Rural Industries Research and Development Corporation Publication, Kingston ACT, AU.

Quilodrán B, Hinzpeter I, Hormazabal E, Quiroz A, Shene C (2010) Docosahexaenoic acid (C22:6n-3, DHA) and astaxanthin production by *Thraustochytriidae* sp. AS4-A1 a native strain with high similitude to *Ulkenia* sp.: Evaluation of liquid residues from food industry as nutrient sources. Enzyme Microb Technol 47 (1-2):24-30

Raghukumar S (1988) *Schizochytrium mangrovei* sp. nov., a thraustochytrid from mangroves in India. Transcripts of the British Mycological Society 90 (4):627-631

Raghukumar S (1992) Bactivory: a novel dual role for thraustochytrids in the sea. Mar Biol 113:165-169

Raghukumar S (2002) Ecology of the marine portists, the Labyrinthulomycetes (thraustochytrids and labyrinthulids). Eur J Protistol 38 (2):127-145

Raghukumar S (2008) Thraustochytrid marine protists: production of PUFAs and other emerging technologies. Mar Biotechnol 10 (6):631-640

Raghukumar S, Anil AC, Khandeparker L, Patil JS (2000) Thraustochytrid protists as a component of marine microbial films. Mar Biol 136 (4):603-609

Rann M (2009) Microalgal biodiesel - A renewable future. Goverment of South Australia. <www.renewablessa.sa.gov.au/files/bioalgae.pdf>. Accessed 23 Jan 2013

Ratledge C (2004) Fatty acid biosynthesis in microorganisms being used for single cell oil production. Biochimie 86 (11):807-815

Ratledge C, Cohen Z (2008) Microbial and algal oils: Do they have a future for biodiesel or as commodity oils? Lipid Technol 20 (7):155-160

Ratledge C (2012) Omega-3 biotechnology: Errors and omissions. Biotechnol Adv 30 (6):1746-1747

Ratledge C, Wynn JP (2002) The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. Adv Appl Microbiol 51:1- 51

Ren L-J, Ji X-J, Huang H, Qu L, Feng Y, Tong Q-Q, Ouyang P-K (2010) Development of a stepwise aeration control strategy for efficient docosahexaenoic acid production by *Schizochytrium* sp.. Appl Microbiol Biotechnol 87 (5):1649-1656

Rendleman J (1978) Metal-polysaccharide complexes-Part I. Food Chem 3 (1):47-79

Rezanka T, Sigler K (2009) Odd-numbered very-long-chain fatty acids from the microbial, animal and plant kingdoms. Prog Lipid Res 48 (3-4):206-238.

Richmond A (2004) Principles for attaining maximal microalgal productivity in photobioreactors: an overview. Hydrobiologia 512:33-37

Rimington C (1931) The carbohydrate complex of the serum proteins: Improved method for isolation and re-determination of structure. Isolation of glucosaminodimannose from proteins of ox blood. Biochem J 25 (4):1062-1071

Rodolfi L, Zittelli GC, Bassi N, Padovani G, Biondi N, Bonini G, Tredici MR (2009) Microalgae for oil: Strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnol Bioeng 102 (1):100-112

Rosa SM, Galvagno MA, Vélez CG (2010a) Adjusting culture conditions to isolate thraustochytrids from temperate and cold environments in southern Argentina. Mycoscience 52:242-252

Rosa SM, Soria MA, Vélez CG, Galvagno MA (2010b) Improvement of a two-stage fermentation process for docosahexaenoic acid production by *Aurantiochytrium limacinum* SR21 applying statistical experimental designs and data analysis. Bioresour Technol 101 (7):2367-2374

Rosegrant MW (2008) Biofuels and grain prices: impacts and policy responses. International Food Policy Research Institute Washington, DC, US. < http://www.ifpri.org/publication/biofuels-and-grain-prices> Accessed 21 Jan 2012

Rye L, Blakey S, Wilson CW (2010) Sustainability of supply or the planet: a review of potential drop-in alternative aviation fuels. Energy Environ Sci 3 (1):17-27

Ryu B-G, Kim K, Kim J, Han J-I, Yang J-W (2012) Use of organic waste from the brewery industry for high-density cultivation of the docosahexaenoic acid-rich microalga, *Aurantiochytrium* sp. KRS101. Bioresour Technol 129:351-359

Saito H (2007) Identification of novel n-4 series polyunsaturated fatty acids in a deep-sea clam, *Calyptogena phaseoliformis*. J Chromatogr 1163 (1-2):247-259

Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 4 (4):406-425

Samarasinghe N, Fernando S, Lacey R, Faulkner WB (2012) Algal cell rupture using high pressure homogenization as a prelude to oil extraction. Renew Energ 48:300-308

Sandford PA (1984) Biotechnology of marine polysaccharide. In: Colwell RR, Pariser ER, Sinksey AJ (eds). McGraw-Hill, New York, US, pp 454–516

Schenk P, Thomas-Hall S, Stephens E, Marx U, Mussgnug J, Posten C, Kruse O, Hankamer B (2008) Second generation biofuels: High-efficiency microalgae for biodiesel production. BioEnergy Research 1 (1):20-43

Scott SD, Armenta RE, Berryman KT, Norman AW (2011) Use of raw glycerol to produce oil rich in polyunsaturated fatty acids by a thraustochytrid. Enzyme. Microb Technol 48 (3):267-272

Sharma S, Raghukumar C, Raghukumar S, Sathe-Pathak V, Chandramohan D (1994) Thraustochytrid and fungal component of marine detritus II. Laboratory studies on decomposition of the brown alga *Sargassum cinereum.* J. Ag. J Exp Mar Biol Ecol 175 (2):227-242

Sheehan J, Camobreco V, Duffield J, Graboski M, Shapouri H (1998a) Life cycle inventory of biodiesel and petroleum diesel for use in an urban bus. Final report. National Renewable Energy Lab., Golden, CO, US

Sheehan J, Dunahay T, Benemann J, Roessler P (1998b) Look Back at the U.S. Department of Energy's Aquatic Species Program: Biodiesel from Algae; Close-Out Report. National Renewable Energy Lab., US Department of Energy. Golden, CO, US

Shirasaka N, Hirai Y, Nakabayashi H, Yoshizumi H (2005) Effect of cyanocobalamin and p-toluic acid on the fatty acid composition of *Schizochytrium limacinum* (Thraustochytriaceae, Labyrinthulomycota). Mycoscience 46 (6):358-363

Siboni N, Rasoulouniriana D, Ben-Dov E, Kramarsky-Winter E, Sivan A, Loya Y, Hoegh-Guldberg O, Kushmaro A (2010) Stramenopile microorganisms associated with the massive coral Favia sp. J Eukaryot Microbiol 57 (3):236-244

Singh A, Ward OP (1996) Production of high yields of docosahexaenoic acid by *Thraustochytrium roseum* ATCC 28210. J Ind Microbiol 16 (6):370-373

Smith MA, Beilin LJ, Mori TA, Oddy WH (2011) Essential fatty acids and mood: a systematic review of observational studies. Am J Food Nutr 1 (1):14-27

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150 (1):76-85

Smith TJ (2000) Squalene: potential chemopreventive agent. Expert Opin Inv Drug 9 (8):1841-1848

Spitzer V (1996) Structure analysis of fatty acids by gas chromatography - Low resolution electron impact mass spectrometry of their 4,4-dimethyloxazoline derivatives - A review. Prog Lipid Res 35 (4):387-408

Spolaore P, Joannis-Cassan C, Duran E, Isambert A, (2006) Commercial applications of microalgae. J Biosci Bioeng 101 (2):87-96

Stephens E, Ross IL, King Z, Mussgnug JH, Kruse O, Posten C, Borowitzka MA, Hankamer B (2010) An economic and technical evaluation of microalgal biofuels. Nat Biotech 28 (2):126-128

Stratton RW, Wong HM, Hileman JI (2011) Quantifying variability in life cycle greenhouse gas inventories of alternative middle distillate transportation fuels. Environ Sci Technol 45 (10):4637-4644

Sun A, Davis R, Starbuck M, Ben-Amotz A, Pate R, Pienkos PT (2011) Comparative cost analysis of algal oil production for biofuels. Energy 36 (8):5169-5179

Sutherland IW (1972) Bacterial Exopolysaccharides. In: Rose AH, Tempest DW (eds) Advances in Microbial Physiology, vol 8. Academic Press, pp 143-213

Sutherland IW (1982) Biosynthesis of microbial exopolysaccharides. In: Rose AH, Morris JG (eds) Advances in microbial physiology, vol 23. Academic Press, pp 79-150

Sutherland IW (1997) Microbial exopolysaccharides-structural subtleties and their consequences. Pure Appl Chem 69 (9):1911-1918

Sutherland IW (1998) Novel and established applications of microbial polysaccharides. Trends Biotechnol 16 (1):41-46

Takahata K, Monobe K, Tada M, Weber PC (1998) The benefits and risks of n-3 polyunsaturated fatty acids. Biosci Biotech Biochem 62 (11):2079-2085.

Tapiero H, Ba GN, Couvreur P, Tew KD (2002) Polyunsaturated fatty acids (PUFA) and eicosanoids in human health and pathologies. Biomed Pharmacother 56:215-222

Unagul P, Assantachai C, Phadungruengluij S, Suphantharika M, Tanticharoen M, Verduyn C (2007) Coconut water as a medium additive for the production of docosahexaenoic acid (C22:6 n3) by *Schizochytrium mangrovei* Sk-02. Bioresour Technol 98 (2):281-287

US DOE (2010a) National algal biofuels technology roadmap. U.S. Department of Energy, Office of Energy Efficiency and Renewable Energy, Biomass Program. <www1.eere.energy.gov/biomass/pdfs/algal_biofuels_roadmap.pdf>. Accessed 18 Mar 2011

US DOE (2010b) Secretary Chu announces nearly $80 million investment for advanced biofuels research and fueling infrastructure. U.S. Department of Energy <http://www1.eere.energy.gov/biomass/news_detail.html?news_id=15733>. Accessed 23 Jan 2013

Van den Hoek C, Mann DG, Jahns HM (1995) Algae. An introduction to phycology. University Press, Cambridge, UK

Vazhappilly R, Chen F (1998) Heterotrophic production potential of omega-3 polyunsaturated fatty acids by microalgae and algae-like microorganisms. Bot Mar 41:553-558

Volkman JK, Barrett SM, Blackburn SI, Mansour MP, Sikes EL, Gelin F (1998) Microalgal biomarkers: A review of recent research developments. Org Geochem 29 (5-7):1163-1179

Wahlen BD, Morgan MR, McCurdy AT, Willis RM, Morgan MD, Dye DJ, Bugbee B, Wood BD, Seefeldt LC (2013) Biodiesel from microalgae, yeast, and bacteria: engine performance and exhaust emissions. Energy Fuels 27 (1):220-228

Wallington T, Kaiser E, Farrell J (2006) Automotive fuels and internal combustion engines: a chemical perspective. Chem Soc Rev 35 (4):335-347

Walters C (2006) The origin of petroleum. In: Hsu C, Robinson P (eds) Practical advances in petroleum processing. Springer, New York, US, pp 79-101

Wang YW, Kim H, Weete JD (2000) Production of docosahexaenoic and pentadecanoic acids by *Thraustochytrium* sp. ATCC26185. Paper presented at the AOCS Annual meeting, San Diego California, USA.

Weiner RM (1997) Biopolymers from marine prokaryotes. Trends Biotechnol 15 (10):390-394

Whyte SK, Cawthorn RJ, McGladdery SE (1994) QPX (Quahaug Parasite X), a pathogen of Northern Quahaug *Mercenaria mercenaria* from the Gulf of St Lawrence, Canada. Dis Aquat Org 19 (2):129-136

Wijffels RH, Barbosa MJ (2010) An outlook on microalgal biofuels. Science 329 (5993):796-799

William B, Craig W, James M (2005) Development of a docosahexaenoic acid production technology using *Schizochytrium*: A historical perspective and update. In: Cohen Z, Ratledge C (eds) Single Cell Oils. AOCS Publishing, , Champaign, IL, US, pp 75-96

Williams A, Wimpenny J (1978) Exopolysaccharide production by *Pseudomonas* NCIB11264 grown in continuous culture. J Gen Microbiol 104 (1):47-57

Wotton RS (2004) The utiquity and many roles of exopolymers (EPS) in aquatic systems. Scientia marina 68 (S1):13-21

Wu ST, Yu ST, Lin LP (2005) Effect of culture conditions on docosahexaenoic acid production by *Schizochytrium* sp. S31. Process Biochem 40 (9):3103-3108

Yamamura R, Shimomura Y (1997) Industrial high-performance liquid chromatography purification of docosahexaenoic acid ethyl ester and docosapentaenoic acid ethyl ester from single-cell oil. J Am Oil Chem Soc 74 (11):1435-1440

Yan D, Lu Y, Chen Y-F, Wu Q (2011) Waste molasses alone displaces glucose-based medium for microalgal fermentation towards cost-saving biodiesel production. Bioresour Technol 102 (11):6487-6493

Yang H-L, Lu C-K, Chen S-F, Chen Y-M, Chen Y-M (2010) Isolation and characterization of Taiwanese heterotrophic microalgae: Screening of strains for docosahexaenoic acid (DHA) production. Mar Biotechnol 12 (2):173-185

Yokochi T, Honda D, Higashihara T, Nakahara T (1998) Optimization of docosahexaenoic acid production by *Schizochytrium limacinum* SR21. Appl Microbiol Biotechnol 49 (1):72-76

Yokoyama R, Honda D (2007) Taxonomic rearrangement of the genus *Schizochytrium* sensu lato based on morphology, chemotaxonomic characteristics, and 18S rRNA gene phylogeny (Thraustochytriaceae, Labyrinthulomycetes): emendation for *Schizochytrium* and erection of *Aurantiochytrium* and *Oblongichytrium* gen. nov. Mycoscience 48 (4):199-211

Yokoyama R, Salleh B, Honda D (2007) Taxonomic rearrangement of the genus *Ulkenia* sensu lato based on morphology, chemotaxonomical characteristics, and 18S rRNA gene phylogeny (Thraustochytriaceae, Labyrinthulomycetes): emendation for *Ulkenia* and erection of *Botryochytrium*, *Parietichytrium*, and *Sicyoidochytrium* gen. nov. Mycoscience 48 (6):329-341

Zanchetta P, Lagarde N, Guezennec J (2003) A new bone-healing material: a hyaluronic acid-like bacterial exopolysaccharide. Calcif Tissue Int 72 (1):74-79

Zhang JY, Yu QT, Liu BN, Huang ZH (1988) Chemical modification in mass spectrometry IV—2-alkenyl-4,4-dimethyloxazolines as derivatives for the double bond location of long-chain olefinic acids. Biol Mass Spectrom 15 (1):33-44

Zhu L, Zhang X, Ren X, Zhu Q (2008) Effects of culture conditions on growth and docosahexaenoic acid production from *Schizochytrium limacinum*. J Ocean Univ China (English Edition) 7:83-88