Characterisation and application of Australian thraustochytrids

by

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Submitted in fulfilment of
the requirements of the degree of
Doctor of Philosophy
University of Tasmania, June 2001

. . .a scientist must also be absolutely like a child. If he sees a thing, he must say that he sees it, whether he thought he was going to see it or not.

See first, think later, then test. But always see first. Otherwise you will only see what you were expecting to see.

Most scientists forget that.

Zarquon the sane, quoted from:

Adams, D. (1984) *So long and thanks for all the fish*, Pan, London

Declaration

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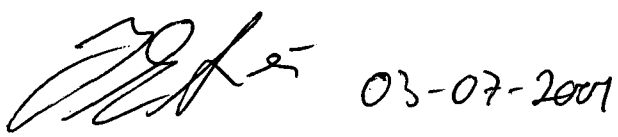

03-07-2001

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Abbreviations

The following abbreviations have been used in this thesis:

18S rDNA	18S ribosomal RNA gene
AA	arachidonic acid (20:4n6)
ACAM	Australian Collection of Antarctic Microorganisms
ACEM	the authors private collection of microorganisms
CV	co-efficient of variation (= 100 x (mean / SD))
DHA	docosahexaenoic acid (22:6n3)
DPAn3	docosapentaenoic acid (n3)
DPAn6	docosapentaenoic acid (n6)
dw	dry weight
EPA	eicosapentaenoic acid (20:5n3)
FAME	fatty acid methyl ester
GC	gas chromatograph/y
IAA	isoamyl alcohol
LCPUFA	long chain ($\geq C_{20}$) polyunsaturated fatty acid
MeOH	methanol
MS	mass spectrometer/y
MUFA	monounsaturated fatty acid/s
n3	fatty acid with the first double bond 3 carbon atoms from the terminal methyl group
n6	fatty acid with the first double bond 6 carbon atoms from the terminal methyl group
PC	phosphatidylcholine
PUFA	polyunsaturated fatty acid/s
SD	standard deviation

SFA	saturated fatty acid/s
TCM	Thraustochytrid Culture Medium
TCMA	Thraustochytrid Culture Medium with added antibiotics
T. Iso	*Isochrysis* sp - clone T. Iso
TFA	total fatty acid/s
x:*y*(n*z*)	fatty acid containing "*x*" carbon atoms and "*y*" double bonds, with the first double bond occurring "*z*" carbon atoms from the terminal methyl group. Double bonds in fatty acids are either in the *cis* (c) geometric configuration (alkyl groups on the same side of the double bond), or in the *trans* (t) configuration (alkyl groups on opposite sides of the double bond). As most double bonds in naturally occurring fatty acids have the *cis* configuration, this configuration is assumed where configuration is undefined.

Publications

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

Lewis, T., Nichols, P.D. and McMeekin, T.A. (2001). Sterol and squalene content of a DHA-producing thraustochytrid: influence of culture age, temperature and dissolved oxygen. *Marine Biotechnology* **in press**.

Lewis, T., Nichols, P.D. and McMeekin, T.A. (2000). Evaluation of extraction methods for recovery of fatty acids from lipid-producing microheterotrophs. *Journal of Microbiological Methods* **43**:107-116.

Lewis, T.E., Nichols, P.D. and McMeekin, T.A. (1999). The biological potential of thraustochytrids. *Marine Biotechnology* **1**:580-587. (Invited review).

Lewis, T.E., Nichols, P.D., Hart, P.R., Nichols, D.S. and McMeekin, T.A. (1998). Enrichment of rotifers (*Brachionus plicatilis*) with eicosapentaenoic acid and docosahexaenoic acid produced by bacteria. *Journal of the World Aquaculture Society* **29**:313-318.

Bowman, J.P., Gosink, J.J., McCammon, S.A., Lewis, T.E., Nichols, D.S., Nichols, P.D., Skerratt, J.H., Staley, J.T. and McMeekin, T.A. (1998). *Colwellia demingiae* sp. nov., *Colwellia hornerae* sp. sov., *Colwellia rossensis* sp. nov. and *Colwellia psychrotropica* sp. nov - psychrophilic Antarctic species with the ability to synthesize docosahexaenoic acid (22:6n3). *International Journal of Systematic Bacteriology*, **48**, 1171-1180.

Nichols, D., Bowman, J., Sanderson, K., Mancuso Nichols, C., Lewis, T., McMeekin, T. and Nichols, P.D. (1999). Developments with Antarctic microorganisms: culture collections, bioactivity screening, taxonomy, PUFA production and cold-adapted enzymes [Review]. *Current Opinion in Biotechnology*, **10(3)**, 240-246.

Abstract

Aspects of the taxonomy, lipid production and aquacultural application of several new polyunsaturated fatty acid (PUFA)-producing microheterotrophs were examined using 18S ribosomal DNA sequencing and fatty acid, sterol and lipid class analyses.

Thraustochytrid-like organisms were isolated from marine environments in south-eastern Tasmania (Australia). A high proportion of strains produced PUFA, especially eicosapentaenoic acid (EPA, 20:5n3) and docosahexaenoic acid (DHA, 22:6n3). The phylogenetic relationships of Tasmanian and type strains were compared, using 18S rDNA sequences and fatty acid profiles.

The effect of different extraction techniques on the recovery of fatty acids from two Tasmanian thraustochytrid strains (ACEM 6063 and ACEM 000A) was examined. Two procedures were used: the extraction of lipids from biomass followed by transesterification of the fatty acids (extraction-transesterification); and the direct transesterification of biomass to produce fatty acid methyl esters (i.e. without the initial extraction step). Results showed that the most efficient direct transesterification method yielded significantly more fatty acids than the most efficient extraction-transesterification method.

Production of biomass and lipid by strain ACEM 6063 under different culture conditions was examined. Biomass production increased with increasing glucose and sea salt concentration, culture temperature, and with low fermenter impeller speeds. The predominant lipid classes were triacylglycerols and phospholipids. The proportion of triacylglycerols increased, and the proportion of phospholipids decreased, with culture age. Lipid content increased with increasing culture age, temperature, glucose and sodium glutamate concentration and dissolved oxygen (DO). Increasing DO levels substantially decreased the proportion of saturated fatty acids and substantially increased the proportion of monounsaturated fatty acids. Twenty sterols, 13 of which were identified, were detected. Predominant were: cholest-5-en-3-ol, 24-ethylcholesta-5,22E-dien-3-ol, 24-methylcholesta-5,22E-dien-3-ol, and two co-eluting sterols – one of which was 24-ethylcholesta-5,7,22-trien-3-ol. Culture age, temperature and DO influenced squalene and sterol content. Total sterols (as a proportion of total lipids) decreased with increasing culture age.

The ability of PUFA-producing thraustochytrids to enrich rotifers (*Brachionus plicatilis*) in these PUFA was examined, and compared to that of two previously isolated PUFA-producing bacteria, which were rich in either EPA or DHA. Enrichment with the EPA-producing bacterium only resulted in rotifer EPA levels increasing from 0.1% to 1.2% of total dry weight (%dw). Similarly, following enrichment with the DHA-producing bacterium only, rotifer DHA levels increased from below detection to 0.1 %dw. When enriched with a mixture of the two bacterial strains, final rotifer EPA- and DHA-levels were 0.5 %dw and 0.3 %dw respectively.

When thraustochytrid strains ACEM 6063 and ACEM 000A were fed to rotifers, no significant differences were found between the fatty acid profile of rotifers enriched with thraustochytrids preserved by different treatments. Significant differences were observed between rotifers fed biomass from individual thraustochytrid strains or a mixture of the two strains. With both strains, the sum of arachidonic acid (AA, 20:4n6), EPA and DHA in rotifers enriched for 6 h reached 1.5-2 %dw in most treatments. There was little change in fatty acid content of rotifers enriched for 24 h compared to 6 h. Rotifers enriched with thraustochytrids contained increased levels of PUFA compared to rotifers enriched with bacteria. Strain specific variation in the PUFA content of the thraustochytrid strains also allowed the PUFA content of thraustochytrid-enriched rotifers to be manipulated to a greater degree than that of bacteria-enriched rotifers. Following 24 h post-enrichment starvation, levels of AA, EPA and DHA in most thraustochytrid-enriched rotifers were still significantly higher than in continuously starved and yeast-fed rotifers, indicating that rotifers had digested and assimilated the microheterotroph fatty acids. The two thraustochytrid strains are considered to be suitable for use in Australian aquaculture, as they provide enrichment levels comparable to those achieved with currently available commercial enrichment diets.

Acknowledgements

I wish to express my heartfelt thanks to my supervisors, Dr Peter Nichols and Prof. Tom McMeekin, for their support and guidance during this journey. Their understanding of the process – allowing me to have my head within carefully, but subtly, monitored boundaries – helped make this study the enjoyable, stimulating and illuminating sojourn it has turned out to be. Gentlemen, Thank You!

The project was supported by the Australian Postgraduate Award scheme, the Australian Fisheries Research and Development Corporation (Project 97/329), the University of Tasmania and Clover Corporation Pty Ltd.

Colleagues and fellow students at the School of Agricultural Science, University of Tasmania, and at CSIRO Marine Research were a constant source of inspiration and friendship. Sharee McCammon and John Bowman patiently guided me into the world of DNA sequencing, and helped refine the final 18S rDNA alignment files and generate the phylogenetic trees. Thanks also to Dr David Ratkowsky for helping me make sense of the numbers, Bill Peterson for keeping the laboratory equipment operational, and to Sally Jones and Jane Bailey for keeping the School running.

Lipid analyses were performed with the help of staff of the Organic Chemistry laboratories of CSIRO Marine Research. I thank particularly Dr Peter Nichols for his patience and time in providing me with the training in analytical lipid chemistry techniques used in this study, and Mina Augerinos and Ben Mooney, who provided knowledge and time to help with sample processing.

Rotifer feeding experiments were conducted with the help of Dr David Nichols, Dr Piers Hart and staff at the Tasmanian Aquaculture and Fisheries Institute aquaculture research facility.

18S rDNA sequence electrophoresis was performed by staff at CSIRO Marine Research, Tasmania, and at Griffith University, Queensland.

Close friends and family were always there when needed. Especially my Mum and Dad, whose love, nurture and interest have provided the perfect foundation for my life and my studies.

But my biggest thanks go to Elke, whose unwavering love, support, and understanding kept me grounded when things went awry, and held me high on the good days. This thesis exists because of Elks.

Chapter 1 Introduction: The biotechnological potential of PUFA-producing bacteria and thraustochytrids

1.1 Abstract

During the past decade, interest in the ability of some heterotrophic microorganisms to produce substantial quantities of lipids has increased. Microbial lipids, in particular polyunsaturated fatty acids (PUFA), have potential commercial applications as nutraceuticals, pharmaceuticals, and feed ingredients for aquaculture. Considerable effort has gone into the isolation of lipid-producing microheterotrophs, and optimisation of their lipid production. Recent studies have shown that some thraustochytrid strains (thraustochytrids are common marine microheterotrophs, taxonomically aligned with heterokont algae: e.g. brown algae and diatoms) can be cultured to produce high biomass, containing substantial amounts of lipid rich in PUFA. It is also evident that cell yield and PUFA production by some thraustochytrid strains can be varied by manipulation of physical and/or chemical culture parameters. At present, fish oils and cultured phototrophic microalgae are the main commercial sources of PUFA. The possible decline of commercial fish stocks, and the relatively complex technology required to commercially produce microalgae, has prompted research into possible alternative sources of PUFA. The culture of thraustochytrids and other PUFA-producing microheterotrophs is seen as one such alternative. Indeed, several thraustochytrid-based products are already on the market, and research into additional applications is continuing. There is also potential for genes responsible for microheterotrophic PUFA production to be cloned and inserted into other organisms, including broad-acre crops, which may allow efficient production of PUFA-rich lipids. Many fish and microalgal oils currently available have relatively complex PUFA profiles, increasing the cost of preparation of high-purity PUFA oils. In contrast, some of the microheterotrophs (microorganisms capable of heterotrophic growth) examined to date have simpler PUFA profiles. If these or other strains can be grown in sufficient quantities and at an appropriate cost, the use of microheterotroph-derived oils may decrease the high expense currently

involved with producing high purity microbial oils. Another possibility is the establishment of facilities in which high purity PUFA could be manufactured using cell-free fatty acid synthetase enzymes isolated from some PUFA-producing bacteria. As more is learned about the health and nutritional benefits of PUFA, demand for PUFA-rich products is expected to increase. Results to date suggest that microheterotrophs, especially thraustochytrids, could form an important part in the supply of such products.

1.2 Introduction

Interest in the nutritional importance of PUFA has increased markedly during the past decade. As PUFA are necessary constituents of cell membranes and of many cell-signalling systems, deficiencies in PUFA can be associated with defects in cellular function, which may lead to disease. A number of studies have further demonstrated that PUFA are essential dietary components for humans (Crawford and Sinclair, 1972; Simopoulos, 1989; Takahata *et al.*, 1998; Valagussa *et al.*, 1999; Crawford *et al.*, in press) and also in aquaculture operations for marine finfish and crustacean larvae (Sorgeloos and Leger, 1992; Castell *et al.*, 1994; D'Abramo, 1997; Takeuchi, 1997).

PUFA are generally classified into two main groups: the omega-6 (ω6 or n6) and omega-3 (ω3 or n3) series. Of the n6 PUFA, arachidonic acid (AA; 20:4n6) is of particular importance, as it is a major precursor of many prostaglandins and eicosanoids. Eicosapentaenoic acid (EPA; 20:5n3) and docosahexaenoic acid (DHA; 22:6n3) are two n3 PUFA which are currently receiving much attention and have been termed "essential" fatty acids. The n3 PUFA are known to decrease the incidence of coronary heart disease, stroke and rheumatoid arthritis (Kinsella, 1987). Evidence on the benefits and risks of n3 PUFA for human health has recently been reviewed by Takahata *et al.* (1998). DHA is essential for normal development of neural tissue in infants, especially in the eyes and brain. The possible role of these PUFA against other disorders (e.g. asthma, dyslexia, depression, some forms of cancer) is also becoming increasingly recognised, although further research is required (Simopoulos, 1989; Takahata *et al.*, 1998).

As the importance of the presence and proportions of various PUFA in the diet of both man and beast becomes better understood, the value of these dietary components to a range of industries also increases.

At present, selected fish oils and microalgal species are utilised as the main industrial sources of PUFA. However, fish oil sources may be unreliable due to the failure or variability of various fisheries. There is concern that insufficient fish oil will be available in the future to meet the expected growth in world demand for n3 oils (Tacon, 1995; Ward, 1995). Phototrophic microalgae are also used to provide PUFA for aquaculture operations (Volkman *et al.*, 1989), with additional application in the production of nutraceuticals (dietary supplements). There is also some doubt that, due to the relatively sophisticated technology required, large-scale cultivation of PUFA-rich phototrophic microalgae will be able to meet this shortfall.

In comparison, the *de novo* synthesis of n3 and n6 PUFA by thraustochytrids and other heterotrophic microorganisms may provide a cheaper and easier means of producing PUFA-rich biomass and oils. In recent years, interest in the use of microheterotrophs as a source of PUFA has increased (Ratledge, 1993). Microheterotrophs do not require some of the elements necessary for the culture of autotrophs (e.g. light, carbon dioxide), and are seen by some as a potential alternative to traditional commercial sources of PUFA. Arachidonic acid has been produced in quantity by some fungi (Sajbidor *et al.*, 1990; Gandhi and Weete, 1991). Certain bacteria have been shown to produce EPA and/or DHA (Nichols *et al.*, 1993; Jostensen and Landfald, 1997). There is a recognised need for alternative sources of PUFA for feeding to both larval and adult aquaculture species. PUFA-producing bacteria and thraustochytrids have been successfully demonstrated as a means to enrich rotifers (*Brachionus plicatilis*; a live-feed organism for finfish larvae) with these fatty acids (Watanabe *et al.*, 1992; Barclay and Zeller, 1996; Nichols *et al.*, 1996).

1.3 PUFA production by microheterotrophs

1.3.1 Thraustochytrids

Thraustochytrids are heterotrophic protists, commonly found in marine and other saline environments, and feed as detritivores (e.g. Porter, 1990; Raghukumar *et al.*,

1994), bactivores (Raghukumar, 1992) and/or parasites (Polglase, 1980; Awaji *et al.*, 1989; Whyte *et al.*, 1994). Thraustochytrids have been found in many different marine systems, and also in non-marine saline systems. Strains have been isolated from Antarctic waters and sea-ice to tropical mangroves, from coastal plankton to biofilms, and from saline environments in and near Great Salt Lake in the USA (Bahnweg and Sparrow, 1974; Amon, 1978; Raghukumar and Gaertner, 1980; Honda *et al.*, 1998; Naganuma *et al.*, 1998; Raghukumar *et al.*, 2000; Lopez-Garcia *et al.*, 2001).

Thraustochytrids were originally thought to be primitive fungi (e.g. Sparrow, 1936; Goldstein, 1963b; Ellenbogen *et al.*, 1969). In spite of thorough investigation, thraustochytrids defied satisfactory classification, being assigned to the Saprolegniales by Dick (1973) and to the redefined Thraustochytriales by Alderman *et al.* (Alderman *et al.*, 1974). Comparative morphologic and taxonomic studies of thraustochytrids indicated that some of the characteristics used to distinguish several thraustochytrid species were too variable to be useful (Booth and Miller, 1968). One suggested reason for such wide morphological variation was the range of nutrient media used to culture the strains from which taxonomic data was compiled (Kazama *et al.*, 1975; Johnson, 1976). As more became known about thraustochytrid ultrastructure and biochemical composition (e.g. Chamberlain, 1980), further suggestions regarding the taxonomic relationship within the thraustochytrid lineage, and between thraustochytrids and other taxa were made (Alderman *et al.*, 1974; Gaertner, 1977; Bahnweg and Jackle, 1986; Porter, 1990). The confused taxonomy of thraustochytrids led Chamberlain and Moss (1988) to state "In terms of phylogeny, there are no clear cut evolutionary routes passing from, or to the thraustochytrids, they stand distinctive . . .".

More recently, 18S ribosomal RNA gene (18S rDNA) sequencing techniques were used to assign 2 thraustochytrid strains to the sub-class Thraustochytridae (Chromista, Heterokonta), aligning them more closely with brown algae and diatoms (Cavalier-Smith *et al.*, 1994). Additional data, based on 18S rDNA sequences of 18 thraustochytrid strains, confirmed this concept, and showed that thraustochytrids formed a monophyletic grouping within the stramenopiles (Honda *et al.*, 1999) [The stramenopile lineage also includes diatoms, chrysophytes, brown algae, opalines, other algae and protozoa (Patterson and Sogin, 2000)]. Honda *et al.* (1999) reported

some correlation between thraustochytrid 18S rDNA sequences and the sugar composition of their cell walls. However, there was no apparent correlation between current diagnostic morphological features and the major phylogenetic lineages revealed by the 18S rDNA sequences (Honda *et al.*, 1999). These authors suggested that morphological characters may not provide reliable taxonomic data, and that further molecular phylogenetic analyses may be necessary to reveal the true evolutionary relationships between members of this group.

Thraustochytrids have become well known for their ability to produce a number of different PUFA. Findlay *et al.* (1986) proposed the use of the PUFA 22:5n6 as a signature lipid for thraustochytrids, as this compound was present as a major fatty acid in all thraustochytrid strains examined from within mangrove detrital systems, but had limited distribution in other marine organisms. Since then, several studies (which will be referred to later in this Chapter) have catalogued the ability of some thraustochytrid strains to produce 1) a relatively high biomass in culture, 2) a high proportion of lipid as part of this biomass, and/or 3) a high proportion of PUFA in the lipid.

The importance of DHA in human and animal nutrition has received a great deal of research attention during the past decade (Simopoulos, 1989; Takahata *et al.*, 1998). This has prompted many studies into alternative sources of PUFA to focus on this particular fatty acid. Most reports concerning the production of PUFA by thraustochytrids have dealt almost exclusively with DHA production (Table 1.1), as this compound is the most abundant PUFA produced by many of the thraustochytrid strains examined to date.

Data presented in Table 1.1 demonstrates the large variation in biomass, lipid and maximum DHA yields obtained for different thraustochytrid strains. For example, *Schizochytrium aggregatum* produced a biomass of 0.9 g L^{-1} after 10 days (Vazhappilly and Chen, 1998), while a biomass of 48 g L^{-1} after 4 days was achieved using *Schizochytrium* sp. SR21 (Yaguchi *et al.*, 1997). Perhaps more importantly, PUFA production by a single strain (*T. roseum* ATCC 28210) cultured under different conditions also showed marked differences. For this strain, a fed-batch flask culture yielded 2100 mg DHA L^{-1} (Singh and Ward, 1996), as compared to an unsupplemented flask culture, which yielded 650 mg DHA L^{-1} (Li and Ward, 1994).

Table 1.1 DHA production by thraustochytrids

Organism	Culture conditions				Biomass ($g\ L^{-1}$)	Total lipid (% dw)	DHA production			Reference
	Age (d)	Temp (°C)	Vessel	Other			%TFA	$mg\ g^{-1}$	$mg\ L^{-1}$	
Schizochytrium sp SR21	2.5	28	fermenter	pH 4	21	50	35	224	4700	Nakahara *et al.*, 1996
Schizochytrium sp SR21	4		fermenter 300 rpm	-	48	77	36	277	13300	Yaguchi *et al.*, 1997
S. limacinum SR21	5	25	flask	-	38	37	33	110	4200	Yokochi *et al.*, 1998
S. aggregatum ATCC 28209	10	25	flask 200 rpm	dark	0.9	-	1.7	30	0.4	Vazhappilly & Chen, 1998
Thraustochytrium aureum ATCC 34304	6	25	flask 300 rpm	light	3.8	16.5	49	70	270	Bajpai *et al.*, 1991a
T. aureum ATCC 34304	6	25	flask 300 rpm	light	4.9	20.3	51	104	511	Bajpai *et al.*, 1991b
T. aureum ATCC 34304	2.5	25	flask	light	5.7	8.1	40	-	-	Iida *et al.*, 1996
T. aureum ATCC 28211	6	25	flask 200 rpm	dark	0.8	-	3.7	50	4.0	Vazhappilly & Chen, 1998
T. roseum ATCC 28210	5	25	flask 250 rpm	light	7.6	18.2	50	87	650	Li & Ward, 1994
T. roseum ATCC 28210	12	25	flask 200 rpm	fed batch	17.1	25	49	115	2100	Singh & Ward, 1996
Thraustochytrium sp. ATCC 20892	4	25	flask 200 rpm	-	2.7	7.3	35	25	68	Singh *et al.*, 1996
Thraustochytrium sp. ATCC 26185	6	28	flask 120 rpm	light	7.5	32	25	-	-	Weete *et al.*, 1997
Strain G13	4	24	Fermenter 750 rpm		14	78	32	250	2.2	Bowles *et al.* 1999

It is beyond the scope of this review to explore in detail the many physicochemical manipulations of culture conditions that have been used in an attempt to influence PUFA production by different thraustochytrid strains. However, data in Table 1.2 gives an indication of how variations in culture conditions can influence the biomass and amount of PUFA produced by various thraustochytrid strains. It is apparent from these results that changes to culture conditions do not have uniform effects on PUFA production by different thraustochytrid strains. Optimisation and manipulation of culture conditions to produce the amounts and types of PUFA required for specific applications are definitely areas that will require extensive research for each strain taken towards commercial production. It will also be important to ascertain the distribution of PUFA in the oil structure. PUFA in natural fish oil triacylglycerols tend to occur in the glycerol-2 position (Ackman, 1990). The thraustochytrid *Schizochytrium limacinum* SR21 has been reported to accumulate phosphatidylcholine (PC) in its polar lipids. Two DHA containing compounds, 1-palmitoyl-2-docosahexaenoyl-PC and 1,2-didocosahexaenoyl-PC, comprised over 70% of the total PC were detected (Yaguchi *et al.*, 1997). The positional chemistry of microbial oils is, nonetheless, not well understood, and may influence metabolism of these oils by target consumers.

Although production of DHA has been the main focus of recent attention, it is evident that some thraustochytrid strains also produce other PUFA. Yokochi *et al.* (1997) suggested the fatty acid profiles could be used to classify DHA-producing thraustochytrids into 6 separate categories: (a) DPAn6 (22:5n6)/DHA, (b) EPA/DHA, (c) EPA/DPAn6/DHA, (d) AA/EPA/DHA, (e) LA (18:2n6)/AA/DPAn6/DHA and (f) LA/AA/EPA/DHA.

Given the diversity of PUFA profiles seen for thraustochytrids examined to date, it is likely that other, more unusual, PUFA will also be discovered from this group of organisms.

Table 1.2 Some factors that influence cell yield and PUFA production by thraustochytrids.

Organism	Culture vessel	Culture conditions, that resulted in changes when varied	Reference
Schizochytrium sp SR21	fermenter	Medium composition Impeller speed (300 rpm; 500 rpm) Impeller shape (propeller-shaped, turbine-shaped) Culture age (62 – 125 h)	Yaguchi *et al.*, 1997
S. limacinum SR21	flask	Medium composition Incubation temperature (10 - 35°C) Seawater concentration (0 – 200% seawater)	Yokochi *et al.*, 1998
T. aureum ATCC 34304	flask fermenter	Medium composition Incubation temperature (15 - 35°C) Initial medium pH (4 – 9) Illumination (light; dark) Inoculum age (24 – 72 h) Culture vessel type (shake flask; fermenter) Culture age (0 – 12 d)	Bajpai *et al.*, 1991b
T. aureum ATCC 34304	flask fermenter	Medium composition Salinity (0 – 200% seawater) Vitamins Culture vessel type (shake flask; fermenter)	Iida *et al.*, 1996
T. roseum ATCC 28210	flask	Medium composition Incubation temperature (5 - 37°C) Initial medium pH (4 – 9) Culture age (0 – 7 d)	Li & Ward, 1994
T. roseum ATCC 28210	flask	Medium composition Batch feeding (4 – 12 d after inoculation) Culture age (2 – 8 d)	Singh & Ward, 1996
Thraustochytrium sp. ATCC 20892	flask	Medium composition Incubation temperature (15 – 35 °C) Incubation temperature shift (initial 25°C; final 15°C) Culture age (1 – 7 d)	Singh *et al.*, 1996

1.3.2 Bacteria

Studies concerning the production of PUFA by bacteria have concentrated largely on comparisons of the relative percentage of PUFA in the total fatty acids (TFA) produced by various strains. Historically, it was thought that heterotrophic bacteria lacked the ability to produce long-chain PUFA (Goldfine, 1972). Since then, however, a wider range of bacteria has been studied, and analytical techniques have improved. This led to the discovery and characterisation of numerous PUFA-producing bacteria [reviewed by Russell and Nichols (1999)]. It should be noted that the misconception that bacteria do not produce PUFA still appears occasionally in the literature (e.g. Zhukova and Kharlamenko, 1999).

Bacteria that produce one or more of the physiologically important long-chain PUFA have now been isolated (Wirsen *et al.*, 1987; Nichols and Russell, 1996; Bowman *et al.*, 1997; Nichols *et al.*, 1997; Watanabe *et al.*, 1997; Yano *et al.*, 1997; Bowman *et al.*, 1998b; Table 1.3). Jostensen and Landfald (1997) also reported isolating PUFA-producing bacteria (EPA up to 18.7%TFA, DHA up to 13.5%TFA) from arctic fish and invertebrates, although data for individual strains was not provided. In contrast to data shown in Table 1.3, none of the DHA-producing bacteria isolated by Jostensen and Landfald (1997) produced EPA, and no DHA-producing bacteria were isolated from fish.

The proportion of unsaturated fatty acids produced by bacteria can be influenced by culture conditions. There is considerable evidence correlating increased proportions of PUFA in bacterial membranes with growth at low temperature and/or high pressure. Salinity has also been shown to influence the proportion of PUFA in bacterial fatty acids (Table 1.3).

Very little quantitative data for bacterial PUFA-production is available. When grown in liquid media, cultures of *Shewanella* spp and *Colwellia* spp can reach about 0.5 - 1 g dry weight (dw) L^{-1} (T. Lewis unpublished data). PUFA levels of up to 1.5 mg g^{-1} dry weight (dw) have been reported (Nichols *et al.*, 1994). These figures indicate that, under currently-used culture conditions, the yield of PUFA from bacterial cultures is relatively low.

Table 1.3 Long chain PUFA production by bacteria cultured under different conditions

Isolate	Source	PUFA (% total fatty acids)			Culture conditions	Reference
		20:4n6	20:5n3	22:6n3		
Alteromonas sp. F1A	deep seawater	-	18, 31	-	10, 2 °C	Wirsen *et al.* (1987)
Alteromonas sp. F1A	deep seawater	-	44, 31	-	450, 1 bar	Wirsen *et al.* (1987)
Colwellia psychrerythraea	Antarctic sea-ice	-	1.5	8.0	10 °C	Bowman *et al.* (1998a)
Photobacterium profundum EA2			4.1, 9.6, 15, 14		15, 10, 4, 2 °C	Allen *et al.*, 1999
Shewanella putrefaciens ACAM 342	Antarctic sea-ice		2.1, 3.2, 1.3		10, 15, 25 °C	Nichols *et al.*, 1994
Shewanella putrefaciens ACAM 342	Antarctic sea-ice		3.2, 0		3.5, 7.0 % NaCl	Nichols *et al.*, 1994
Shewanella hanedai ACAM 585	Antarctic sea-ice	-	20	-	10 °C	Bowman *et al.* (1997)
Vibrio sp. CCUG 35308	intestine of arctic fish		4.4, 9.8, 11		15, 5, 0 °C	Jostenson and Landfald 1996
ACAM 456 [a]	Antarctic sea-ice	-	16	-	2 °C	Nichols and Russell (1996)
ACAM 651[b]	Antarctic sea-ice	1.4, 2.7, 2.2, 1.9	2.6, 6.5, 12, 12	-	15, 10, 4, 2 °C	Nichols *et al.* (1997)
56D1	intestine of deep-sea fish	(0.7)[c]	1.2	21		Yano *et al.* (1997)
SCRC-21406	intestine of deep-sea fish, Japan Sea	-	0.8	23		Watanabe *et al.* (1997)

a: subsequently classified as *Shewanella gelidimarina* (Bowman *et al.*, 1997)
b: subsequently classified as *Psychroflexus torquis* (Bowman *et al.*, 1998b)
c: position of first double bond not specified

1.4 The market for PUFA

It is anticipated that the market in which microheterotroph-based oils could have the most impact will be that currently occupied by PUFA-rich oils derived from marine fish. As such, some consideration of this market is warranted. Market data included in this section has been obtained from published papers, where available, and also from industry association and company websites and from market research information provided by Australian industry (Clover Corporation Pty Ltd, New South Wales, Australia).

The current and potential world market for fish oil-based products spans a number of sectors: from unprocessed, oil-rich biomass for animal feeds, to high quality food-grade oils for use as food additives and nutraceuticals and to very high purity oils and even individual fatty acids for use in the pharmaceutical industry.

At present, the major commercial source of PUFA-rich oils is fish oil. Annual worldwide production of fish oil has remained stable at about 1.3 million t for the past 10 years or so, and is unlikely to rise (Tacon, 1997). Pike and Barlow (1999) estimated that inclusion of fish oil in aquaculture feeds will rise from 708,000 t in 2000 to 955,000 t in 2010. This could well result in a worldwide under-supply of fish oil, leading to increased demand for fish oil alternatives.

Fish oil is included in aquaculture feeds as a source of both dietary energy and PUFA (New and Csavas, 1995). There is considerable research occurring worldwide in an effort to find alternatives to fishmeal and fish oil in aquaculture feeds. However, this research is tempered by the obligate dietary requirement of many marine finfish species for long chain PUFA (LCPUFA: e.g. EPA and DHA). It seems likely that cheap, plant- or animal-derived oils, which often contain low levels of LCPUFA, will be used increasingly as an alternative source of energy in some aquaculture feeds. If such substitution does occur, sufficient LCPUFA to meet the dietary requirements of cultured aquaculture species may be required from other sources. Typically, many cultured marine species require around 1-2% (w/w) LCPUFA in their diets (e.g. Rees *et al.*, 1994; Salhi *et al.*, 1994). Pike and Barlow (1999) estimated that marine aquaculture finfish species will require about 3×10^6 t of feed in 2010. These figures point to a potential demand, for these species alone, for at least 30,000 t of LCPUFA per annum.

The imprecise boundaries surrounding the nutraceutical market make estimating the size of this market sector more difficult. Sales of marine supplement oils were in the order of US$55 million in the USA in 1996, and represented 20% of sales from health food retail outlets (Molyneaux and Chong, 1998). In the United Kingdom, fish oils account for approximately 29% (US$140 million) of the total annual market for nutraceuticals (Mukherjee, 1999). Analysis of consumer awareness and knowledge indicates n3 PUFA rate highly in both market perception and potential market success.

The western European market for infant formula increased from 81,500 t in 1988 to 103,933 t in 1994. There is an increasing trend for infant formula manufacturers to include PUFA-rich oils in their products. Typical inclusion levels of PUFA-rich oils are designed to achieve a final DHA concentration in dry infant formula of 0.1-0.2% (w/w). Extrapolating these figures suggests a potential annual demand in the European infant formula market for up to 100-200 t of DHA (Mr G Drummond, Clover Corporation, Australia *pers. comm.*)

Already a number of food and beverage products enriched with DHA or other PUFA are on the market. Mukherjee (1999) reported the availability of products such as enriched spreads, breads, eggs and soft drinks in Europe and Japan. Bread enriched with refined tuna oil as a source of LCPUFA is achieving substantial market penetration in Australia. As awareness, by both consumers and regulators, of the importance of adequate levels of PUFA in our diet increases, it can be assumed that demand for a greater range of PUFA-enriched products will increase.

1.5 The potential of PUFA-producing microheterotrophs

1.5.1 Thraustochytrids

Large-scale culture of thraustochytrids has real potential to be developed further as a commercial source of PUFA. If and where thraustochytrid-derived products are to fit into the market will be determined by our ability to produce, refine and/or enrich the oils to meet market specifications.

Thraustochytrids are already being used for commercial production of PUFA-rich products. A patented method for growing *Schizochytrium* strain (Barclay, 1992) is the basis for two products marketed for enriching rotifers (*Brachionus* spp) and brine shrimp (*Artemia* spp) with PUFA, prior to feeding these organisms to cultured finfish larvae (Barclay and Zeller, 1996; Aquafauna Biomarine Inc 2000; Sanders Brine Shrimp Company 2000). These products have entered the market in direct competition with microalgal and fish-oil based products. It is possible, however, that thraustochytrids may offer some advantage over other oils as sources of PUFA for aquaculture. Many aquaculture species require proportionally more DHA than EPA in their diet (Narciso *et al.*, 1999). The PUFA profiles of many thraustochytrids fit

this criterion, while most oils from the fishmeal industry contain more EPA than DHA.

Other uses for thraustochytrid oil are being actively explored. Monsanto (Monsanto, 2000) is producing *Schizochytrium* sp.-derived oil under a cooperative technology agreement with Golden Circle Farms (Golden Circle Farms, 2000). This oil is currently being used as a feed ingredient for laying hens to produce DHA-enriched eggs, and is under investigation for other food applications (Fitch Haumann, 1999; Franklin *et al.*, 1999).

Ratledge (1993) stated that an increase in demand for pure PUFA preparations (e.g. oils containing EPA or DHA only) is likely to be the main driving force behind any commercial success of microbial oils. Oils derived from fish and microalgae generally have a complex fatty acid (total and polyunsaturated) profile, and do not readily lend themselves to the isolation of high purity (> 98%) fatty acids. On the other hand, oils produced by some microheterotrophs have relatively simple fatty acid profiles (e.g. *Schizochytrium limacinum* SR21; Yokochi *et al.*, 1998) and may well be more amenable to cost effective refinement.

Development of economically viable technologies for the production of microbial PUFA for aquaculture, livestock and human diets is the subject of intense worldwide research at present. Given the potential for significant economic gain by those funding the research, much of the data and results being generated have not been released to the scientific community. However, selected information is available via the Internet (e.g. Aquafauna Bio Marine Inc., 2000; Avecia Life Science Molecules, 2000; Golden Circle Farms, 2000; Monsanto, 2000).

1.5.2 Bacteria

From the data presented in Table 1.3, it appears that bacteria *per se* do not have much potential as a renewable source of PUFA-rich biomass. Culture conditions for PUFA-producing bacteria, as reported in the literature, are similar to those for thraustochytrids. Many thraustochytrids, however, produce higher biomass, containing higher lipid levels, which in turn are richer in PUFA than any bacteria reported to date.

There are, however, other ways that PUFA-producing bacteria could be used in the development of new sources of PUFA. Bacteria (prokaryotes) have a smaller genome than thraustochytrids (eukaryotes), making them a potentially easier subject for genetic research. Current research aimed at determining the nucleotide sequence of the genes associated with PUFA-production by *Vibrio marinus* [now *Moritella marina* (Urakawa *et al.*, 1998)] (Morita *et al.*, 1999; Tanaka *et al.*, 1999), and *Shewanella* sp. (Takeyama *et al.*, 1997) is progressing. The EPA synthesis gene cluster from a *Shewanella* sp. has been cloned and expressed in a marine cyanobacterium, *Synechococcus* sp. (Takeyama *et al.*, 1997). Similarly, a patent has been lodged relating to the cloning and expression of "*V. marinus*" and *S. putrefaciens* PUFA synthesis genes in plants (Facciotti *et al.*, 1998). The potential therefore exists for the genes responsible for PUFA production in bacteria to be expressed by other organisms, including oil-producing seed crops (e.g. canola). Successful expression of PUFA producing enzymes in canola, or similar, crops could lead to cost effective broad-acre production of PUFA-rich biomass.

Notwithstanding their low lipid levels, bacteria may have a role to play as a PUFA source for some aquaculture species. Incorporation of dietary n3 PUFA has been found to be more efficient in some aquaculture species if the PUFA are from phospholipid rather than triacylglycerol (Izquierdo *et al.*, 2000). Bacterial PUFA are found predominantly in phospholipid (membrane lipids) (Harwood and Russell, 1984), and may therefore become an important renewable PUFA source for some aquaculture species, either by inclusion in manufactured feed, or via rotifers or other live-feed organisms.

1.5.3 Conclusion

Microheterotrophs, especially thraustochytrids, are clearly a new and potentially competitive player in the PUFA market. Considerable work is required, however, before the production of oil from these organisms significantly increases its share of the market for PUFA-rich products. To achieve this aim, the following key stages need to be negotiated:

First is the collection, screening and maintenance of PUFA-producing strains. Several strains with potential for the commercial production of DHA-rich oils have already been isolated. However, if microheterotrophs are isolated and optimised that

produce higher yields, more attractive PUFA-profiles or other less common but sought after PUFA, demand for these isolates and compounds may well increase.

Second, efficiency of PUFA production must be optimised. The types and amounts of PUFA produced by individual microheterotroph strains are susceptible to manipulation by varying culture conditions. Enhancement of PUFA profiles using molecular techniques may also be considered. Different markets will provide demand for strains that produce high levels of PUFA measured either in terms of biomass (i.e. mg PUFA g^{-1} cell mass) or volume (i.e. mg PUFA L^{-1} culture medium).

Third, appropriate conditions for long-term storage of microbial cells and/or their products must be determined. The form and stability of microheterotroph biomass and/or oils will be major factors in determining the suitability of these products for use as food additives.

Finally, oil extraction and refinement technologies must be developed to meet market demands for cost-effective and safe trophic transfer of PUFA to the target consumer/s. The bottom line for the biotechnological future of microheterotroph-oils will be their competitiveness against other LCPUFA-rich oils. Examples given above indicate large-scale culture of thraustochytrids for commercial purposes is, or will soon be, economically feasible. However, the commercial success of value-added oil products is yet to be proven.

Although this review has concentrated on PUFA production, the high growth rates and biomass already achieved with some strains suggests that microheterotrophs could have wider use as cell factories for the generation of other products. Further research and development with the PUFA-producing microheterotrophs is necessary to allow increased transfer of this knowledge to the biotechnology and associated industries. Similarly, increased market acceptance of, and demand for, specific n3 products, including a range of nutraceuticals and functional foods, will require increased knowledge of specific nutritional and health benefits of these oils.

1.6 Aim of this study

The aim of this study was to isolate PUFA-producing thraustochytrids from Australian habitats, to characterise these strains, and to evaluate the potential application of selected strains for use in aquaculture live-feeds.

The approach used to achieve this aim was as follows:

Presumptive thraustochytrids were isolated from local (Tasmania, Australia) marine habitats, using appropriate modifications of techniques described in the literature. The profile of fatty acids produced under controlled conditions was obtained for all isolates. Further characterisation of selected isolates was performed using 18S ribosomal DNA gene sequence data (Chapter 2).

Existing analytical methods were modified to enable rapid, quantitative extraction of fatty acids from selected strains (Chapter 3). The modified extraction methods were then used during trials in which the influence of physical and chemical culture conditions on biomass and lipid (lipid class, fatty acids and sterols) production by one strain were investigated (Chapters 4 and 5).

The degree to which rotifers (*Brachionus plicatilis*) - a live feed organism commonly used in aquaculture - can be enriched with PUFA from these strains is examined, and compared to enrichment of rotifers using PUFA-producing bacteria. The results from rotifer enrichment trials are related to current knowledge of dietary PUFA requirements of marine aquaculture species (Chapters 6 and 7).

Chapter 2 Isolation and characterisation of new Australian thraustochytrids

2.1 Abstract

Seventy four thraustochytrid strains were isolated from marine environments in south-eastern Tasmania (Australia). Whole cell fatty acid profiles were obtained for these strains and from 4 recognised thraustochytrid strains obtained from the American Type Culture Collection (ATCC). A strain dissimilarity matrix was formed using standard euclidean distance analysis of the fatty acid profiles; an incremental sum of squares sorting strategy placed the strains into 10 groups. Differences in the relative proportions of DHA in the fatty acid profiles were primarily responsible for the initial separation of the strains into two groups. The mean level of 22:6n3 in the groups formed by this separation were 56% and 17 % of the total fatty acids, respectively. Subsequent separations of strains within these two groups were largely influenced by fatty acids constituting relatively minor proportions (≤ 10%) of the total fatty acids. Nucleotide sequence data was obtained for the 18S ribosomal DNA gene (18S rDNA) from 15 of the new Australian strains and from one non-viable sample of a *Schizochytrium* sp.-based aquaculture feed (Algamac-2000). Evolutionary distances of thraustochytrid strains were calculated from 18S rDNA sequences using a maximum likelihood algorithm. A phylogenetic tree was then generated using a neighbour-joining strategy. There was some correlation between the groups formed by fatty acid and 18S rDNA data. All strains from the "high" 22:6n3 group from the fatty acid profile analysis were grouped in one cluster of the 18S rDNA tree. Only one strain from the "low" 22:6n3 group was grouped in this cluster. The other strains from the "low" 22:6n3 group were spread amongst other branches of the phylogenetic tree. Phylogenetic analysis of 18S rDNA data, including data for 18 thraustochytrid strains and 1 clone from the literature, indicated a high relative diversity amongst the new Australian strains. The Algamac *Schizochytrium* sp. strain, which is already undergoing commercial development, and *Schizochytrium limacinum* SR21, a strain reported to have commercial potential as a source of 22:6n3-rich biomass, clustered closely together. This indicates that this lineage has potential as a pool of 22:6n3-producing strains. Two Australian strains

also clustered closely with these two strains. One of these strains (ACEM 6063) and another strain (ACEM 000A) both produced relatively large amounts of lipid-rich biomass, and were selected for further study.

2.2 Introduction

In any study involving the isolation and application of new microbial strains, it is important to characterise those strains as precisely as possible. As discussed in Chapter 1, a definitive characterisation of thraustochytrids has been elusive. The evolutionary relationships of thraustochytrids to other microorganisms are now starting to become clear, but the relationships within the thraustochytrid taxon, and between thraustochytrids and other stramenopiles (e.g. diatoms), are still obscure (Meon-van der Staay *et al.*, 2001). Commonly used morphological characteristics appear to be too variable to be useful in ascertaining fine taxonomic differences (Honda *et al.*, 1999), while the usefulness of chemotaxonomic characteristics will depend on identifying a consistent correlation with known phylogeny.

Recent studies of thraustochytrids have involved investigations into the ability of these organisms to produce PUFA, especially 22:6n3. It is therefore perhaps not surprising that fatty acid profiles have been suggested as means of grouping thraustochytrids (Ellenbogen *et al.*, 1969; Findlay *et al.*, 1986; Yokochi *et al.*, 1997). Yokochi *et al.* (1997) suggested using PUFA content to group thraustochytrids into 6 categories. However, no strict criteria were given with this suggestion, leaving its value open to interpretation. With the advent and development of rapid DNA sequencing techniques (Saiki *et al.*, 1988), it is now possible to determine phylogenetic relationships of thraustochytrid using data for nucleotide sequences from eukaryote 18S ribosomal RNA genes (18S rDNA) (Cavalier-Smith *et al.*, 1994). Comparison of thraustochytrid fatty acid profiles with their 18S rDNA sequences will determine whether such chemotaxonomic techniques have the potential to be useful tools in the elucidation of thraustochytrid phylogeny.

The work described in this chapter had four main aims: 1 - to isolate thraustochytrid strains from Australian marine systems, and maintain these strains in pure culture for further study; 2 - to use fatty acid profile data to determine chemotaxonomic relationships between newly isolated Australian thraustochytrid strains and recognised type strains; 3 - to use 18S rDNA sequence data to determine the

phylogenetic relationship between some of the newly isolated Australian thraustochytrid strains (those for which sufficient biomass was obtained) and those reported in the literature (Honda *et al.*, 1999; Lopez-Garcia *et al.*, 2001); 4 - to examine the correlation between these two methods for determining relationships amongst thraustochytrid strains.

2.3 Material and Methods

2.3.1 Isolation and maintenance of thraustochytrid-like organisms

Sediment and seaweed were collected from marine environments around the southern coast of Tasmania (Australia). Initial samples were incubated at 20°C on solid Thraustochytrid Culture Medium (TCM) supplemented with antibiotics (TCMA). TCM was prepared by autoclaving all the ingredients shown in Table 2.1 (except the vitamin solution) at 108°C for 20 minutes, cooling the mixture to about 50°C and then aseptically adding 1 mL L^{-1} of the vitamin solution (Table 2.2). An antibiotic solution (Table 2.3; 20 mL L^{-1}) was aseptically added to TCM to prepare TCMA. It should be noted that sodium glutamate was selected as the defined nitrogen source in TCM, as this compound has been found to support relatively high biomass and 22:6n3 production by a variety of thraustochytrid strains (e.g. Goldstein, 1963a; Bahnweg, 1979; Bajpai *et al.*, 1991a; Bajpai *et al.*, 1991b; Singh *et al.*, 1996).

Inoculated TCM agar plates were examined daily by light microscopy. Colonies considered to be neither bacteria, yeast nor filamentous fungi were aseptically transferred to fresh TCMA plates and incubated at 20°C. All colonies growing on the secondary (TCMA) plates were streaked onto TCM (without antibiotics) agar plates to check for purity. Pure cultures were again subcultured onto TCM plates and incubated at 20°C for 7 days. An early maintenance protocol used in this study involved storing cultures at 2°C. However, storage at this temperature was found to be associated with the loss of viability of many strains. Subsequently, cultures were stored at 10°C.

Table 2.1 Thraustochytrid Culture Medium (TCM [1])

Component	Amount
Bacteriological peptone (L37; Oxoid, UK)	5 g
Glucose	10 g
Sodium glutamate	5 g
Yeast extract (L21; Oxoid, UK)	2 g
KH_2PO_4	100 mg
$NaHCO_3$	100 mg
$MnCl_2.4H_2O$	8.6 mg
$FeCl_3.6H_2O$	3.0 mg
$ZnSO_4.7H_2O$	1.3 mg
$CoCl_2.6H_20$	0.3 mg
$CuSO_4.5H_2O$	0.2 mg
Sea salts (S9883: Sigma, USA)	32 g
Distilled H_2O	1000 mL
Agar (Grade 3: Leiner Davis, Australia) 2	10 g
Vitamin solution (see Table 2.2)	1 mL

1: adapted from Iida et al. (1996) and Singh and Ward (1996)
2: Agar only used for solid media

Table 2.2 Vitamin solution [1] used in Thraustochytrid Culture Medium (Table 2.1)

Component	Amount
Pyridoxine hydrochloride	20 mg
Thiamin hydrochloride	10 mg
Calcium pantothenate	10 mg
p-Aminobenzoic acid	10 mg
Riboflavin	10 mg
Nicotinamide or nicotinic acid	10 mg
Biotin	4 mg
Folic acid	4 mg
Vitamin B_{12}	0.2 mg
Distilled H_2O	100 mL

1: Stirred until dissolved, filter sterilised and stored at –20°C (adapted from Staley *et al.*, 1992)

Table 2.3 Antibiotic solution [1] added to TCM (20 mL L^{-1},Table 2.1) for initial isolation of presumptive thraustochytrids

Component	Amount
Penicillin	100,000 units
Streptomycin	250 mg
Distilled H_2O	100 mL

1: Filter sterilise into sterile bottle, store in freezer (from Raghukumar, 1992)

Cultures of thraustochytrid type strains were obtained from the American Type Culture Collection (ATCC) (USA) and maintained on TCM plates as above. These strains were *Thraustochytrium* sp. ATCC 26185, *Schizochytrium aggregatum* Goldstein et Belsky ATCC 28209, *Thraustochytrium roseum* Goldstein ATCC 28210 and *Thraustochytrium aureum* Goldstein ATCC 34304.

2.3.2 Preparation, extraction and analysis of fatty acid methyl esters (FAME)

The fatty acid profiles of thraustochytrids are known to vary considerably with variations in culture media and conditions. The following standard culture protocol was therefore used for the 74 newly isolated Australian strains and 4 ATCC type strains examined in this study. Actively growing cells from pure thraustochytrid colonies were used to inoculate freshly prepared TCM agar plates. Following culture at 20°C for 5 days, cultures were checked for purity by light microscopy. A sample of fresh cells from each plate was then aseptically transferred into clean, 10 mL screw-top test tubes containing a fresh transesterification solution, consisting of methanol, hydrochloric acid and chloroform (10:1:1 v/v/v, 3 mL). Cells were suspended in this solution by vortex mixing and transesterified at 90°C for 60 min, after which the tubes were removed from heat and cooled. Water (Milli Q Plus, Millipore SA, France; 1 mL) was then added to each tube and the FAME extracted using a hexane:chloroform 4:1 (v/v) solution.

FAME analyses were obtained using a Hewlett Packard 5890 GC equipped with a 50 m x 0.32 mm internal diameter cross-linked HP5 methyl silicone (0.17 μm film thickness) fused-silica capillary column, an HP7673A autosampler, a split/splitless injector, and flame ionization detector (Nichols *et al.*, 1996). Peak areas were recorded and quantified using Millenium 32 v3.05.01 (Waters Corporation, USA). Mass spectrometric data was obtained using either a Fisons MD800 (Fisons Instruments, UK) or a GCQ (Thermoquest, USA) GC-mass spectrometer (GC-MS), operated as described in Nichols et al. (1996). Component identification was by comparison of retention time and MS data with that obtained for authentic and laboratory standards

Blank controls (i.e. containing no sample) were incorporated into this study. No fatty acids were recovered from any of the blank controls.

2.3.3 Extraction, purification, amplification and sequencing of 18S ribosomal RNA genes

Sufficient biomass for extraction of 18S ribosomal RNA genes (18S rDNA) was obtained from 15 of the 74 Australian strains originally isolated for this study, and from one non-viable sample of the commercially available thraustochytrid-based aquaculture feed Algamac 2000 (Aquafauna BioMarine, USA). All viable strains were grown in TCM broth for 7-10 days at 20°C, harvested by centrifugation, washed in 1% (w/v) NaCl, freeze-dried and stored at -20°C prior to extracting the genomic DNA. All materials that came into contact with cells, DNA and DNA products during the process of extracting, amplifying and sequencing the 18S rDNA were sterile. Total genomic DNA was extracted by a modification of the method developed by Murray and Thompson (1980). Freeze-dried cells from each strain were dispersed in 700 µL extraction buffer [0.7 M NaCl, 1% (w/v) cetyltrimethylammonium bromide, 50 mM Tris-HCl (pH 8), 10 mM EDTA, 1% (w/v) ß-mercaptoethanol] in a 2.0 mL bead beating tube. Glass beads (0.1 mm diameter, ~0.5 g) were added to the cell suspension, and the mixture shaken (Mini Bead Beater, BioSpec Products, USA) at 3,800 rpm for 4 x 10 s. Tubes were placed on ice for at least 10 s between each pulse. The mixture was then incubated at 55°C for 30 min. Extracts were emulsified, using gentle inversion, with an equal volume of 24:1 (v/v) chloroform:iso-amyl alcohol (24:1 $CHCl_3$:IAA) and centrifuged at 10,000 x g for 10 min. The upper (aqueous) layer was transferred by pipette into a 1.5 mL eppendorf tube, to which an equal volume of 24:1 $CHCl_3$:IAA and a 0.1 volume of a saline CTAB solution [10% (w/v) CTAB, 0.7M NaCl] were added. This mixture was emulsified by gentle inversion and centrifuged at 10,000 x g for 10 min. The upper layer was transferred by pipette into a new tube, to which an equal volume of phenol:chloroform (Amresco, USA) was added. This mixture was emulsified by gentle inversion and centrifuged at 8,000 x g for 10 min. The upper layer was transferred by pipette into a new tube, to which an equal volume of isopropanol was added, and mixed by gentle inversion. To precipitate DNA, this solution was held at -20°C overnight before centrifugation at 20,000 x g for 25 min at 4°C. Following removal of the supernatant, a further 800 µL ethanol (-20°C) was added and the sample centrifuged at 20,000 x g for 25 min at 4°C. The supernatant was again removed using a pipette, leaving the DNA precipitate in the tube. The precipitate was

air-dried at room temperature, resuspended in 50 µL 4% (w/v) RNAse (Sigma, USA) and incubated at 37°C for 30 min before storing at -20°C.

Standard polymerase chain reaction (PCR) protocols were followed to amplify 18S rDNA from the genomic DNA extracts, using the oligonucleotide PCR primers 18S001 and 18S13 (Table 2.4). PCR reactions were performed using the HotStart Taq Master Mix kit (Qiagen, USA) according to the manufacturer's instructions. Each PCR reaction mixture contained 25 µL HotStart Master Mix, 1 µL of a 50 pM μL^{-1} solution of each primer, 2 - 8 µL of genomic DNA extract and sterile water (Milli Q Plus, Millipore SA, France) to a final volume of 20 µL. The PCR amplification was performed using a DNA Engine PCT-200 (M J Research Inc, USA) with the following thermocycling conditions: 95°C for 15 min, 30 cycles of (94°C for 1 min, 52°C for 1 min and 72°C for 1 min), 72° for 10 min, then holding at 4°C. Purity of PCR products were examined on electrophoresis gels containing 1% (w/v) agarose in TAE buffer (40 mM Tris, 40 mM glacial acetic acid, 1 mM disodium EDTA, pH 8) and 1 µg mL^{-1} ethidium bromide. Where necessary, PCR product was re-amplified to provide sufficient amounts for sequencing. In these cases, 0.1 µL of the original PCR product was used with the same reaction mixture and thermocycling profile as described above. Unincorporated dNTP and primers were removed from PCR products using QIAquick-spin purification columns (Qiagen, Germany).

Table 2.4 Oligonucleotide primers [1] used for amplification and sequencing

Code	Synthesis direction	Sequence	Reference
18S001	forward	5′-AACCTGGTTGATCCTG CCAGTA-3′	Honda *et al.* (1999)
18SmidF	forward	5′-CAAGTCTGGTGCCAGC AGCC-3′	this study
18S13	reverse	5′-CCTTGTTACGACTTCAC CTTCCTCT-3′	Honda *et al.* (1999)

1: Primers were obtained from Life Technologies Pty Ltd, Australia

PCR sequencing reactions were performed using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA). Each sequencing reaction contained purified PCR product (1-5 μL); primer (1 μL); BigDye buffer (4 μL); BigDye reaction mix (4 μL) and sterile Milli Q water to a final volume of 20 μL. Primers used for sequencing reactions were 18S001, 18SmidF and 18S13 (Table 2.4). Sequencing reactions were performed using a DNA Engine PCT-200 (M J Research Inc, USA) with the following thermocycling conditions: 96°C for 10 min, 26 cycles of (50°C for 5 min and 60°C for 4 min), then holding at 4°C.

Each sequencing reaction mix was transferred to a 1.5 mL tube, to which 4 μL of 3M sodium acetate (pH 4.6) and 60 μL of absolute ethanol (-20°C) were added. Tubes were placed on ice for 20 min and centrifuged at 20,000 x g for 30 min at 4°C. The supernatant was removed with a pipette and the remaining pellet rinsed with 250 μL 70% ethanol (-20°C), followed by centrifugation at 20,000 x g for 10 minutes at 4°C. After removing the supernatant with a pipette, the precipitate was dried in a vacuum centrifuge (DNA mini, Heta, Denmark), and stored at -20°C. Electrophoresis and analysis was performed on an automated sequencer (A377, Applied BioSystems, USA).

2.3.4 Statistical analyses

The fatty acid profiles of the thraustochytrid strains were analysed using the software TAXON PC (v1.0 beta 3). A thraustochytrid strain dissimilarity matrix was generated using a Standardised Euclidean Distance analysis of all fatty acids occurring at ≥ 0.1% of the total fatty acids (%TFA) (Kohring *et al.*, 1994). This matrix was clustered into 10 groups using an Incremental Sum of Squares sorting strategy, allowing a chemotaxonomic dendrogram of thraustochytrid strains to be generated.

Sequence electrophoretograms were examined using the software Sequence Navigator v1 (PE Applied Biosystems, USA) to resolve ambiguous base positions. Corrected sequences were manually aligned against published 18S rDNA thraustochytrid sequences (Honda *et al.*, 1999; Lopez-Garcia *et al.*, 2001). Phylogenetic analysis of aligned sequences was done using the software suite

PHYLIP v 3.57c (Felsenstein, 1993). Evolutionary distances were determined using the Maximum Likelihood algorithm in the module DNADIST, and the phylogenetic tree constructed using the neighbour-joining method in the module NEIGHBOR.

2.4 Results

2.4.1 Fatty acid data

The fatty acid profiles of the newly isolated thraustochytrids and thraustochytrid type strains are shown in Table 2.5. Strains in Table 2.5 are ordered in the 10 fatty acid groups formed by the Taxon cluster analysis (described in Section 2.3.4). A dendrogram depicting the cluster analysis is shown in Figure 2.1. Those fatty acids exerting ≥ 10% of the relative influence of all fatty acids on each separation (S1-S9, Figure 2.1) are shown in Table 2.6. Variations in the relative proportions (%TFA) of DHA and AA in all strains were the major factors influencing the first separation (S1). Mean levels of DHA and AA in strains grouped in the upper branch of the first separation (S1, Figure 2.1) were 56 and 0.7 %TFA respectively. Comparative values for strains grouped in the lower branch of S1 were 17 and 8.6 %TFA respectively (Table 2.6). The mean EPA level of strains in the upper and lower branches of S1 were 5.0 and 12 %TFA respectively.

There was no overlap between the groups of fatty acids exerting ≥ 10% of the relative influence of all fatty acids on separations S3, S5, S8, S9 within the upper branch of S1 in Figure 2.1 and those exerting similar influence on separations S2, S4, S6, S7 within the lower branch of S1 (Table 2.6). Separations amongst strains within the upper branch of S1 (S3, S5, S8, S9) were caused by differences in relatively minor (< 10 %TFA) components of the TFA in those strains. The only PUFA substantially influencing separations within the upper branch of S1 (18:4n3 and C_{22} PUFA) were present at very low levels. Mean levels for 18:4n3 in the upper and lower branches of S8 were < 0.1% and 0.3 %TFA respectively, and for the upper and lower branches of S9 were 1.2% and < .1 %TFA respectively. Mean levels for C_{22} PUFA in the upper and lower branches of S5 were 0.9% and < 0.1 %TFA respectively (Table 2.6).

Table 2.5 Fatty acid composition (% of total fatty acids [1]) of thraustochytrid strains

Fatty acid / Strain [2]	12:0	14:0	15:0	16:1 n9	16:1 n7	16:1 n7t	16:1 n5	16:0	17:0	18:3 n6	18:4 n3	18:2 n6	18:1 n9	18:1 n7	18:0	20:4n 6	20:5n 3	20:3n 6	20:4n 3	20:2n 6	20:1 n9+11	20:0	22:5n 6	22:6n 3	22:4n 6	22:5n 3	C_{22} PUFA
Fatty acid group 1 [3]																											
6063	0.2	9.6	2.2		3.2		0.2	27.8	0.5			0.1		7.3	0.8	1.0	2.0	0.1	0.4				5.2	38.1	0.1	0.7	
ATCC 26185	0.1	5.3	3.1		1.2		0.2	23.6	1.0	0.1		0.2		5.5	0.6	1.6	3.1	0.2	0.4				9.2	41.9	0.3	0.9	
Fatty acid group 2																											
0007		1.8					2.4	21.5									3.2						1.3	67.2		0.7	1.9
0011		1.7					2.8	20.4									4.1						5.5	65.6			
0012		1.6	0.4				2.6	22.1									2.5						1.1	67.8		0.7	1.3
0013		2.5	0.4				2.9	26.3									1.7						1.1	63.7		0.7	0.8
0019		0.9	0.6			1.5	1.6	21.0							0.6	0.3	0.5						16.8	53.4	0.5	1.5	0.7
Fatty acid group 3																											
0020		2.1						21.8				0.5	0.4	0.2	1.1	0.5	4.5	0.3	0.5				2.7	64.1		1.4	
0021		2.8						25.8				0.8	0.8		1.4	0.5	4.0	0.4	0.3				2.1	60.3		0.8	
6000	0.4	3.7			0.2		0.1	25.7				0.4	0.3	0.4	0.6	0.3	4.4	0.2	0.6			0.1	1.9	59.4	0.2	0.7	
6001	0.4	3.9			0.1		0.2	29.6		0.2		0.6	0.7	0.3	1.0	0.4	3.7	0.3	0.4			0.1	2.1	54.5	0.3	1.0	
6002	0.5	4.1			0.3			26.7		0.2		0.7	0.6	0.5	1.0	0.4	5.6	0.2	0.4			0.1	1.6	55.6	0.3	0.9	
6003	0.5	3.9		0.1	0.2		0.2	29.5		0.7		3.2	2.9	0.2	3.4	1.0	7.6	1.3	0.6	0.2		0.2	1.2	41.4	0.3	1.0	
6004	0.5	4.4			0.2		0.2	26.6		0.2		0.9	0.9	0.4	1.2	0.5	6.0	0.3	0.5			0.1	1.3	54.6	0.1	0.6	
6005	0.3	3.5	0.3					25.7				0.2	0.2		0.7	0.3	3.7	0.6	0.4			0.1	2.3	60.1	0.4	0.7	
6006	0.5	4.5	0.1		0.2		0.1	32.5		0.3		0.8	1.0	0.2	1.8	0.6	5.8	0.3	0.6			0.1	1.3	48.0	0.2	0.6	

Table 2.5 (continued)

Fatty acid / Strain [2]	12:0	14:0	15:0	16:1 n9	16:1 n7	16:1 n7t	16:1 n5	16:0	17:0	18:3 n6	18:4 n3	18:2 n6	18:1 n9	18:1 n7	18:0	20:4n 6	20:5n 3	20:3n 6	20:4n 3	20:2n 6	20:1 n9+11	20:0	22:5n 6	22:6n 3	22:4n 6	22:5n 3	C_{22} PUFA
6007	0.5	4.3	0.1		0.2			31.7		0.3		1.3	1.2	0.2	2.3	0.6	5.6	0.5	0.5			0.2	1.5	47.5	0.3	0.8	
6008	0.4	3.7	0.2		0.2			24.1		0.1		0.3	0.3	0.3	0.6	0.3	4.5	0.3	0.5				2.0	60.5	0.3	0.9	
6009	0.3	3.6	0.1		0.2			30.3		0.2		0.7	1.1		1.0	0.4	4.4	0.4	0.6			0.2	1.4	53.7	0.3	0.7	
6010	0.4	3.5	0.1				0.1	28.3		0.1		0.6	0.6	0.2	1.5	0.4	5.7	0.2	0.5			0.1	1.2	54.5	0.2	0.9	
6011	0.5	4.2			0.1			25.1		0.2		0.5	0.4	0.5	0.7	0.3	6.3	0.2	0.4			0.1	1.3	57.7	0.2	0.8	
6012	0.5	3.3			0.3			21.5		0.2		0.9	0.5	0.7	0.6	0.4	8.6	0.2	0.5				1.2	59.3	0.2	1.0	
6013	0.5	3.2	0.1		0.3			21.4		0.2		0.9	0.5	0.7	0.6	0.5	8.6	0.4	0.5				1.2	59.0	0.2	1.0	
6016	0.4	3.4	0.1		0.2		0.1	27.4		0.4		1.2	1.1	0.3	1.6	0.7	5.3	0.8	0.7			0.1	1.9	52.7	0.3	1.0	
6019	0.3	3.4			0.2		0.3	28.7		0.1		0.3	0.2	0.3	0.6	0.2	3.1	0.2	0.5			0.1	1.6	58.5	0.1	0.7	
6024	0.4	3.6			0.2		0.3	25.8		0.1		0.4	0.3	0.4	0.6	0.3	4.1	0.2	0.6			0.1	1.4	60.1		0.6	
6025	0.4	3.6	0.1		0.2		0.2	26.2		0.2		0.6	0.3	0.3	0.8	0.4	4.6	0.4	0.7				1.5	58.1		0.9	
6026	0.4	3.6		0.1	0.2		0.3	25.6		0.3		1.4	0.8	0.4	1.1	0.7	6.9	0.8	0.7			0.2	1.3	53.8	0.1	0.8	
6027	0.4	3.0			0.1			21.9				0.3	0.2	0.7	0.5	0.2	5.9		0.4				1.3	63.7		0.9	
6028	0.4	2.7					0.3	22.0				0.3	0.2	0.4	0.4	0.3	4.5	0.1	0.6				1.7	64.3		1.0	
6030	0.4	3.2	0.1		0.1		0.3	27.2		0.1		0.6	0.5	0.3	0.7	0.4	4.1	0.3	0.5			0.1	1.4	58.6		0.6	
6031	0.4	3.3					0.5	25.8		0.1		0.4	0.3	0.3	0.8	0.3	3.9	0.2	0.5			0.1	1.5	60.0		1.2	
6033	0.5	3.2			0.2		0.3	22.6		0.2		0.9	0.6	0.4	0.9	0.6	7.8	0.5	0.7				1.0	58.0		1.0	
6034	0.4	3.6			0.2		0.5	25.6		0.2		0.8	0.5	0.3	1.1	0.4	4.2	0.3	0.5			0.1	1.3	59.1		0.6	
6035	0.4	4.3			0.1		0.8	28.8		0.2		0.5	0.4	0.3	0.9	0.3	3.5	0.1	0.3			0.2	1.3	56.6		0.6	
6036	0.7	4.6		0.1	0.3		0.7	25.3		0.5		1.5	1.1	0.3	1.4	0.8	7.3	0.5	0.5			0.2	1.0	51.7		1.1	
6037	0.5	4.3			0.1		0.5	31.3		0.2		0.7	0.8	0.2	1.3	0.3	4.2	0.2	0.3			0.2	1.3	52.2	0.1	0.8	
6038	0.4	3.6			0.1		0.5	26.1		0.2		1.2	0.7	0.3	1.4	0.5	4.6	0.2	0.4			0.2	1.1	57.1		0.7	
6039	0.4	3.4					0.2	26.3		0.1		0.5	0.4	0.3	0.9	0.3	4.9	0.2	0.5			0.1	1.3	58.9	0.1	0.8	

Table 2.5 (continued)

Fatty acid	12:0	14:0	15:0	16:1 n9	16:1 n7	16:1 n7t	16:1 n5	16:0	17:0	18:3 n6	18:4 n3	18:2 n6	18:1 n9	18:1 n7	18:0	20:4n 6	20:5n 3	20:3n 6	20:4n 3	20:2n 6	20:1 n9+11	20:0	22:5n 6	22:6n 3	22:4n 6	22:5n 3	C_{22} PUFA
Strain [2]																											
6041	0.4	4.3			0.2		0.5	30.7		0.2		0.7	0.6	0.3	1.3	0.3	3.6	0.3	0.5			0.2	1.4	53.3		0.7	
6042	0.5	4.4	0.1				0.4	27.8		0.1		0.3	0.3	0.1	0.7	0.3	3.2	0.2	0.4				1.4	58.7		0.8	
6043	0.5	4.2			0.2		0.4	25.7		0.2		0.6	0.5	0.3	0.8	0.3	4.6	0.2	0.5			0.1	1.0	58.5	0.1	0.7	
6045	0.4	3.1	0.1		0.1		0.1	20.1		0.2		0.9	0.4	0.5	0.6	0.5	7.0	0.2	0.4				1.6	62.4	0.2	0.9	
6046	0.5	4.5			0.2		0.2	31.1		0.5		1.2	1.1	0.4	1.5	0.6	6.3	0.5	0.5			0.2	1.2	48.3	0.2	0.8	
6047	0.3	3.0			0.1			24.1		0.1		0.3	0.3	0.4	0.6	0.2	4.4	0.1	0.4				2.0	62.2	0.1	0.7	
6048	0.3	3.4			0.1		0.5	26.4		0.1		0.3	0.3	0.3	0.7	0.2	3.4	0.2	0.5			0.1	1.6	60.4		0.7	
Fatty acid group 4																											
6040	0.5	3.2	0.2		0.3	0.1	0.6	20.2		0.2	1.2	0.4	1.0	1.6	1.0	0.4	5.4	0.1	0.4	0.9			1.3	60.0	0.1	0.8	
Fatty acid group 5																											
6050		1.9	1.5	0.1	1.1		0.3	24.0	0.5			1.0	3.3	0.3	2.6	6.5	12.4	2.4	1.5	2.6		0.2	4.0	29.2	1.6	3.0	
ATCC 28209		1.2	1.1		0.5		0.1	23.6	0.3	0.1		0.2	1.3	1.1	0.2	2.3	5.5	1.0	0.3	0.1			5.2	54.2	0.5	0.9	
ATCC 28210		1.1	0.7		0.2	0.1		24.2	0.2	0.2	0.1	0.4	0.9	0.8	0.6	3.3	6.3	0.6	0.3				7.0	51.2	0.7	0.9	
ATCC 34304		1.3	0.4		0.3			25.2	0.2	0.1	0.2	0.2	0.4	0.7	1.1	3.4	7.8	0.4	0.5				7.0	49.3	0.5	0.8	
Fatty acid group 6																											
6029		0.7	0.1	6.8	0.4		0.1	17.6	0.2		0.1	1.6	9.0	0.2	0.6	3.4	15.3	1.3	2.2	8.5	2.3		0.3	24.4	0.2	4.0	0.4
6032		0.7	0.4	8.0	0.4		0.1	16.0	0.3		0.1	1.3	12.9	0.2	0.5	1.6	15.4	1.0	3.2	7.1	4.5		0.1	22.6		3.3	0.2
Fatty acid group 7																											
0005		1.1	0.4	0.3	1.4			21.5		1.3		6.7	22.4	1.2	4.0	3.0	5.6	0.7		0.6			3.2	21.5	3.2	1.5	0.5
0006		2.9		0.3				22.8		0.5		4.5	10.5	1.4	3.1	9.1	5.5	1.3	0.2	1.2			20.2	10.7	4.3	0.7	0.7

Table 2.5 (continued)

Fatty acid / Strain [2]	12:0	14:0	15:0	16:1 n9	16:1 n7	16:1 n7t	16:1 n5	16:0	17:0	18:3 n6	18:4 n3	18:2 n6	18:1 n9	18:1 n7	18:0	20:4n 6	20:5n 3	20:3n 6	20:4n 3	20:2n 6	20:1 n9+11	20:0	22:5n 6	22:6n 3	22:4n 6	22:5n 3	C_{22} PUFA
0017		1.5		0.4				25.5		0.3		1.4	15.5	1.4	5.1	5.9	5.9	0.9	0.2	0.5	0.5	0.2	15.8	14.1	3.7	1.2	
6018		0.6			0.3			33.4		0.5	0.3	2.2	11.3	1.5	4.0	3.6	5.0	0.8	0.1	0.4	0.1	0.1	17.0	16.4	1.5	0.4	
6020		1.2		0.6	0.1			21.8		0.5	0.3	0.8	9.4	1.4	4.2	5.4	9.6	0.5	0.3	0.1	0.1		20.9	18.7	3.0	1.0	
6049	0.1	3.1		0.2	0.3		0.5	32.0		0.9	0.3	10.3	12.7	1.3	3.6	3.1	3.8	5.6	0.6	0.7		0.4	6.5	9.9	3.3	0.8	
Fatty acid group 8																											
0008		2.4	0.5	0.7	0.6	1.6		15.5				1.5	0.9	0.3	0.5	12.1	17.6	3.0	0.7	1.2			17.8	20.8	0.3	2.1	
0009		3.2	0.5	0.3	0.5	3.2		25.6				0.8	0.7	0.4	1.5	7.2	17.4	1.8	0.7	1.0			13.9	19.0	0.4	2.1	
0014		2.2	0.4	0.8	0.7	1.3		16.2				2.4	2.8	0.3	0.8	14.7	13.1	3.7	0.7	1.4			14.4	20.7	0.5	3.1	
0015		2.3	0.4	0.4	0.7	1.3		15.6				1.4	1.7		1.1	9.9	16.6	2.7	0.9	1.2			13.8	27.2	0.7	2.1	
0016		2.7	0.5	0.4	0.9	1.4		19.4				1.2	3.6	0.3	2.6	10.1	10.8	2.6	0.6	0.9			15.3	21.7	0.6	4.5	
6014	0.2	3.8	0.5	0.5	0.9			23.4				1.7	7.0	0.1	2.7	7.0	12.2	1.4	1.2	0.9	0.4	0.1	11.5	20.4	0.4	3.2	
6015	0.2	3.9	0.4	0.5	0.6			20.0		0.2		1.8	1.4	0.1	1.1	12.4	13.9	1.5	0.8	0.7	0.1		10.6	26.6	0.4	2.2	
6017		3.9	0.6	0.6	0.7		0.2	17.7		0.1	0.1	2.7	1.7	0.2	0.7	12.4	16.8	1.9	1.1	1.0	0.2		12.8	20.6	0.4	2.9	
6021		3.6	0.5	0.5	0.5			19.7		0.2		1.6	1.1		0.7	12.6	19.8	1.5	0.9	0.8			12.0	20.9	0.4	2.3	
6023		3.4	0.7	0.5	0.5			17.3		0.2		2.1	0.7	0.1	0.8	15.5	14.2	2.3	1.1	1.1			15.3	21.6	0.4	1.8	
6044		3.2	0.6	0.3	0.4		0.1	20.6		0.2		1.1	0.6	0.2	0.8	11.0	17.3	1.6	1.1	1.1			13.6	23.3	0.3	2.2	
Fatty acid group 9																											
000A		4.1			1.9			26.4				4.0	4.9		8.7	5.8	17.0	2.8	1.8	3.9			0.3	9.5	0.7	8.1	
0001		4.5			3.3			30.1				2.3	5.4		8.6	6.6	13.5	1.7	0.4	3.9			0.3	11.0	0.9	7.4	
0002		4.7			3.5			28.6				1.3	3.7		8.2	5.2	13.9	1.8	0.6	3.7			0.3	13.0	0.9	10.6	
0003		5.1			6.3			23.5				1.2	5.4		5.2	11.3	15.3	1.2	0.2	2.4			0.4	12.8	1.4	8.4	
6022	0.3	7.5	0.1		4.2	0.2		46.3				3.3	3.0	0.3	6.8	13.6	0.6	1.1		2.1		0.2	4.1	3.4	2.2	0.4	

Table 2.5 (continued)

Fatty acid	12:0	14:0	15:0	16:1 n9	16:1 n7	16:1 n7t	16:1 n5	16:0	17:0	18:3 n6	18:4 n3	18:2 n6	18:1 n9	18:1 n7	18:0	20:4n 6	20:5n 3	20:3n 6	20:4n 3	20:2n 6	20:1 n9+11	20:0	22:5n 6	22:6n 3	22:4n 6	22:5n 3	C_{22} PUFA
Strain [2]																											
Fatty acid group 10																											
0004					3.1			14.5				2.1	43.0		5.9	15.5	5.3	3.0	0.2	5.5	1.9						
0010		11.4						16.9				0.8	25.3	0.6	2.4	1.2	9.2	1.1	1.0	1.8	2.1	0.3		20.6	0.3	4.9	
0018		1.2			0.2			21.1				2.0	3.1	4.7		13.1	13.2	3.7	0.6	9.7	0.3		1.1	22.8	1.3	1.9	

1: Only components comprising ≥ 1% of total fatty acids are shown

2: Strains ATCC 26185, 28209, 28210 and 34304 were obtained from the American Type Culture collection as *Thraustochytrium* sp., *Schizochytrium aggregatum* Goldstein et Belsky, *Thraustochytrium roseum* Goldstein and *Thraustochytrium aureum* Goldstein, respectively. All other strains were isolated during the course of this study.

3: Group numbers are those shown in Figure 2.1.

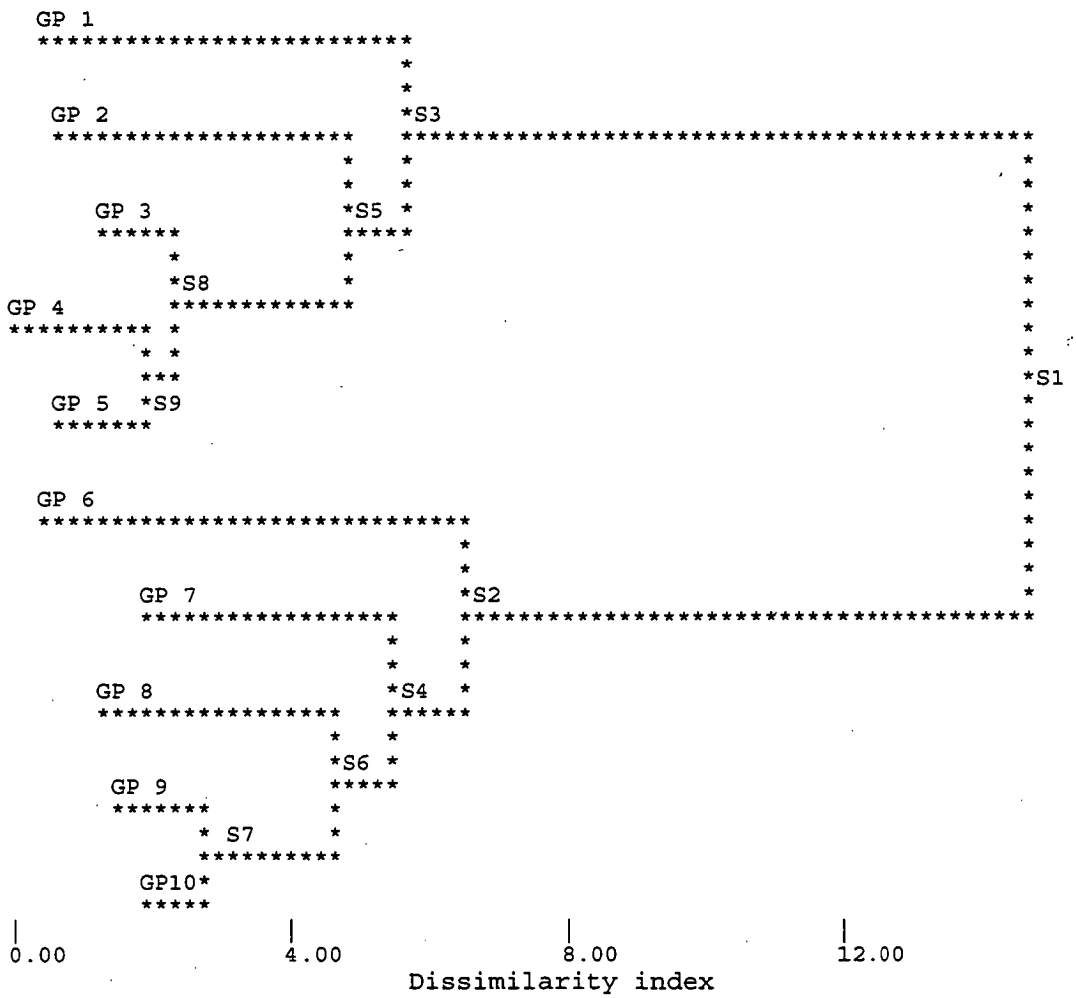

Figure 2.1 Australian and type thraustochytrid strains clustered into 10 groups based on incremental sum of squares sorting of a standard euclidean distance dissimilarity matrix of fatty acid profiles (created using TAXON v 1.0 beta)

Separations (S1 – S9) are numbered according to decreasing dissimilarity index; see Table 2.5 for information regarding fatty acid profiles for strains in fatty acid groups 1-10; and Table 2.6 for information regarding which fatty acids substantially influenced separations

Table 2.6 Fatty acids that exerted substantial [a] influence on separations S1 - S9 in Figure 2.1

Separation	Fatty acid	Relative contribution to each separation (%)	Mean level in upper [b] branch (%TFA)	Mean level in lower [b] branch (%TFA)
S1	22:6n3	15	56	17
	20:4n6	11	0.7	8.6
Upper branch of S1 in Figure 2.1				
S3	18:1n7	30	6.4	0.4
	15:0	29	2.7	0.1
	17:0	26	0.8	0.1
S5	16:1n5	41	2.5	0.2
	C_{22} PUFA	30	0.9	<0.1
S8	18:4n3	26	<0.1	0.3
	17:0	17	<0.1	0.2
	15:0	15	<0.1	0.8
	12:0	13	0.4	0.1
S9	18:4n3	75	1.2	0.1
Lower branch of S1 in Figure 2.1				
S2	16:1n9	33	7.4	0.3
	20:1n9+11	22	3.4	0.2
	20:4n3	17	2.7	0.6
S4	18:3n6	22	0.7	0.1
	22:4n6	21	3.2	0.7
S6	18:0	17	1.2	5.7
	22:5n6	16	14	0.8
	16:1n7	13	0.7	2.8
S7	18:1n9	18	4.5	24
	16:0	16	31	18
	16:1n7	14	3.8	1.1
	22:5n3	13	7.0	2.3
	18:0	13	7.5	2.8
	20:1n9+11	11	<0.1	1.4

a: fatty acids which each contributed to ≥ 10% of the total difference at each separation
b: "upper" and "lower" refer to the relative positions of the two branches of each separation in Figure 2.1

Thus only small differences in the means of 18:4n3 at S8 and S9 and C_{22} PUFA at S5 influenced the observed separations (Table 2.6). Similarly, the mean levels of most of the fatty acids exerting substantial influence on the separations within the lower branch of S1 (S3, S4, S6, S9) occurred at < 10 %TFA. Of the fatty acids that exerted substantial influence on the additional separations within the lower branch of S1, only DPAn6 (14 and 0.8 %TFA in the upper and lower branches of S6 respectively) and 16:0 (31 and 18 %TFA in the upper and lower branches of S7 respectively) occurred at mean levels of > 10 %TFA. Several PUFA substantially influenced one or more of the additional separations (S2, S4, S6, S7) within the lower branch of S1 (Table 2.6). However, apart from DPAn6 (mean levels of 14 and 0.8 %TFA in the upper and lower branches of S6 respectively), all these PUFA were relatively minor (*i.e.* < 10 %) components of the TFA in these groups.

2.4.2 18S rDNA data

A phylogenetic tree generated from 18S rDNA sequence data from this study (15 Australian strains and a sample of Algamac-2000), and 18 thraustochytrid strains (Honda *et al.*, 1999) and 1 thraustochytrid clone (Lopez-Garcia *et al.*, 2001) from the literature is shown in Figure 2.2. Sequences for two additional Australian strains - ACEM 6003 and ACEM 6030 - were omitted as they contained many unresolved base positions, therefore distorting the final tree. The partial sequences for these two strains (not shown), grouped them within Cluster A (Figure 2.2).

The 15 newly isolated Australian strains from which 18S rDNA was extracted were distributed across most of the thraustochytrid phylogenetic tree (Figure 2.2). The majority (9) of the Australian strains examined were grouped in Cluster A (Figure 2.2). The nearest known relative of Cluster A was *Thraustochytrium kinnei* KMPB 1694b. Strain ACEM 0005 clustered nearest to *Japonochytrium* sp. ATCC 28207. Strains ACEM 6018 and 6063 clustered closely with both *Schizochytrium limacinum* SR21 and the *Schizochytrium* sp. used in the production of Algamac-2000. Strain ACEM 6032 clustered closest with *Schizochytrium minutum* KMPB N-BA-27. Strains ACEM 000A and ACEM 0004 clustered closely with each other, but were widely separated from the other strains contained in this tree (Figure 2.2).

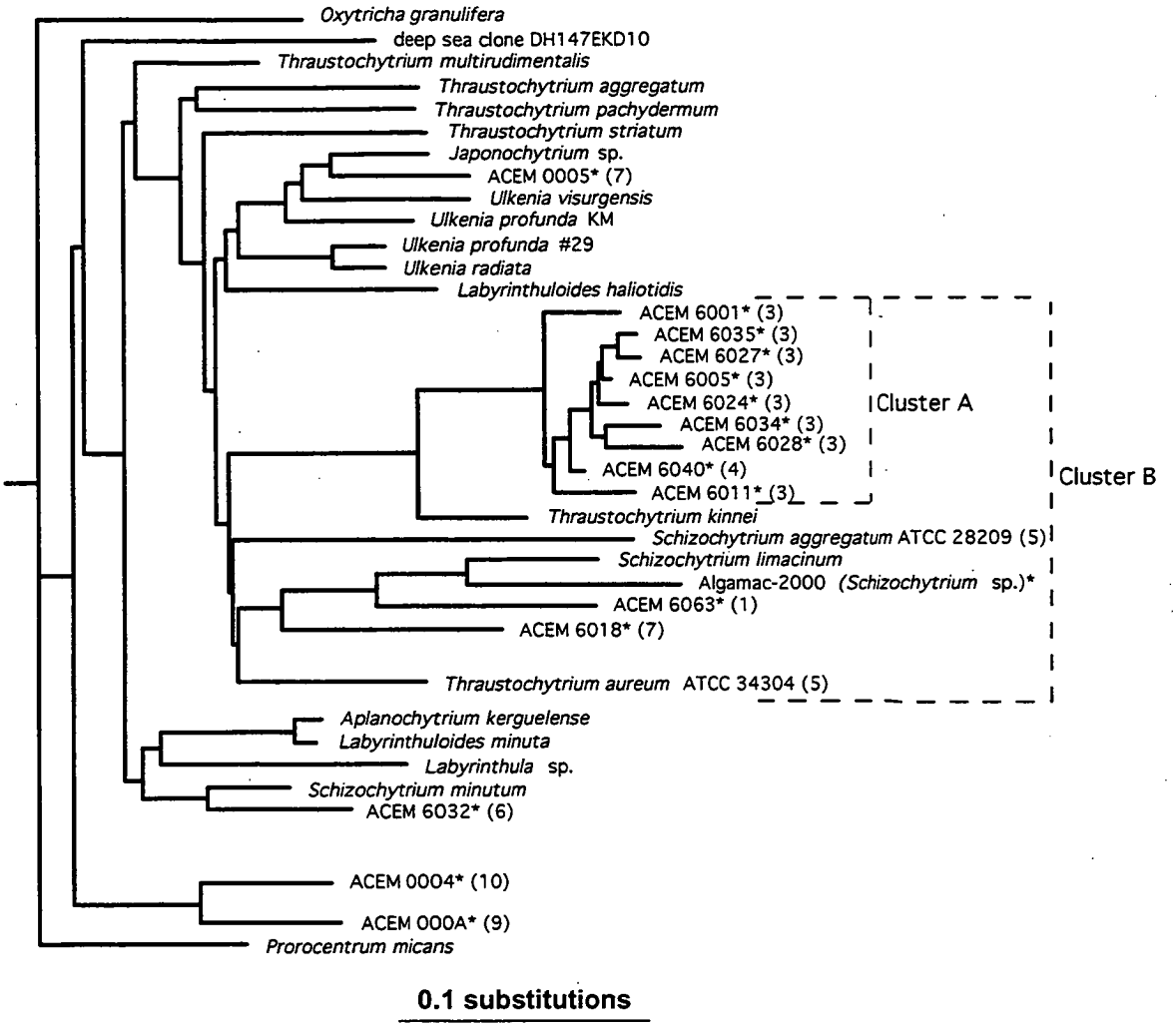

Figure 2.2 Thraustochytrid 18S rDNA tree, with *Prorocentrum micans* and *Oxytricha granulifera* used as outgroups. Evolutionary distances were determined using a maximum likelihood algorithm. The tree was constructed using a neighbour-joining strategy (created using PHYLIP v 3.5c)

Notes:
- asterisks indicate strains for which 18S rDNA sequences were determined for this study
- numbers in brackets indicate the groups to which strains were assigned, based on fatty acid profiles (Figure 2.1, Table 2.5)
- scale bar = maximum likelihood distance for 0.1 substitutions / 100 nucleotides

2.5 Discussion

As outlined in Chapter 1, some thraustochytrid strains have the potential to be used as sustainable commercial sources of DHA and other PUFA. It is therefore interesting to note that the relative level of DHA was one of the most influential factors in separating thraustochytrids strains based on whole cell fatty acid profiles of 5 day old thraustochytrid plate cultures. The mean levels of the nutritionally important PUFA AA, EPA and DHA in the strains grouped in the upper branch of S1 were 0.7, 5.0 and 56 %TFA respectively (Figure 2.1). Corresponding values in the lower branch of S1 were 8.6, 12.0 and 18 %TFA respectively (Figure 2.1). From a nutritional point of view, this is potentially important information. It is becoming clear that not only is the total amount of n3 PUFA in, for example, human and finfish diets important, but it is considered beneficial for the diets to contain high ratios of n3 : n6 PUFA and DHA : EPA (e.g. Takahata *et al.*, 1998; Sargent *et al.*, 1999). On average, PUFA derived from strains in the upper branch of S1 would be better suited to provide these requirements than strains in the lower branch of S1 (Figure 2.1).

It is interesting to note that strains ACEM 6063 and ATCC 26185 formed a two-member group based on the presence of DHA and DPAn6 at levels of ≥ 5 %TFA (Table 2.7). These two strains also formed a two-member group based on their overall fatty acid profiles (Table 2.5). However, the relative level of DPAn6 in these strains did not substantially influence the separation of this group from other strains (Table 2.6, Figure 2.1).

Fatty acid, including PUFA, profiles have been used in attempts to distinguish microorganisms for some time (e.g. Korn *et al.*, 1965; Shaw, 1966). Separation of taxa was largely based on the presence or absence of specific fatty acids. Ellenbogen *et al.* (1969) reported that the major fatty acids of *Thraustochytrium roseum* and *Schizochytrium aggregatum* were 16:0, 18:0 and DHA, while those for *Thraustochytrium aureum* were 16:0, DPAn6 and DHA. More recently, it has been suggested that thraustochytrids can be mainly divided into 6 categories, based on their PUFA profiles (Yokochi *et al.*, 1997). As mentioned in Section 2.2 (Page 18) these categories were defined by the occurrence of LA, AA, EPA, DPAn6 and DHA in the total fatty acids. In the present study, only three of these five PUFA (AA, DPAn6, DHA) were substantially involved with any of the separations observed in

the fatty acid profile-derived dendrogram (Figure 2.1, Table 2.6). The lack of correlation between the categories put forward by Yokochi *et al.* (1997) and the results presented in this Chapter suggests that more work is required before a scheme for categorising thraustochytrid strains on PUFA content can be routinely applied.

The intrinsic variability of fatty acid profiles of thraustochytrids grown under various culture conditions makes comparison of fatty acid data provided by different studies difficult. Such comparisons will be possible only when all strains being compared are cultured under the same conditions. Yokochi *et al* (1997) state that the strains they used when forming their PUFA category concept were not all cultured using the same medium. This information raises some doubts about the validity of the categories suggested by those authors. During the present study, care was taken to culture all strains under the same conditions (e.g. media composition, light levels, temperature, culture duration) to reduce the influence of variables that are known to influence fatty acid profiles of thraustochytrids. No strict criteria were used to define the categories proposed by Yokochi *et al* (1997) (D. Honda, Konan University, Japan, *pers. comm.)*, making direct relation of these categories to the data in this study difficult. When the Australian and type strains were grouped depending on the presence of one or more specific PUFA at levels of ≥ 5 %TFA, a pattern different to that described by Yokochi *et al.* (1997) appeared (Table 2.7). Out of a total of 13 PUFA categories determined by this method, only 4 of the 6 categories proposed by Yokochi *et al* (1997) were represented.

Findlay *et al.* (1986) suggested that the fatty acid 22:5n6 had the potential to be a signature marker for thraustochytrids. However, the presence of high levels of 22:5n6 in thraustochytrid whole cell fatty acids is by no means a standard trait. The range of 22:5n6 contained in the Australian strains cultured for this study ranged from < 0.1 to 21 %TFA.

These comparisons highlight the potential difficulties with trying to categorise thraustochytrids by their PUFA profiles. There is a similar lack of correlation between many conventional thraustochytrid taxonomic characters and their 18S rDNA sequences, although thraustochytrid 18S rDNA data correlated better with cell wall sugar compositions than with other parameters (Honda *et al.*, 1999).

Table 2.7 Distribution of strains that contained only the nominated PUFA at levels of ≥ 5% of total fatty acids after culture on TCM at 20°C for 5 days

PUFA	Strain
22:5n6, 22:6n3 [1]	ACEM 6063, ATCC 26185
20:5n3, 22:6n3 [1]	0010, 0011, 0019, 6002, 6003, 6004, 6006, 6007, 6010, 6011, 6012, 6013, 6016, 6026, 6027, 6033, 6036, 6040, 6045, 6046
20:5n3, 22:5n6, 22:6n3 [1]	6018, ATCC 28109, ATCC 28210, ATCC 34304
20:4n6, 20:5n3, 22:6n3 [1]	6050
20:4n6, 20:5n3, 22:5n6, 22:6n3	0006, 0008, 0009, 0014, 0015, 0016, 0017, 6020, 6014, 6015, 6017, 6021, 6023, 6044
18:2n6, 20:3n6, 22:5n6, 22:6n3	6049
20:4n6, 20:5n3, 20:2n6, 22:6n3	0018
20:4n6, 20:5n3, 22:6n3, 22:5n3	000A, 0001, 0002, 0003
20:4n6, 20:5n3, 20:2n6	0004
18:2n6, 20:5n3, 22:6n3	0005
20:5n3, 20:2n6, 22:6n3	6029, 6032
22:6n3	0007, 0012, 0013, 0020, 0021, 6000, 6001, 6005, 6008, 6009, 6019, 6024, 6025, 6028, 6030, 6031, 6034, 6035, 6037, 6038, 6039, 6041, 6042, 6043, 6047, 6048
20:4n6	6022

1: Groups which correspond to categories proposed by Yokochi *et al.* (1997).

In this study, there was some correlation between the groups formed using 18S rDNA sequence data and whole cell fatty acid data. However, the low number of strains for which both fatty acid profiles and 18S rDNA sequences were available must temper this observation to some degree. The largest grouping from the 18S rDNA data (Cluster A, Figure 2.2) all belonged to fatty acid group 3, except for one strain that belonged to fatty acid group 4. There was very little difference between the mean fatty acid profiles of these two groups. The fatty acids that were largely responsible for their separation (18:4n3, 17:0, 15:0, 12:0) all occurred at levels of < 1.0 %TFA in both groups (Table 2.6, S8). No strains from fatty acid groups 3 and 4 occurred anywhere else in the 18S rDNA tree, indicating the relative specificity of the fatty acid profiles of those strains. Other correlations between the 18S rDNA and fatty acid profile trees were less apparent when viewed on the scale in which Figure 2.1 is presented (*i.e.* a 10-group solution). However, when Figure 2.1 is viewed as a 2-group solution (*i.e.* with strains grouped on the basis of S1 only), all strains found within the upper arm of S1 were also found in Cluster B in Figure 2.2. Only one strain found within the lower arm of S1 in Figure 2.1 (strain ACEM 6018 - fatty acid group 7) was present in Cluster B. The presence of this strain in Cluster B is an interesting anomaly. Strain ACEM 6018 was morphologically similar to strain ACEM 6063 (data not shown), but it's fatty acid profile contained only 16% DHA (c.f. 38% DHA for strain ACEM 6063). Other strains from the lower arm of S1 (Figure 2.1) were distributed more widely amongst the 18S rDNA tree (Figure 2.2). Strain ACEM 0005 (fatty acid group 7) was widely separated from strain ACEM 6032 (fatty acid group 6). Strains ACEM 000A and ACEM 0004 (fatty acid groups 9 and 10 respectively), clustered together in a 2-member group that was widely separated from all other strains (Figure 2.2).

Despite the correlations between fatty acid profiles and 18S rDNA profiles found in this study, scope exists for closer agreement between the two data sets. It is likely that the clusters formed by a given set of thraustochytrid strains based on fatty acid or PUFA profiles will change depending on the conditions under which the strains are cultured. Thus, the possibility exists that a specific set of culture conditions could give rise to fatty acid or PUFA profiles that more closely fit phylogenetic data. However, at present, there appears to be insufficient understanding of the influence of environmental conditions on thraustochytrid fatty acid profiles to achieve this goal. Compilation and careful analysis of such data will be necessary before fatty

acid profiles can be used to provide a detailed picture of the evolutionary relationships between different thraustochytrid strains.

Comparison of 18S rDNA sequence data from the new Australian thraustochytrid strains, with that currently available from the literature reveals that the Australian strains represent a wide cross-section of the currently described thraustochytrid taxon (Figure 2.2). This indicates that the isolation protocol used in this study was sufficiently non-selective to allow a wide diversity of thraustochytrids to be isolated. Of the 74 thraustochytrid strains originally isolated for this study, only 15 were still viable when work towards DNA sequence analysis started. No strains from two of the 10 fatty acids groups (groups 2 and 8) shown in Table 2.5, and only limited numbers of strains from the other groups, could be grown for 18S rDNA sequence analysis. Loss of strain viability during long-term maintenance of thraustochytrid cultures by continual subculture is a recognised problem amongst thraustochytrid researchers (D. Honda, Konan University, Japan; C. Leander, University of Georgia, USA; D. Frampton, CSIRO Marine Research, Australia; *pers. comm.*).

It is interesting to note the apparent close phylogenetic relationship between strain ACEM 6063, *Schizochytrium limacinum* SR21 and the DNA extracted from a sample of the commercial product Algamac-2000 (which is based on a proprietary *Schizochytrium* sp. Strain). Honda *et al.* (1999) suggested that *Schizochytrium limacinum* SR21 represents a separate lineage from all other thraustochytrids. The data presented in the present study suggest that strain ACEM 6063, the Algamac-2000 *Schizochytrium* sp. strain, and possibly strain ACEM 6018 belong to the same lineage. This relationship places strains ACEM 6063 and ACEM 6018 in a group of organisms showing particular potential as sources of 22:6n3-rich biomass. Fermenter culture studies have shown *Schizochytrium limacinum* SR21 has produced biomass up to 48 g dry weight L^{-1}, and up to 270 mg 22:6n3 g^{-1} dry weight (Nakahara *et al.*, 1996; Yaguchi *et al.*, 1997; Yokochi *et al.*, 1998). The *Schizochytrium* sp on which Algamac-2000 is based is presumably undergoing continued development as a commercial source of 22:6n3. The apparent correlation between members of the *Schizochytrium limacinum* SR21 lineage and high rates of 22:6n3-rich biomass suggests that other strains from the same lineage could have potential as commercially viable sources of 22:6n3. It remains to be determined how more strains from this lineage can be targeted for isolation and development.

Strain ACEM 6063 was isolated from a single colony, from one of a large number of plates used to isolate the original suite of strains obtained during this study. Similarly *Schizochytrium limacinum* SR21 was just one of about 400 thraustochytrid strains isolated during a sampling trip to the Yap Islands in Micronesia (Nakahara *et al.*, 1996). In both cases, all strains had to be screened for fatty acid and biomass production before strains worthy of closer examination could be identified. Targeted isolation strategies, as discussed by Bull *et al.* (1992), could markedly improve the efficiency of isolating additional closely related strains. Analysis of the 18S rDNA sequences of thraustochytrid strains notable for their high production of DHA-rich biomass could reveal specific oligonucleotide sequences. If that were the case, it would be feasible to construct appropriate DNA probes, enabling rapid screening of environmental samples for the presence of these patterns. Isolation and screening of viable strains need only proceed for those samples yielding positive results for the DNA probes. If this technique proves effective for strains related to *Schizochytrium limacinum* SR21, it should also work for other thraustochytrid lineages. Accumulation of sufficient DNA sequence and fatty acid profile data would be a first step towards development of strategies to target the isolation of thraustochytrids that produce other fatty acids of interest.

Data presented in this Chapter demonstrate that samples obtained from Australian marine environments contained a wide diversity of thraustochytrids. Preliminary data (see Chapter 3) indicated that two new strains - ACEM 000A and ACEM 6063 – were able to produce substantial quantities of lipid-rich biomass. Of these, strain ACEM 6063 was of primary interest due to its ability to produce substantial amounts of the nutritionally important PUFA 22:6n3. Subsequent research in this thesis describes the development and application of these 2 strains.

Chapter 3 Evaluation of extraction methods for recovery of fatty acids from lipid-producing thraustochytrids

3.1 Abstract

The effect of different extraction techniques on the recovery of fatty acids from freeze-dried biomass of two lipid-producing thraustochytrids was examined. Two procedures were used: the extraction of lipids from biomass followed by transesterification of the fatty acids (extraction-transesterification); and the direct transesterification of biomass to produce fatty acid methyl esters (i.e. without the initial extraction step). Variable factors in the extraction-transesterification experiment were the sequence in which solvents were added to the samples, the relative amount of methanol in the solvent mix, and sonication of biomass while in the solvent mix. Variable factors in the direct transesterification experiment were sample size, and reaction duration. Statistical analysis of data (level of significance $\alpha = 0.05$) showed that: 1) extraction of total fatty acids prior to transesterification was significantly more efficient when solvents were added in the order of increasing polarity; 2) neither sonication nor increasing the proportion of methanol in the extraction solvent significantly affected extraction of fatty acids prior to transesterification; 3) efficiency of direct transesterification of fatty acids increased significantly with reaction time; 4) efficiency of direct transesterification of fatty acids was not significantly affected by sample size; 5) the most efficient method for extraction of fatty acids prior to transesterification yielded significantly less fatty acids than the most effective direct transesterification method. While this study examined only two strains, results suggest that fatty acid analysis methodology for microheterotrophs under consideration for biotechnological exploitation requires optimisation and validation.

3.2 Introduction

As discussed in Chapter 1, interest in the ability of some heterotrophic microorganisms to produce substantial quantities of lipids is increasing. Microbial lipids [in particular PUFA, but also monounsaturated (MUFA) and saturated (SFA) fatty acids] have potential commercial applications as nutraceuticals, pharmaceuticals, and feed ingredients for aquaculture. Considerable effort has gone into the isolation of, and optimisation of lipid production by, oleaginous strains (Ratledge, 1993; Leman, 1997).

To determine the quantity and types of lipid produced by microheterotrophs, the lipids need to be extracted from the biomass in a form that can be analysed quantitatively. A common lipid extraction method is that of Bligh and Dyer (1959), in which lipids are extracted from biological material using a combination of chloroform and methanol in a ratio such that a single phase is formed with the water in the sample. Dilution of the solvent mixture with water and chloroform causes separation into two phases, with the lower (chloroform) phase containing all the extracted lipids. Further modifications of this method, including addition of water to the solvent mixture when extracting lipids from dry samples (White *et al.*, 1979), sample sonication (Dunstan *et al.*, 1992), adding water to lyophilised samples before the non-polar solvents (Dunstan *et al.*, 1993) and increasing the proportion of methanol in the extraction solvent mixture (Smedes and Askland, 1999) have also been used.

Other solvent systems and methods have also been evaluated for prokaryotes and eukaryotes. While a hexane-isopropanol solvent system was found to be effective for the bacterium *Pseudomonas atlantica* (Guckert and White, 1988), this system gave lower recovery of lipids in the green alga *Chlorella* (Guckert *et al.*, 1988). Pressurised accelerated hot solvent extraction (ASE) of soil, air and water samples has been tested using a modified Bligh and Dyer solvent mix (Macnaughton *et al.*, 1997). Compared to the Bligh and Dyer extraction performed at ambient temperature, recovery of lipids using ASE was similar for vegetative biomass, soil and water samples, but was higher for spores and air-borne biomass.

Extracted lipids are usually transesterified to form fatty acid methyl esters, allowing identification and quantification of fatty acids using gas-chromatographic techniques.

This process has been used regularly to quantify lipid content of microheterotroph cells (e.g. Sajbidor *et al.*, 1990; Bajpai *et al.*, 1991b; Gandhi and Weete, 1991; Iida *et al.*, 1996).

In studies concerning lipid production by microheterotrophs, some researchers have dispensed with the initial lipid extraction step, in favour of single-step (extraction-transesterification) methods (e.g. Eroshin *et al.*, 1996; Nakahara *et al.*, 1996; Yokochi *et al.*, 1998; Allen *et al.*, 1999). A sequential direct saponification-acid hydrolysis-esterification procedure has also been used to recover more effectively cyclopropane and hydroxylated fatty acids from bacterial samples (Mayberry and Lane, 1993). The concept of direct transesterification of fatty acids in samples has received attention over the years, primarily due to the substantial reduction in both time and solvents that this technique offers. Several studies have discussed the relative efficiency of direct transesterification and lipid extraction followed by transesterification. Increased recovery of fatty acids (from adipose tissue and milk, and lactic acid bacteria respectively) by direct transesterification techniques has been reported (Lepage and Roy, 1984; Dionisi *et al.*, 1999). However, direct transesterification did not change fatty acid recovery from plant tissue and infant formulae respectively (Garces and Mancha, 1993; Bohnert *et al.*, 1997).

These results strongly suggest that before true comparison of data from different sources can be made, it is important to consider the methods used to extract and analyse fatty acids from biological samples, including microorganisms. If deviations from a standard method give rise to significantly different results, as has been suggested by Smedes and Askland (1999), then direct comparisons of lipid production will not be possible. Similarly, it is important to determine the most effective extraction and analysis method for microheterotrophs, so that lipid content and composition can be determined accurately.

In this Chapter, the effects of some variations of both the Bligh and Dyer method (at ambient temperature) and direct transesterification procedures on the efficiency of extraction of fatty acids from freeze-dried biomass of two lipid-producing thraustochytrids were examined.

3.3 Materials and Methods

3.3.1 Analytical reagents

All reagents used were of analytical standard. Chloroform, methanol, hexane and hydrochloric acid were obtained from Mallinckrodt (USA). Deionised water was produced using a Milli Q Plus (Millipore SA, France) water treatment system.

3.3.2 Thraustochytrid culture and cell preparation

Two lipid-producing thraustochytrids (strains ACEM 6063 and ACEM 000A), isolated from coastal marine sediment in southeastern Tasmania (Australia), were used. Biomass was produced in 500 mL, screw topped Erlenmeyer flasks, containing 150 mL of Thraustochytrid Culture Medium or variants (see Chapter 2), incubated at 20°C on a rotary-shaking table (GIO Gyrotary Shaker, New Brunswick, USA) at 200 rpm. Cells of ACEM 6063 were harvested after 10 days incubation. Cells of ACEM 000A were harvested after 3 and 5 days incubation.

Cells were harvested by centrifugation (J2-21M/E centifuge, JA 14 rotor, Beckman USA) at 8000 x g for 15 minutes in 250 mL polyethylene centrifuge tubes. Following centrifugation, the supernatant was discarded, the cell pellet resuspended in 100 mL 1.0% NaCl (w/v), and re-centrifuged. The second supernatant was discarded and the cell pellet frozen overnight at -30°C. Frozen biomass was freeze dried (chamber temperature -110°C, Mini Ultra Cold, Dynavac Australia; pressure $< 7 \times 10^{-1}$ mbar, RV3 vacuum pump, Edwards High Vacuum International) for 15 hours and subsequently stored in a sealed glass container at -30°C. Preliminary experiments showed that freeze-drying ACEM 000A and ACEM 6063 cells prior did not influence the amount of lipid recovered from the biomass, nor the fatty acid profile of the extracted lipid. Biomass used in the experiments described below came from a single batch of each strain.

3.3.3 Preparation of fatty acid methyl esters (FAME)

Two procedures were used to prepare FAME from freeze-dried biomass: a) extraction followed by transesterification; b) direct transesterification (Figure 3.1). The two procedures and variants were all performed in duplicate and are described below.

3.3.3.1 Extraction-transesterification

Freeze-dried ACEM 6063 cells were weighed (100 ± 10 mg) into clean, 100 mL screw-top test tubes. A total of 80 mL of solvent was then added in a predetermined sequence, with each sample being vortex mixed for 15 seconds following the addition of each solvent (Table 3.1). Selected samples were sonicated (Labsonic 1510 Ultrasonicator, Braun, Germany) by immersing a clean probe directly into the cell-solvent mixture and supplying the probe with 100 W (peak) for 2 x 1 minute (Table 3.1).

ACEM 6063 biomass-solvent mixtures were left in the tubes for about 18 hours, after which they were quantitatively transferred into clean glass separatory funnels. Each tube was rinsed (2 x 5 mL) with a mixture containing the same ratio of extraction solvents as was used for that tube, with the rinse mixture being added to the appropriate separatory funnel.

Freeze-dried ACEM 000A cells, from 3- and 5-day old cultures, were weighed (100 ± 10 mg) into glass separatory funnels, to which a total of 114 mL solvents were added in the sequence: chloroform, methanol, water to achieve a final chloroform:methanol:water ratio of 1:2:0.8 (v/v/v). Samples were shaken for 15 seconds immediately following the addition of each solvent, and allowed to stand for about 18 hours, with occasional shaking by hand.

Phase separation of the biomass-solvent mixtures in the separatory funnels was achieved by adding chloroform and water to each separation funnel to obtain a final chloroform:methanol:water ratio of 1:1:0.9 (v/v/v). A known portion of each total lipid extract recovered from the lower chloroform phase in the separatory funnel was used for FAME preparation and analysis.

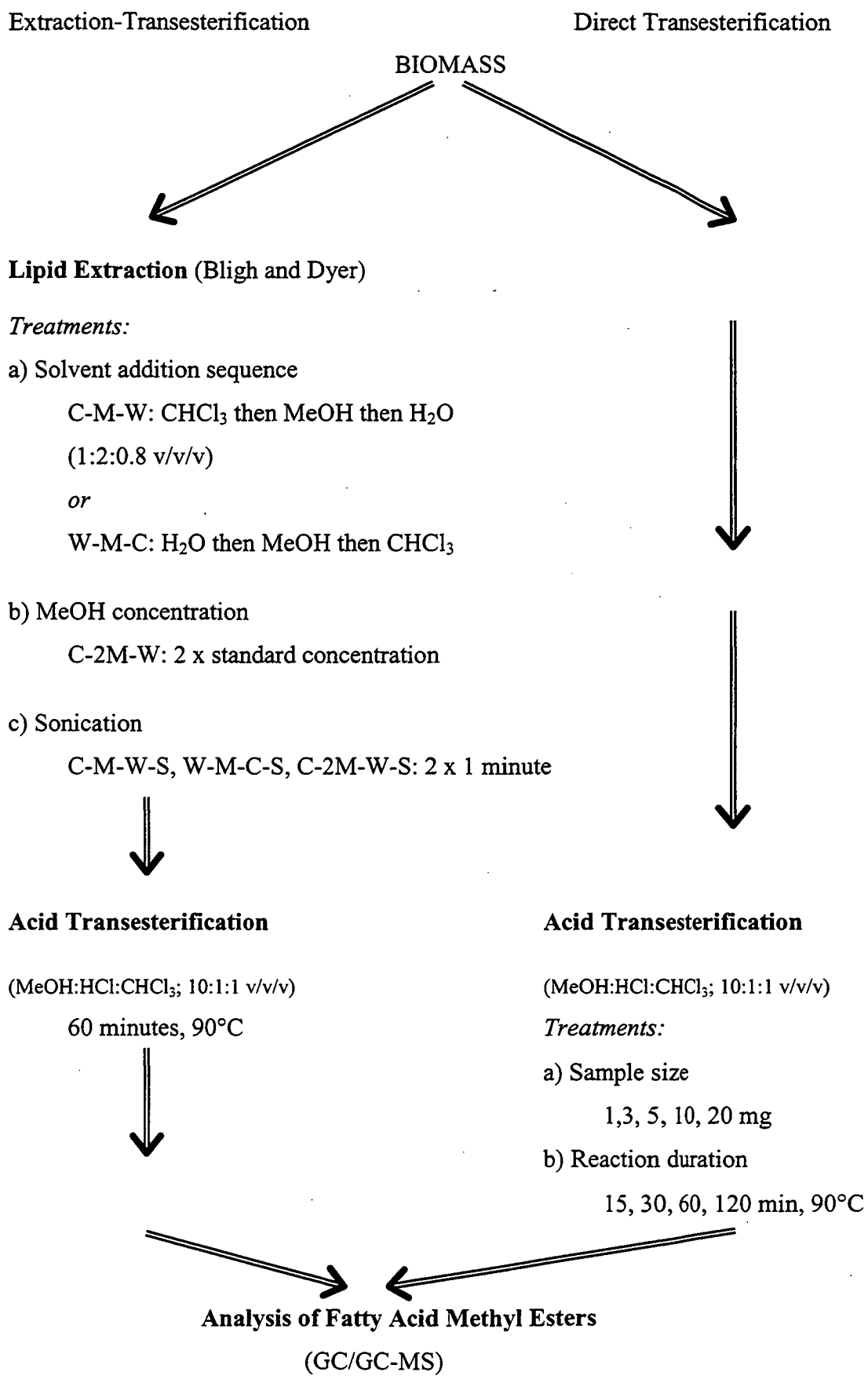

Figure 3.1 Flow diagram of lipid extraction methods

Portions of the total lipid extracts were transferred into clean 10 mL, screw-top test tubes, to which a fresh solution of methanol:hydrochloric acid:chloroform (10:1:1 v/v/v, 3 mL) was added for reaction at 90°C for 60 minutes.

3.3.3.2 Direct transesterification

Freeze-dried ACEM 6063 cells were weighed (desired weight ± 10%) into duplicate clean, 10 mL screw-top test tubes (Table 3.2), to which a fresh solution of the transesterification reaction mix [methanol:hydrochloric acid:chloroform (10:1:1 v/v/v, 3 mL)] was added. Cells were suspended in this solution by vortex mixing and immediately placed at 90°C for transesterification. Reaction times varied between 15 and 120 minutes (Table 3.2).

Freeze-dried ACEM 000A cells were weighed (50 ± 5 mg) into duplicate, clean 10 mL, screw-top test tubes, to which a fresh solution of the transesterification reaction mix was added. Cells were suspended in this solution by vortex mixing and reacted at 90°C for 60 minutes.

3.3.4 Extraction and analysis of FAME

Transesterification reaction tubes were removed from heat after the appropriate reaction time, and cooled. Water (1 mL) was then added to each tube and the FAME extracted (hexane:chloroform, 4:1 v/v, 3 x 2 mL). For gas chromatographic (GC) analysis, samples were diluted with chloroform containing a known concentration of 19:0 FAME as the internal injection standard. Analyses of the FAME were performed with a Hewlett Packard 5890 GC equipped with a 50 m x 0.32 mm internal diameter cross-linked HP5 methyl silicone (0.17 μm film thickness) fused-silica capillary column and flame ionisation detector (Nichols *et al.*, 1996). Peak areas were recorded and quantified using the software package Millenium 32 v3.05.01 (Waters Corporation, USA). Confirmation of FAME identity was performed on a MD 800 (Fisons, United Kingdom) or GCQ (Thermoquest, USA) GC-mass spectrometers (GC-MS) (Nichols *et al.*, 1996). Component identification was by comparison of retention time and MS data with that obtained for authentic and laboratory standards.

Blank controls (i.e. containing no sample) were used in each experiment. No fatty acids were recovered from any of the controls.

3.3.5 Statistical analyses

Statistical analyses of data were performed using the General Linear Models Procedure of the software package SAS System for Windows v 6.12 (SAS Institute Inc. USA). Differences between individual means were deemed to be significant at $\alpha = 0.05$.

3.4 Results

3.4.1 Extraction-transesterification experiment - strain ACEM 6063

The amount of fatty acids extracted from ACEM 6063 by various extraction-transesterification treatments is shown in Table 3.1.

The sequence in which solvents were added to samples resulted in significant differences in the amount of total fatty acids (TFA), SFA and PUFA extracted. The amount of MUFA extracted was not affected significantly by changing the solvent addition sequence (Table 3.1).

Neither sonication (2 x 1 minute) nor doubling the relative amount of methanol in the extraction solvent mixture resulted in significant differences between any of the parameters examined (Table 3.1). Similarly, there were no significant differences in the proportions of SFA, MUFA or PUFA extracted by any of the treatments (Table 3.1).

3.4.2 Direct transesterification experiment - strain ACEM 6063

The amount of fatty acids extracted from ACEM 6063 by direct transesterification is shown in Table 3.2. Variations in sample size had no significant influence on the amount of TFA, SFA, MUFA or PUFA extracted from freeze-dried biomass of ACEM 6063.

Table 3.1 Fatty acid recovery from duplicate samples of ACEM 6063 by extraction-transesterification (expressed as both mg g^{-1} dry weight and % total fatty acids)

Treatment	Solvent addition sequence	Solvent ratio (v/v)	Fatty acid recovery						
		C : M : W	sum TFA	sum SFA	sum MUFA	sum PUFA	% SFA	% MUFA	%PUFA
W-M-C	WMC [2]	1 : 2 : 0.8	261, 256 [a]	121, 118 [a]	3.6, 3.3	131, 140 [a]	47, 45	1.4, 1.2	51, 54
W-M-C-S [1]	WMC	1 : 2 : 0.8	290, 235[a]	125, 100 [a]	6.9, 2.7	158, 132 [a]	43, 43	2.4, 1.2	55, 56
C-M-W	CMW [3]	1 : 2 : 0.8	348, 352 [b]	150, 150 [b]	4.3, 4.4	186, 197 [b]	45, 43	1.2, 1.3	54, 56
C-M-W-S	CMW	1 : 2 : 0.8	333, 353 [b]	143, 162 [b]	8.3, 4.9	182, 186 [b]	42, 46	2.5, 1.4	55, 53
C-2M-W	CMW	1 : 4 : 0.8	336, 327 [b]	157, 147 [b]	4.7, 4.8	175, 174 [b]	47, 45	1.4, 1.5	52, 53
C-2M-W-S	CMW	1 : 4 : 0.8	328, 363 [b]	151, 170 [b]	6.3, 11.3	171, 182 [b]	46, 47	1.9, 3.1	52, 50

Values in each column with different superscripts are significantly different ($p<0.05$)

1: W: water; M: methanol; C: chloroform; S denotes sample sonicated for 2 x 1 minute

2: water then methanol then chloroform

3: chloroform then methanol then water

Table 3.2 Fatty acid recovery from duplicate samples of ACEM 6063 by direct transesterification (expressed as mg g^{-1} dry weight)

Treatment	Reaction time (minutes)	Nominal sample size (mg)	Fatty acid recovery		
			sum SFA	sum MUFA	sum PUFA
15/5	15	5	46, 53	1.3, 1.7	45, 52
30/1	30	1	150, 136	3.6, 3.3	157, 153
30/5	30	5	123, 118	3.7, 3.9	136, 131
30/20	30	20	120, 114	3.3, 3.2	117, 112
60/1	60	1	178, 192	4.3, 5.6	201, 218
60/3	60	3	188, 179	5.2, 4.9	225, 209
60/5	60	5	201, 151	7.8, 5.6	188, 210
60/10	60	10	214, 198	5.5, 5.4	220, 204
60/20	60	20	199, 204	5.7, 6.0	182, 208
120/1	120	1	195, 210	6.7, 5.3	157, 205
120/5	120	5	238, 207	9.0, 8.7	157, 220
120/20	120	20	206, 224	5.8, 6.4	206, 213

Reaction time significantly influenced the amount of fatty acids extracted from the biomass. Significant increases in the amount of TFA, SFA, MUFA and PUFA recovered from the biomass were observed between 15 and 30, and 30 and 60 minutes reaction time (Table 3.3). There was also a significant increase in the amount of MUFA extracted between 60 and 120 minutes reaction time. However, the amounts of TFA, SFA and PUFA extracted did not increase further nor vary significantly between 60 and 120 minutes reaction time.

The proportions of SFA and PUFA extracted from ACEM 6063 biomass by direct transesterification were not significantly affected by reaction time (Table 3.3). The proportion of MUFA extracted by this method was significantly affected by reaction

time, although no clear trend was evident (Table 3.3). It is interesting to note that the proportion of all fatty acid classes extracted by direct transesterification became more variable with increasing reaction time (Table 3.3).

Table 3.3 Fatty acid recovery from replicate samples of ACEM 6063 by direct transesterification (expressed as both mg g^{-1} dry weight and % total fatty acids)

Reaction time	Fatty acid recovery (mean±CV [1])						
	TFA	sum SFA	sum MUFA	sum PUFA	% SFA	% MUFA	% PUFA
15 (n=2) [2]	100±10 [a]	50±9 [a]	1.5±16 [a]	49±11 [a]	50±1	1.5±4 [ab]	49±1
30 (n=6)	265±12 [b]	127±11 [b]	3.5±7 [b]	134±14 [b]	48±3	1.3±12 [b]	51±3
60 (n=10)	403±5 [c]	191±9 [c]	5.6±16 [c]	206±7 [c]	47±6	1.4±17 [b]	51±6
120 (n=6)	413±7 [c]	213±7 [c]	7.0±22 [d]	193±15 [c]	52±8	1.7±23 [a]	47±10

Values in each column with different superscripts were deemed to be significantly different at α= 0.05
1: Coefficient of Variation (100 x standard deviation / mean)
2: combined data for all direct transesterification samples reacted for 15, 30, 60 or 120 minutes

3.4.3 Comparison of extraction-transesterification and direct transesterification methods

3.4.3.1 Strain ACEM 6063

Pooled data for all samples extracted by direct transesterification with a reaction time of 60 minutes was compared with that for all extraction-transesterification samples from which fatty acids were extracted by adding solvents in the sequence chloroform-methanol-water (N.B. the latter were also transesterified at 90°C for 60 minutes). The amounts of major fatty acids (those comprising > 2% TFA in most samples) recovered from ACEM 6063 are shown in Table 3.4. The amounts of TFA, SFA and PUFA extracted by direct transesterification were all significantly higher than those extracted by extraction-transesterification.

Table 3.4 Fatty acid recovery from replicate samples of ACEM 6063 by extraction-transesterification and by direct transesterification (expressed as both mg g^{-1} dry weight and % total fatty acids)

	Major [1] fatty acids								Mean fatty acid recovery						
	14:0	15:0	16:0	18:0	20:4 n6	20:5 n3	22:5 n6	22:6 n3	TFA	sum SFA	sum MUFA	sum PUFA	% SFA	% MUFA	% PUFA
extraction-transesterification [2]															
Mean (n=8)	36	3.8	109	4.2	3.9	5.0	45	115	342 [a]	155 [a]	6.1	182 [a]	45	1.8	53
CV [3]	9	5	5	12	3	5	5	5	4	6	41	5	3	38	3
direct transesterification [4]															
Mean (n=10)	48	4.8	130	4.8	4.8	6.0	50	131	403 [b]	191 [b]	5.6	206 [b]	47	1.4	51
CV	14	10	10	9	8	10	11	6	5	9	16	7	6	17	6

Values for mean fatty acid content in each column with different superscripts are significantly different ($p<0.05$)

1: fatty acids comprising >2% total fatty acids in most samples. Other fatty acids detected were: 12:0, 14:1, i15:1, i15:0, a15:0, 15:1, i16:0, 16:1n9, 16:1n7, 16:1n7t, 16:1n5, 17:0, 18:3n6, 18:4n3, 18:2n6, 18:1n9, 18:1n7, 20:3n6, 20:4n3, 20:2n6, 20:0, 22:4n6, 22:5n3, C22PUFA

2: combined data for all extraction-transesterification samples extracted by adding solvents in the sequence chloroform:methanol:water

3: Coefficient of Variation (100 x standard deviation / mean)

4: combined data for all direct transesterification samples reacted for 60 minutes

Neither the amount of MUFA extracted, nor the proportions of SFA, MUFA or PUFA in the extracts varied significantly between these two methods (Table 3.4).

Co-efficients of variation (CV) of data obtained from the two groups of pooled data showed that the amounts of TFA, SFA and PUFA extracted were less variable than the amounts of MUFA extracted. The CV for MUFA extracted by direct transesterification was less than that for MUFA extracted by extraction-transesterification. The CV's for TFA, SFA and PUFA were all slightly higher in the direct transesterification experiment than in the extraction-transesterification experiment (Table 3.4).

3.4.3.2 Strain ACEM 000A

The amounts of major fatty acids (those comprising > 2% TFA in most samples) recovered from ACEM 000A are shown in Table 3.5. Samples from both 3-day old (low lipid) and 5-day old (high lipid) cultures extracted by direct transesterification yielded significantly more TFA, SFA, MUFA and PUFA than those from which lipid was extracted prior to transesterification.

There were also significant differences in the proportion of MUFA and PUFA, but not SFA extracted from ACEM 000A by the two methods. Fatty acids recovered from ACEM 000A by direct transesterification contained proportionally more MUFA and less PUFA than those recovered by extraction-transesterification (Table 3.5).

3.5 Discussion

Results from this study indicate that deviations from a standard lipid extraction method (Bligh and Dyer, 1959) significantly influenced the amount of fatty acids recovered from freeze-dried ACEM 6063 and ACEM 000A biomass.

The amount of TFA, SFA and PUFA extracted from ACEM 6063 by various extraction-transesterification methods was significantly affected by the sequence in which solvents were added to the samples. Adding solvents in the order chloroform-methanol-water resulted in an increase in extraction efficiency when compared to adding the solvents in the reverse order.

Table 3.5 Fatty acid recovery from duplicate samples of 3- (low lipid) and 5- (high lipid) day old cultures of ACEM A by extraction-transesterification and by direct transesterification (expressed as both mg g^{-1} dry weight and % total fatty acids)

	Major [1] fatty acids											Fatty acid recovery						
Culture age (days)	14:0	16:1 n7	16:0	18:2 n6	18:1 n9	18:0	20:4 n6	20:5 n3	20:2 n6	22:6 n3	22:5 n3	TFA	sum SFA	sum MUFA	sum PUFA	% SFA	% MUFA	% PUFA
extraction-transesterification																		
3	0.6, 0.7	0.6, 0.5	7.1, 8.2	0.6, 0.6	0.8, 0.9	1.3, 1.7	1.3, 1.1	5.3, 5.1	0.6, 0.7	3.3, 3.1	1.2, 1.4	25, 24^{c}	11, 9.0^{c}	1.5, 1.3^{d}	13, 13^{c}	43, 38^{ab}	5.8, 5.7^{d}	52, 57^{c}
5	2.5, 2.0	2.2, 1.7	16, 13	5.8, 4.5	8.9, 7.3	2.9, 2.9	2.7, 2.0	12, 10	1.1, 2.1	6.1, 4.9	3.1, 2.6	56, 66^{b}	18, 21^{b}	9.8, 11^{b}	28, 33^{b}	32, 32^{a}	18, 17^{b}	50, 51^{bc}
direct transesterification																		
3	3.4, 3.1	1.2, 1.3	20, 19	1.7, 1.8	3.3, 2.9	6.3, 4.8	2.2, 1.7	8.3, 8.9	2.3, 2.1	6.4, 6.4	4.2, 4.3	58, 63^{b}	27, 28^{b}	4.2, 6.2^{c}	27, 27^{b}	46, 47^{b}	7.2, 9.8^{c}	47, 43^{ab}
5	6.6, 8.8	4.0, 5.2	33, 44	14, 18	24, 32	9, 12	3.6, 4.9	17, 22	6.5, 8.2	7.6, 9.6	7.1, 8.7	181, 137^{a}	66, 49^{a}	38, 28^{a}	77, 59^{a}	37, 36^{a}	21, 21^{a}	42, 43^{a}

Values for mean TFA, SFA, MUFA and PUFA content in each column with different superscripts are significantly different ($p<0.05$)

1: fatty acids comprising >2% total fatty acids in most samples. Other fatty acids detected below this value were: 12:0, 15:0, 16:1n9, 16:1n5, 16:1, 17:0, 18:3n6, 18:3n3, 18:1n7, 20:3n6, 20:4n3, 20:1n11,9, 20:1n7, 20:0, 22:5n6, 22:4n6, C22PUFA

Similar results have been reported by Smedes and Askland (1999). These authors suggested that increased efficiency by some multi-step extractions (i.e. those in which the non-polar component/s of a solvent system are mixed with samples before the polar components) could be explained in terms of cell disruption prior to lipid extraction. Initial contact with the non-polar solvents could weaken the association between lipids and cell structures, prior to lipid dissolution in the mono-phasic system. Indeed, the presence of methanol in these solvent systems disrupts lipid-protein bonds, as well as allowing the chloroform (the effective lipid solvent) to wet the sample (Lovern, 1942; Lovern, 1965). Dunstan *et al.* (1993) reported an increased lipid recovery from freeze-dried oyster tissue following rehydration of the tissue prior to extraction by the Bligh and Dyer method, although freeze-drying had no apparent influence on the fatty acid profile of the lipid extract.

In the extraction-transesterification experiment using heterotrophic microorganisms, neither sonication nor doubling the proportion of methanol in the solvent mixture significantly affected the amounts of SFA or PUFA extracted. In contrast, Smedes and Askland (1999) found the effect of solvent addition sequence on extraction of lipid from fresh fish was over-ridden by increasing the proportion of methanol in the extraction mixture. This difference could be due to differences in cell structure of the two types of biomass and/or differences in sample treatment before analysis [i.e. freeze-dried thraustochytrid biomass in this study; fresh fish flesh in Smedes and Askland (1999)].

The amount of MUFA recovered in the extraction-transesterification experiment was not affected significantly by any of the variations used. This lack of correlation appears to be due to the relatively high variability in the MUFA data. However, the proportion of MUFA in the sample was relatively minor and had little effect on the overall lipid yield. For experiments where the proportion of MUFA in the sample is of critical importance to the interpretation of results, care should be taken to select a method that efficiently extracts MUFA from all samples.

When freeze-dried cells of strain ACEM 6063 were directly transesterified, variations in sample size (within the range examined in this study) did not significantly influence the total amount or the relative proportion of fatty acids recovered. This also indicated the freeze-dried biomass was quite homogenous.

It is apparent, however, that the duration of the transesterification reaction had a significant effect on the amount of fatty acids extracted. Data indicated that a reaction time of 60 minutes at 90°C was sufficient to extract the vast majority of SFA and PUFA from these samples. Although a reaction time of 60 minutes has been used frequently (e.g. Bohnert *et al.*, 1997; Sonnichsen and Muller, 1999), it is evident that optimum reaction time is sample specific. Rodriguez-Ruiz *et al.* (1998) reported similar lipid recovery after transesterification reaction times of 60 and 10 minutes, for both cod-liver oil and freeze-dried macroalgae. However, Garces and Mancha (1993) increased lipid recovery from sunflower seeds with increasing reaction times up to two hours.

The proportion of SFA and PUFA, and individual fatty acids from these two groups, extracted from ACEM 6063 by direct transesterification did not vary significantly, indicating that there was no selective extraction of these two fatty acid classes attributable to different reaction times.

The recovery of MUFA from strain ACEM 6063 by direct transesterification increased steadily between 15 and 120 minutes reaction time, and did not plateau at 60 minutes reaction time. The proportion of MUFA also increased significantly between reaction times of 15 and 120 minutes. This indicated that the MUFA-containing lipid in these samples was not as easily extracted as the SFA- or PUFA-containing lipid, and that a reaction time of at least 120 minutes was necessary to allow full transesterification of MUFA from these samples.

For the two strains of thraustochytrid examined, greater recovery of fatty acids was achieved using direct transesterification than by using extraction-transesterification. These observations support the suggestion by Smedes and Askland (1999) that cell disruption plays a role in the efficiency of lipid extraction from some samples. The transesterification reaction mix used in this study contained about 8% (v/v) chloroform and was highly acidic: conditions that could be expected to cause severe disruption to cell integrity (Lovern, 1942; Bligh and Dyer, 1959; Lovern, 1965).

3.5.1 Conclusion

This study has demonstrated that the total amount of fatty acids recovered from two lipid-producing thraustochytrids increased by about 30% by adding solvents to the biomass in order of increasing, as opposed to decreasing, polarity.

It has also been shown that by directly transesterifying the thraustochytrid biomass, instead of transesterifying a portion of extracted lipid, the total amount of fatty acids extracted was significantly greater than for the most efficient extraction-transesterification extraction method. This increase in extraction efficiency differed between the two thraustochytrid strains examined, and varied from 15-20% for strain ACEM 6063 and about 150% for strain ACEM 000A.

One consequence of using direct transesterification for fatty acid analyses is that lipid class data cannot be obtained. For samples where lipid class data is required, extraction of the lipid fraction prior to transesterification is obviously required. Biotechnologists and analysts should be aware, however, that if a portion of the total lipid extract is subsequently used for transesterification and fatty acid analysis, the data could underestimate the true fatty acid content of the biomass.

Results show that the efficiency of lipid recovery from different thraustochytrids can vary substantially between extraction techniques. This is an important observation for researchers involved in the field of microheterotrophic fatty acid production and analysis. It appears that, given the wide variation in extraction efficiencies revealed by these results, meaningful data for other microheterotrophs under consideration for biotechnological applications will require careful choice and validation of analysis methodology.

Chapter 4 Biomass and lipid production by thraustochytrid strain ACEM 6063: influence of physical and chemical parameters

4.1 Abstract

Thraustochytrid strain ACEM 6063, which produces lipid rich in n3 PUFA, was grown under different culture conditions to ascertain their influence on biomass and lipid production. Biomass production increased with initial glucose concentration up to 200 g L^{-1}, initial sea salt concentration up to 32 g L^{-1}, culture temperature up to 25°C and when fermenter impeller speed was set to a maximum of 200 rpm, as opposed to a maximum of 750 rpm. Lipid content increased as cultures approached peak biomass. Lipid content also increased with increasing culture temperature up to 25°C, initial glucose concentration up to 100 g L^{-1}, initial sodium glutamate concentrations up to 10 g L^{-1} and with dissolved oxygen (DO) concentration up to > 40% saturation. The predominant lipid classes in oil from ACEM 6063 were triacylglycerol and phospholipid. As cultures aged, the proportion of triacylglycerol increased to about 90% of the total lipid. Increasing DO levels from < 5% to > 40% saturation substantially decreased the proportion of saturated fatty acids and substantially increased the proportion of monounsaturated fatty acids in ACEM 6063 biomass. Culture conditions were identified under which a 7 day old ACEM 6063 cultures could be grown to produce up to 23 g dry weight L^{-1}, 45% (dry weight) lipid and 10% (dry weight) DHA. This level of DHA production is amongst the highest reported for thraustochytrids.

4.2 Introduction

The increasing interest in the nutritional role of n3 fatty acids discussed in Chapter 1 is fuelling research into novel and sustainable sources of these fatty acids. Recent studies have shown that one group of microheterotrophs, the thraustochytrids, can be cultured to produce high biomass, containing substantial amounts of lipid rich in a number of polyunsaturated fatty acids (PUFA), including DHA. Thraustochytrids are a monophyletic protist group, and are taxonomically aligned to heterokont algae (Cavalier-Smith *et al.*, 1994; Honda *et al.*, 1999). Thraustochytrids are commonly isolated from marine environments (Raghukumar *et al.*, 1990; Raghukumar *et al.*, 2000). Although generally considered to have a obligate requirement for Na^+ (Siegenthaler *et al.*, 1967; Amon, 1978), at least one strain has been reported to grow in media containing negligible amounts of Na^+ (Yokochi *et al.*, 1998).

Chemical and physical factors previously shown to influence biomass and lipid production by several thraustochytrid strains include: medium composition, batch feeding, salinity, initial medium pH, inoculum age, culture age, temperature, illumination, culture vessel (flask, fermenter) and fermenter impeller shape and speed (see Chapter 1).

As the number of reports regarding thraustochytrids has increased, it has become apparent that biomass and lipid production by these organisms is strain specific. However, there is only limited data regarding the systematic evaluation of the influence of culture manipulation of fatty acid production by thraustochytrids. From the little data presented to date, it appears that strain selection will be an important factor in determining the commercial potential of thraustochytrids (Bowles *et al.*, 1999). The response of thraustochytrid strains to variations in culture conditions also appears to be strain specific, indicating that optimisation of PUFA production will require individual attention to be given to each promising strain or group of closely related strains. In this Chapter, the influence of physical and chemical culture parameters on biomass and lipid production by thraustochytrid strain ACEM 6063 are examined.

4.3 Materials and Methods

4.3.1 Strain isolation and maintenance

Thraustochytrid strain ACEM 6063 was isolated from marine sediments in southeastern Tasmania (Australia), and maintained by monthly subculture as described in Section 2.3.1 (page 19).

4.3.2 Culture experiments

A series of culture experiments were performed to examine the influence of physical and chemical culture parameters on biomass and lipid production by ACEM 6063. Initial experiments to examine the influence of culture age, media composition and inoculum characteristics were performed in 500 mL screw top Erlenmeyer flasks. Subsequent experiments to examine the influence of glucose concentration, physical culture conditions and dissolved oxygen levels were performed in a BioFlo IIC fermenter (New Brunswick, USA). Methodology for these experiments is detailed in Sections 4.3.2.1 and 4.3.2.2, below.

4.3.2.1 Flask culture experiments

Inocula for all flask culture experiments were prepared using the following method. A maintenance culture of strain ACEM 6063 was inoculated onto TCM agar and incubated at 20°C for 5 days. Once strain purity had been confirmed, actively growing cells were used to inoculate 150mL of TCM, modified as appropriate, in 500 mL screw top Erlenmeyer flasks. Flasks were incubated at 20°C on a rotary-shaking table (GIO Gyrotary Shaker, New Brunswick, USA) at 200 rpm. During incubation, flask lids were kept loose to facilitate gas exchange. After incubation for the appropriate time, culture purity was confirmed by light microscopy, and the culture immediately used to inoculate experimental media.

Experimental cultures were grown in flasks as described above. All cultures were sampled as required by aseptically drawing 10 mL from each flask and transferring the sample directly into sterile, 15 mL screw-top plastic centrifuge tubes (Greiner, Germany). Cells were immediately centrifuged (5000 x g, 10 min: EasySpin, Sorval Instruments, USA), resuspended in 10 mL 1.0% (w/v) NaCl, and recentrifuged. Cell pellets were frozen, freeze-dried (chamber temperature -110°C, Mini Ultra Cold,

Dynavac Australia; pressure < 7 x 10^{-1} mbar, RV3 vacuum pump, Edwards High Vacuum International) for 15-20 h, weighed and then stored at -30°C.

Biomass, calculated as g dry weight L^{-1}, was determined using the following equation, which allowed for the dry weight of the small volume of NaCl wash solution still associated with the centrifuged cells.

$$\text{Biomass (g dry weight } L^{-1}) = 1000 * \{[T_{dw} - ((T_{ww} - T_{dw}) * W_{dw})] / S_{vol}\}$$

Where: T_{dw} = total dry weight of sample (g)

T_{ww} = total wet weight (centrifuged) of sample (g)

W_{dw} = dry weight of 1 mL of the NaCl wash solution (g)

S_{vol} = sample volume (mL)

Flask Experiment 1: Initial glucose concentration and culture age

Ten flasks (5 treatments, in duplicate) containing 150 mL of TCM supplemented to contain 10, 50, 100, 150 and 200 g L^{-1} glucose were inoculated with 5% (v/v) inoculum, prepared as described on page 61. Flasks were incubated on a rotary shaker (200 rpm) at 20°C. Cells were sampled regularly until peak biomass had been reached.

Flask Experiment 2: Sea salt concentration

Four flasks containing 150 mL of TCM, modified to contain 1, 8, 15 and 30 g L^{-1} sea salts (S9883: Sigma, USA), were inoculated (5% v/v) as described on page 61, and incubated on a rotary shaker (200 rpm) at 20°C. Each flask was sampled regularly until peak biomass had been reached.

Flask Experiment 3: Glucose and sodium glutamate concentration

Thirty two flasks (16 treatments, in duplicate) containing 150 mL of TCM, modified to contain all combinations of 50, 100, 150 and 200 g L^{-1} glucose and 1, 2, 5 and 10 g L^{-1} sodium glutamate, were inoculated with 5% (v/v) inoculum. For this experiment, two flasks of inoculum were prepared as described on page 61. The contents of these flasks were combined prior to use to provide a homogenous

inoculum. Experimental flasks were incubated on a rotary shaker (200 rpm) at 20°C for 10 days.

Flask Experiment 4: Inoculum size and age

Twenty four flasks (12 treatments, in duplicate) containing 150 mL of TCM, modified to contain 100 g L^{-1} glucose, were inoculated with 1, 5 and 10% (v/v) of 1-, 2-, 5- and 10-day old inocula, prepared as described on page 61. Two inoculum cultures were used for this experiment. Each inoculum culture was used to inoculate one of the duplicate flasks for each treatment on each day. Flasks were incubated on a rotary shaker (200 rpm) at 20°C for 10 days.

4.3.2.2 Fermenter culture experiments

Fermenter culture experiments were conducted in a BioFlo IIC fermenter (New Brunswick, USA). Two, four-bladed paddle impellers (tip diameter 6.2 cm), were used to mix the fermenter cultures. Both impellers were attached to a central axle, which in turn was connected to the stirring motor. Impellers were situated on the axle at $^1/_3$ and $^2/_3$ the depth of the culture medium in the fermenter chamber.

Inocula for fermenter culture experiments were prepared as described for flask culture experiments in Section 4.3.2.1 (page 61). Inocula (5% v/v) were aseptically transferred into the fermenter using sterile disposable syringes fitted with large bore needles (18 gauge, Terumo, USA). Inocula were drawn into, and expelled from, the syringes very slowly, to minimise the possibility of cell damage due to pressure changes and/or shear forces. Culture temperatures were controlled to within 1°C using a microprocessor-controlled water jacket. Dissolved oxygen levels were controlled semi-automatically, using a combination of impeller revolution speed (via microprocessor-controlled feedback from the oxygen probe) and air/oxygen supply (manual control). Aeration, at a maximum flow rate of 1.0 L min^{-1}, was supplied using 0.2 µm-filtered air (Midisart 2000, Sartorius, Germany) for low DO cultures, or with a manually-controlled mixture of air and oxygen (Industrial Grade O_2, BOC Gases, Australia) for high DO cultures. All gas supplied to the fermenter was bubbled through sterile distilled water, to minimise evaporative losses from the culture media, before delivery to the fermenter culture chamber via four 0.5 mm diameter holes situated at the base of the culture vessel. Sterile antifoam solution

(Foamaster LQ217, Henkel, Australia) was added automatically as required. A maximum of about 1 mL of antifoam was used in each fermenter culture.

Culture conditions for each fermenter experiment are shown in Table 4.1. It should be noted that the fermenter culture system used was not able to supply sufficient oxygen to maintain high DO levels in ACEM 6063 cultures grown at 25°C.

During each fermenter experiment, samples (typically 10 mL) were aseptically collected through the fermenter sample port. Cells were centrifuged, freeze-dried, weighed and stored as described in Section 4.3.2.1.

4.3.3 Lipid extraction, fractionation and analysis

Two procedures were used to extract lipids from freeze-dried ACEM 6063 biomass: the extraction of lipids using a modified (White *et al.*, 1979; Chapter 3) Bligh and Dyer (1959) solvent extraction; and the direct transesterification of biomass to produce fatty acid methyl esters (FAME) (i.e. without the initial extraction step) (as discussed in Chapter 3). Direct transesterification was used to obtain fatty acid data. Total lipid extracts obtained using the modified Bligh and Dyer extraction method were used to obtain lipid class data.

Table 4.1 Culture conditions used in ACEM 6063 fermenter culture trials

Treatment code	Initial glucose concentration ($g\ L^{-1}$)	Dissolved oxygen [1]	Temperature (°C)	Maximum impeller speed [2]
10H20H	10	High	20	High
50H20H	50	High	20	High
50L20L	50	Low	20	Low
50H20L	50	High	20	Low
100L15L	100	Low	15	Low
100H15L	100	High	15	Low
100L20L	100	Low	20	Low
100H20L	100	High	20	Low
100L25L	100	Low	25	Low

1: Dissolved oxygen: High (H) = > 40% saturation, Low (L) = < 5% saturation
2: Maximum impeller speed: High (H) = 750 rpm, Low (L) = 200 rpm

4.3.3.1 Fatty acid analyses

Freeze-dried cells were weighed into clean 10 mL screw-top test tubes to which a fresh solution of the transesterification reaction mix [methanol:hydrochloric acid:chloroform (10:1:1 v/v/v, 3 mL)] was added. Cells were suspended in this solution by vortex mixing and immediately placed at 90°C for transesterification. Tubes were removed from heat after 60 minutes and cooled. Water (1 mL) was then added to each tube, and the FAME extracted (hexane:chloroform, 4:1 v/v, 3 x 2 mL). For gas chromatographic (GC) analysis, samples were diluted with chloroform containing a known concentration of 19:0 FAME as the internal injection standard. Analyses of the FAME were performed with a Hewlett Packard 5890 GC equipped with a 50 m x 0.32 mm internal diameter, cross-linked, HP5 methyl silicone (0.17 μm film thickness) fused-silica capillary column and flame ionisation detector (Nichols *et al.*, 1996). Peak areas were recorded and quantified using the software package Millenium 32 v3.05.01 (Waters Corporation, USA). Confirmation of FAME identity was performed on MD 800 (Fisons, United Kingdom) or GCQ (Thermoquest, USA) GC-mass spectrometers (GC-MS) (Nichols *et al.*, 1996). Component identification was by comparison of retention time and MS data with that obtained for authentic and laboratory standards.

Blank controls (i.e. containing no sample) were used in each experiment. No fatty acids were recovered from any of the controls.

4.3.3.2 Lipid class analyses

Lipids were extracted from freeze-dried biomass using a one-phase chloroform-methanol-water (1:2:0.8 v/v/v) extraction. Solvents were added to the freeze-dried biomass in order of increasing polarity, as this technique increased lipid recovery from ACEM 6063 biomass (as discussed in Chapter 3). The biomass-solvent mixtures were left overnight. Phase separation was achieved the next day by adding chloroform and water to obtain a final chloroform-methanol-water ratio of 1:1:0.9 (v/v/v). Lipids were recovered from the lower chloroform phase. Solvents were removed under vacuum prior to the extracted lipids being stored in chloroform under N_2 at -20°C.

Lipid class data 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). Aliquots (1 μL) from a known volume of each extract were applied onto duplicate Iatroscan chromarods using disposable micro-pipettes. Chromarods were developed using a hexane - diethyl ether- acetic acid (60:17:0.2 v/v/v) solvent system in a sealed glass tank containing pre-extracted filter paper. After development, the chromarods were oven-dried at 60°C for 10 min and analysed immediately. Peak areas were recorded and quantified using the chromatography software DAPA (Scientific Software, Kalamunda, Western Australia).

4.3.4 Statistical analyses

Statistical analyses of data were performed using the General Linear Models Procedure of the software package SAS System for Windows v 6.12 (SAS Institute Inc. USA). Differences between individual means were deemed to be significant at $\alpha = 0.01$.

4.4 Results

Profiles of the major fatty acid components of ACEM 6063 oil have been provided in Chapter 2 and Chapter 3. These profiles were largely unchanged by the culture conditions used in the current Chapter. Hence, the fatty acid data presented in this Chapter is that for the proportions of SFA, MUFA, PUFA and DHA (%TFA), and the total fatty acid content (mg g^{-1} dry weight) of ACEM 6063 cultures.

4.4.1 Flask culture experiments

4.4.1.1 Flask Experiment 1: Initial glucose concentration and culture age

Maximum initial growth rates of flask-cultured ACEM 6063 in which the culture medium was 10, 50, 100 or 150 g L^{-1} were very similar to each other (~2 g L^{-1} d^{-1}), while that of the culture containing an initial glucose concentration of 200 gL^{-1} was slightly lower (~1.7 g L^{-1} d^{-1}) (Figure 4.1).

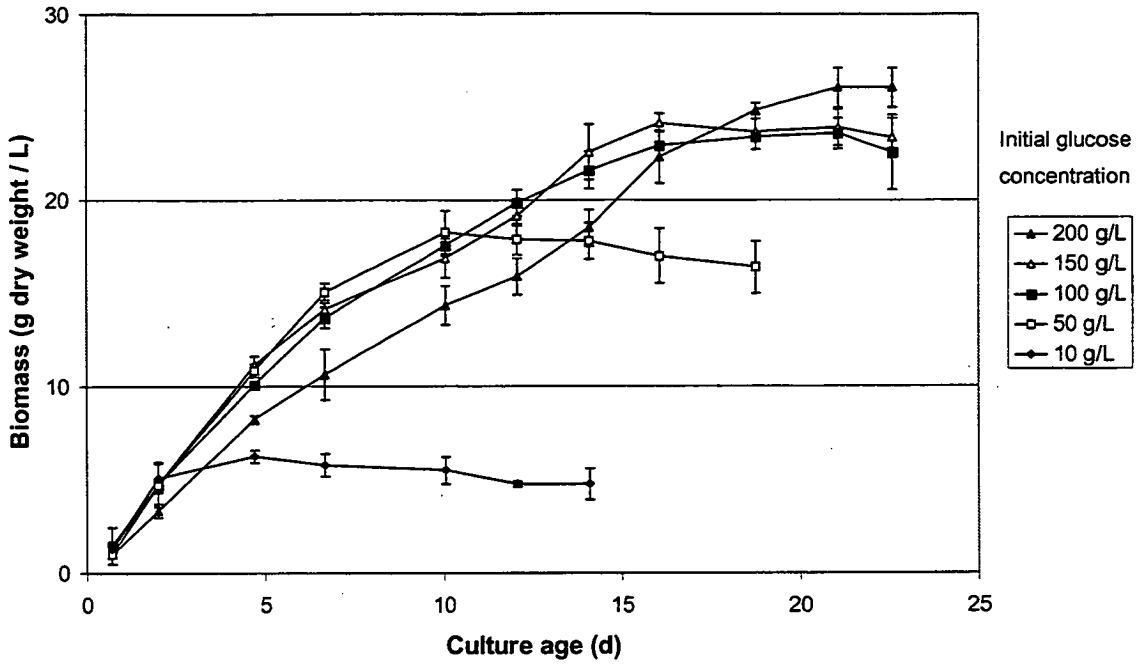

Figure 4.1 Biomass production by flask-cultured ACEM 6063 grown in TCM broth in response to variations in initial glucose concentration (mean±SD: n=2)

Initial glucose concentration substantially influenced the peak biomass obtained by the flask cultures (Figure 4.1). Mean (n=2) peak biomasses for cultures in which the initial glucose concentration was 10, 50, 100, 150 and 200 g L^{-1} were 6, 18, 23, 24 and 26 g dry weight L^{-1} respectively. The mean (n=2) age of these cultures when they reached peak biomass were 5, 10, 16, 16 and 21 days respectively (Figure 4.1). There was little variation in either biomass or fatty acid content between duplicate flasks.

The relative proportions of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) in flask-cultured ACEM 6063 cells at peak biomass were not substantially influenced by initial glucose concentration (Table 4.2). The proportions of these fatty acid classes in ACEM 6063 biomass were, however, influenced by culture age (Table 4.2).

Table 4.2 Fatty acid content and proportion of fatty acid classes and DHA of duplicate flask cultures of ACEM 6063 as influenced by initial glucose concentration and culture age

Initial glucose concentration ($g\ L^{-1}$)	Culture age (d)	Fatty acids (% total fatty acids [1])				Total fatty acids (mg g^{-1} dry weight)
		sum SFA	sum MUFA	sum PUFA	DHA	
10	2	24, 26	2.1, 2.2	68, 70	59, 63	72, 78
	5*	36, 41	1.7, 2.0	57, 60	38, 42	87, 93
	10	38, 44	1.4, 1.6	54, 59	35, 39	81, 89
50	2	24, 28	3.7, 4.9	66, 70	55, 57	82, 88
	5	32, 38	0.5, 1.3	57, 61	40, 43	118, 136
	10*	42, 46	0.8, 1.7	51, 53	34, 36	225, 241
	14	43, 47	1.5, 2.0	50, 53	32, 36	272, 283
100	2	23, 28	1.3, 1.4	58, 66	53, 57	102, 117
	5	43, 47	0.7, 1.1	52, 57	35, 39	268, 284
	12*	39, 44	1.5, 2.0	54, 60	34, 39	366, 382
	16	43, 47	1.0, 1.8	51, 56	29, 33	407, 424
150	2	25, 33	1.1, 2.2	66, 71	56, 65	78, 92
	5	39, 48	1.9, 2.3	46, 58	34, 46	217, 233
	16*	36, 45	1.3, 2.1	48, 59	26, 36	379, 400
	19	40, 49	1.1, 2.0	47, 53	22, 34	393, 409
200	2	28, 39	0.6, 1.0	58, 72	54, 62	83, 95
	5	41, 52	0.1, 0.9	43, 53	37, 46	255, 276
	21*	39, 48	0.8, 1.4	47, 57	27, 36	386, 404

*: day on which peak biomass was reached
1: unidentified fatty acids have not been included

The proportion of SFA increased from 25-33% of the total fatty acids (TFA) in 2-day old cultures, to 38-44 %TFA on the day each culture reached peak biomass. The proportion of MUFA was very low in all samples, and varied between 0.5 and 4.2 %TFA. There was no apparent correlation between the proportion of MUFA in the samples and culture age (Table 4.2). The relative proportions of PUFA and DHA decreased with culture age, but did not appear to be influenced by initial glucose concentration. PUFA levels decreased from 62-69 %TFA in 2-day old cultures to 52-58 %TFA at peak biomass. Similarly, DHA levels decreased from 55-61 %TFA in 2-day old cultures to 31-40 %TFA at peak biomass. Higher levels of DHA at peak biomass were seen in those cultures that reached peak biomass earlier (Table 4.2). Fatty acid content of ACEM 6063 biomass was influenced by both culture age and initial glucose concentration. The TFA content of biomass increased with culture age, ranging from 75-109 mg g^{-1} dry weight in 2-day old cultures to 90-417 mg g^{-1} dry weight at or just after reaching peak biomass. Fatty acid content at peak biomass increased as initial glucose concentrations increased from 10-100 g L^{-1}, with the maximum of 417 mg g^{-1} occuring at 100 g L^{-1}. The TFA content then plateaued, with no further increase observed at initial glucose concentrations of 150 and 200 g L^{-1} (Table 4.2).

4.4.1.2 Flask Experiment 2: Sea salt concentration

Maximum growth rates of flask-cultured ACEM 6063 in which the sea salt concentration in the culture medium was 4, 8, 16 and 32 g L^{-1} were very similar to each other (~ 2 g L^{-1} d^{-1}) (Figure 4.2). Sea salt concentration, however, clearly influenced the peak biomass obtained by these cultures. Peak biomass achieved by cultures in which the sea salt concentration was 4, 8, 16 and 32 g L^{-1} were 4, 12, 18 and 23 g dry weight L^{-1} respectively. The age of these cultures when they reached peak biomass were 2, 6, 12, and 14 days respectively (Figure 4.2).

The proportion of SFA in cultures at peak biomass tended to increase (32-49 %TFA) with increasing sea salt concentration, while the proportions of PUFA and DHA tended to decrease (64-44 %TFA and 41-29 %TFA respectively) (Table 4.3).

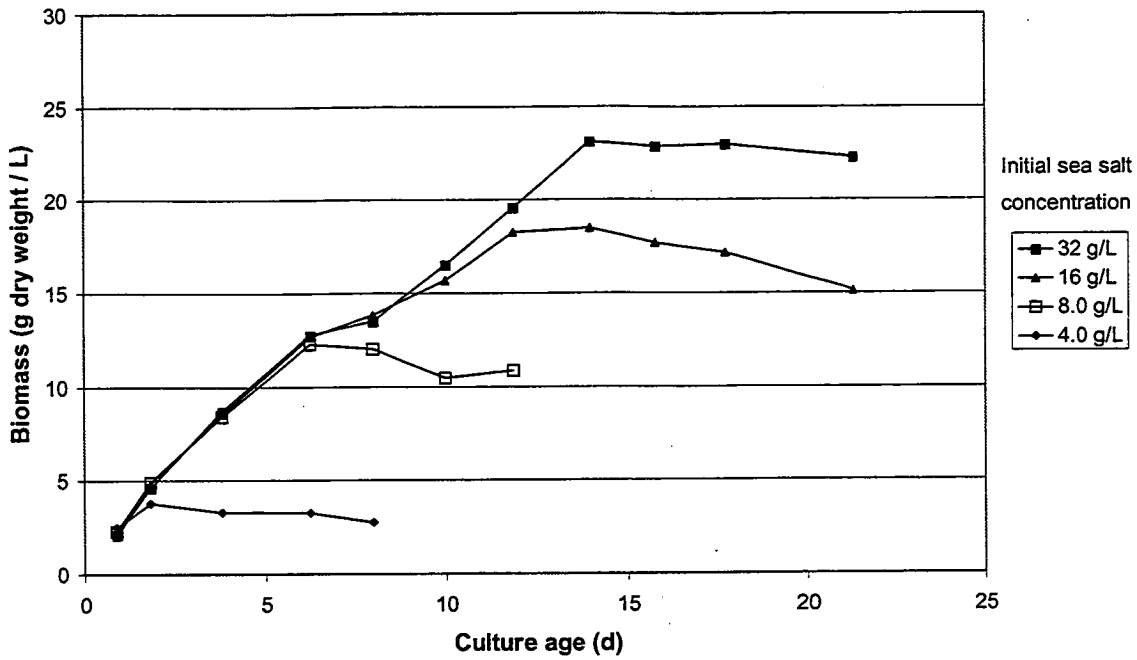

Figure 4.2 Biomass production by flask-cultured ACEM 6063 grown in TCM broth in response to variations in initial sea salt (S9883: Sigma, USA) concentration

Table 4.3 Fatty acid content, proportion of fatty acid classes and DHA, and biomass at peak biomass in flask-cultured ACEM 6063 in response to different sea salt concentrations

Initial sea salt [1] concentration ($g\ L^{-1}$)	Culture age (d)	Fatty acids (% total fatty acids [2])				Total fatty acids ($mg\ g^{-1}$ dry weight)
		sum SFA	sum MUFA	sum PUFA	DHA	
4	2	32	2.0	64	41	85
8	6	44	0.5	52	30	237
16	12	38	8.0	52	31	317
32	14	49	2.9	44	29	341

1: S9883: Sigma, USA
2: unidentified fatty acids have not been included

The fatty acid content of ACEM 6063 biomass at the time each culture reached peak biomass also increased with increasing sea salt concentration (85-341 mg g^{-1} dry weight) (Table 4.3). These trends are consistent with those observed in relation to culture age in Flask Experiment 1.

4.4.1.3 Flask Experiment 3: Glucose and sodium glutamate concentration

The relative proportions of SFA, MUFA, PUFA and DHA (%TFA), the fatty acid content (mg g^{-1} dry weight), and the absolute biomass (g dry weight L^{-1}) in 10-day old flask cultures of ACEM 6063, grown with different initial concentrations of glucose and sodium glutamate, are shown in Table 4.4. Initial glucose concentration had a slight, but statistically significant, effect on the relative proportions of SFA and MUFA in the biomass. The influence of initial glucose concentration on the absolute biomass of these 10-day old flask cultures was also significant (Table 4.5). There was, however, no obvious trend in the relationship between SFA levels and initial glucose concentration. The relative proportion of MUFA in cultures that received an initial glucose concentration of 200 g L^{-1} was significantly higher than in the other cultures (3.3 %TFA c.f. ~1.5 %TFA). The absolute biomass in 10-day old cultures grown with an initial glucose concentration of 200 g L^{-1} was significantly lower than in the other cultures (9.0 g dry weight L^{-1} c.f. ~15 g dry weight L^{-1}) (Table 4.5). Statistical analysis detected no significant interaction between the effects of initial glucose and sodium glutamate concentrations on any of the production parameters shown in Table 4.5.

Initial sodium glutamate concentration had a slight, but statistically significant, effect on the relative proportions of SFA, MUFA, PUFA and DHA in ACEM 6063 biomass. Initial sodium glutamate concentration also influenced the absolute fatty acid content of the biomass and absolute biomass in each flask (Table 4.6). The relative proportion of SFA in the biomass in 10-day old cultures with an initial sodium glutamate concentration of 1 g L^{-1} (60 %TFA) was higher than in those with an initial sodium glutamate concentration of 5, 10 and 50 g L^{-1} (51-56 %TFA). The proportion of MUFA in biomass varied between 1.3 and 2.3 %TFA, but with no obvious trend in relation to initial sodium glutamate concentration.

Table 4.4 Fatty acid content, proportion of fatty acid classes and DHA, and biomass of duplicate 10-day old flask cultures of ACEM 6063 in response to different initial glucose and sodium glutamate concentrations

Initial glucose ($g L^{-1}$)	Initial sodium glutamate ($g L^{-1}$)	Fatty acids (% total fatty acids [1])				Total fatty acids (mg g^{-1} dry weight)	Biomass (g dry weight L^{-1})
		sum SFA	sum MUFA	sum PUFA	DHA		
50	1	59, 63	1.6, 1.7	33, 34	20, 21	189, 225	11, 14
50	5	51, 53	1.2, 1.4	42, 44	25, 27	213, 231	12, 18
50	10	52, 55	1.0, 1.1	39, 40	24, 26	176, 203	16, 20
50	50	54, 56	1.4, 1.6	38, 40	21, 23	124, 127	14, 15
100	1	58, 59	2.2, 2.3	36, 38	22, 23	248, 257	13, 14
100	5	49, 52	1.3, 1.4	42, 44	27, 28	205, 217	16, 17
100	10	53, 53	1.1, 1.2	39, 40	23, 26	188, 215	14, 17
100	50	53, 54	1.3, 1.7	39, 40	21, 25	126, 143	12, 18
150	1	58, 61	1.1, 1.3	35, 37	20, 22	207, 240	10, 12
150	5	57, 58	1.7, 1.8	36, 37	22, 24	191, 224	13, 18
150	10	56, 57	1.2, 1.5	38, 39	22, 25	201, 207	18, 21
150	50	54, 54	1.7, 1.7	38, 40	22, 25	135, 144	10, 12
200	1	60, 61	3.6. 3.9	31, 32	19, 20	224, 271	8.1, 11
200	5	47, 55	2.1, 3.9	36, 44	22, 27	183, 205	5.8, 11
200	10	58, 61	1.5, 1.8	33, 34	19, 21	174, 186	11, 14
200	50	38, 41	4.3, 4.6	46, 49	23, 26	72, 82	4.4, 6.2

1: unidentified fatty acids have not been included

Table 4.5 Fatty acid content, proportion of fatty acid classes and DHA, and biomass of 10-day old flask cultures of ACEM 6063 in response to variations in initial glucose concentration (mean±SD; n=8)

Initial glucose concentration ($g\ L^{-1}$)	Fatty acids (% total fatty acids [1])				Total fatty acids ($mg\ g^{-1}$ dry weight)	Biomass (g dry weight L^{-1})
	sum SFA	sum MUFA	sum PUFA	DHA		
50	58±4.2 ^{ab}	1.4±0.3 ^{a}	36±4.2	23±2.9	186±42	14±2.3 ^{a}
100	56±2.4 ^{bc}	1.5±0.4 ^{a}	37±2.7	25±2.0	200±49	15±2.4 ^{a}
150	59±2.0 ^{a}	1.5±0.3 ^{a}	36±1.5	23±1.3	194±37	14±3.7 ^{a}
200	55±8.9 ^{c}	3.3±1.2 ^{b}	34±6.6	22±2.5	174±71	9.0±2.8 ^{b}

values in each column with different superscripts are significantly different ($\alpha = 0.01$)
1: unidentified fatty acids have not been included

Table 4.6 Fatty acid content, proportion of fatty acid classes and DHA, and biomass of 10-day old flask cultures of ACEM 6063 in response to variations in initial sodium glutamate concentration (mean±SD; n=8)

Initial sodium glutamate concentration ($g\ L^{-1}$)	Fatty acids (% total fatty acids [1])				Total fatty acids ($mg\ g^{-1}$ dry weight)	Biomass (g dry weight L^{-1})
	sum SFA	sum MUFA	sum PUFA	DHA		
1	60±2.1 ^{a}	2.2±1.1 ^{ab}	35±2.1 ^{a}	21±1.7 ^{a}	232±21 ^{a}	12±1.9 ^{a}
5	53±3.0 ^{b}	1.9±0.8 ^{b}	41±2.8 ^{b}	25±2.1 ^{b}	209±12 ^{b}	14±3.7 ^{ab}
10	56±2.7 ^{b}	1.3±0.3 ^{c}	38±3.0 ^{c}	24±2.2 ^{bc}	194±11 ^{b}	17±3.1 ^{b}
50	51±7.1 ^{b}	2.3±1.5 ^{a}	41±3.9 ^{b}	23±1.0 ^{c}	119±29 ^{c}	11±3.9 ^{a}

values in each column with different superscripts are significantly different ($\alpha = 0.01$)
1: unidentified fatty acids have not been included

PUFA levels did not vary greatly between treatments, but were significantly higher in 10-day old cultures with an initial sodium glutamate concentration of 5 and 50 g L^{-1} (41 %TFA) than in cultures with an initial sodium glutamate concentration of 1 and 10 g L^{-1} (35 and 38 %TFA, respectively). Similarly, DHA levels did not vary greatly, but were slightly higher in cultures with initial sodium glutamate concentrations of 5 and 10 g L^{-1} (~ 25 %TFA respectively) than in cultures with initial sodium glutamate concentrations of 1 and 50 g L^{-1} (21 and 23 %TFA, respectively). Fatty acid content of biomass of 10-day old ACEM 6063 cultures decreased from 232 mg g^{-1} dry weight in cultures with an initial sodium glutamate concentration of 1 g L^{-1}, to 119 mg g^{-1} weight in cultures with an initial sodium glutamate concentration of 50 g L^{-1}. Total biomass increased from 12 g dry weight L^{-1} in cultures with an initial sodium glutamate concentration of 1 g L^{-1}, to 17 g L^{-1} in cultures with an initial sodium glutamate concentration of 10 g L^{-1}, and decreased again to 11 g L^{-1} in cultures with an initial sodium glutamate concentration of 50 g L^{-1} (Table 4.6).

4.4.1.4 Flask Experiment 4: Inoculum size and age

The proportions of SFA, MUFA, PUFA and DHA (%TFA), the fatty acid content (mg g^{-1} dry weight) of, and the amount of biomass (g dry weight L^{-1}) in 10-day old flask cultures of ACEM 6063 cultured using inocula of different proportions (relative to the volume of the media being inoculated) and different ages are shown in Table 4.7. There were no significant differences in any of these parameters attributable to differences between the two cultures used as the experimental inocula for this experiment.

The proportion of inoculum, relative to the volume of medium being inoculated, did not significantly influence any of the parameters shown in Table 4.7. The age of the inoculum (*i.e.* the time after the inoculum itself was inoculated) significantly influenced the relative proportions of SFA, PUFA and DHA in ACEM 6063 biomass, as well as the absolute amount of biomass in each flask (Table 4.8). The relative proportion of SFA in the biomass was highest in cultures for which 1- and 2-day old inocula were used (~70 %TFA) and was significantly lower in cultures for which 5- and 10-day old inocula were used (65 and 60 %TFA respectively) (Table 4.8).

Table 4.7 Fatty acid content, proportion of fatty acid classes and DHA, and biomass of duplicate 10-day old flask cultures of ACEM 6063 in response to variations in inoculum size and age

Inoculum		Fatty acids (% total fatty acids [1])				Total fatty acids (mg g^{-1} dry weight)	Biomass (g dry weight L^{-1})
Proportion (% v/v)	Age (d)	sum SFA	sum MUFA	sum PUFA	DHA		
1	1	67, 76	1.5, 3.3	19, 30	10, 19	167, 220	14, 20
5	1	63, 62	1.3, 1.9	34, 34	20, 21	184, 203	13, 18
10	1	82, 78	2.1, 2.4	14, 18	7.2, 9.6	155, 157	13, 19
1	2	63, 68	2.4, 2.7	28, 33	17, 19	210, 238	17, 20
5	2	64, 68	2.5, 2.9	24, 27	17, 17	179, 217	18, 19
10	2	69, 70	2.0, 2.1	25, 26	15, 15	182, 210	19, 21
1	5	62, 68	1.8, 2.0	27, 34	17, 21	231, 248	18, 19
5	5	61, 63	2.0, 2.1	32, 33	19, 19	204, 221	17, 19
10	5	61, 64	2.0, 2.1	29, 35	22, 24	138, 189	15, 18
1	10	55, 59	1.3, 1.9	38, 39	23, 25	201, 290	17, 19
5	10	59, 63	2.2, 2.7	34, 37	21, 23	244, 248	18, 21
10	10	59, 61	3.1, 3.9	30, 33	20, 22	130, 195	16, 18

1: unidentified fatty acids have not been included

Table 4.8 Fatty acid content, proportion of fatty acid classes and DHA, and biomass of 10-day old flask cultures of ACEM 6063 in response to variations in inoculum age (mean±SD; n=6)

Inoculum age (d)	Fatty acids (% total fatty acids [1])				Total fatty acids (mg g^{-1} dry weight)	Biomass (g dry weight L^{-1})
	sum SFA	sum MUFA	sum PUFA	DHA		
1	72±8.5 [a]	2.1±0.7	23±8.8 [a]	15±6.2 [a]	181±26	16±3.7 [a]
2	68±2.8 [a]	2.4±0.3	25±2.8 [a]	17±1.5 [ab]	206±22	21±1.1 [b]
5	65±2.3 [b]	2.0±0.1	30±3.0 [b]	20±1.9 [bc]	205±39	18±1.4 [ab]
10	60±2.6 [c]	2.5±0.9	32±3.3 [b]	22±1.7 [c]	218±55	18±1.7 [ab]

values in each column with different superscripts are significantly different ($\alpha = 0.01$)
1: unidentified fatty acids have not been included

The relative proportions of PUFA and DHA in 10-day old flask cultures of ACEM 6063 were lowest in cultures for which 1-day old inocula were used (23 and 15 %TFA respectively), and highest in cultures for which 10-day old inocula were used (32 and 22 %TFA respectively). The absolute amount of biomass was lowest in cultures for which 1-day old inocula were used (16 g L^{-1}), and highest in cultures for which 2-day old inocula were used (21 g L^{-1}). Relative to the use of 2-day old inoculum, there was a slight but insignificant decrease in biomass production in cultures for which 5- and 10-day old inocula were used (18 g L^{-1} for both) (Table 4.8).

4.4.1.5 Cell morphology

Cell morphology in all flask cultures followed the following pattern. About 24 h after inoculation, cultures consisted of clumps containing up to several hundred 10-12 µm diameter cells. Many motile cells (ovoid: ~5 µm long, ~ 2 µm wide) were often seen at or around this time. Between this stage and when the cultures reached peak biomass, the cultures were predominantly composed of spherical clusters (up to 75 µm diameter) of 10-12 µm diameter cells, with some smaller clumps and some single

cells also being present. As the cultures reached peak biomass, the spherical clusters began to break up into smaller clumps and single cells. After peak biomass was reached, > 95% of the cells were present either singly or in clumps of < 5 cells. Individual cell size also tended to decrease with culture age, with most cells at the time peak biomass was reached having a diameter of about 5 µm.

4.4.2 Fermenter Culture

4.4.2.1 Biomass production

Cultures grown using high impeller speeds (cultures 10H20H and 50H20H: see Table 4.1) grew at similar rates to each other (~3.5 g dry weight L^{-1} d^{-1}) for the first 2 days following inoculation (Figure 4.3). Culture 10H20H reached peak biomass (5.5 g dry weight L^{-1}) at this time (Figure 4.3). Culture 50H20H grew more slowly (~ 1 g dry weight L^{-1} d^{-1}) after this time, and reached peak biomass (11 g dry weight L^{-1}) after a total of 5 days incubation (Figure 4.3).

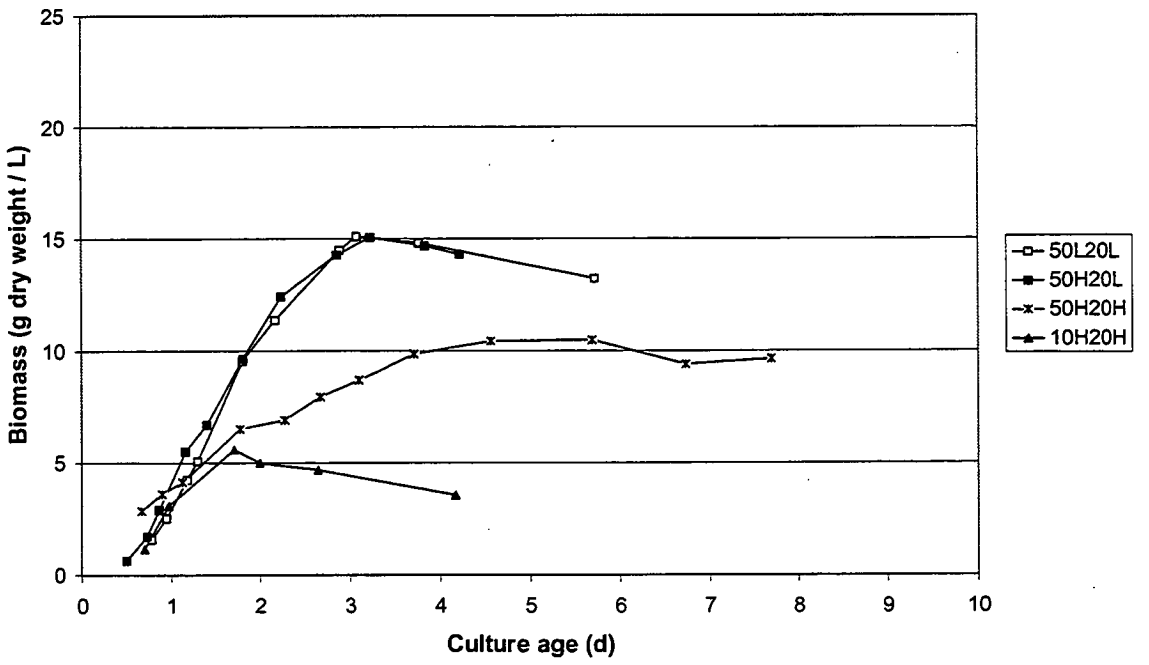

Figure 4.3 Biomass production by fermenter-cultured ACEM 6063 grown under treatments 10H20H, 50H20H, 50H20L and 50L20L

Treatment notation: 10, 50: initial glucose concentration (g L^{-1}); L, H: low (<5%), high (>40%) dissolved oxygen level in the culture medium; 20: incubation temperature (°C); L, H: low (max 200), high (max 750) impeller speed (rpm)

The maximum growth rates of cultures grown with an initial glucose concentration of 50 g L^{-1} and low impeller speed (cultures 50L20L and 50H20L) were very similar to each other (~6.5 g dry weight L^{-1} d^{-1}) (Figure 4.3), with peak biomass (15 g dry weight L^{-1}) being reached in 3 days.

The maximum growth rates of cultures grown with an initial glucose concentration of 100 g L^{-1}, at 20 and 25°C, and with low impeller speeds (cultures 100L20L, 100H20L and 100L25L) were also similar to each other (~4.5 g dry weight L^{-1} d^{-1}), with peak biomass (21-23 g dry weight L^{-1}) being reached in 7-8 days (Figure 4.4).

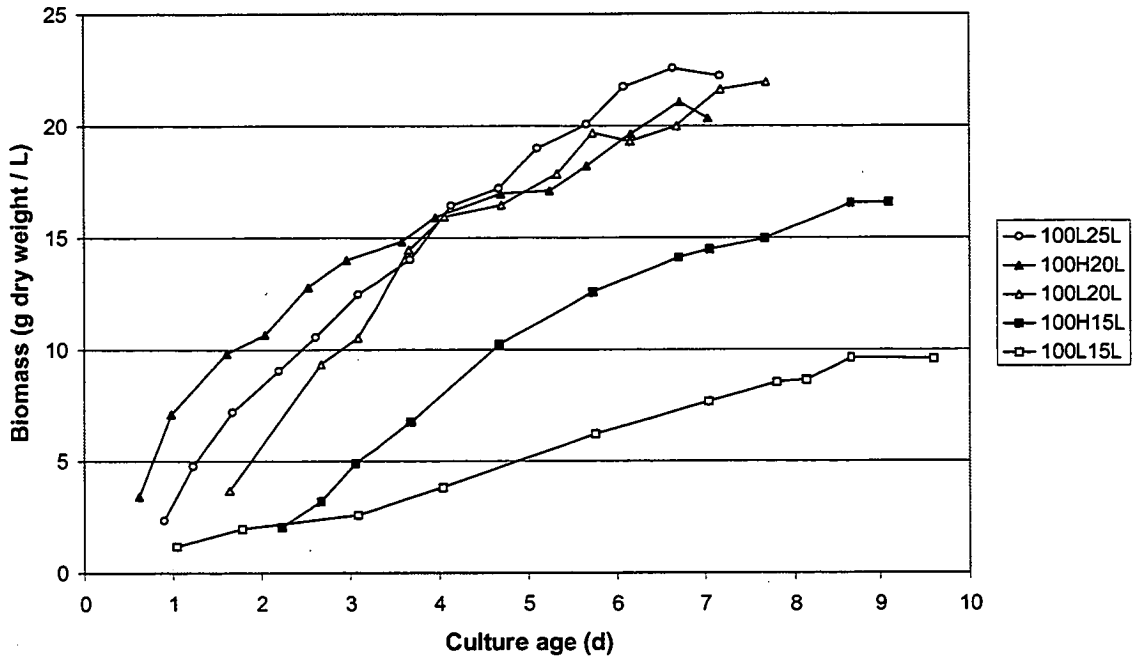

Figure 4.4 Biomass production by fermenter-cultured ACEM 6063 grown under treatments 100H15L, 100H15H, 100L20L, 100H20L and 100H25L

Treatment notation: 100: initial glucose concentration (g L^{-1}); L, H: low (<5%), high (>40%) dissolved oxygen level in the culture medium; 15, 20, 25: incubation temperature (°C); L: low (max 200), high (max 750) impeller speed (rpm)

The maximum growth rate of culture 100H15L (initial glucose concentration 100 g L^{-1}, grown at high DO levels, at 15°C and with low impeller speeds) was similar to those of cultures 100L20L, 100H20L and 100L25L (Figure 4.4). Culture 100H15L, however, appeared to undergo a longer lag time following inoculation than cultures

100L25L, 100H20L and 100L20L, and reached a peak biomass of 17 g dry weight L^{-1} (c.f. peak biomasses of 21-23 g dry weight L^{-1} for cultures 100L25L, 100H20L and 100L20L) after 8-9 days incubation. Culture 100L15L (initial glucose concentration 100 g L^{-1}, grown at low DO levels, at 15°C and with low impeller speeds) exhibited a similar increased lag time to, but grew more slowly than, culture 100H15L. Culture 100L15L grew at a maximum rate of ~1.1 g dry weight L^{-1} d^{-1}, and reached a peak biomass of 10 g dry weight L^{-1} after 9 days (Figure 4.4).

4.4.2.2 Fatty acid and lipid production

The proportions of SFA, MUFA, PUFA and DHA (%TFA), and the fatty acid content (mg g^{-1} dry weight) of fermenter cultures of ACEM 6063 cultured under a range of treatments involving initial glucose concentration, dissolved oxygen level, temperature and impeller speed are shown in Table 4.9. These data indicate that increasing the initial glucose concentration did not substantially influence the relative proportions of SFA, MUFA, PUFA or DHA in fermenter-cultured ACEM 6063 biomass. Increasing the initial glucose concentration from 10 to 100 g L^{-1} did, however, result in an increase in fatty acid content from 81 to about 450 mg g^{-1} dry weight.

Increasing the level of dissolved oxygen in the culture media from < 5% saturation (termed "low") to > 40% saturation (termed "high") influenced fatty acid production by ACEM 6063. The major changes were that SFA levels decreased from 54-61 %TFA to 33-38 %TFA and that MUFA levels increased substantially from 1.9-5.2 %TFA to 28-35 %TFA. In addition to these major changes in fatty acid content, PUFA levels decreased from 31-41 %TFA to 27-30 %TFA; DHA levels decreased from 24-30 %TFA to 20-23 %TFA and fatty acid content increased from 60-282 mg g^{-1} dry weight to 123-457 mg g^{-1} dry weight (Table 4.9).

The fatty acid content (mg g^{-1} dry weight) of ACEM 6063 biomass for cultures 100L15L, 100L20L and 100L25L (grown at 15, 20 and 25°C respectively, at low DO levels, with initial glucose concentration of 100 g L^{-1} and at low impeller speed) were 60, 282 and 404 mg g^{-1} dry weight, respectively.

Table 4.9 Fatty acid content of fermenter-cultured ACEM 6063 at peak biomass under different culture conditions

Treatment [1]	Fatty acids (% total fatty acids [2])				Total fatty acids (mg g^{-1} dry weight)
	sum SFA	sum MUFA	sum PUFA	DHA	
10H20H	32	33	33	20	81
50H20H	31	36	30	24	102
50L20L	57	2.3	39	27	121
50H20L	38	28	30	23	155
100L15L	54	1.9	41	30	60
100H15L	35	34	27	20	123
100L20L	61	5.2	31	24	282
100H20L	33	35	27	20	457
100L25L	56	4.9	34	22	404

1: Treatment notation:
10, 50, 100: initial glucose concentration (g L^{-1})
L, H: low (<5%), high (>40%) dissolved oxygen level in the culture medium
15, 20, 25: incubation temperature (°C)
L, H: low (max 200), high (max 750) impeller speed (rpm)
2: unidentified fatty acids have not been included

Similarly, the total fatty acid content (mg g^{-1} dry weight) of ACEM 6063 biomass for cultures 100H15L and 100H20L (grown at 15 and 20°C respectively, at high DO levels, with initial glucose concentration of 100 g L^{-1} and at low impeller speed) were 123 and 457 mg g^{-1} dry weight, respectively (Table 4.9). The only direct comparison that could be made of the effects of impeller speed on lipid production was between cultures 50H20H and 50H20L. In this case, lower impeller speed appeared to result in a higher absolute level of total fatty acids (155 c.f 102 mg g^{-1} dry weight). Impeller speed did not appear to substantially influence the relative proportions of SFA, MUFA or PUFA in ACEM 6063 biomass (Table 4.9).

The predominant lipid classes produced by ACEM 6063 under the conditions specified in Table 4.1 were triacylglycerols and phospholipids (Table 4.10). The

relative proportion of triacylglycerols increased from 38-76% to 69-93% of the total lipids during the four days immediately prior to cultures reaching peak biomass. The relative proportion of phospholipids decreased from 23-59% to 5-29% of the total lipids during the same time. Neither culture temperature nor DO levels had any obvious influence on triacylglycerol or phospholipid levels in ACEM 6063 biomass. Wax esters, sterols and free fatty acids were present in all samples, but at levels less than 3, 2 and 2% of the total lipids respectively (Table 4.10). The levels of wax esters and free fatty acids showed no obvious trends in relation to culture age, temperature or DO levels. The relative proportion of sterols in the lipid fraction tended to decrease with culture age.

4.4.3 Cell morphology

Cell morphology for all fermenter cultures except 10H20H and 50H20H followed the pattern described for flask culture in Section 4.4.1.5 (page 76). In cultures 10H20H and 50H20H, however, the spherical clusters of cells typically seen in actively growing flask cultures of ACEM 6063 were evident only during the first 1-2 days. The spherical clusters in both these cultures broke up well before peak biomass was reached - an occurrence not seen in other cultures of ACEM 6063.

Table 4.10 Lipid-classes produced by fermenter-cultured ACEM 6063 biomass when grown under different culture conditions (duplicate analyses of single samples)

Treatment [1]	Sampling time [2]	Lipid class (% total lipids)				
		triacylglycerol	phospholipid	wax ester	free fatty acid	sterol
100L15L	4	38, 43	54, 60	1.7, 2.0	0.2, 0.2	1.0, 1.2
	2	67, 72	23, 29	2.7, 3.5	0.1, 0.2	0.9, 1.0
	0	68, 69	28, 30	1.7, 2.1	0.0, 0.1	0.5, 0.9
100H15L	4	38, 37	59, 60	0.8, 0.9	0.0, 0.0	1.8, 1.8
	2	77, 79	19, 21	1.4, 1.8	0.0, 0.0	0.6, 0.7
	0	80, 81	15, 17	2.1, 2.3	0.2, 0.3	0.7, 1.1
100L20L	4	56, 60	34, 36	2.4, 3.2	1.9, 1.9	1.8, 2.0
	2	94, 95	4.3, 4.4	0.6, 0.8	0.0, 0.3	0.5, 0.7
	0	92, 94	4.3, 5.6	0.9, 1.0	0.1, 0.4	0.6, 0.8
100H20L	4	64, 65	33, 33	0.9, 1.6	0.0, 0.8	0.9, 1.1
	2	68, 82	15, 28	1.6, 2.7	0.1, 0.5	0.6, 0.8
	0	71, 81	16, 26	2.1, 2.8	0.2, 0.2	0.5, 0.6
100L25L	4	74, 78	21, 25	0.4, 0.5	0.0, 0.1	0.5, 0.5
	2	84, 88	11, 16	0.5, 0.5	0.0, 0.0	0.3, 0.4
	0	90, 93	5.4, 8.3	0.7, 0.8	0.0, 0.0	0.4, 0.4

1: Treatment notation:
100: initial glucose concentration (g L^{-1})
L, H: low (<5%), high (>40%) dissolved oxygen level in the culture medium
15, 20, 25: incubation temperature (°C)
L: low (max 200) impeller speed (rpm)
2: days prior to the culture reaching peak biomass

4.5 Discussion

This study has demonstrated that changes to both physical and chemical culture conditions can substantially influence biomass and lipid production by thraustochytrid strain ACEM 6063.

Biomass levels in both flask and fermenter cultures increased at linear rates with increasing culture age, until peak biomass was reached, and then decreased slightly after that time. Similar growth curves have been reported for *T. aureum* ATCC34304 (Bajpai *et al.*, 1991b), *T. roseum* ATCC 28210 (Singh and Ward, 1996), *Thraustochytrium* sp ATCC 20892 (Singh *et al.*, 1996) and *Schizochytrium* sp SR21 (Yaguchi *et al.*, 1997; subsequently identified as *Schizochytrium limacinum* SR21 by Honda *et al.*, 1998). These data indicate that, unlike bacteria and other microheterotrophs, thraustochytrids growing in liquid media do not replicate in truly exponential fashion during times of maximum growth rate. The linear growth rate observed in thraustochytrid cultures could be due to the apparent complexity of the life history of thraustochytrids (e.g. Booth and Miller, 1968; Bahnweg and Jackle, 1986; Azavedo and Corral, 1997; Honda *et al.*, 1998).

As ACEM 6063 cultures aged, the total fatty acid content of the biomass increased, and the relative proportion of DHA decreased. This trend continued past the time when culture reached peak biomass in all cultures, except for one flask culture grown with an initial glucose concentration of 10 g L^{-1}. The lipid contents of three thraustochytrid strains have all been reported to increase, and the proportion of DHA to decrease, as the cultures approached peak biomass. Following attainment of peak biomass these trends reversed substantially [*T. aureum* ATCC 34304 (Bajpai *et al.*, 1991b), *T. roseum* ATCC 28210 (Singh and Ward, 1996) and *Thraustochytrium* sp. ATCC 20892 (Singh *et al.*, 1996)]. Unsaturated fatty acid content of many microorganisms has been reported to decrease with culture age (Ratledge, 1989).

The type of culture vessel, and the physical conditions within culture vessels, also influenced growth rate, peak biomass and lipid content of ACEM 6063 cultures. Maximum growth rates in fermenter cultures were about 2-3 greater than those in flask cultures. The reasons for this disparity are unclear, but do not appear to be related to dissolved oxygen levels in the media. Growth rates of flask and fermenter cultures of *T. aureum* ATCC 34304, grown under conditions comparable to the

present study, were reported to be similar to each other (Bajpai *et al.*, 1991b), indicating that responses to different culture conditions by thraustochytrids are strain specific. The fatty acid content and the relative proportions of SFA, MUFA and PUFA in flask cultures (which had low DO levels, data not shown) and the fermenter cultures grown at low DO levels were quite similar. This indicates that the type of culture vessel did not substantially influence lipid production by ACEM 6063 cells.

Dissolved oxygen levels in fermenter cultures substantially influenced lipid production by ACEM 6063. Increasing DO levels from < 5% saturation to > 40% saturation resulted in absolute fatty acid levels increasing by > 30%, and the proportions of SFA, MUFA and PUFA decreasing by > 30%, increasing by > 700% and decreasing by > 20% respectively. These findings suggest that DO levels will be important for the optimisation of oil production by ACEM 6063. To the author's knowledge, no other reports have examined the influence of DO levels on fatty acid production by thraustochytrids. Increased oxygen availability has, however, been shown to increase the degree of unsaturation in the fatty acids produced by other eukaryotic microorganisms (Erwin, 1973; Beach, 1973 cited in Jiang and Chen, 2000).

Maximum impeller rotation speed influenced the peak biomass reached by fermenter cultures of ACEM 6063. Rotation speeds of up to 200 rpm did not appear to impede growth or disrupt the normal morphological development of ACEM 6063. Impeller speeds of up to 750 rpm did, however, result in a decrease in growth rate, a lower peak biomass and abnormal morphological development of the cultures. Impeller type and rotation speed have also been reported to influence growth rate and peak biomass of *Schizochytrium* sp. SR21 cultures, and DHA production by *T. aureum* cultures (Yaguchi *et al.*, 1997), indicating that these strains are also susceptible to the high shear forces which can be generated in fermenters. Peak biomass reached by ACEM 6063 cultures grown using comparable media in flasks or in the fermenter with low impeller rotation speeds, were similar to each other. Other studies have found that peak biomass reached in fermenter cultures were different to those reached in flask cultures [fermenter biomass < flask biomass for *T. aureum* ATCC 34304 (Bajpai *et al.*, 1991b; Iida *et al.*, 1996); fermenter biomass > flask biomass for *Schizochytrium* sp. SR21 (Yaguchi *et al.*, 1997)].

Culture temperature also influenced biomass and lipid production by ACEM 6063. Maximum growth rates and peak biomass reached by fermenter cultures grown at 20°C and 25°C were very similar, and were higher than those of fermenter cultures grown at 15°C. ACEM 6063 grows substantially slower at 35° than at 25°C (data not shown). Other thraustochytrid strains have been shown to grow fastest between 20°C and 30°C, with growth rates declining rapidly at super-optimal temperatures. (Bajpai *et al.*, 1991b; Li and Ward, 1994; Singh *et al.*, 1996; Yokochi *et al.*, 1998). The fatty acid content (mg g^{-1}) of ACEM 6063 increased with culture temperature up to 25°C. Culture temperature did not appear to influence the proportions of SFA or MUFA in the lipid, while the proportions of PUFA and DHA appeared to decrease slightly with increasing culture temperature. Higher incubation temperatures have been shown to decrease the content of unsaturated fatty acids in other microheterotrophs (Ratledge, 1989; Nichols *et al.*, 1994). Lipid content of two other thraustochytrids has also been shown to increase with increasing culture temperature (Bajpai *et al.*, 1991b; Singh *et al.*, 1996). These two studies also reported that lipid content decreased in cultures grown at super-optimal temperatures, and it is likely that ACEM 6063 would follow this trend.

The initial concentration of sea salts used in the culture media influenced the peak biomass reached by cultures of ACEM 6063, but not the growth rate. Maximum peak biomass was reached in cultures grown at the highest salt concentration tested – the equivalent of 100% seawater. Yokochi *et al.* (1998) reported that *Schizochytrium limacinum* SR21 could grow at 0% seawater, with maximum growth being reached at 50% seawater and being maintained up to 200% seawater. The salinity tolerance of *T. aureum* ATCC 34304 (Iida *et al.*, 1996) was markedly different to *Schizochytrium limacinum* SR21 and ACEM 6063. *T. aureum* ATCC 34304 growth was completely inhibited at 0% seawater, peaked at 50%, decreased at 100%, and was again completely inhibited at 200% seawater. As cultures of *Schizochytrium limacinum* SR21 and *T. aureum* ATCC 34304 were all harvested after a set period (5 and 4 days respectively), it is not possible to tell whether variations in seawater concentration influenced only their growth rate, only their peak biomass, or both. Data for ACEM 6063 culture suggests that peak biomass in these cultures is being limited by depletion of a trace nutrient/s provided by the sea salt mixture. Identification of such limiting micronutrient/s could help increase peak biomass in ACEM 6063 cultures. Lipid content at peak biomass of flask-cultured ACEM 6063 cells appeared to be

related to the initial sea salt concentration in the media. These differences, however, are consistent with changes related to the culture age at peak biomass, and may not be a direct response to the actual sea salt concentration. This argument is supported by the observation that there was no significant difference in the DHA yields from *T. roseum* ATCC 28210 when sodium chloride levels in the culture media were decreased from 25 g L^{-1} to 10 g L^{-1} (Singh and Ward, 1996).

Inoculum age was found to have significant effects on both biomass and lipid production by 10-day old ACEM 6063 cultures. Increasing inoculum age was associated with decreases in the proportion of SFA, increases in the proportions of PUFA and DHA, and increases in absolute biomass in these cultures. This is in contrast to data reported for *T. aureum* ATCC 34304, which indicated that lipid content of the biomass decreased markedly when 2- or 3-day old inocula were used instead of a 1-day old inoculum (Bajpai *et al.*, 1991b).

Although the influence of initial culture pH was not examined in this study, the initial pH of TCM was about 6.6, and rose to 7.1-7.4 during the course of fermenter cultures (data not shown). The initial pH of TCM is within the range reported to be optimal for biomass and DHA production by other thraustochytrids (Bajpai *et al.*, 1991b; Li and Ward, 1994), and other DHA producing microheterotrophs (Jiang and Chen, 2000).

Initial glucose concentration influenced peak biomass and lipid production in ACEM 6063 cultures, although differences in lipid content became more apparent after cultures reached peak biomass. Peak biomass levels increased, but the increments decreased, as initial glucose concentrations increased from 10 g L^{-1} to 200 g L^{-1}. This indicates that other factors were limiting biomass production at the higher glucose concentrations. Similar data has been presented for thraustochytrid strain G13 (Bowles *et al.*, 1999). It is interesting to note that ACEM 6063 grew well in media with an initial glucose concentration of 200 g L^{-1}. Other thraustochytrids have been reported to have maximum growth at glucose concentrations of only 20 g L^{-1} (*T. aureum* ATCC 34304: Bajpai *et al.*, 1991a) and 40 g L^{-1} (*Thraustochytrium* sp. ATCC 20892: Singh *et al.*, 1996; strain G13: Bowles *et al.*, 1999). Measurements for these strains were made after 6, 4 and 4 days respectively, and it may be that cultures provided with higher initial glucose concentrations would have reached higher biomass given more time. *Schizochytrium limacinum* SR21 is, to date, the only

thraustochytrid reported to obtain peak biomass levels substantially greater than those reached by ACEM 6063 in this study (Yaguchi *et al.*, 1997). As mentioned in Chapter 2, *Schizochytrium limacinum* SR21 is, to the author's knowledge, the only thraustochytrid strain reported to grow well at the high initial glucose concentrations (i.e. ≥100 g L^{-1}) preferred by ACEM 6063 (c.f 30-40 g L^{-1} for several other strains: e.g. Kendrick and Ratledge, 1992; Singh *et al.*, 1996; Bowles *et al.*, 1999). It is possible that the *Schizochytrium* sp. strain from which Algamac-2000 is produced would also thrive under such high glucose concentrations, given its apparent phylogenetic relationship with ACEM 6063 and *Schizochytrium limacinum* SR21 (Chapter 2).

Modifications of initial glucose levels resulted in only minor changes in the relative proportions of SFA and MUFA in ACEM 6063 biomass, and no significant changes in the proportions of PUFA or DHA. Glucose concentration has also been reported to have little or no influence on the proportion of DHA in biomass of *T. aureum* ATCC 34304 (Bajpai *et al.*, 1991a), *Thraustochytrium* sp. ATCC 20892 (Singh *et al.*, 1996), *T. roseum* ATCC 28210 (Singh and Ward, 1996), *Schizochytrium limacinum* SR21 (Yaguchi *et al.*, 1997; Yokochi *et al.*, 1998) and strain G13 (Bowles *et al.*, 1999). In all cases, however, glucose concentration had a substantial effect on both peak biomass and lipid content of the biomass, consequently influencing overall DHA production by these strains.

The initial concentration of sodium glutamate influenced biomass and lipid content of 10-day old flask cultures of ACEM 6063. Biomass increased when the initial sodium glutamate concentration increased from 1 to 10 g L^{-1}, and decreased with a further increase of the initial sodium glutamate concentration from 10 to 50 g L^{-1}. This is in contrast to biomass production of *T. aureum* ATCC 34304, which was highest with an initial sodium glutamate concentration of 20 g L^{-1} (Iida *et al.*, 1996). Initial sodium glutamate concentration had only a minor influence on the relative proportions of SFA, MUFA, PUFA and DHA in ACEM 6063 lipid, although total absolute lipid levels decreased substantially as initial sodium glutamate concentration increased. There was no apparent interaction between initial concentrations of glucose and sodium glutamate on either biomass or lipid production by ACEM 6063. Although sodium glutamate was not the only source of nitrogen in the media, this result suggests the carbon:nitrogen ratio in the media had no significant effect on

either biomass or lipid production by ACEM 6063. A high C:N ratio has been reported to be generally favourable for microbial growth and lipid production, the latter usually being triggered by N depletion (Yongmanitchai and Ward, 1989; Singh *et al.*, 1996; Yokochi *et al.*, 1998; Bowles *et al.*, 1999).

The lipid of ACEM 6063 biomass was predominated by triacylglycerol and phospholipid, with small amounts of free fatty acid, wax ester and sterol (see Chapter 5 for a description of sterol production by ACEM 6063). The proportion of triacylglycerols in the total lipids of ACEM 6063 increased with culture age, and reached > 90% of the total lipids in some cultures at peak biomass. These results, when considered in conjunction with decreases in the proportion of DHA in the total fatty acids with age, indicate that the triacylglycerol fraction of ACEM 6063 lipids contained a relatively lower proportion of DHA than the phospholipid fraction. This characteristic has also been reported for *Schizochytrium* SR21 (Yaguchi *et al.*, 1997). Thraustochytrid strain G13 was also found to have a predominantly triacylglycerol-based oil (Bowles *et al.*, 1999).

Conditions in which thraustochytrid strain ACEM 6063 cultures have produced biomass up to 23 g dry weight L^{-1} biomass and triacylglycerol-rich oil up to 450 mg g^{-1} dry weight have been ascertained. Cell biomass contained up to 130 mg g^{-1} dry weight DHA, allowing potential production of 3 g DHA L^{-1}. This level of DHA production is amongst the highest reported for thraustochytrids (c.f. 2.2 g L^{-1}: Bowles *et al.*, 1999; 13.3 g L^{-1}: Yaguchi *et al.*, 1997).

Another important consideration when assessing the efficiency of thraustochytrid production facilities is the dry weight content of DHA in the biomass. If thraustochytrid biomass is to be used as a source of DHA for aquaculture feeds (as investigated in Chapter 7), it is important for the biomass to contain as much DHA as possible. In these cases, it could well be important to choose culture conditions that maximise biomass DHA content, even if this compromises overall biomass production and/or DHA production as measured on a volumetric basis.

It is likely that further changes to culture conditions, including other combinations of the initial types and concentrations of macro- and micro-nutrients and sea salts and/or additional doses of nutrient (e.g. (Singh and Ward, 1996)) will result in further enhancement of growth rates and/or peak biomass for ACEM 6063. Such

results for laboratory-scale production bolster the argument that thraustochytrids are amongst the most productive known sources of microbial PUFA (see Chapter 1). If and when culture conditions, including media, can be provided to allow production of PUFA-rich oils and/or biomass at a quality and price acceptable to market demands, then thraustochytrids will become a viable source of these products. Indeed, several DHA-rich, thraustochytrid-based products have already appeared on the aquaculture market, and research into additional applications is continuing. As research into this group of organisms continues, it is likely that such strains will be further developed to expand their use as commercially viable sources of PUFA-rich oils for use in nutraceuticals and functional foods.

In Chapter 2, it was suggested that ACEM 6063 is closely related to the highly productive *Schizochytrium limacinum* SR21 and also to the *Schizochytrium* sp. used in the commercial product Algamac-2000. This relationship, together with the biomass and DHA production data reported for ACEM 6063 in this Chapter, support the argument that ACEM 6063 may be amenable to culture in less defined media and conditions, to provide DHA-rich biomass and/or oil a price sufficient to allow entry into the PUFA market.

Chapter 5 Sterol and squalene content of strain ACEM 6063: influence of culture age, temperature and dissolved oxygen

5.1 Abstract

Thraustochytrid strain ACEM 6063, rich in n3 PUFA, was cultured at 15°C and 20°C in media with high (> 40%) and low (< 5%) dissolved oxygen (DO), and at 25°C in low DO media. Samples were taken 4, 2 and 0 days before each culture reached peak biomass (T_{-4}, T_{-2} and T_p respectively). Twenty sterols, 13 of which were identified, were detected. Predominant were: cholest-5-en-3β-ol, 24-ethylcholesta-5,22E-dien-3β-ol, 24-methylcholesta-5,22E-dien-3β-ol, and two co-eluting sterols – one of which was 24-ethylcholesta-5,7,22-trien-3β-ol. These 4 sterols comprised 50-90% of total sterols. Cultures grown at high DO contained simpler sterol profiles than those grown at low DO. Only the 4 sterols mentioned above were present at > 3% of total sterols in high DO cultures. In low DO cultures, up to 6 additional sterols were present at > 3% of total sterols. Culture age, temperature and DO influenced squalene and sterol content. Total sterols (as a proportion of total lipids) decreased with increasing culture age. If organisms such as ACEM 6063 are to be used for commercial production of lipid products for human consumption, both their sterol content and factors influencing sterol production need to be characterised thoroughly.

5.2 Introduction

Although PUFA production by a number of thraustochytrid strains has been well documented, there has been (to the author's knowledge) only one report discussing the production of sterols by these organisms (Weete *et al.*, 1997). Sterols are membrane lipids that are produced by all microeukaryotes, as well as by a limited number of bacteria (e.g. Jones *et al.*, 1994; Volkman *et al.*, 1998; Schouten *et al.*, 2000). The first major step in sterol synthesis is the oxygenation of squalene to form (3S)-squalene epoxide (3S-squalene-2,3-oxide), which is then cyclised by one of two enzymes to form either lanosterol or cycloartenol. These two compounds then undergo a series of additional reactions to yield further sterol products (Torssell, 1983; Jones *et al.*, 1994).

Those sterols commonly found in food plants (phytosterols: e.g. brassicasterol, sitosterol, campesterol, stigmasterol; Harwood and Russell, 1984) are generally regarded as safe for human consumption, and are even believed to confer some positive health benefits (e.g. Moghadasian and Frohlich, 1999). Regulatory authorities, however, treat the presence of uncommon and/or unknown sterols in food products very warily. This wariness is based on knowledge that, when ingested, sterols may influence metabolic functions (Nichols *et al.*, 1998), and therefore evidence confirming the safety of novel sterols is required.

In this study, the sterol composition of the DHA-producing thraustochytrid ACEM 6063 has been examined in detail. The effects of culture temperature, dissolved oxygen concentration and culture age on the squalene and sterol content of biomass of this strain have also been determined. This was a first step towards understanding the factors that influence sterol production by a PUFA-producing thraustochytrid.

5.3 Material and Methods

5.3.1 Analytical reagents

All reagents used were of analytical standard. Chloroform, methanol, hexane and hydrochloric acid were obtained from Mallinckrodt (USA). Deionised water was produced using a Milli Q Plus (Millipore SA, France) water treatment system.

5.3.2 Microheterotroph culture and cell preparation

Biomass of ACEM 6063 to be used for lipid analysis was produced under ambient light conditions in a BioFlo IIC fermenter (New Brunswick, USA) containing an initial volume of 2 L of TCM (Section 2.3.1, page 19) supplemented to contain an initial glucose concentration of 100 g L^{-1}. Culture parameters varied were temperature and dissolved oxygen (DO). Culture temperatures (15°C; 20°C; 25°C) were controlled to within 1°C using a microprocessor-controlled water jacket. Dissolved oxygen levels (low: < 5% saturation; high: > 40% saturation) were controlled semi-automatically, using a combination of impeller revolution speed (via microprocessor-controlled feedback from the oxygen probe) and air/oxygen supply (manual control). The maximum impeller speed in all fermenter cultures was limited to 200 rpm, as ACEM 6063, like some other thraustochytrids (Yaguchi *et al.*, 1997) grow suboptimally when exposed to higher impeller speeds (see Chapter 4). Aeration was supplied using 0.2 µm-filtered (Midisart 2000, Sartorius, Germany) air for low DO cultures, or a mixture of air and oxygen (Industrial Grade O_2, BOC Gases, Australia) for high DO cultures. The air/oxygen mixture was delivered via 0.5-mm diameter holes situated at the base of the culture vessel. The culture system used was not able to supply sufficient oxygen to maintain high DO levels in cultures grown at 25°C.

Fermenter cultures were sampled daily from inoculation until 4 days after peak biomass (mg dry weight L^{-1}) was reached. Samples (10 mL) were aseptically drawn from the culture chamber directly into sterile, 15 mL screw-top plastic centrifuge tubes (Greiner, Germany). Cells were immediately centrifuged (5000 x g, 10 min: EasySpin, Sorval Instruments, USA), resuspended in 10 mL 1.0% (w/v) NaCl, and recentrifuged. Cell pellets were frozen, freeze-dried (chamber temperature -110°C, Mini Ultra Cold, Dynavac Australia; pressure < 7 x 10^{-1} mbar, RV3 vacuum pump, Edwards High Vacuum International) for 15 hours, weighed and then stored at -30°C. Culture biomass (mg dry weight L^{-1}) was determined daily using freeze-dried samples, as described in Section 4.3.2.1 (page 61).

5.3.3 Sterol extraction, fractionation and analysis

Analyses were performed on ACEM 6063 samples collected on the day each culture reached peak biomass, and 2 and 4 days prior to that day (T_p, T_{-2} and T_{-4} respectively). Lipids were extracted, in duplicate from each sample, using a one-phase chloroform-methanol-water (1:2:0.8 v/v/v) extraction (White *et al.*, 1979). Solvents were added to the freeze-dried biomass in order of increasing polarity, as this technique led to an increased lipid recovery from ACEM 6063 (Chapter 3). Phase separation was achieved the next day by adding chloroform and water to obtain a final chloroform-methanol-water ratio of 1:1:0.9 (v/v/v). Lipids were recovered from the lower chloroform phase. Solvents were removed under vacuum prior to the extracted lipids being stored in chloroform under N_2 at -20°C.

The sterol content of the various ACEM 6063 biomass samples was determined by analysing a portion of each total lipid extract using thin layer chromatography – flame ionisation detection (Iatroscan Mk V TH-10, Iatron Laboratories, Japan) as described in Section 4.3.3.2 (page 65).

To obtain the non-saponifiable lipids (which included the squalene and sterol fractions), portions of the total lipid extracts were transferred into 10 mL, screw-top test tubes. A fresh solution of 5% (w/v) potassium hydroxide in methanol-water (4:1 v/v) was added to each tube for reaction (saponification) at 60°C for 3 hours. After the addition of water (1 mL), the resultant non-saponifiable neutral lipids were extracted with hexane-chloroform (4:1 v/v, 3 x 2 mL) (Bakes and Nichols, 1995). Sterols in the non-saponifiable neutral lipid fraction were analysed by gas chromatography of their trimethylsilyl ether (TMS) derivatives. Steryl-TMS derivatives were prepared by reacting a portion of the non-saponifiable neutral lipid with an excess of N,O-bis(trimethylsilyl) trifluoroacetamide (Alltech, USA) at 60°C for one hour (Bakes and Nichols, 1995). Gas chromatographic data was obtained with a Hewlett Packard 5890 GC equipped with a 50 m x 0.32 mm internal diameter cross-linked HP5 methyl silicone (0.17 μm film thickness) fused-silica capillary column, an HP7673A autosampler, a split/splitless injector and flame ionization detector (Nichols *et al.*, 1996). Peak areas were recorded and quantified using Millenium 32 v3.05.01 (Waters Corporation, USA). Mass spectrometric data was obtained using a GCQ (Thermoquest, USA) GC-mass spectrometer (GC-MS), operated as described in Nichols *et al.* (1996). Component

identification was by comparison of retention time and MS data with that obtained for authentic and laboratory standards, including lipid from *Methylosphaera hansonii* (Schouten *et al.*, 2000).

Blank controls (i.e. fresh, cell-free culture medium) were incorporated into this study. No sterols were detected in any of the controls.

5.4 Results

Twenty sterols, 13 of which have been identified, were recovered from ACEM 6063 biomass (Figure 5.1, Table 5.1). The levels of sterols exceeding 3% of the total sterol fraction in each sample are shown in Table 5.2. The two major sterols in all samples were cholest-5-en-3β-ol and 24-ethylcholesta-5,22E-dien-3β-ol (sterols B and J respectively, Table 5.2). These two sterols accounted for 35–80% of the total sterols.

Cultures grown at 15°C-high DO and 20°C-high DO contained only sterols B, J, E (24-methylcholesta-5,22E-dien-3β-ol) and M (a mixture of two sterols co-eluting at a relative retention time of 1.58– one of which was 24-ethylcholesta-5,7,22-trien-3β-ol) at levels > 3% of the total sterols (Table 5.2). Cultures grown at 15°C-low DO, 20°C-low DO and 25°C-low DO also contained these sterols, but at slightly lower levels than in the high DO cultures. The low DO cultures also contained up to 6 of the following sterols at levels of > 3% of total sterols: cholest-8(9)-en-3β-ol, 5α-cholest-7-en-3β-ol, 24-methylcholesta-5,24(28)-dien-3β-ol, 24-ethylcholesta-5, 24(28)E-dien-3β-ol, 24-ethylcholesta-5, 24(28)Z-dien-3β-ol, and 4 unidentified sterols.

Absolute squalene content (expressed as mg g^{-1} dry weight; mean, n=2) in ACEM 6063 biomass varied under different temperature and dissolved oxygen parameters, and with time (Figure 5.2). Cultures grown at high DO contained lower levels of squalene than those grown at low DO. Cultures grown at 15°C-high DO all contained ≤ 0.01 mg g^{-1} squalene. Cultures grown at 20°C-high DO contained 0.01, 0.02 and 0.01 mg g^{-1} squalene at T_{-4}, T_{-2} and T_p respectively. Squalene content of cultures grown at 15°C-low DO decreased sequentially from T_{-4} to T_{-2} to T_p (1.8, 1.5 and 1.2 mg g^{-1} respectively).

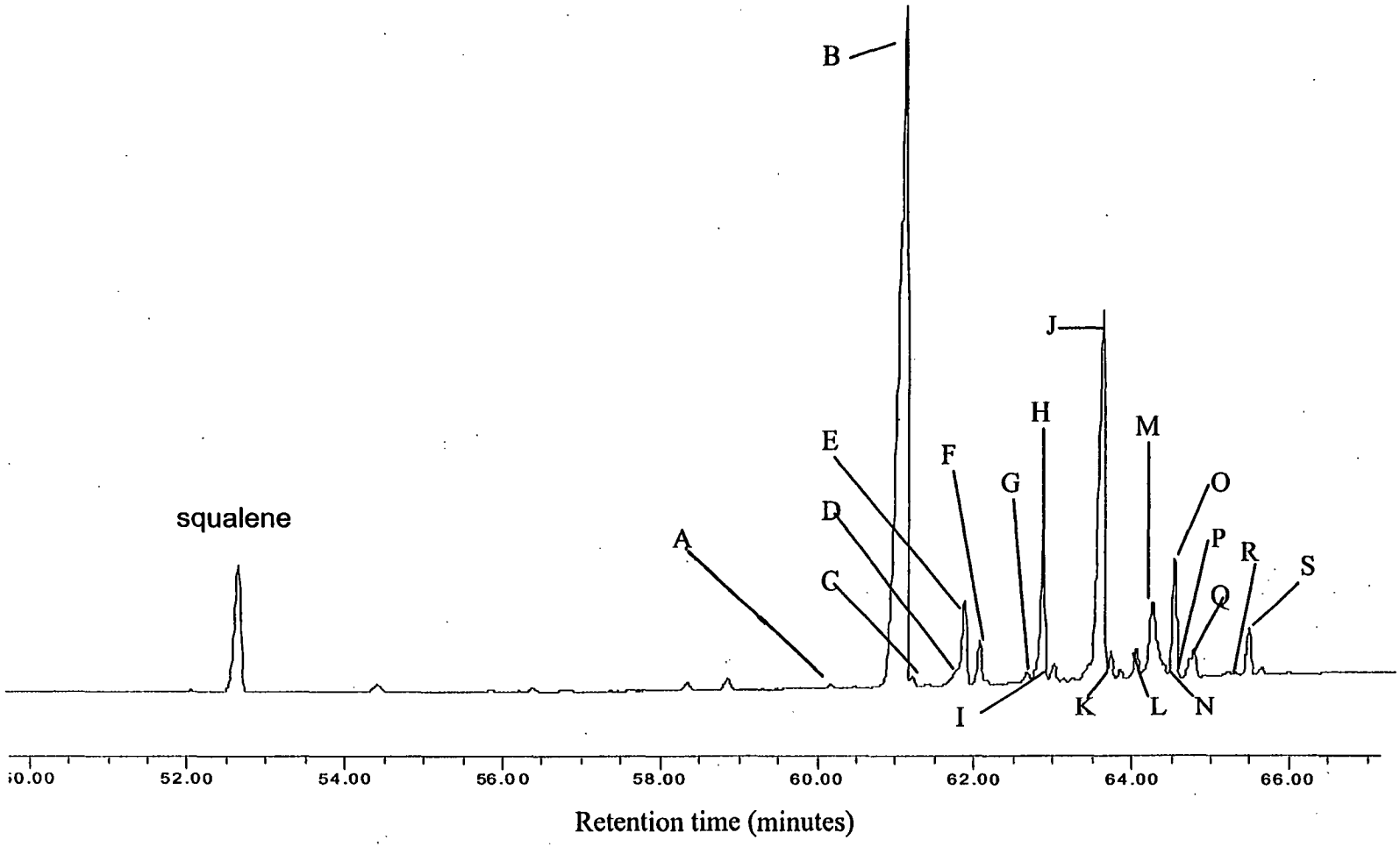

Figure 5.1 Representative partial gas chromatogram of squalene and sterols (as OTMS ethers) recovered from thraustochytrid strain ACEM 6063 (H5 capillary column; peak labels refer to Table 5.1)

Table 5.1 Sterols recovered from the thraustochytrid strain ACEM 6063

Code	Name	Trivial name [1]	RRT [2]	M+	Base peak	Major ion fragments (*m/z*)
A	cholesta-5, 22-dien-3β-ol	22-dehydrocholesterol	0.87	456	366	351, 327, 255, 129
B	cholest-5-en-3β-ol	cholesterol	1.00	458	368	443, 353, 329, 255, 247
C	cholest-8(9)-en-3β-ol	24-dihydrozymosterol	1.06	458	458	443, 368, 353, 255, 229, 213
D	cholesta-5, 24-dien-3β-ol	desmosterol	1.11	456	207	441, 366, 343, 253
E	24-methylcholesta-5, 22E-dien-3β-ol	brassicasterol / crinosterol	1.14	470	255	445, 380, 365, 340, 129
F	5α-cholest-7-en-3β-ol	lathosterol	1.18	458	458	443, 368, 353, 255, 229, 213
G [3]	4-methylcholest-8(14)-en-3β-ol		1.29	472	472	457, 382, 367, 227
H	24-methylcholesta-5,24(28)-dien-3β-ol	24-methylenecholesterol	1.32	470	296	455, 386, 341, 253
I [4]	Unknown 1		1.34	472	472	457, 382, 367, 227
J	24-ethylcholesta-5, 22E-dien-3β-ol	stigmasterol / poriferasterol	1.45	484	394	379, 355, 255, 129
K	Unknown 2		1.47	484	227	470, 394, 365
L [5]	Unknown 3		1.50	470	343	455, 386, 253
M [6]	Unknown 4, 24-ethylcholesta-5,7,22-trien-3β-ol		1.58	484/ 482	211	394, 377, 351, 253, 225
N [7]	4,4-dimethylcholesta-8(14),24-dien-3β-ol		1.60	484	241	469, 379, 357, 227
O	24-ethylcholesta-5, 24(28)E-dien-3β-ol	fucosterol	1.63	484	296	386, 371, 355, 281, 257
P [8]	Unknown 5		1.67	484	241	469, 394, 379, 357, 227
Q	24-ethylcholesta-5, 24(28)Z-dien-3β-ol	isofucosterol	1.68	484	296	386, 371, 281, 257
R	Unknown 6		1.78	470	357	400, 379, 267, 227
S [9]	Unknown 7		1.81	484/ 498	343	469, 409,393, 386, 365, 253

1: C24 stereochemistry not determined for sterols E and J
2: Relative Retention Time – calculated using cholesterol (1.00) and fucosterol (1.63) (Jones *et al.*, 1994)
3: structure IV from Schouten *et al.* (2000)
4: possibly the Δ8(9) analogue of G
5: shares major ion fragments with Unknown 7
6: mixed peak containing a C29 sterol with 2 double bonds [M+: 484] and 24-ethylcholesta-5,7,22-trien-3β-ol.
7: structure I from Schouten *et al.* (2000)
8: possibly the Δ8(9),24 analogue of N
9: mixed peak, shares major ion fragments with Unknown 3

Table 5.2 Sterol composition [1] of thraustochytrid strain ACEM 6063 cultured under different temperature and dissolved oxygen parameters (expressed as % of total sterols)

	Sterol [2] (mean, n=2)													
Treatment [3]	B	C	E	F	H	I	J	M	O	P	Q	R	S	Total [4]
$15LT_{-4}$	25		3.2			4.7	14	13	9.4	4.1	3.9	3.1	12	92
$15LT_{-2}$	22					4.4	13	14	8.2	5.2	3.2		19	89
$15LT_{p}$	27	6.9				3.3	18	11	11				11	88
$15HT_{-4}$	51		9.5				22	5.6						88
$15HT_{-2}$	54		8.1				25	4.7						92
$15HT_{p}$	55		7.9				25	5.2						93
$20LT_{-4}$	41		5.0	8.5			21	5.0	3.3				7.3	91
$20LT_{-2}$	42		3.1		3.2		16	4.5	4.2				12	85
$20LT_{p}$	50		3.8		5.4		19	4.3	4.3				7.2	94
$20HT_{-4}$	52		6.3				28	5.4						92
$20HT_{-2}$	55		6.9				26	5.0						93
$20HT_{p}$	53		6.5				26	5.2						91
$25LT_{-4}$	44						19	13	5.2				7.7	89
$25LT_{-2}$	41						18	13	3.5				13	89
$25LT_{p}$	47		4.6				19	13	3.2				4.3	91

1: Sterols comprising > 3% of the total sterol fraction (mean data, n=2)
2: see Table 5.1 for sterol identifications
3: Treatment notation:
15, 20, 25: incubation temperature (°C);
L, H: low (<5%), high (>40%) dissolved oxygen level in the culture medium;
T_{-4}, T_{-2}, T_{p}: sampled 4, 2 and 0 days prior to peak biomass being reached
4: Cumulative total of major sterols as a % of total sterols

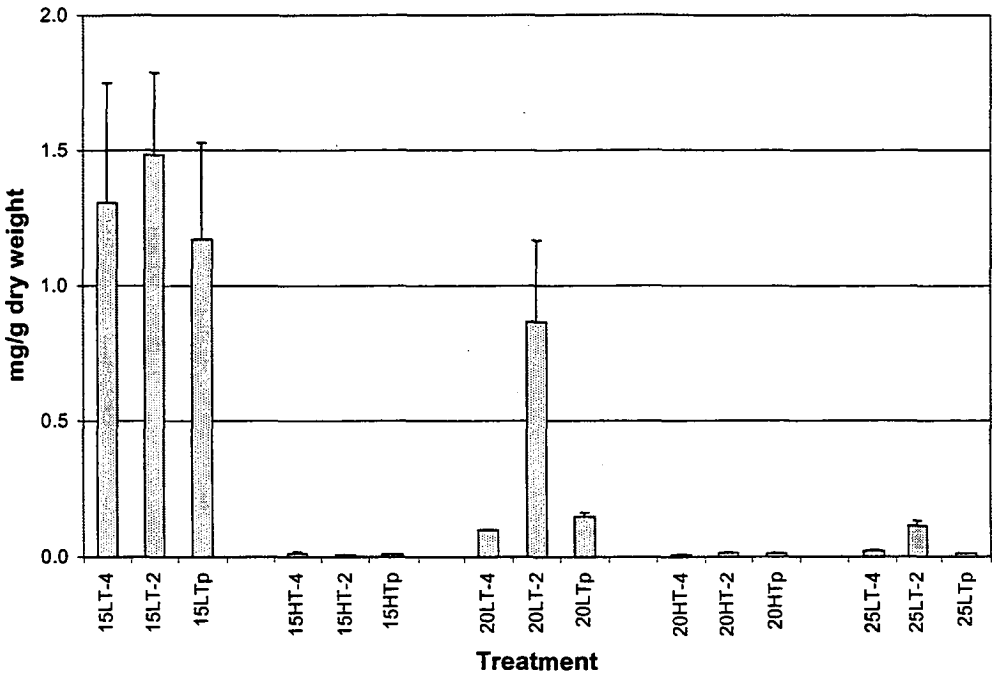

Figure 5.2 Squalene content of thraustochytrid strain ACEM 6063 biomass cultured under different temperature and dissolved oxygen parameters [expressed as mean±SD (n=2) mg g^{-1} of dry weight]

Treatment notation:

15, 20, 25: incubation temperature (°C);

L, H: low (<5%), high (>40%) dissolved oxygen level in the culture medium;

T-4, T-2, Tp: sampled 4, 2 and 0 days prior to peak biomass being reached

At each sample time (T_{-4}, T_{-2} and T_p), the culture grown at 20°C-low DO contained less squalene (0.1, 0.7 and 0.2 mg g^{-1} respectively) than that grown at 15°C-low DO. Similarly, at each sample time (T_{-4}, T_{-2} and T_p), the culture grown at 25°C-low DO contained less squalene (0.02, 0.1 and 0.01 mg g^{-1} respectively) than that grown at 20°C-low DO. The three cultures grown at 20°C and 25°C all contained the highest squalene concentrations at T_{-2}, whereas the two cultures grown at 15°C contained the highest squalene concentration at T_{-4}.

Total sterol content for ACEM 6063 biomass cultured under different temperature and dissolved oxygen parameters, and sampled at T_{-4}, T_{-2} and T_p is shown in Figure 5.3. The relative level of sterols in the total lipid extracted from ACEM 6063 decreased between T_{-4} (0.5-2.4 % total lipids) and T_p (0.4-0.9 % total lipids). Sterol concentrations in cultures grown at 15°C-low DO were lower than for those grown at 15°C-high DO (0.4-0.6 mg g^{-1} c.f. 0.8-1.2 mg g^{-1}). Sterol concentration in cultures grown at 20°C increased markedly between T_{-4} and T_{-2}, and then decreased slightly between T_{-2} and T_p. At T_{-4}, both cultures grown at 20°C contained a similar amount of sterol to those grown at 15°C-high DO (~1.0 mg g^{-1}). However, at T_{-2}, sterol content in cultures grown at 20°C had increased markedly (reaching 2.2 mg g^{-1} for low DO cultures, and 3.2 mg g^{-1} for high DO cultures). Sterol levels for both low and high DO cultures grown at 20°C at T_p (1.7 mg g^{-1} and 2.2 mg g^{-1} respectively) were slightly lower than those at T_{-2}. Absolute sterol concentration in the 25°C-low DO culture increased substantially between T_{-4} and T_{-2}, and increased slightly between T_{-2} and T_p (0.6, 1.4 and 1.5 mg g^{-1} respectively).

Cultures grown at 20°C and 25°C reached a peak biomass of 21-23 g dry weight L^{-1} after incubation for 7-8 days. Cultures grown at 15°C reached a peak biomass of 17 and 10 g dry weight L^{-1} (high DO and low DO cultures respectively) after incubation for 9 days.

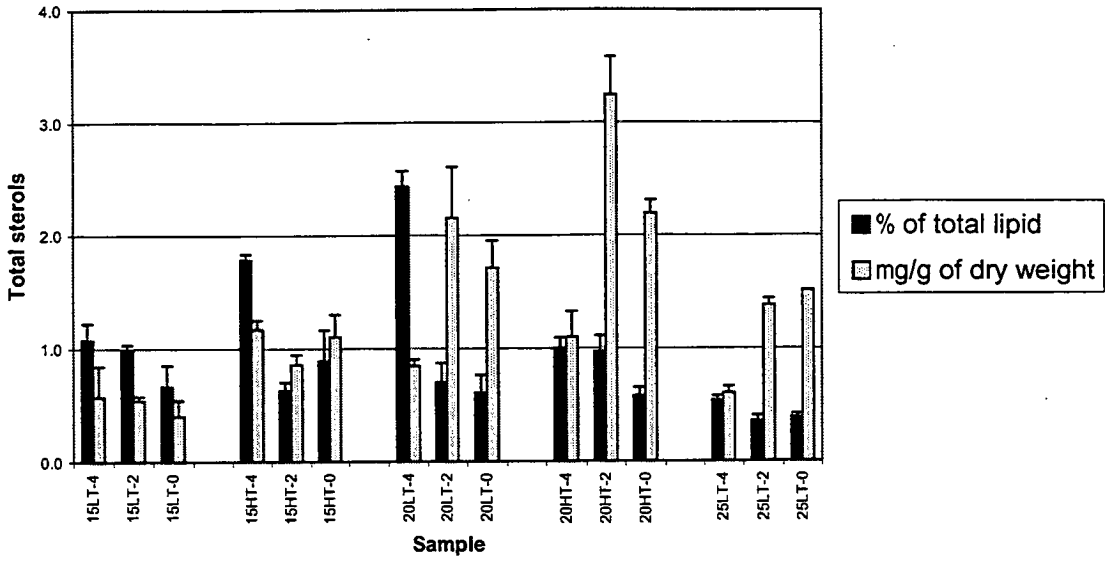

Figure 5.3 Total sterol content of thraustochytrid strain ACEM 6063 biomass cultured under different temperature and dissolved oxygen parameters [expressed as mean±SD (n=2) of % of total lipid and mg g^{-1} of dry weight]

Treatment notation:

15, 20, 25: incubation temperature (°C);

L, H: low (<5%), high (>40%) dissolved oxygen level in the culture medium;

T-4, T-2, Tp: sampled 4, 2 and 0 days prior to peak biomass being reached

5.5 Discussion

This study has demonstrated that changes to both physical and chemical culture conditions can influence squalene and sterol production by the thraustochytrid ACEM 6063. Changes in sterol levels, measured as a proportion of the total lipid fraction, were observed in cells cultured at different temperatures and dissolved oxygen concentrations. The proportion of sterols in the total lipid fractions of all treatments decreased with culture age, even when absolute sterol levels increased. Although there was no clear pattern to these changes, it is likely that they were brought about by variations in the relative amount of other lipid classes, in particular triacylglycerols, produced by ACEM 6063. As sterols are associated with cell membranes (Harwood and Russell, 1984), this result indicates ACEM 6063 produced proportionally less membrane lipids with increasing culture age. Similar findings have been reported for microalgae, some of which are thought to be the closest relatives of thraustochytrids (Cavalier-Smith *et al.*, 1994; Honda *et al.*, 1999). In a review on microalgal biomarkers, Volkman *et al.* (1998) reported that the proportion of microalgal membrane lipids generally increases during logarithmic growth, while neutral lipids (free fatty acids, triacylglycerols, ketones and hydrocarbons) are highest during the stationary phase (*i.e.* after achieving peak biomass).

The concentration of dissolved oxygen in the culture media influenced the squalene and total sterol concentration in ACEM 6063. A clear inverse relationship was seen between the squalene concentration in the different cultures and the DO levels at which those cultures were grown. Squalene requires oxygenation to form (3S)-squalene epoxide (3S-squalene-2,3-oxide), before being cyclised to form either lanosterol or cycloartenol (Jones *et al.*, 1994). It is proposed that at low DO levels, the efficiency of the conversion of squalene into sterols by ACEM 6063 was reduced. This observation could also explain the high squalene levels (63% of total non-saponifiable lipids) in another thraustochytrid (*Thraustochytrium* sp ATCC 26185) reported by Weete *et al.* (1997). The cultures described by Weete *et al.* (1997) were grown in Erlenmeyer flasks (100 mL medium in 250 mL flasks) on a rotary shaker at 120 rpm. These conditions are similar to those known to produce very low oxygen levels (< 5% DO) in actively growing thraustochytrid cultures (data not shown). Cultures grown at high DO contained higher total sterol concentrations than equivalent cultures grown at low DO.

Incubation temperature also influenced the absolute amount of squalene and total sterols in ACEM 6063 cells. An inverse relationship between absolute squalene content and culture temperature was observed. This was especially evident for cultures grown at low DO levels. While there also seemed to be an inverse correlation between squalene and sterol content at different oxygen levels (as discussed earlier), no correlation was evident at different culture temperatures. As was the case for culture DO, variations in culture temperature had a greater impact on total squalene content than on total sterol content.

The influence of culture age on squalene and total sterol content of ACEM 6063 biomass varied with incubation temperature. Squalene content of cells grown at 15°C did not vary substantially between T_{-4} and T_p. However, squalene content of cells grown at 20°C and 25°C did vary with culture age, and all showed the same pattern: squalene content increased between T_{-4} and T_{-2}, and decreased between T_{-2} and T_p. Total sterol concentration in the various ACEM 6063 cultures followed a similar trend to squalene content, especially for samples collected at T_{-4} and T_{-2}. In cultures grown at 15°C, sterol content at T_p was similar to those at T_{-2} and T_{-4}. In cultures grown at 20°C and 25°C, the total sterol content increased between T_{-4} and T_{-2}, and either decreased slightly or remained the same between T_{-2} and T_p.

Data presented here indicate a relationship between the absolute levels of squalene and total sterols in ACEM 6063 biomass. However, the degree of variation in squalene content between low and high DO cultures was much greater than that for sterols. There was also some discrepancy between concentration of squalene and total sterols in samples incubated at 20°C and 25°C and collected at T_{-2} and T_p, with the substantial decreases in squalene content not coinciding with similarly substantial decreases in total sterols. Although further work is required, the following sequence could at least partially explain these observations: a) from inoculation until T_{-2}, squalene production is sufficient to meet demands for sterol production; b) between T_{-2} and T_p, squalene production cannot meet this demand, resulting in a decrease in squalene reserves.

Culture DO appears to have had a greater influence on the relative amounts of individual sterols (expressed as a % of total sterols) than culture temperature or age. Although all sterols listed in Table 5.1 were detected in all samples, many were often present at very low levels (e.g. $\leq$ 1% of total sterols), especially in the high DO cultures. Cultures grown at high DO had a less diverse profile of those sterols comprising > 3% of the total sterols than low DO cultures. The high DO cultures were dominated by 4 or 5 sterols: cholest-5-

en-3β-ol, 24-ethylcholesta-5,22E-dien-3β-ol, 24-methylcholesta-5,22E-dien-3β-ol, and a mixture of two sterols – one of which was 24-ethylcholesta-5,7,22-trien-3β-ol - co-eluting at the relative retention time of 1.58. These sterols were present in similar proportions in all high DO cultures and comprised about 90% of the total sterols in each culture. The low DO cultures also contained these same sterols, but in lower proportions, together with several other sterols – again combining to a total of about 90% of the total sterols. One consistent difference between the sterols in high and low DO cultures was the relatively high levels of an unidentified sterol (sterol S: a mixture of two unidentified sterols, co-eluting at the relative retention time of 1.81) in all low DO cultures. These two sterols were also observed, but at much lower levels, in the high DO cultures. In all low DO cultures, the relative abundance of these two sterols was highest at T_{-2}.

Although the influence of culture media on sterol production was not examined, it is likely that sterol content of ACEM 6063 would also be influenced by changes to the media. Weete *et al.* (1997) reported that by omitting basal salts from the culture medium, the non-saponifiable lipid content of *Thraustochytrium* sp. ATCC 26185 decreased by 68%. Unfortunately, no data was presented on the sterol profiles obtained under these different culture conditions.

The individual sterol profiles of ACEM 6063 were similar to those reported in the only other study (known to the author) of sterol production by a thraustochytrid. Weete *et al.* (1997) reported that *Thraustochytrium* sp. ATCC 26185 contained cholest-5-enol, 24-methylcholesta-5,22-dienol, 24-methylcholesta-5,24,(28)-dienol, 24-ethyl-cholesta-5,22-dienol and 24-ethyl cholesta-5,7,22-trienol at levels of 41.3%, 7.6%, 1.5%, 12.4% and 21% respectively. In the present study, the same sterols (sterols B, E, H, J and a proportion of M) were detected at levels of 22-55%, 3-10%, < 3-5%, 13-28% and a proportion of 5-14% respectively. Weete *et al.* (1997) also reported 8 unknown sterols to a total of 17% of total sterols. It is interesting to note that neither lanosterol nor cylcoartenol, the two primary sterols formed by the cyclisation of 3S-squalene-2,3-oxide (Torssell, 1983), were detected in the present study or that of Weete *et al.* (1997). Assuming that one or both of these primary sterols would have been produced, it appears that, in these organisms, lanosterol and/or cylcoartenol occurred only as intermediates during the formation of other sterols.

Microalgae and other eukaryotic microorganisms produce a wide variety of sterols, some of which have been used as chemotaxonomic markers (e.g. Volkman *et al.*, 1989).

However, Volkman *et al.* (1998) also offered a caveat on comparisons of microalgal sterol compositions for taxonomic purposes, citing environmental conditions as a source of significant changes in many species. Given the results presented in this study, it appears that variations in some environmental conditions can also significantly influence the sterol profile of thraustochytrids. This indicates that taxonomic comparisons of thraustochytrids using sterol profiles should only be made with care. Notwithstanding these comments, the similarities of the sterol profiles of *Thraustochytrium* sp. ATCC 26185 presented by Weete *et al.* (1997) and those of ACEM 6063 in this study indicate a degree of taxonomic similarity between these two organisms. This inference is strengthened by the comment made by Weete *et al.* (1997) that unpublished sterol compositions for four other *Thraustochytrium* spp. were very similar to that of *Thraustochytrium* sp. ATCC 26185. It may be that the minor sterols will provide further taxonomic insight into the relationships between members of this group of organisms.

This Chapter presents, for the first time, an examination of sterol and squalene content of thraustochytrid biomass produced under different culture conditions. Results indicate that total squalene and sterol content were influenced to varying degrees by culture age, temperature and dissolved oxygen concentration. An interaction between incubation temperature and culture age was also noted; in that sterol content (mg g^{-1} dry weight) increased markedly between T_4 and T_2 in cultures grown at 20°C and 25°C, but did not increase in cultures grown at 15°C. This study represents an early step in the process of understanding sterol production by thraustochytrids. Further research will be needed to more carefully examine any interactions between the influence of major environmental variables on sterol content and composition. Some of the sterols identified in ACEM 6063 and in *Thraustochytrium* sp. ATCC 26185 (Weete *et al.*, 1997) are the same as those found in many common food plants (Harwood and Russell, 1984), and, as such, could be regarded as safe for human consumption. However, these two thraustochytrids also contain low levels of other unusual or unidentified sterols, which will require further characterisation and study before their nutritional effects can be determined. Whether understanding of thraustochytrid sterol production is required for taxonomic or nutraceutical purposes, the importance of describing the effect/s of physical and chemical culture parameters on sterol production by these organisms is likely to increase.

Chapter 6 Enrichment of rotifers (*Brachionus plicatilis*) with eicosapentaenoic acid and docosahexaenoic acid produced by bacteria

6.1 Abstract

Two bacterial strains, rich in either EPA (*Shewanella gelidimarina* ACAM 456) or DHA (*Colwellia psychroerythrus* ACAM 605) were tested for their ability to enrich rotifers (*Brachionus plicatilis*) in these polyunsaturated fatty acids. Rotifers were exposed for 24 hours to each bacterial strain and to a mixture of the two strains. They were then harvested and their fatty acid compositions were analysed and compared to those of rotifers that had been either starved or fed yeast (*Saccharomyces cerevisiae*) or microalgae (*Tetraselmis suecica*) in 2L glass flasks. Exposure to 1.4 x 10^9 cells mL^{-1} of the EPA-producing bacterium only resulted in rotifer EPA levels increasing from 0.1% to 1.2% of total dry weight (%dw). Similarly, following exposure to 1.0 x 10^9 cells mL^{-1} of the DHA-producing bacterium only, rotifer DHA levels increased from below detection to 0.1 %dw. When exposed to a mixture of the two bacterial strains, containing 7.0 x 10^8 cells mL^{-1} of the EPA producer and 5.0 x 10^8 cells mL^{-1} of the DHA producer, the rotifers' final EPA- and DHA-levels were 0.5 %dw and 0.3 %dw respectively. Although feeding strategies need refining, these results show, for the first time, that rotifers can be enriched with DHA from bacteria, and that rotifers can be enriched simultaneously with both DHA and EPA from different bacterial strains.

6.2 Introduction

Polyunsaturated fatty acids are essential components of aquaculture diets, and must be supplied through artificial food chains (Rimmer *et al.*, 1994). Provision of dietary PUFA, especially the n3 fatty acids EPA and DHA, is essential for normal growth and development of the larvae of many aquaculture species (e.g. Furuita *et al.*, 1996b). Fatty acids are usually provided to larvae through live diets, usually rotifers (e.g. *Brachionus plicatilis*) or brine shrimp (*Artemia* sp.) (Ostrowski and Divakaran, 1990), or via manufactured diets. As adults, many aquaculture species are reared on manufactured diets that must also contain PUFA (e.g. New and Csavas, 1995).

At present, only selected fish oils and microalgal species are utilised as large-scale industrial sources of PUFA (New and Csavas, 1995). While bacteria have previously been considered for use in aquaculture, their perceived lack of essential PUFA was seen as a major deficiency (Brown *et al.*, 1996). It is now evident, however, that certain strains of bacteria and autotrophic microalgae do produce high levels of PUFA (e.g. Bowman *et al.*, 1998a). Utilisation of such PUFA-producing microheterotrophs for aquaculture diets, either as extracts or directly into the food chain, is now an expanding area of interest (Brown *et al.*, 1996). Several EPA- and/or DHA-producing bacterial strains (Watanabe *et al.*, 1992; Intriago and Jones, 1993; Nichols *et al.*, 1996), and one DHA-rich thraustochytrid strain (Barclay and Zeller, 1996) have been used to enrich rotifers with these PUFA. Indeed, several DHA-rich, thraustochytrid-based products have already appeared on the aquaculture market, and research into additional applications is continuing.

The recent isolation of Antarctic bacteria that produce EPA (Nichols and Russell, 1996) and DHA (Bowman *et al.*, 1998a) provides a timely opportunity to develop novel biotechnological processes for the production of these essential dietary components. Preliminary experiments in which an EPA-producing Antarctic bacterial strain was fed to rotifers have been reported (Nichols *et al.*, 1996). These experiments demonstrated that rotifers could be enriched with EPA from bacteria. Importantly, results indicated a higher enrichment of EPA (i.e. ratio of % EPA in enriched rotifers : % EPA in the bacteria) than has been reported for many studies using microalgae as the source of EPA (e.g. Whyte and Nagata, 1990). Calculations

presented by Nichols *et al.* (1996) also suggest the potential for bacteria to produce PUFA at a greater rate than some microalgal species.

The experimental protocol for this study was based on that of Nichols *et al.* (1996), who found that, during a time-course feeding experiment, EPA content in rotifers increased with duration of exposure (up to the end point of 24 h) to the EPA-producing bacterial strain ACAM 456. The present study was designed to determine: 1) if rotifers could be similarly enriched with DHA derived from another bacterial strain (ACAM 605) previously found to be rich in DHA, and 2) whether these DHA- and EPA-producing bacteria could be used simultaneously to enrich rotifers with both of these essential PUFA.

6.3 Materials and Methods

6.3.1 Bacterial strains

Bacterial strains ACAM 456 and ACAM 605 (*Shewanella gelidimarina* and *Colwellia psychroerythrus* respectively) are facultatively anaerobic, Gram-negative, non-sporulating pleomorphic rods (Bowman *et al.*, 1997; Bowman *et al.*, 1998a). Both strains are psychrophilic, with optimum growth temperatures of 16.5 °C and 12 °C respectively. For this experiment they were grown at 10 °C, with generation times of 184 and 334 minutes. These strains were selected for this feeding experiment due to their ability to synthesise relatively high amounts (compared to other bacteria) of EPA (ACAM 456) or DHA (ACAM 605).

6.3.2 Bacterial culture

The two bacterial strains were grown in 50 mL of Zobell's broth (ZB) (Zobell, 1946) for 2 days (ACAM 456) and 3 days (ACAM 605) at 10 °C. Four 10 mL aliquots of each 50 mL broth culture were used to inoculate each of four sealed 2 L glass flasks containing 1.8 L of ZB. These cultures were incubated at 10 °C for 2 days (ACAM 456) and 3 days (ACAM 605), with agitation and aeration provided by the addition of ~ 2 L min^{-1} of 0.2 µm-filtered air to each flask. All cultures were harvested on the same day by centrifugation (3500 x g, 15 min). Cell pellets were pooled in minimal volumes of sterile 0.85% (w/v) saline. Total bacterial cell numbers were calculated

using light microscopy and a glass counting chamber (Weber Scientific, England). Cell concentrates were stored overnight at 2 °C.

6.3.3 Rotifer culture

Rotifers were from a population continuously cultured, with aeration and overhead fluorescent lighting, in 2000 L fiberglass cylindrical containers. Culture water was maintained at 21 to 25‰ salinity and at 21 °C. Rotifers were fed three times daily with compressed bakers yeast (*Saccharomyces cerevisiae*) at the rate of 0.6 gm yeast 10^{-6} rotifers. Supplemental feeding of the rotifer culture, with *Tetraselmis suecica*, occurred every second day, with the microalga added to a final concentration of about 1 x 10^5 cells mL^{-1}. Rotifer density was determined from the mean count of five 1 mL aliquots from each culture.

Twelve hours prior to the start of the feeding trial, sufficient rotifers were removed from culture, washed with clean culture water and placed in clean, aerated culture water at a concentration of about 300 rotifers mL^{-1}. These rotifers were not fed again before the start of the feeding trial.

6.3.4 Rotifer feeding and harvest

The feeding trial was performed using twelve 2 L glass flasks, maintained at 21 °C, and gently aerated. Flasks were randomly positioned within the experimental system. Bacteria, microalgae, yeast (6 duplicate treatments), as indicated in Table 6.1, were added to experimental flasks containing 1.4 L of 25‰ seawater at 21 °C. A control treatment of starved rotifers was also included. Finally, 100 mL of a suspension of the starved rotifers was added to each flask, to yield a final rotifer concentration of 200 rotifers mL^{-1}. A 9 mL aliquot was obtained from each flask 24 hours after addition of rotifers. Aliquots were added to 10 mL of neutral buffered formalin (NBF) (Ajax Chemicals, USA) in clean test tubes and stored at 4 °C prior to counting. Final counts of rotifers still actively swimming in each flask were obtained by counting rotifers from five x 1 mL aliquots of each NBF fixed sample.

Twenty four hours after the addition of rotifers, a 1 L sample was obtained from each flask. Each sample was transferred onto a clean silk screen (mesh size 63 µm), washed 3 times with ~ 1 L 25‰ seawater and transferred to a clean beaker using a minimal amount of 25‰ seawater. Washed rotifers were harvested by filtration onto

premuffled (450 °C, 24 h) glass-fibre filters (Whatman GF/F) and stored at -20 °C. Two 100 mL samples of the initial unfed rotifer culture and five 5 mL samples of the original bacterial concentrates were harvested prior to the start of the feeding trial. These samples were filtered and frozen as described above.

6.3.5 Extraction, fractionation and analysis of FAME

Lipids were extracted from bacteria and rotifer samples using a modified one-phase chloroform:methanol:water (1:2:0.8 v/v/v) extraction (White *et al.*, 1979). A portion of the total-lipid extract recovered from the lower chloroform phase was transmethylated by reaction for 1 h at 80 °C with methanol:hydrochloric acid:chloroform (10:1:1 v/v/v, 3 mL). Fatty acid methyl esters (FAME) were extracted and diluted with chloroform containing a known concentration of either 19:0 or 23:0 FAME as the internal injection standard. Analyses of the FAME were performed with a Hewlett Packard 5890 GC equipped with a 50 m x 0.32 mm internal diameter cross-linked methyl silicone (0.17 μm film thickness) fused-silica capillary column and flame ionization detector (Nichols *et al.*, 1996). Fatty acid percentages (Table 6.1) represent mean values of duplicate samples. Fatty acid concentration data (expressed as percent of total rotifer dry weight) were calculated using the final abundance of rotifers in each culture along with values obtained for dry weight of both fed- and starved-rotifers. Confirmation of FAME identity was performed on a Fisons MD 800 (Manchester, United Kingdom) GC-mass spectrometer (GC-MS) (Nichols *et al.*, 1996). Component identification was by comparison of retention time and MS data with that obtained for authentic and laboratory standards.

6.4 Results

Under the culture conditions used, ACAM 456 produced EPA to a level of 14.8±1.1% (mean±SD) of total fatty acids, while ACAM 605 produced 5.9±0.5% and 0.7±0.03% DHA and EPA respectively. Major components of the fatty acid fractions from rotifers are shown in Table 6.1. Statistical analysis (ANOVA) of final levels of EPA and DHA in rotifers, expressed as either %TFA or %dw, showed significant differences between treatments at $\alpha = 0.0001$ (EPA %TFA; DHA %TFA; DHA %dw) and $\alpha = 0.005$ (DHA %dw). Treatment means were grouped according to Fishers LSD test (SAS System for Windows, version 6.12, SAS Institute Incorporated, USA) (Table 6.1).

EPA content (%TFA and %dw) in those rotifers fed ACAM 456 only was significantly greater than in all other rotifers in this trial. Rotifers fed *T. suecica* contained either significantly lower (%TFA) or similar (%dw) levels of EPA than those fed a mixture of ACAM 456 and 605. Rotifers from these three treatments contained significantly higher EPA (%TFA and %dw) than in any of the remaining treatments.

DHA content (%TFA and %dw) in those rotifers fed a mixture of ACAM 456 and 605 was significantly higher than for all other rotifers in this trial. DHA levels (%dw) in rotifers from all other treatments were not significantly different from each other. Except as noted above, rotifers fed ACAM 605 only had significantly higher levels of DHA (%TFA) than rotifers from remaining treatments.

The numbers of healthy rotifers (i.e. those remaining in the water column at the conclusion of the feeding trial) varied considerably (Table 6.1). However, those rotifers exposed to ACAM 605 only, had a significantly lower survival rate than all other groups ($\alpha = 0.05$).

Table 6.1 Initial cell counts, final rotifer counts and EPA and DHA levels in duplicate samples of rotifers either starved or fed bacteria, microalgae or yeast for 24 h (means of values within each column with the same superscript letter are not significantly different)

Treatment	Initial cell count (mL^{-1})	Rotifer count (post-trial)	% total fatty acids		% dry weight (w/w)	
			EPA	DHA	EPA	DHA
Starved (pre-trial)		-	0.7, 0.7 ^{ef}	0, 0 ^{c}	0.1, 0.1 ^{c}	0, 0 ^{b}
Starved (post-trial)		173, 138 ^{a}	0.9, 0.7 ^{e}	0, 0 ^{c}	0.1, 0.1 ^{c}	0, 0 ^{b}
ACAM 456	1.4×10^{9}	181,155 ^{a}	6.8, 6.9 ^{a}	0, 0 ^{c}	1.1, 1.3 ^{a}	0, 0 ^{b}
ACAM 605	1.0×10^{9}	17, 102 ^{b}	1.2, 1.0 ^{d}	1.1, 1.6 ^{b}	0.1, 0.1 ^{c}	0.1, 0.1 ^{b}
ACAM 456 + 605	$7 \times 10^{8} + 5 \times 10^{8}$	166, 168 ^{a}	3.5, 3.3 ^{b}	1.7, 2.1 ^{a}	0.6, 0.4 ^{b}	0.4, 0.2 ^{a}
T. suecica	4.3×10^{5}	206, 198 ^{a}	2.0, 2.0 ^{c}	0.1, 0.1 ^{c}	0.4, 0.4 ^{b}	0, 0 ^{b}
S. cerevisiae	2×10^{8}	196, 207 ^{a}	0.5, 0.5 ^{f}	0, 0 ^{c}	0.1, 0.1 ^{c}	0, 0 ^{b}

Table 6.2 Major[1] fatty acid composition (mean % total fatty acid; n=2) of the feed bacterial cultures and rotifers either starved or fed bacteria, microalgae or yeast for 24 h

	Bacterial strain		Rotifer diet						
Fatty acid	ACAM 456	ACAM 605	Starved (pre-trial)	Starved	ACAM 456 only	ACAM 605 only	ACAM 456 and 605	*T. suecica*	*S. cerevisiae*
16:0	13.6	24.5	6.2	6.0	7.1	10.1	8.1	11.2	6.1
18:0	0.5	0.6	3.9	3.8	2.6	3.2	2.5	3.2	3.4
16:1(n-7)c	16.2	17.2	23.5	23.4	20.5	19.4	20.4	12.5	26.4
18:1(n-9)c	5.0	5.5	29.2	28.6	14.9	13.6	13.2	19.0	28.9
18:2(n-6)	1.7	1.9	6.6	7.0	3.9	3.0	3.6	15.7	6.5
20:4(n-3)	0.0	0.0	1.8	2.0	1.4	0.8	1.3	2.4	1.4
20:5(n-3)	14.6	0.2	0.7	0.8	6.9	1.1	3.4	2.0	0.5
22:6(n-3)	0.0	5.9	0.0	0.0	0.0	1.4	1.9	0.1	0

1: Fatty acids detected but not included in this Table: 12:0, 13:0, 14:0, 15:0, 17:0, i13:0, i14:0, i15:0, a15:0, 14:1, i15:1, i16:0, br17:1, i17:1, br19:1, 15:1n6, 16:1n9c, 16:1n7t, 16:1n5c, 17:1n6c, 17:1n8c, 18:1n5c, 18:1n7c, 19:1, 20:1n7c, 20:1n9c, 18:2n9, 22:1, 24:1, C_{16} PUFA, 18:3, 18:4n3, 20:2n6, C_{20} PUFA, 20:4n6, 22:5n3

6.5 Discussion

Interest in microorganisms, including bacteria and yeasts, for use in aquaculture diets is increasing (Watanabe *et al.*, 1992; Intriago and Jones, 1993; Nichols *et al.*, 1996). Brown *et al.* (1996) investigated 7 strains of marine bacteria and 6 strains of yeast as food for bivalves. One major finding from Brown *et al.* (1996) and similar work (Phillips, 1984) was that, although most strains provided high levels of good quality protein, they lacked detectable levels of essential PUFA, including EPA and DHA.

Maximum EPA incorporation by rotifers in this study (6.9 %TFA; 1.2 %dw) was similar to results presented by other studies in which EPA-producing bacteria were used as feed-stock for rotifers (Watanabe *et al.*, 1992). These authors reported EPA incorporation to a level equivalent to 1.8 %dw after exposing rotifers to 10^9 cells mL^{-1} of the marine bacterium SCRC-6370 for 12 hours. Nichols *et al.* (1996) reported a maximum EPA enrichment equivalent to 1.4 %dw for rotifers following 24 h exposure to 10^8 cells mL^{-1} of ACAM 456.

EPA enhancement of rotifers in the present study decreased significantly, from 1.2 %dw to 0.5 %dw, as the concentration of ACAM 456 to which rotifers were exposed decreased from 1.4×10^9 cells mL^{-1} to 7×10^8 cells mL^{-1}. These data support conclusions reached by Watanabe *et al.* (1992) and Nichols *et al.* (1996) that there is a close relationship between the concentration of EPA-producing bacteria and the level of EPA incorporation by rotifers exposed to those bacteria.

It should be noted that the maximum EPA enhancement of rotifers reported by Nichols *et al.* (1996) was equivalent to 5.8 %dw. However, differences in protocols for estimating bacterial concentrations are evident (*i.e.* direct microscopic cell counts in this study; colony forming units in Nichols *et al.* (1996)). Comparison of the two techniques reveals that the concentration of 10^8 cells mL^{-1} of ACAM 456 as measured by Nichols *et al.* (1996) was equivalent to the concentration of 10^9 cells mL^{-1} used in the present study (T. Lewis and D. Nichols, University of Tasmania, unpublished data).

The apparent efficiency of incorporation of DHA increased when the concentration of DHA-producing bacteria to which rotifers were exposed decreased from 1×10^9 cells mL^{-1} to 5×10^8 cells mL^{-1} (0.1 %dw c.f. 0.3 %dw; Table 6.1). The survival of

rotifers exposed to 1.0 x 10^9 cells mL^{-1} of ACAM 605 for 24 h was significantly lower than for any other treatment (α= 0.05) (Table 6.1). These results suggest that conditions in flasks containing ACAM 605 only were detrimental to rotifer survival, and may have led to the low uptake of bacterial DHA by rotifers. Barclay and Zeller (1996) reported DHA enrichment of rotifers to 1.4 %dw using a spray-dried *Schizochytrium* sp. This result is about 5-fold higher than the 0.3 %dw achieved in the present study.

The present study provides the first report of trophic transfer of DHA from bacteria to rotifers. The successful simultaneous transfer of DHA and EPA, both derived from bacteria, to rotifers has also been demonstrated. These results represent a unique step in the development of bacteria-based feeds for the aquaculture industry. Although thraustochytrids are considered to be a richer source of microbial PUFA than bacteria (Chapter 6 c.f. Chapter 7), there still may be a role for bacterial PUFA producers in the area of rotifer enrichment. As discussed in Section 1.5.2 (page 13) some aquaculture species assimilate PUFA more readily if it is provided in the form of phospholipid rather than triacylglycerol. Bacterial lipids are primarily phospholipid (Harwood and Russell, 1984), while thraustochytrid lipids are mainly triacylglycerol (Bowles *et al.*, 1999; Chapter 4). It may be that PUFA-producing bacteria will be used as a source of PUFA-rich phospholipid in some areas of the aquaculture industry.

Chapter 7 Enrichment of rotifers (*Brachionus plicatilis*) with polyunsaturated fatty acids produced by thraustochytrids

7.1 Abstract

Two Australian thraustochytrid strains, rich in polyunsaturated fatty acids, were preserved by different treatments and tested for their ability to enhance rotifers (*Brachionus plicatilis*) in these essential nutrients. No significant differences were found between the fatty acid profile of rotifers enriched with fresh, frozen, freeze-dried or air-dried biomass. Significant differences were observed, however, between rotifers fed different diets, consisting of biomass from individual strains or of a mixture of the two strains. After 6 h enrichment, fatty acid profiles of rotifers reflected those of their enrichment diets. With both strains, the sum of AA; EPA and DHA in rotifers enriched for 6 h reached 1.5-2% of dry rotifer weight in most treatments. DHA:EPA ratios varied between 0.3:1 and 6:1, depending on the diet. There was little change in fatty acid content of rotifers enriched for 24 h compared to 6 h. Following a 24 h post-enrichment starvation period, levels of AA, EPA and DHA in most enriched rotifers were still significantly higher than in continuously starved and yeast-fed rotifers, indicating that rotifers had digested and assimilated the microheterotroph fatty acids. Variations in rotifer survival between treatments were associated with the strain of microheterotroph used, the conditions under which one thraustochytrid strain was cultured, and the density of enrichment diet to which rotifers were exposed. The results indicate that the two strains would be suitable for use in Australian aquaculture, as they provide enrichment levels comparable to those achieved with currently available commercial enrichment diets.

7.2 Introduction

The long-chain (C_{20} and C_{22}) polyunsaturated fatty acid content of larval marine finfish and prawn diets is crucial for their normal growth and development (Watanabe *et al.*, 1983; Sorgeloos and Leger, 1992; Rainuzzo *et al.*, 1994a; Rees *et al.*, 1994; Takeuchi *et al.*, 1996; Zheng *et al.*, 1996). Specific PUFA requirements vary between and also within species, depending on the total lipid content and relative proportions of fatty acids in the diet (Sargent *et al.*, 1999). Typical values, however, are in the order of about 1-2% of feed dry weight (Izquierdo, 1996). Rotifers (*Brachionus plicatilis*) are used extensively as live prey in marine larviculture operations (Lubzens *et al.*, 1989). Under typical mass-rearing conditions using yeast as feed, rotifers generally lack sufficient PUFA to meet larval dietary requirements (Watanabe *et al.*, 1983). As such, rotifers usually need to be enriched with PUFA immediately prior to being fed to larvae.

The fatty acid composition of rotifers largely reflects that of their diet, and much effort has gone into development of formulations to efficiently enrich rotifers with PUFA (Watanabe *et al.*, 1983; Sorgeloos and Leger, 1992). Recent emphasis has been on diets containing high levels of DHA, and high ratios of DHA to EPA, as these have been shown to be important pre-requisites for normal larval development of many species of interest to the aquaculture industry (Takeuchi, 1997; Sargent *et al.*, 1999; Izquierdo *et al.*, 2000). There is also an increasing awareness of the dietary importance of other fatty acids, including AA (Sargent *et al.*, 1999). As more is learnt about the fatty acid requirements of aquaculture species, it is likely that species-specific inclusion levels for AA and other fatty acids in live- and manufactured-feeds will become more clearly defined.

As discussed in Chapter 6, utilisation of PUFA-producing microheterotrophs for aquaculture diets, either as extracts or via direct insertion into the food chain, is now an expanding area of interest. The work described in this Chapter was designed to evaluate the potential for rotifer PUFA content to be manipulated using enrichment diets consisting of pure and mixed biomass of strains ACEM 000A and ACEM 6063. These two strains were selected for their different fatty acid profiles (ACEM 6063 – high DHA, low AA and EPA; ACEM 000A – low DHA, moderate AA and EPA), and the ability of ACEM 000A to produce biomass containing relatively high and

low lipid levels under different culture conditions. Additional aims were to determine whether different thraustochytrid biomass preservation techniques would affect the degree of PUFA enrichment in rotifers, and whether fatty acids from the microheterotroph biomass would be incorporated into rotifer biomass or would remain within the microheterotroph cells.

7.3 Materials and Methods

7.3.1 Microheterotroph culture

Biomass of ACEM 000A, containing low oil levels (total fatty acids about 85 mg g^{-1}) was used in Trial 1. Biomass of ACEM 000A and ACEM 6063, containing high oil levels (total fatty acid levels about 550 and 340 mg g^{-1} respectively), was used in Trial 2. Biomass was produced in 500 mL, screw-topped Erlenmeyer flasks, containing 150 mL of Thraustochytrid Culture Medium broth (TCM; Chapter 2) supplemented to contain initial glucose concentrations of either 50 or 100 g L^{-1}. Cultures were incubated at 20°C on a rotary-shaking table (GIO Gyrotary Shaker, New Brunswick, USA) at 200 rpm for 7 (ACEM 6063) and 10 (ACEM 000A) days.

Cells were harvested by centrifugation (J2-21M/E centifuge, JA 14 rotor, Beckman USA) at 8000 x g for 15 minutes in 250 mL polyethylene centrifuge tubes. Cell pellets were washed in 100 mL 1.0% NaCl (w/v), re-centrifuged and the second supernatant discarded. Cell pellets for each strain were combined and then divided into sub-samples, each of which was exposed to one of the four different treatments described below.

- Fresh (FS) cells: stored overnight in a clean glass container at 2°C;
- Frozen (FZ) cells: stored in a clean glass container at -30°C;
- Freeze-dried (FD) cells: frozen at -30°C, freeze-dried (chamber temperature -110°C, Mini Ultra Cold, Dynavac Australia; pressure < 7 x 10^{-1} mbar, RV3 vacuum pump, Edwards High Vacuum International) for 15-18 hours and stored in a sealed glass container at -30°C.

These cells were weighed before and after drying to determine the dry-weight equivalent of the FS and FZ cells;

- Air-dried (AD) cells: spread thinly in a 20 cm diameter glass dish, dried overnight at 60°C, and stored in a sealed glass container at -30°C.

7.3.2 Rotifer culture

Rotifers were from a population continuously cultured as described in Section 6.3.3 (page 108).

7.3.3 Rotifer feeding and harvest

The same experimental protocol was used for Trials 1 and 2.

Eighteen hours prior to the start of each feeding trial, rotifers were removed from their culture tanks, washed with clean culture water and placed in clean, aerated culture water at a concentration of about 300 rotifers mL^{-1}. These rotifers were not fed again before the start of the feeding trial.

Each feeding trial was performed in 2 L glass flasks, maintained at 21 °C, and gently aerated. Flasks were randomly positioned within the experimental system. Biomass of ACEM 000A, ACEM 6063, a commercial thraustochytrid-based enrichment diet (Algamac-2000, Aquafauna BioMarine, USA), fresh microalgae (*Isochrysis* sp: clone T. Iso, strain CS-177/00, CSIRO Marine Research, Hobart) and yeast (*Saccharomyces cereviseae*) (Table 7.1 and Table 7.2) were added to the flasks, which were then filled to 1.5 L with 25‰ seawater at 21°C. Finally, 100 mL of a concentrated suspension of the starved rotifers was added to each flask, to yield a final rotifer concentration of 200 rotifers mL^{-1}.

Six hours after the addition of rotifers (T6), a 10 mL aliquot of actively swimming rotifers was obtained from the water column of each flask. Aliquots were added to 10 mL of neutral buffered formalin (NBF) (Ajax Chemicals, USA) in clean test tubes and stored at 4 °C prior to counting. Rotifer density was determined from the mean of 5 x 1 mL aliquots from each NBF fixed sample.

Table 7.1 Initial concentrations of enrichment diets used in Trial 1

	Enrichment diet						
	ACEM 000A (mg dry weight equivalent L^{-1})				Algamac (mg L^{-1})	T. Iso[1] (cells mL^{-1})	Yeast[2] (mg L^{-1})
Treatment[3]	Fresh	Frozen	Freeze -dried	Air-dried			
A FS	100	0	0	0	0	0	0
A FZ	0	100	0	0	0	0	0
A FD	0	0	100	0	0	0	0
A AD	0	0	0	100	0	0	0
Algamac	0	0	0	0	100	0	0
T. Iso	0	0	0	0	0	5×10^5	0
Yeast	0	0	0	0	0	0	100
Starved	0	0	0	0	0	0	0

1: T. Iso = *Isochrysis* sp, clone T. Iso
2: Yeast = Saccharomyces cerevisiae
3: A = ACEM 000A; FS = fresh cells; FZ = frozen cells; FD = freeze-dried cells; AD = air-dried cells

At the same time, a 350 mL sample was obtained from each flask. Each sample was washed (3 x ~500 mL 25‰ seawater) on a clean silk screen (mesh size 63 µm), and transferred to a clean beaker using a minimal amount of 25‰ seawater. Washed rotifers were harvested by filtration onto pre-weighed and muffled (450 °C, 24 hr) glass-fibre filters (GF/F 47 mm, Whatman, USA) and stored at -20 °C. Duplicate samples of the initial unfed rotifer culture (100 mL each) and of each component of the enrichment diets were collected prior to the start of each feeding trial. These samples were filtered and frozen as described above.

Flasks were then refilled to 1.6 L with 25‰ seawater and a second ration of feed (40% of the original ration) was added.

Eighteen hours later (T24), a second 350 mL sample (for fatty acid analysis) and one 10 mL aliquot (for counting) were obtained from each flask, and processed as described above.

Table 7.2 Initial concentrations of enrichment diets used in Trial 2

	Enrichment diet								
	ACEM 000A (mg dry weight equivalent L^{-1})		ACEM 6063 (mg dry weight equivalent L^{-1})				Algamac (mg L^{-1})	T. Iso[1] (cells mL^{-1})	Yeast[2] (mg L^{-1})
Treatment[3]	Frozen	Freeze-dried	Fresh	Frozen	Freeze-dried	Air-dried			
A FZ	100	0	0	0	0	0	0	0	0
A FD	0	100	0	0	0	0	0	0	0
A FD x2	0	200	0	0	0	0	0	0	0
A FD + T. Iso	0	100	0	0	0	0	0	5×10^5	0
A FD + 6063 FD	0	100	0	0	100	0	0	0	0
6063 FS	0	0	100	0	0	0	0	0	0
6063 FZ	0	0	0	100	0	0	0	0	0
6063 FD	0	0	0	0	100	0	0	0	0
6063 FD x2	0	0	0	0	200	0	0	0	0
6063 AD	0	0	0	0	0	100	0	0	0
6063 FD + T. Iso	0	0	0	0	100	0	0	5×10^5	0
Algamac	0	0	0	0	0	0	100	0	0
Algamac x2	0	0	0	0	0	0	200	0	0
T. Iso	0	0	0	0	0	0	0	5×10^5	0
Yeast	0	0	0	0	0	0	0	0	100
Starved	0	0	0	0	0	0	0	0	0

1: T. Iso = *Isochrysis* sp, clone T. Iso
2: Yeast = Saccharomyces cerevisiae
3: A = ACEM 000A; 6063 = ACEM 6063; FS = fresh cells; FZ = frozen cells; FD = freeze-dried cells; AD = air-dried cells; x2= double the standard enrichment rate (200 c.f. 100mg L^{-1})

After obtaining these samples, rotifers from 1.2 L from each flask were washed as described above and transferred into a clean flask, which was then filled to 1.6 L with isothermal 25‰ seawater. No food was added at this time.

Twenty four hours later (T48), a final 500 mL sample (for fatty acid analysis) and one 10 mL aliquot (for counting) were obtained from each flask, and processed as described above.

All filters were freeze-dried, weighed and stored at –20 °C prior to analysis.

7.3.4 Lipid extraction, fractionation and analysis

Lipids were extracted, fractionated and analysed as described in Section 6.3.5 (page 109), except that confirmation of FAME identity was achieved using either an MD 800 (Fisons, United Kingdom) or a GCQ (Thermoquest, USA) GC-mass spectrometer (GC-MS).

7.3.5 Statistical analyses

Statistical analyses of data were performed using the General Linear Models Procedure of the software package SAS System for Windows v 6.12 (SAS Institute Inc. USA). Differences between individual means of replicate samples were deemed to be significant at $\alpha = 0.05$.

7.4 Results

7.4.1 Enrichment diets

The major fatty acids in each component of the enrichment diets used in Trials 1 and 2 are shown in Table 7.3 and Table 7.4. The different treatments (fresh, frozen, freeze-dried, air-dried) of ACEM 000A and ACEM 6063 biomass did not significantly alter the fatty acid profiles of either strain. The fatty acid profile of ACEM 000A, however, was markedly different between the 2 trials. The proportion of saturated fatty acids (SFA) decreased from 61% of total fatty acids (TFA) in Trial 1 to 23 %TFA in Trial 2, while the proportion of monounsaturated fatty acids (MUFA) increased from 12 to 54 %TFA.

Table 7.3 Mean (n=2) fatty acid[1] composition (% total fatty acids except where noted) of enrichment diets used in Trial 1

	Enrichment diet						
Fatty acid	A FS[2]	A FZ	A FD	A AD	Algamac	T. Iso	Yeast
14:0	11	12	11	6.0	17	12	
16:1n7	2.9	3.5	2.9	2.8	4.9	5.5	27.1
16:0	38	38	37	40	38	15	14
18:3n6						3.0	
18:4n3						13	
18:2n6						11	20
18:1n9	6.4	6.5	6.1	7.1		23	30
18:0	13	13	14	15			4.9
20:4n6	6.2	6.3	6.3	7.0			
20:5n3	3.5	3.7	3.7	3.9			
20:1n9+11	2.1	2.1	2.1	2.1			
22:5n6					13		
22:6n3	4.8	4.6	4.8	4.3	21	8.2	
22:5n3	7.2	6.4	7.4	5.9			
sum SFA[3]	61	62	61	61	56	27	19
sum MUFA[4]	12	12	12	12	5.9	31	59
sum PUFA[5]	26	26	27	26	37	41	22
sum other	0.7	0.5	1.1	0.9	1.0	0.5	0.3
TFA[6] mg g^{-1}	82	96	81	63	440	84	6.8

1: fatty acids comprising >2% of the mean total fatty acids. Other fatty acids detected below this value included: 12:0, i14:0, 14:1n9, 14:1n7, 14:1n5, i15:0, a15:0, 15:1, 15:0, 16:1n9, 17:1, 17:0, 18:3n3 (co-eluted with 18:1n9), 18:1n7, 19:1, 20:2, 20:3n6, 20:4n3, 20:2n6, 20:1n7, 20:0, 22:4n6, C22PUFA

2: A = ACEM 000A; FS = fresh cells; FZ = frozen cells; FD = freeze-dried cells; AD = air-dried cells; T. Iso = *Isochrysis* sp, clone T. Iso; Yeast = *Saccharomyces cerevisiae*

3, 4, 5, 6: SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; TFA = total fatty acids

Table 7.4 Mean (n=2) fatty acid[1] composition (% total fatty acids except where noted) of enrichment diets used in Trial 2

	Enrichment diet								
Fatty acid	A FZ[2]	A FD	6063 FS	6063 FZ	6063 FD	6063 AD	Algamac	T. Iso	Yeast
14:0	3.9	3.4	14	14	13	14	15	16	
16:1n9								2.6	
16:1n7	3.4	3.1					4.9	4.8	30
16:0	15	14	34	33	35	38	42	12	14
18:3n6								2.4	
18:4n3								22	
18:2n6	2.4	2.3						7.4	20
18:1n9	20	21						15	29
18:1n7	25	26						2.7	2.1
18:0	4.2	4.8							5.4
20:4n6	4.8	5.0							
20:5n3	5.2	4.7							
20:1n9+11	4.9	4.9							
22:5n6			11	13	12	12	12		
22:6n3			28	29	28	26	21	7.7	
22:5n3	5.2	5.0							
sum SFA[3]	23	22	52	50	50	54	58	29	19
sum MUFA[4]	53	55	2.7	1.8	2.2	2.4	5.9	27	60
sum PUFA[5]	23	23	45	48	47	43	36	43	20
sum other	0.6	0.6	0.5	0.5	0.8	1.0	0.4	1.0	0.9
TFA[6] mg g^{-1}	557	544	346	342	330	353	533	84	5.7

1: fatty acids comprising >2% of the mean total fatty acids. Other fatty acids detected below this value included: 12:0, i14:0, 14:1n9, 14:1n7, 14:1n5, i15:0, a15:0, 15:1, 15:0, i16:0, 17:1, 17:0, 20:3n6, 20:4n3, 20:2n6, 20:0, 22:4n6, C22PUFA

2: A = ACEM 000A; 6063 = ACEM 6063; FS = fresh cells; FZ = frozen cells; FD = freeze-dried cells; AD = air-dried cells; T. Iso = Isochrysis sp, clone T. Iso; Yeast = Saccharomyces cerevisiae

3, 4, 5, 6: SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; TFA = total fatty acids

The proportion of PUFA was essentially the same in both trials. These changes in fatty acid profile were due to modifications of culture conditions. The fatty acid profile of biomass of ACEM 6063, which was used in Trial 2 only, comprised about 50% SFA, 2% MUFA and 48% PUFA. The fatty acid profile of the commercially-available Algamac cells used in both trials was 58% SFA, 6% MUFA and 36% PUFA. The proportions of SFA, MUFA and PUFA in the T. Iso cells were 29%, 27% and 43% respectively. The proportions of SFA, MUFA and PUFA in the yeast cells were about 19%, 60% and 20% respectively.

In Trial 1, the absolute level of TFA (mg g^{-1} dry weight) in air-dried cells of ACEM 000A was significantly lower than in the other ACEM 000A cells (63 mg g^{-1} c.f 81-96 mg g^{-1}). The absolute TFA content of ACEM 000A used in Trial 2 was about 6-fold higher (550 mg g^{-1}) than that used in Trial 1. This increase in lipid content coincided with the marked shift in the relative proportions of SFA and MUFA mentioned earlier. The TFA content of ACEM 6063 cells was between 330 and 353 mg g^{-1}. The absolute amount of TFA in Algamac, T. Iso and yeast cells was about 530, 84 and 6 mg g^{-1} respectively.

7.4.2 Trial 1

The fatty acid compositions of rotifers sampled during Trial 1 are shown in Table 7.5 and Table 7.6. Although there were slight differences in the proportions of SFA, MUFA and PUFA in rotifers fed the different ACEM 000A treatments at different time points, the same general trends were evident. The proportion (%TFA) of SFA in all rotifers fed ACEM 000A increased between the start of the trial (T0) and T6, and then decreased slightly between T6 and T48. The proportion of MUFA decreased between T0 and T6, and did not vary substantially between T6 and T48. The proportion of PUFA in ACEM 000A-fed rotifers increased slightly between T0 and T6, and again between T6 and T48.

Table 7.5 Mean (n=2) fatty acid[1] composition (% total fatty acids except where noted) of rotifers before enrichment, and after 6 and 24 h enrichment with ACEM 000A and a further 24 h starvation [Trial 1]

		Enrichment diet											
		A FS[2]			A FZ			A FD			A AD		
Fatty acid	T0[3]	T6	T24	T48	T6	T24	T48	T6	T24	T48	T6	T24	T48
14:0		5.9	4.4		5.1	4.1	2.3	5.3	4.2	2.6	4.8	3.6	2.0
16:1n7	15	12	9.1	8.5	11	9.8	8.4	12	8.6	8.9	11	9.4	8.6
16:0	9.5	19	16	16	18	18	14	18	17	15	18	17	16
18:2n6	13	8.1	7.1	8.1	8.1	7.8	7.7	8.4	7.0	8.0	8.2	7.3	8.0
18:1n9	26	16	15	16	17	15	15	17	14	16	17	15	16
18:1n7	5.5	3.2	3.3	4.2	3.2	3.5	3.9	3.3	3.2	3.9	3.2	3.6	4.0
18:0	5.2	7.4	6.2	6.3	7.5	6.8	5.8	7.1	6.6	6.3	7.4	6.9	6.4
20:4n6		4.9	6.8	7.4	5.7	7.0	7.7	5.6	7.2	7.3	5.6	7.0	7.3
20:5n3		2.7	4.0	4.4	3.1	3.9	4.3	3.0	3.7	3.8	3.0	3.7	3.8
20:4n3	2.8												
20:1n9+11	3.4	2.2	2.8	3.1	2.3	2.4	3.0	2.1	2.6	3.2	2.5	2.8	2.9
22:6n3		2.4	3.8	3.0	2.8	3.1	3.3	2.5	3.4	2.8	3.1	3.0	2.6
22:4n6				2.3			2.6			2.2			2.3
22:5n3		3.9	7.6	7.5	4.2	5.9	8.1	3.6	6.9	7.3	4.3	6.6	7.2
sum SFA[4]	19^{a}	34^{b}	28^{c}	25^{c}	32^{b}	30^{b}	24^{a}	32^{b}	29^{ab}	26^{ab}	32^{b}	29^{b}	26^{ab}
sum MUFA[5]	55^{a}	35^{b}	33^{b}	34^{b}	35^{b}	33^{b}	34^{b}	36^{b}	31^{b}	35^{b}	36^{b}	34^{b}	34^{b}
sum PUFA[6]	26^{a}	30^{a}	38^{b}	40^{b}	32^{ab}	36^{bc}	41^{c}	31^{ab}	37^{ab}	39^{b}	32^{ab}	37^{bc}	39^{c}
sum other	1.1	0.8	0.8	0.6	0.7	1.6	0.7	0.7	2.5	0.9	0.6	0.6	1.0
TFA[7] mg g^{-1}	34^{a}	48^{b}	39^{b}	22^{c}	44^{b}	33^{a}	28^{c}	52^{b}	33^{b}	32^{c}	48^{b}	38^{b}	28^{c}

1: fatty acids comprising >2% of the mean total fatty acids. Other fatty acids detected below this value included: 12:0, 14:1n9, 14:1n7, 14:1n5, i15:0, a15:0, 15:1, 15:0, i16:0, 16:1n9, i17:0, 17:0, 18:3n6, 18:4n3, 18:3n3 (co-eluted with 18:1n9), 19:1, 20:3n6, 20:1n9+11, 20:2n6, 20:0, 22:5n6

2: A = ACEM 000A; FS = fresh cells; FZ = frozen cells; FD = freeze-dried cells; AD = air-dried cells

3: T0 = before enrichment; T6 = enriched for 6 h; T24 = enriched for 24 h; T48 = starved for 24 h following 24 h enrichment

4, 5, 6, 7: SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; TFA = total fatty acids. Values for SFA, MUFA, PUFA and TFA with different superscripts within each treatment were deemed to be significantly different at $\alpha = 0.05$ (values for T0 all have the superscript "a")

Table 7.6 Mean (n=2) fatty acid[1] composition (% total fatty acids except where noted) of starved rotifers and those fed Algamac, Isochrysis sp (clone T. Iso) or yeast for 6 and 24 h and starved for a further 24 h [Trial 1]

	Enrichment diet											
	Algamac			T Iso[2]			Yeast			Starved		
Fatty acid	T6[3]	T24	T48	T6	T24	T48	T6	T24	T48	T6	T24	T48
14:0	11	4.9	2.0	8.2	8.8	5.2						
16:1n7	7.5	7.3	8.4	11	8.9	7.8	22	25	20	16	15	12
16:0	25	15	20	10	9.0	11	9.1	7.8	7.9	10	8.1	11.2
18:3n6				2.7	2.8	2.4						
18:4n3				8.7	12	6.6						
18:2n6	3.0	3.2	4.5	13	13	15	10	7.2	7.9	14	14	14
18:3n3				2.6	3.9	3.2						
18:1n9	5.8	6.4	11	18	15	16	29	30	29	25	26	24
18:1n7		3.0	4.9	3.3	3.5	4.2	5.1	5.5	5.7	5.5	5.0	6.5
18:0	2.0	2.8	5.2	2.6	2.0	3.6	5.2	4.3	5.2	5.6	5.9	7.4
20:4n6		2.7	3.6								2.4	2.6
20:5n3		3.4	4.0			2.6						2.1
20:4n3					3.8	5.7				2.4	3.2	2.7
20:1n9+11				2.0	2.6	3.2	3.4	3.8	4.2	3.6	4.4	3.8
22:5n6	15	11	4.7									
22:6n3	19	30	17	5.0	6.1	4.2						
sum SFA[4]	38^b	23^c	29^c	22^a	21^a	21^a	18^b	15^b	16^b	20^a	17^a	17^a
sum MUFA[5]	17^b	19^b	28^c	37^b	31^c	34^c	61^b	68^b	62^b	53^a	52^a	53^a
sum PUFA[6]	45^b	54^c	42^b	40^b	48^c	44^b	20^b	16^c	20^b	27^a	30^a	29^a
sum other	0.4	4.2	1.4	0.7	0.5	1.0	1.0	1.1	1.8	1.0	0.8	0.7
TFA[7] mg g^{-1}	128^b	66^b	23^c	59^a	90^b	55^a	32^a	34^a	30^a	33^a	19^b	11^b

1: fatty acids comprising >2% of the mean total fatty acids. Other fatty acids detected below this value included: 12:0, 14:1n9, 14:1n7, 14:1n5, i15:0, a15:0, 15:1, 15:0, i16:0, 16:1n9, i17:0, 17:0, 19:1, 20:3n6, 20:1n9+11, 20:2n6, 20:0, 22:5n6, 22:4n6, 22:5n3

2: T. Iso = *Isochrysis* sp, clone T. Iso; Yeast = *Saccharomyces cerevisiae*

3: T6 = enriched for 6 h; T24 = enriched for 24 h; T48 = starved for 24 h following 24 h enrichment

4, 5, 6, 7: SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; TFA = total fatty acids. Values for SFA, MUFA, PUFA and TFA with different superscripts within each treatment were deemed to be significantly different at $\alpha = 0.05$ (values for T0 all have the superscript "a")

The proportion of SFA in the Algamac-fed rotifers increased markedly between T0 and T6, decreased between T6 and T24, and was stable between T24 and T48 (Table 7.6). The proportion of MUFA decreased substantially between T0 and T6, was stable between T6 and T24, and increased between T24 and T48. The proportion of PUFA in these rotifers rose between T0 and T6, and between T6 and T24, and decreased between T24 and T48.

The proportion of SFA in the T. Iso-fed rotifers did not change substantially between T0 and T48. The proportion of MUFA decreased between T0 and T6, and between T6 and T24, and was stable between T24 and T48. The proportion of PUFA rose between T0 and T6, and T6 and T24, and decreased slightly between T24 and T48. The proportion of SFA in the yeast-fed rotifers decreased slightly between T0 and T6 and was relatively stable between T6 and T48. The proportion of MUFA increased substantially between T0 and T6, and was stable between T6 and T48. The proportion of PUFA in these rotifers decreased between T0 and T6, decreased again between T6 and T24, and increased slightly between T24 and T48. There were no significant variations in the proportions of SFA, MUFA or PUFA in the starved rotifers during the course of this trial.

Absolute TFA levels in all treatments except yeast-fed and starved rotifers increased between T0 and T6. At T6, all rotifers fed ACEM 000A contained similar amounts of TFA (44 – 52 mg g^{-1} dry weight). Algamac- and T. Iso-fed rotifers contained significantly more TFA (128 and 59 mg g^{-1} respectively) than the other treatments. TFA levels in yeast-fed and starved rotifers (32 and 33 mg g^{-1} respectively) were similar to those in rotifers at T0.

At T24, absolute TFA levels in ACEM 000A-fed rotifers had decreased slightly and were all between 33 and 39 mg g^{-1}. TFA levels in Algamac-fed rotifers had decreased to 66 mg g^{-1}, while those in T. Iso-fed rotifers had increased to 90 mg g^{-1}. Total fatty acid levels in yeast-fed and starved rotifers were 34 and 19 mg g^{-1} respectively.

At T48, TFA levels in rotifers fed ACEM 000A, Algamac and yeast were all similar (22 – 32 mg g^{-1}), while T. Iso-fed rotifers contained 55 mg g^{-1}. The TFA level in starved rotifers had decreased to 11 mg g^{-1}.

At T6, the highest AA levels (2 – 3 mg g^{-1}) were found in rotifers enriched with ACEM 000A and Algamac (Figure 7.1). AA levels in all other rotifers varied between 0.5 mg g^{-1} (yeast-fed rotifers) and 1 mg g^{-1} (T. Iso-fed rotifers). Highest EPA levels (2 mg g^{-1}) were found in Algamac-fed rotifers, and varied between 0.4 mg g^{-1} (yeast-fed rotifers) and 1.5 mg g^{-1} [ACEM 000A (freeze-dried)-fed rotifers] for the other treatments. Highest DHA levels (25 mg g^{-1}) were found in Algamac-fed rotifers. DHA levels in all other rotifers varied between 0.1 mg g^{-1} (yeast-fed rotifers) and 3 mg g^{-1} (T. Iso-fed rotifers).

At T24, the highest AA levels (2 – 3 mg g^{-1}) were found in rotifers enriched with ACEM 000A (Figure 7.1). AA levels in all other rotifers varied between 0.4 mg g^{-1} (yeast-fed rotifers) and 2 mg g^{-1} (Algamac-fed rotifers). Highest EPA levels (2 mg g^{-1}) were found in Algamac-fed rotifers. EPA levels in all other rotifers varied between 0.3 mg g^{-1} (yeast-fed rotifers) and 2 mg g^{-1} [ACEM 000A (fresh)-fed rotifers].

At T48, the highest AA levels (2 – 3 mg g^{-1}) were found in rotifers enriched with ACEM 000A (Figure 7.1). AA levels in all other rotifers varied between 0.3 mg g^{-1} (starved rotifers) and 1 mg g^{-1} (T. Iso-fed rotifers). Highest EPA levels (ca 1 mg g^{-1}) were found in ACEM 000A- and Algamac-fed rotifers. EPA levels in yeast-fed and starved rotifers were 0.4 mg g^{-1} and 0.2 mg g^{-1} respectively. Highest DHA levels (4 mg g^{-1}) were found in Algamac-fed rotifers. DHA levels in all other rotifers varied between < 0.1 mg g^{-1} (starved rotifers) and 2 mg g^{-1} (T. Iso-fed rotifers).

The density of rotifers in each flask was assessed at each sampling time (Figure 7.2). At T6, rotifer numbers had decreased markedly (c.f. 200 mL^{-1} at T0) for all treatments. At this time, the density of starved rotifers and T. Iso-fed rotifers (117 and 96 rotifers mL^{-1} respectively) were significantly higher than for all other treatments (59 - 84 rotifers mL^{-1}).

At T24, rotifer numbers had again decreased, although this was only significant for the starved rotifers and those fed Algamac and T. Iso. The density of Algamac-fed rotifers (31 rotifers mL^{-1}) was significantly lower than for all other treatments (50 – 75 rotifers mL^{-1}). At T48, rotifer density had decreased significantly (c.f. counts at T24) in treatments fed ACEM 000A or yeast. Differences in rotifer density between treatments at T48 were not significant.

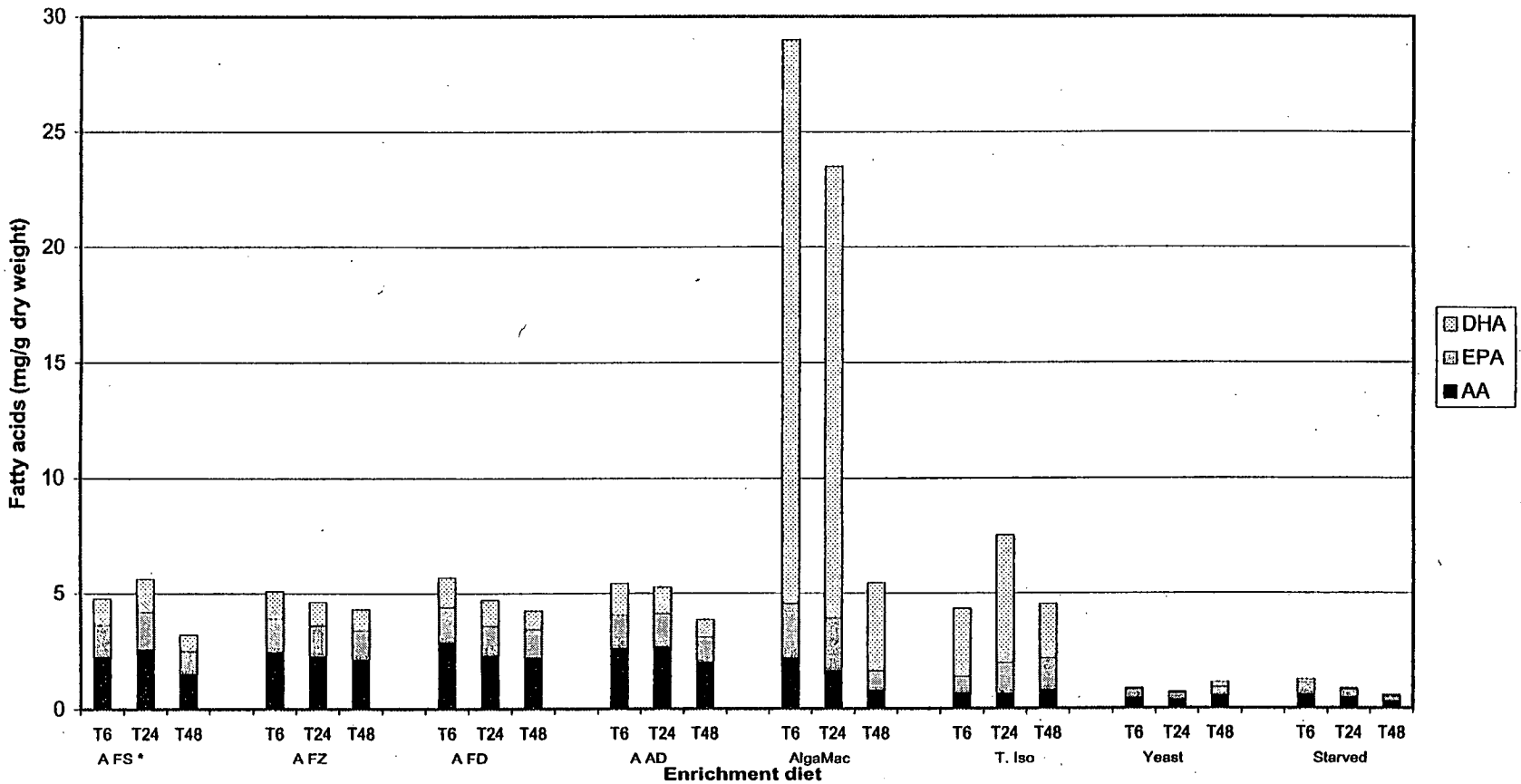

Figure 7.1 Mean amounts (mg g^{-1} dry weight; n=2) of 20:4n6 (AA), 20:5n3 (EPA) and 22:6n3 (DHA) in rotifers following 6 (T6) and 24 (T24) h enrichment and a further 24 h starvation (T48) [Trial 1]

*: A = ACEM 000A; FS = fresh cells; FZ = frozen cells; FD = freeze-dried cells; AD = air-dried cells; T. Iso = *Isochrysis* sp, clone T. Iso; Yeast = *Saccharomyces cerevisiae*

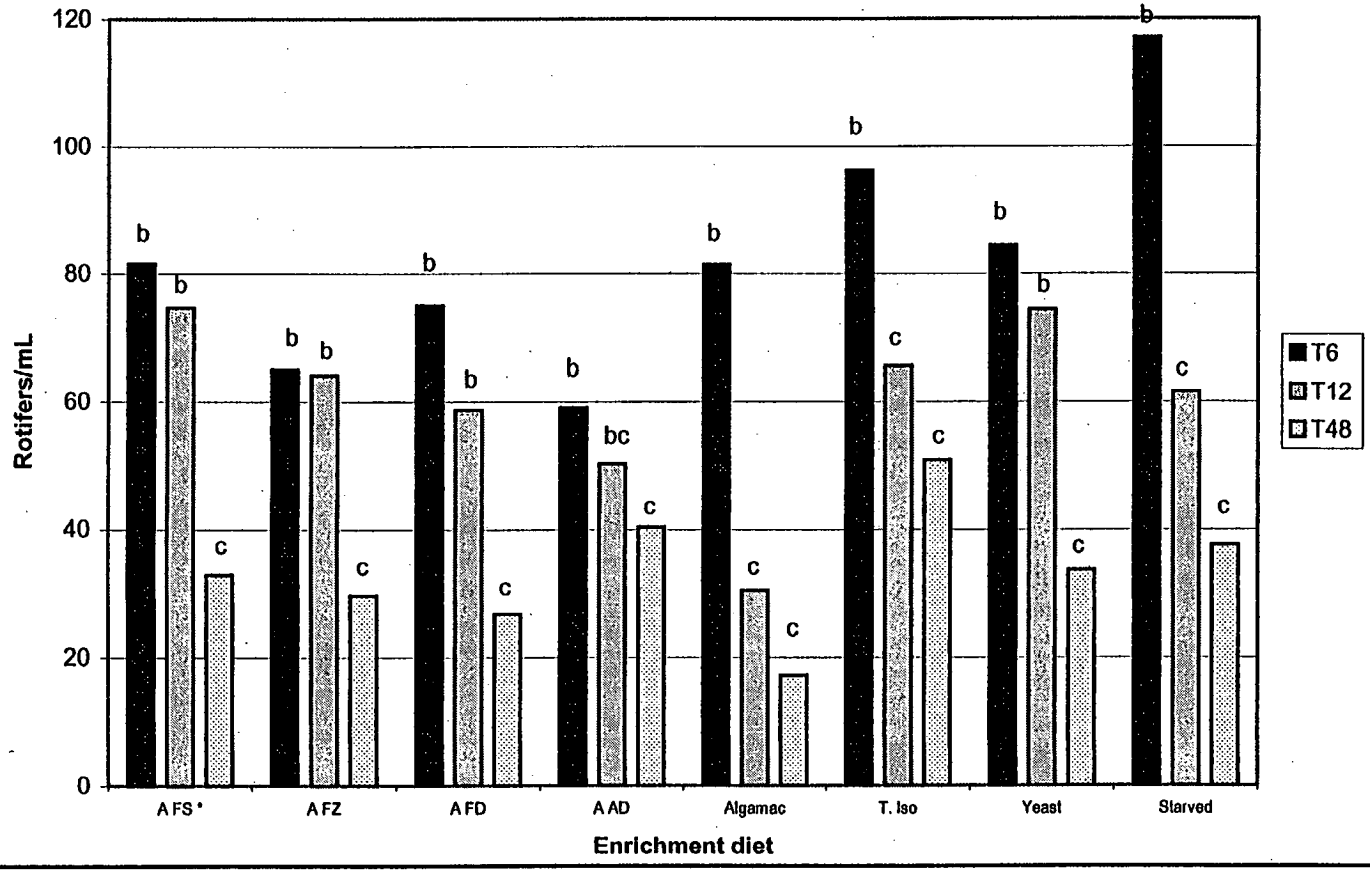

Figure 7.2 Mean rotifer density (number mL^{-1}; n=2) following 6 (T6) and 24 (T24) h enrichment and a further 24 h starvation (T48) [Trial 1]

*: A = ACEM 000A; FS = fresh cells; FZ = frozen cells; FD = freeze-dried cells; AD = air-dried cells; T. Iso = *Isochrysis* sp, clone T. Iso; Yeast = *Saccharomyces cerevisiae*
Values with different letters within each treatment are significantly different ($p < 0.05$) [values for T0 (200 rotifers mL^{-1}) all have the letter "a"]

7.4.3 Trial 2

The fatty acid compositions of rotifers sampled during Trial 2 are shown in Table 7.7, Table 7.8 and Table 7.9. The proportion (%TFA) of SFA in all rotifers fed frozen and freeze-dried ACEM 000A biomass at 100 mg L^{-1} dry weight equivalent did not vary substantially between T0 and T48. The proportion of MUFA in these rotifers was variable, but did not change substantially when compared to levels at T0. The proportion of PUFA in rotifers fed frozen ACEM 000A cells did not vary substantially between T0 and T48. The proportion of PUFA contained in rotifers fed freeze-dried ACEM 000A cells at 100 mg L^{-1} was stable between T0 and T6, increased between T6 and T24, and was stable between T24 and T48.

The proportion of SFA in rotifers fed freeze-dried cells of ACEM 000A at 200 mg L^{-1} remained stable between T0 and T24, and increased between T24 and T48. The proportion of MUFA in these rotifers was stable between T0 and T24, and decreased between T24 and T48. The proportion of PUFA did not vary substantially between T0 and T48. The proportions of SFA, MUFA and PUFA in rotifers fed the ACEM 000A-T. Iso and ACEM 000A-ACEM 6063 mixtures showed similar trends to those for rotifers fed only freeze-dried ACEM 000A cells at 100 mg L^{-1}.

The proportions of SFA, MUFA and PUFA in rotifers fed either ACEM 6063 only, or the ACEM 6063-T. Iso mixture were similar to each other. Between T0 and T6, the proportions of SFA increased, MUFA decreased and PUFA increased. The fatty acid profiles of these rotifers remained quite stable between T6 and T48. The proportions of SFA, MUFA and PUFA in Algamac-fed rotifers showed similar trends to those of ACEM 6063-fed rotifers. The proportion of SFA in T. Iso-fed rotifers did not vary substantially between T0 and T6, and increased slightly between T6 and T48. The proportion of MUFA decreased slightly, and the proportion of PUFA increased slightly, between T0 and T6. Neither varied substantially between T6 and T48. The proportions of SFA and MUFA in yeast-fed rotifers did not vary substantially between T0 and T48. The proportion of PUFA decreased slightly between T0 and T6, and did not vary substantially between T6 and T48. There were no substantial variations in the proportions of SFA, MUFA or PUFA in starved rotifers between T0 and T48.

Table 7.7 Mean (n=2) fatty acid[1] composition (% total fatty acids except where noted) of rotifers before enrichment, and after 6 and 24 h enrichment with ACEM 000A and ACEM 6063 and a further 24 h starvation [Trial 2]

		Enrichment diet															
		A FZ[2]			A FD			A FD x2			A FD+ T. Iso			A FD + 6063 FD		
Fatty acid	T0[3]	T6	T24	T48	T6	T24	T48	T6	T24	T48	T6	T24	T48	T6	T24	T48
14:0		3.5	2.9	2.4	3.4	3.0	2.1	3.1	2.5		3.6	3.5	2.7	3.2	3.3	2.8
16:1n7	16	6.4	6.3	6.9	6.4	6.1	5.7	4.9	3.8	4.6	6.4	5.9	5.9	6.4	6.4	6.1
16:0	11	14	13	11	13	13	9.2	13	17	18	12	12	10	12	12	11
18:2n6	9.3		11	11		8.2	12		7.4	6.4		17	12		11	12
18:1n9	27	15	33	33	11	22	31	15	37	29	19	24	32	14	31	31
18:1n7	3.6	29		2.2	30	2.5	2.0	29		2.9	27	2.7	2.2	28		
18:0	5.0	4.3	4.4	4.8	4.3	4.2	3.8	4.0	6.3	11.9	3.8	3.8	3.7	3.8	3.4	3.4
20:4n6		6.6	5.9	6.5	6.8	9.6	7.8	7.0	4.7	3.5	5.5	5.8	5.9	6.3	5.6	6.0
20:5n3	3.0	6.6	5.2	4.9	6.8	8.8	6.3	7.5	3.8	2.9	5.3	5.4	4.7	6.9	5.7	5.1
20:4n3	3.0															
20:3n6						2.3	2.2						2.0			
20:2n6			3.5	2.9	2.0	4.4	3.3		2.9			3.6	3.3		2.9	2.8
20:1n9+11	4.9	2.8			2.8			4.9	3.3		4.6			2.9		
20:0	3.4			2.8			2.6			3.6			2.5			2.8
22:6n3						2.5								2.8	3.2	
22:5n3		3.6	3.1	3.4	4.1	5.6	4.4	4.3	2.9	2.7	3.5	3.7	4.0	3.8	3.1	3.3
sum SFA[4]	20^a	21^a	20^a	21^a	21^a	20^a	18^a	20^a	26^a	37^b	20^a	20^a	20^a	19^a	19^a	21^a
sum MUFA[5]	53^a	54^a	44^a	45^a	51^a	34^b	41^a	55^a	47^a	40^c	58^a	36^b	42^a	52^a	42^b	41^b
sum PUFA[6]	26^a	24^a	35^a	34^a	28^a	45^b	40^b	25^a	27^a	23^a	22^a	43^b	37^b	29^a	38^a	38^a
sum other	0.6	1.0	0.9	0.5	1.0	1.0	0.6	1.0	1.0	0.7	1.0	0.9	0.5	1.0	1.0	0.8
TFA[7] mg g^{-1}	27^a	142^b	111^b	43^a	136^b	107^b	46^a	104^b	111^b	19^a	148^b	100^b	80^c	157^b	52^c	48^c

1: fatty acids comprising >2% of the mean total fatty acids. Other fatty acids detected below this value included: 12:0, i14:0, 14:1n9, 14:1n7, 14:1n5, i15:1, i15:0, a15:0, 15:1, 15:0, i16:0, 16:1n9, 16:1n7t, 16:1n5, 17:1, 17:0, 18:4n3, 20:3n6, 20:4n3, 22:5n6, 22:4n6, C22 PUFA

2: A = ACEM 000A; 6063 = ACEM 6063; FS = fresh cells; FZ = frozen cells; FD = freeze-dried cells; AD = air-dried cells; x2= double the standard enrichment rate (200 c.f. 100mg L^{-1}); T. Iso = *Isochrysis* sp, clone T. Iso

3: T0 = before enrichment; T6 = enriched for 6 h; T24 = enriched for 24 h; T48 = starved for 24 h following 24 h enrichment

4, 5, 6, 7: SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; TFA = total fatty acids. Values for SFA, MUFA, PUFA and TFA with different superscripts within each treatment were deemed to be significantly different at α= 0.05 (values for T0 all have the superscript "a")

Table 7.8 Mean (n=2) fatty acid[1] composition (% total fatty acids except where noted) of rotifers after 6 and 24 h enrichment with ACEM 6063 and a further 24 h starvation [Trial 2]

	Enrichment diet																	
	6063 FS[2]			6063 FZ			6063 FD			6063 FD x2			6063 AD			6063 FD + T. Iso		
Fatty acid	T6[3]	T24	T48	T6	T24	T48	T6	T24	T48	T6	T24	T48	T6	T24	T48	T6	T24	T48
14:0	5.2	5.9	3.7	6.0	8.4	4.3	5.1	4.4	3.3	5.7	5.4	3.7	5.5	4.5	3.3	6.6	6.9	5.3
16:1n7	8.5	7.4	6.3	7.6	9.2	6.6	9.3	7.3	7.9	8.1	8.2	8.1	8.6	6.9	7.8	9.8	7.9	7.7
16:0	18	22	19	21	19	18	18	17	18	19	16	17	20	18	17	17	18	17
18:4n3																5.6	3.8	2.1
18:2n6	5.1	3.1	3.0	4.7	2.9	3.3	6.0	3.9	4.6	4.9	3.7	4.5	5.4	3.7	4.5	6.8	5.0	5.8
18:1n9	13	12	9.9	12	12	11	15	11	14	13	11	13	14	11	13	16	12	13
18:1n7		2.9	4.1		4.3	4.0		3.5	4.9		3.5	4.5		3.2	4.1	2.2	3.6	5.0
18:0	2.9	4.9	4.2	2.9	4.6	3.9	3.0	3.3	4.5	2.9	3.2	4.5	3.0	3.1	4.0	3.1	3.1	4.0
20:4n6			2.8			2.9		2.7	3.2		2.7	3.4		2.4	3.1		2.2	3.0
20:5n3	3.4	2.9	3.9	3.0		4.0	3.4	4.5	4.3	3.3	5.0	4.7	3.1	3.9	4.2	3.1	4.1	4.6
20:4n3	2.2	3.0		2.1			2.3	2.2	2.2	2.2	2.3	2.6	2.1		2.1	2.7	3.4	4.2
20:1n9+11	2.5	2.2	2.7	2.3	2.8	2.4	3.0	2.0	2.6	2.4	2.4	2.9	2.7	2.1	2.7	2.9	2.2	2.9
22:5n6	6.9	6.7	7.6	7.1	3.7	7.5	5.7	7.5	5.3	7.0	6.9	4.9	6.2	7.9	6.3	3.5	4.8	4.1
22:6n3	19	16	19	19	17	19	16	21	13	20	20	13	17	21	16	11	14	12
22:4n6			2.4			2.4			2.0						2.1			
sum SFA[4]	28^b	34^b	29^b	31^b	35^b	28^b	27^a	26^a	27^b	29^b	26^b	28^c	29^b	27^b	26^b	28^b	30^c	28^c
sum MUFA[5]	28^b	26^b	26^b	26^b	32^b	25^b	30^b	25^b	32^b	27^b	26^b	31^b	29^b	24^b	30^b	33^b	27^c	31^c
sum PUFA[6]	44^b	39^b	45^b	42^b	32^b	46^b	42^b	49^c	40^b	44^b	47^b	41^c	41^b	48^c	44^b	38^b	42^b	41^b
sum other	0.8	0.7	0.2	0.8	0.6	0.6	0.8	0.8	0.4	0.8	0.8	0.5	0.8	0.8	0.5	0.8	0.8	0.5
TFA[7] mg g^{-1}	68^b	53^b	38^a	55^b	37^b	26^a	77^b	52^b	29^a	85^b	40^c	22^a	76^b	53^c	25^a	79^b	46^c	27^a

1: fatty acids comprising >2% of the mean total fatty acids. Other fatty acids detected below this value included: 12:0, i14:0, 14:1n9, 14:1n7, 14:1n5, i15:1, i15:0, a15:0, 15:1, 15:0, i16:0, 16:1n9, 16:1n7t, 16:1n5, 17:1, 17:0, 20:3n6, 20:2n6, 20:0, 22:5n3, C22PUFA

2: 6063 = ACEM 6063; FS = fresh cells; FZ = frozen cells; FD = freeze-dried cells; AD = air-dried cells; x2= double the standard enrichment rate (200 c.f. 100mg L^{-1}); T. Iso = *Isochrysis* sp, clone T. Iso

3: T6 = enriched for 6 h; T24 = enriched for 24 h; T48 = starved for 24 h following 24 h enrichment

4, 5, 6, 7: SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; TFA = total fatty acids. Values for SFA, MUFA, PUFA and TFA with different superscripts within each treatment were deemed to be significantly different at α= 0.05 (values for T0 all have the superscript "a")

Table 7.9 Mean (n=2) fatty acid[1] composition (% total fatty acids except where noted) of starved rotifers and those fed Algamac, Isochrysis sp (clone T. Iso) or yeast following 6 and 24 h enrichment and a further 24 h starvation [Trial 2]

	Enrichment diet														
	Algamac			Algamac x2[2]			T. Iso			Yeast			Starved		
Fatty acid	T6[3]	T24	T48	T6	T24	T48	T6	T24	T48	T6	T24	T48	T6	T24	T48
14:0	9.2	7.8	4.2	9.2	7.4	3.9	4.3	4.6	3.9		2.1				
16:1n7	7.2	7.0	5.5	6.5	6.6	4.1	13.4	11.9	11.1	20.1	20.2	18.3	15.8	15.0	14.4
16:0	23.9	28.0	24.0	24.2	21.6	22.0	9.6	10.9	10.8	10.5	9.4	9.1	10.5	10.5	10.5
18:4n3							8.6	7.0	5.8	2.1	2.4	2.7		2.5	2.7
18:2n6	4.2	3.1	3.0	3.8	3.0	2.4	10.4	9.8	9.8	8.0	3.6	6.9	10.1	9.9	9.3
18:1n9	9.9	10.9	9.7	9.6	10.5	9.4	24.1	23.7	22.7	30.3	31.0	29.3	28.4	28.3	26.3
18:1n7		2.9	3.1		2.8	2.3	2.7	4.0	5.5	3.4	4.5	5.5	3.4	4.3	6.2
18:0	2.5	3.9	6.0	2.4	3.3	7.0	3.7	4.1	4.2	4.9	4.9	4.8	4.9	4.9	5.1
20:4n6			2.7			2.8									
20:5n3	2.2	2.2	3.0	2.3	2.8	2.7	2.9	2.8	3.1	2.4	2.6	2.4	3.1	3.0	2.8
20:4n3							3.2	3.7	3.5	2.6	2.2	2.4	3.1	2.5	2.4
20:1n9+11		2.1	4.2		2.4	5.5	4.1	3.5	3.7	4.3	4.3	4.0	4.5	3.9	4.3
20:0															
22:5n6	7.4	5.5	4.5	6.8	7.4	4.5									
22:6n3	19.9	14.9	10.7	21.7	19.9	11.0	2.4	2.2	2.2						
22:4n6			5.4			8.3									
sum SFA[4]	37^{b}	41^{b}	38^{b}	37^{b}	34^{b}	37^{b}	19^{a}	22^{b}	22^{b}	19^{a}	19^{a}	18^{a}	19^{a}	20^{a}	20^{a}
sum MUFA[5]	22^{b}	25^{b}	26^{b}	21^{b}	23^{b}	25^{c}	47^{b}	46^{b}	46^{b}	60^{a}	62^{a}	60^{a}	55^{a}	54^{a}	54^{a}
sum PUFA[6]	40^{a}	33^{a}	37^{a}	41^{b}	42^{b}	39^{a}	34^{b}	32^{b}	32^{b}	20^{b}	19^{b}	22^{b}	26^{a}	26^{a}	25^{a}
sum other	0.9	0.8	0.2	0.9	0.9	0.5	0.7	0.6	0.3	0.7	0.6	0.3	0.6	0.5	0.6
TFA[7] mg g^{-1}	111^{b}	32^{a}	17^{a}	94^{b}	57^{a}	13^{c}	43^{a}	25^{a}	27^{a}	31^{a}	28^{a}	18^{a}	29^{a}	27^{a}	12^{b}

1: fatty acids comprising >2% of the mean total fatty acids. Other fatty acids detected below this value included: 12:0, i14:0, 14:1n9, 14:1n7, 14:1n5, i15:1, i15:0, a15:0, 15:1, 15:0, i16:0, 16:1n9, 16:1n7t, 16:1n5, 17:1, 17:0, 20:3n6, 20:2n6, 22:5n3, C22PUFA

2: x2= double the standard enrichment rate (200 c.f. 100mg L^{-1}); T. Iso = *Isochrysis* sp, clone T. Iso; Yeast = *Saccharomyces cerevisiae*

3: T6 = enriched for 6 h; T24 = enriched for 24 h; T48 = starved for 24 h following 24 h enrichment

4, 5, 6, 7: SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; TFA = total fatty acids. Values for SFA, MUFA, PUFA and TFA with different superscripts within each treatment were deemed to be significantly different at $\alpha = 0.05$ (values for T0 all have the superscript "a")

At T6, all fed rotifers contained higher absolute levels of TFA (mg g^{-1} dry rotifer weight) than at T0. Rotifers fed only ACEM 000A contained TFA levels of between 104 and 142 mg g^{-1}. Rotifers fed the ACEM 000A-T. Iso or ACEM 000A-ACEM 6063 mixtures contained 148 and 157 mg g^{-1} respectively. Rotifers fed ACEM 6063 (alone or mixed with T. Iso) contained TFA levels between 55 and 85 mg g^{-1}. Algamac-fed rotifers contained TFA levels of about 100 mg g^{-1}. The lowest TFA levels at T6 were found in T. Iso-fed, yeast-fed and starved rotifers (43, 31, and 29 mg g^{-1} respectively).

At T24, highest absolute TFA levels were found in rotifers fed only ACEM 000A or the ACEM 000A-T. Iso mixture (100 – 111 mg g^{-1}). TFA levels in all other treatments were quite variable, and ranged from 25 – 57 mg g^{-1}.

At T48, absolute TFA levels in all rotifers had decreased markedly from those recorded at T24. The highest TFA level was found in rotifers fed the ACEM 000A-T. Iso mixture (80 mg g^{-1}). TFA levels in all other treatments were again quite variable, ranging between 13 and 48 mg g^{-1}.

The absolute levels of AA, EPA and DHA (mg g^{-1} dry weight) in rotifers at the three time points are shown in Figure 7.3. At T6, the highest AA levels (7 – 10 mg g^{-1}) were found in ACEM 000A-fed rotifers. All other rotifers contained AA levels between 0.4 mg g^{-1} (yeast-fed rotifers) and 1 mg g^{-1} [ACEM 6063 and Algamac-fed rotifers]. Similarly, the highest EPA levels (8 – 11 mg g^{-1}) were found in ACEM 000A-fed rotifers. Other rotifers contained EPA between 0.8 mg g^{-1} (yeast-fed rotifers) and 3 mg g^{-1} [ACEM 6063 (freeze-dried)-fed rotifers]. The highest DHA levels at T6 (~ 20 mg g^{-1}) were found in Algamac-fed rotifers. Rotifers fed only ACEM 6063, and the ACEM 6063-T. Iso mixture, contained DHA levels of between 9 and 17 mg g^{-1}. Levels of DHA in the other samples varied between < 0.1 mg g^{-1} (yeast-fed rotifers) and 4 mg g^{-1} (rotifers fed the ACEM 000A-ACEM 6063 mixture).

At T24 (Figure 7.3), the highest absolute AA levels (5 – 10 mg g^{-1}) were found in rotifers fed only ACEM 000A. AA levels in all other samples were between 0.4 mg g^{-1} (T. Iso-fed rotifers) and 3 mg g^{-1} (rotifers fed the ACEM 000A-ACEM 6063 mixture). Absolute EPA levels were highest in rotifers fed only ACEM 000A or the ACEM 000A-T. Iso mixture (4 – 9 mg g^{-1}).

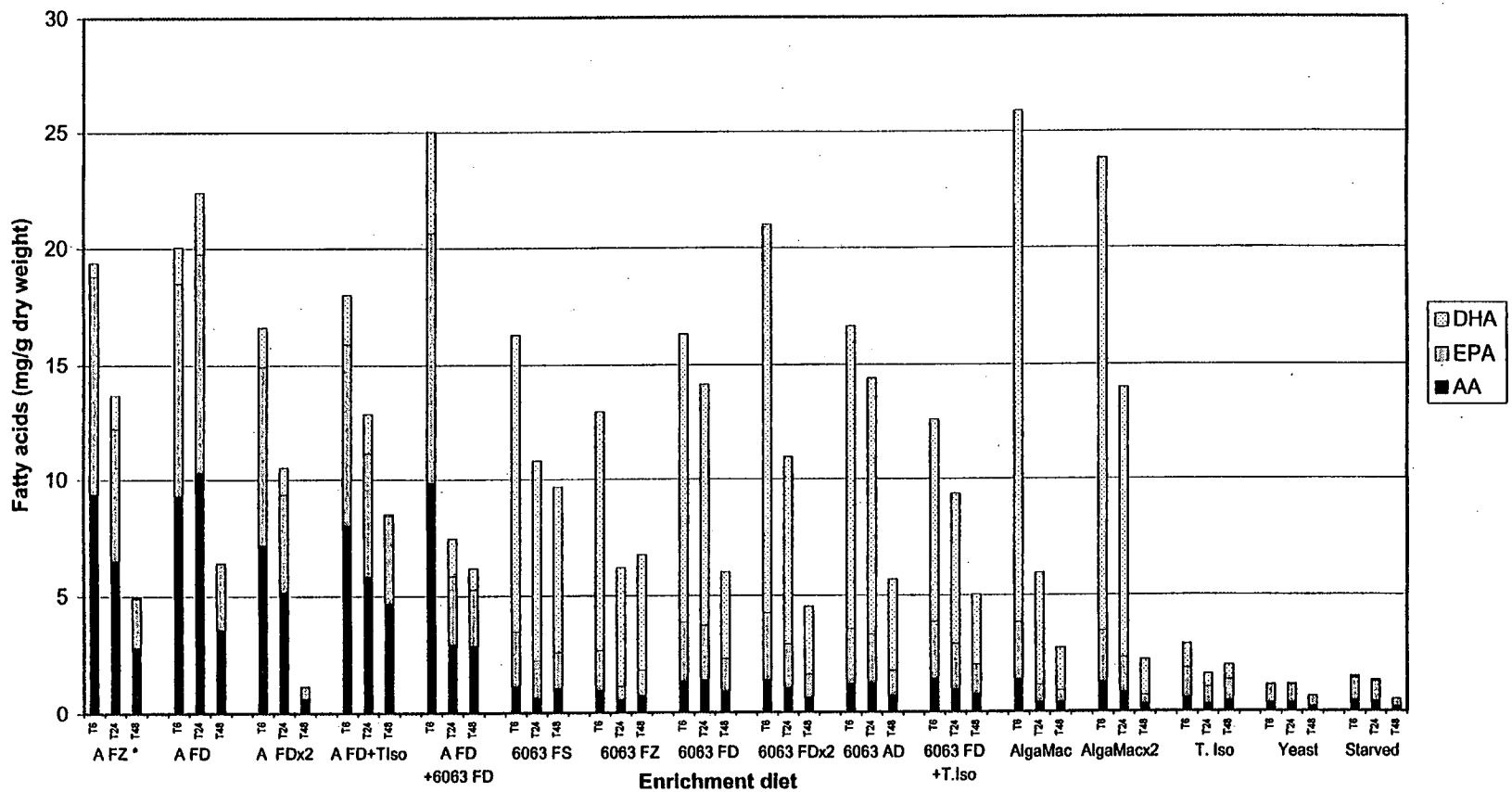

Figure 7.3 Mean amounts (mg g^{-1} dry weight; n=2) of 20:4n6 (AA), 20:5n3 (EPA) and 22:6n3 (DHA) in rotifers following 6 (T6) and 24 (T24) h enrichment and a further 24 h starvation (T48) [Trial 2]

*: A = ACEM 000A; 6063 = ACEM 6063; FS = fresh cells; FZ = frozen cells; FD = freeze-dried cells; AD = air-dried cells; x2= double the standard enrichment rate (200 c.f. 100mg L^{-1}); T. Iso = *Isochrysis* sp, clone T. Iso; Yeast = *Saccharomyces cerevisiae*

EPA levels in other rotifers varied between 0.7 mg g^{-1} (yeast- and T. Iso-fed rotifers) and 3 mg g^{-1} (rotifers fed the ACEM 000A-ACEM 6063 mixture). Absolute DHA levels were highest in rotifers fed only ACEM 6063, the ACEM 6063-T. Iso mixture, and Algamac (7 – 11 mg g^{-1}). DHA levels in all other rotifers varied between 0.1 mg g^{-1} (yeast-fed, T. Iso-fed and starved rotifers) and 3 mg g^{-1} [ACEM 000A (freeze-dried)-fed rotifers].

At T48 (Figure 7.3), absolute AA, EPA and DHA levels had all fallen from those at T24. The highest AA levels were found in rotifers fed frozen or freeze-dried ACEM 000A at 100 mg L^{-1} dry weight equivalent, the ACEM 000A-T. Iso mixture, and the ACEM 000A-ACEM 6063 mixture (3 – 5 mg g^{-1}). All other rotifers contained AA levels between 0.2 mg g^{-1} (Algamac-fed rotifers) and 1 mg g^{-1} [ACEM 6063 (fresh)-fed rotifers]. The highest EPA levels at T48 were in rotifers fed frozen or freeze-dried ACEM 000A at 100 mg L^{-1} dry weight equivalent, the ACEM 000A-T. Iso mixture, and the ACEM 000A-ACEM 6063 mixture (2 – 4 mg g^{-1}). All other rotifers contained EPA levels of < 2 mg g^{-1}. Absolute DHA levels were highest in rotifers fed only fresh ACEM 6063 (7 mg g^{-1}). Rotifers fed only frozen, freeze-dried or air-dried ACEM 6063, or the ACEM 6063-T.Iso mixture contained between 3 and 5 mg g^{-1}. All other rotifers contained DHA at levels between < 0.1 mg g^{-1} and 2 mg g^{-1} (yeast- and Algamac-fed rotifers respectively).

The density of rotifers was assessed at each sampling time (Figure 7.4). At T6, rotifer densities had decreased markedly (48 – 75 mL^{-1} c.f. 200 mL^{-1} at T0) in all treatments. There were no significant differences between rotifer densities in any of the treatments at T6. Rotifer densities in all treatments decreased significantly between T6 and T24. At T24, density was highest amongst starved rotifers and those fed ACEM 6063 (excluding rotifers fed the ACEM 000A-ACEM 6063 mixture), yeast and T. Iso (30 – 51 rotifers mL^{-1}). Counts for the other treatments varied between 3 and 19 rotifers mL^{-1}. Rotifer counts for all treatments at T48 showed no significant change from those at T24. At T48, density was highest for starved rotifers and those fed ACEM 6063 (excluding rotifers fed the ACEM 000A-ACEM 6063 mixture), yeast and T. Iso (31 – 53 rotifers mL^{-1}). Counts for the other treatments varied between 2 and 20 rotifers mL^{-1}. The lowest counts were seen in treatments fed either freeze-dried ACEM 000A or Algamac at 200 mg L^{-1} (2 and 5 mL^{-1} respectively).

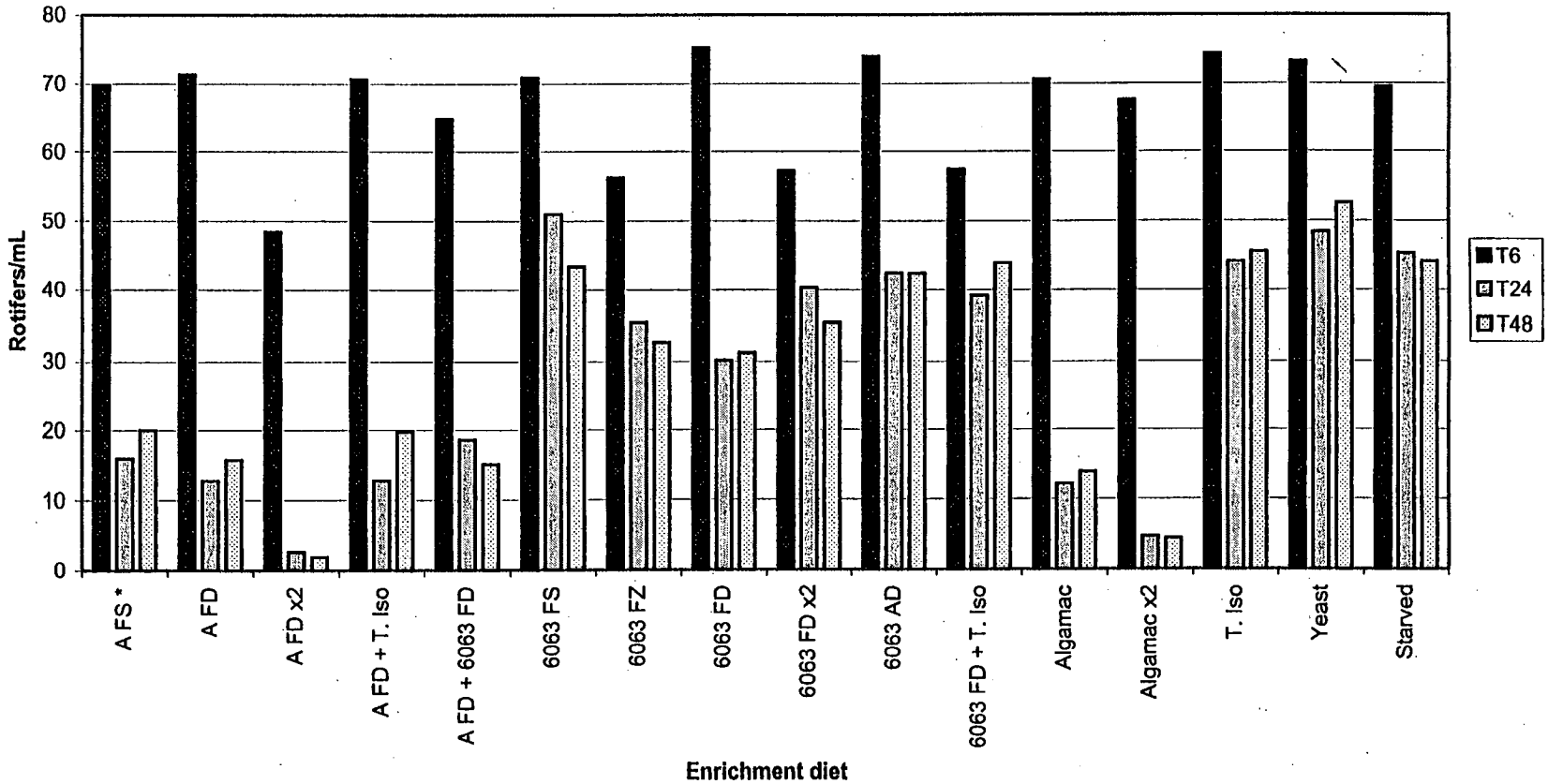

Figure 7.4 Mean rotifer density (number mL^{-1}; n=2) following 6 (T6) and 24 (T24) h enrichment and a further 24 h starvation (T48) [Trial 2]

*: A = ACEM 000A; 6063 = ACEM 6063; FS = fresh cells; FZ = frozen cells; FD = freeze-dried cells; AD = air-dried cells; x2= double the standard enrichment rate (200 c.f. 100mg L^{-1}); T. Iso = *Isochrysis* sp, clone T. Iso; Yeast = *Saccharomyces cerevisiae*
For all treatments, rotifer densities at T0 (200 rotifers ml^{-1}) were significantly higher ($p<0.05$) that at T6, densities at T6 were significantly higher that at T24, and densities at T24 were not significantly different from values at T48

7.5 Discussion

This study has shown that the microheterotroph strains ACEM 000A and ACEM 6063 can be used to enhance PUFA content of rotifers to levels nutritionally significant for many aquaculture species. It is probable, however, that production costs would inhibit the use of fresh thraustochytrid biomass for enriching rotifers in all but the largest facilities. A more likely scenario would be a central production facility supplying preserved biomass to a diversity of customers. Preservation techniques that could be used include freezing and spray-drying (Barclay and Zeller, 1996). In this study, no significant differences were found in the fatty acid profile of either ACEM 000A or ACEM 6063 biomass treated in four different ways (fresh, frozen, freeze-dried, air-dried). In Trial 1, the absolute amount of fatty acids detected in the air-dried ACEM 000A biomass was slightly, but significantly, lower than in the other forms of this strain. In Chapter 3, it was demonstrated that the amount of fatty acids extracted from fresh ACEM 000A and ACEM 6063 biomass was influenced significantly by the extraction method. It is likely that the method by which ACEM 000A (or other microheterotroph) biomass is preserved would also influence fatty acid extraction efficiency.

Changes in the relative proportions of fatty acids in rotifers during the first 6 h of enrichment reflected the fatty acid composition of their enrichment diets. Changes in fatty acid profiles between T6 and T24 were generally minor, and were considered more likely to be affected by prevailing culture conditions than by the enrichment diets. These observations support the views that lipid content of rotifers correlates well with the lipid composition of their food (Ben-Amotz *et al.*, 1987; Frolov *et al.*, 1991; Rainuzzo *et al.*, 1994b), and that all major changes to fatty acid profiles of enriched rotifers occur during the first 6-24 h of enrichment (Olsen *et al.*, 1989; Rainuzzo *et al.*, 1994b; Rodriguez *et al.*, 1996; Yoshimatsu *et al.*, 1997). No consistent changes in fatty acid profiles were observed during the 24 h starvation period (T24 - T48) for any of the treatments in this study. It has previously been reported that the fatty acid profile of rotifers enriched with lipids from easily digestible diets (e.g. yeast, fish oil and/or some microalgae) remained fairly stable during starvation periods of up to 5 days (Lubzens *et al.*, 1985; Olsen *et al.*, 1993). This indicates that rotifers do not preferentially catabolise either total SFA, total MUFA or total PUFA during starvation periods. Gut passage times for *B. plicatilis*

have been reported to be in the order of 30-40 minutes at 20-22°C (Korstad *et al.*, 1989). It is therefore likely that, in the present study, any undigested food would have been excreted well before the end of the 24 h starvation period. Thus, if the diets used in the current trial had proved indigestible, rotifer fatty acid profiles would have been expected to return to pre-enrichment levels following 24 h starvation, as undigested food was excreted. The stability of the fatty acid profiles of rotifers fed ACEM 000A and ACEM 6063 during the post-enrichment starvation period indicates that fatty acids from these microheterotrophs had been digested and assimilated by the rotifers.

The absolute amounts of fatty acids accumulated by rotifers during the enrichment period also reflected those in the enrichment diets. There were no significant differences in the absolute TFA levels in rotifers fed biomass from single strains that had been exposed to different post-harvest preservation treatments. This indicates that freezing, freeze-drying or air-drying did not alter the fatty acid content of the microheterotroph biomass, even though analyses indicated that air-dried ACEM 000A contained a lesser absolute amount of fatty acids than the other treatments. When the increase in average absolute TFA levels in starved rotifers fed single-component (including the high- and low-TFA ACEM 000A biomass) enrichment diets for 6 h were plotted against absolute TFA levels in their diet, a linear correlation was found (R^2= 0.90; Figure 7.5). Barclay and Zeller (1996) used spray-dried *Schizochytrium* sp. biomass, which contained about 320 mg g^{-1} TFA, to enrich rotifers. The increase in absolute TFA level in rotifers after 8 h enrichment (75 mg g^{-1}) in that study also correlated well with the linear line fitted to the single component enrichment diet data. A data point for rotifers fed the EPA-producing Antarctic bacterium ACAM 456 for 24 hours (derived from data presented in Chapter 6) has also been included in Figure 7.5. This data point lies above the trendline in Figure 7.5. However, it should be noted that this data was for rotifers that had been enriched for 24 hours, as compared with the 6-8 hours enrichment for other rotifers represented in Figure 7.5. A previous study (Nichols *et al.*, 1996) demonstrated that the absolute amount of EPA in rotifers increased by a factor of about 3.5 between 6 and 24 hours exposure to ACAM 456. It would therefore be expected that, after 6 hours enrichment, the absolute TFA levels in the rotifers examined in Chapter 6 would have been lower than was observed after 24 hours enrichment – thus correlating more closely with the derived trendline in Figure 7.5.

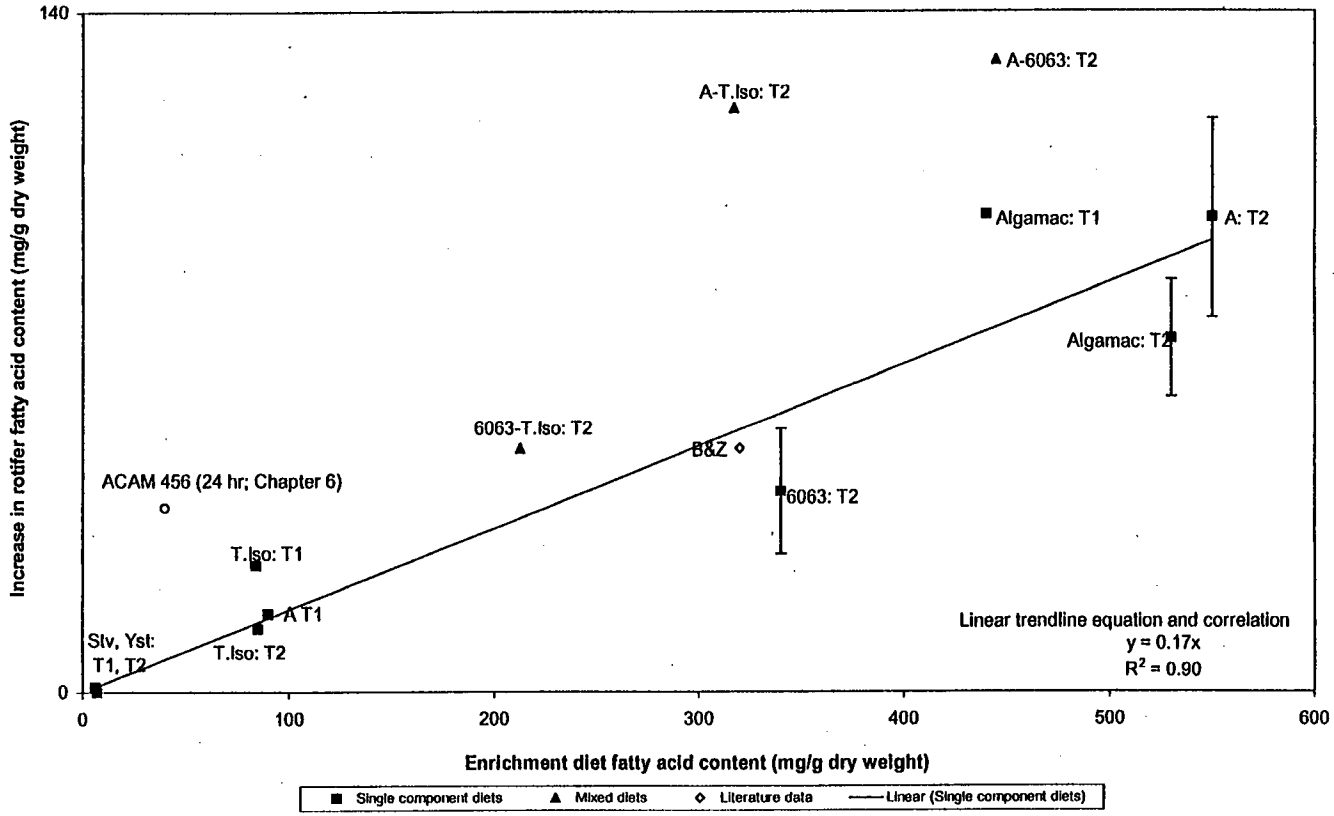

Figure 7.5 Relationship between total fatty acid content (mg g^{-1} dry weight) of rotifers (starved or fed for 6-8 h) and total fatty acid concentration of single component enrichment diets. [Literature data and that for bacterial or mixed diets were not used for calculating the fitted line; error bars (± SD) are shown for data points that are the mean of several samples].

A = ACEM 000A; 6063 = ACEM 6063; T. Iso = *Isochrysis* sp, clone T. Iso; Yst = *Saccharomyces cerevisiae*; T1 = Trial 1; T2 = Trial 2; B&Z = data from Barclay and Zeller (1996); ACAM 456 – EPA-producing bacterial strain fed to rotifers for 24 hours (see Chapter 6)

This analysis suggests that, using knowledge of the fatty acid content of enrichment diets, changes in the fatty acid content and profile of rotifers following short-term enrichment with single component diets can be predicted.

Data for rotifers fed mixed diets did not correlate as consistently with the fitted line in Figure 7.5. To calculate the "average" absolute TFA content of the mixed diets, it was assumed that the rotifers ingested an equal weight of each of the components of their diet. The value, based on these "average" TFA contents, for rotifers fed the ACEM 6063-T. Iso mixture generally fitted the correlation for single diets. The data for mixed diets containing ACEM 000A, however, lie somewhat above the linear fitted line, indicating that the "averaging" model did not fit well for diets containing ACEM 000A. Absolute levels of AA, EPA and DHA detected in rotifers fed mixed diets containing ACEM 000A (Figure 7.3) also do not support the concept that ingestion of equal amounts of the components occurred with these particular diets. It may be that these rotifers preferentially ingested ACEM 000A ahead of ACEM 6063 and T. Iso, to levels similar to rotifers fed ACEM 000A only, and also ingested small amounts of ACEM 6063 and T. Iso. It therefore seems likely that, in this study, there was some degree of selective ingestion of different dietary components by the rotifers. Selective intake of food by rotifers has been reported previously—based on size (Heerkloss and Hlawa, 1995), or whether the food particles are alive or not (Chotiyaputta and Hirayama, 1978). Reasons for the apparent selectivity in the present study are not clear, although particle size may have influenced ingestion efficiency. ACEM 000A and ACEM 6063 cells used in this study were about 10-15 μm and 5-10 μm in diameter respectively, while T. Iso cells were about 5 μm long. If particle size did influence ingestion rates, then it would appear that the rotifers favoured the larger particles. However, this suggestion does not agree with results from a study of particle ingestion by *B. plicatilis* (Heerkloss and Hlawa, 1995), which showed a preference for smaller particles (2 μm) ahead of larger particles (3-10 μm). One implication of feed selectivity by rotifers is that the degree of enrichment elicited by different combinations of dietary components must be determined by direct experimentation, rather than by extrapolation from data obtained from single component diets.

Absolute TFA levels in rotifers fell substantially during the 24 h starvation period (T24 – T48), with the rate of loss generally being proportional to the TFA level at T24. Similar results were reported by Olsen *et al.* (1993). Absolute TFA levels at T48 were generally similar to those at T0, notwithstanding the levels reached at T6 and T24. The main exceptions to this trend were rotifers fed 200 mg L^{-1} of either ACEM 000A or Algamac, which had very low absolute TFA levels at T48. Comparison of rotifer density at T48 strongly suggests that the health of these rotifers had been severely compromised by these two diets.

The potential of new diets for enriching rotifers with PUFA for aquaculture must be evaluated with species-specific requirements for these essential fatty acids in mind. The absolute dietary requirements for total or specific PUFA has been assessed for a number of marine finfish larvae, and are often about 1.5-2 % of feed dry weight [e.g. Gilthead seabream ~2.1% n3 PUFA (Rees *et al.*, 1994); cod ~2.1% DHA (Zheng *et al.*, 1996); Red sea bream ~1.6% DHA (Furuita *et al.*, 1996a); Yellowtail ~2% DHA (Furuita *et al.*, 1996b)]. Similar levels were achieved in many of the ACEM 000A and ACEM 6063-based treatments in Trial 2 of this study.

Recent developments in the understanding of essential PUFA requirements of fish have been recently reviewed by Sargent *et al.* (1999). Evaluation of larval requirements for AA, EPA and DHA in terms of relative, as well as absolute, amounts is now considered necessary. For example, optimum dietary ratio of DHA:EPA:AA for sea bass larvae is about 2:1:1 (w/w/w), while that for turbot and halibut larvae is about 2:1:0.1 (w/w/w) (Sargent *et al.*, 1999). It appears from these data that rotifers enriched for 6 h with ACEM 000A and ACEM 6063 will provide appropriate levels of PUFA for many larvae, and that ACEM 6063 can provide what are currently accepted as preferred DHA levels and DHA:EPA ratios for many fish and prawn larvae. It may be that further research will point to increased requirement of some larvae for AA or other fatty acids. For instance, adult prawns generally have higher AA levels than many fish (Yearsley *et al.*, 1999), and AA has been found to be essential for some, but not all, prawn species (Deering *et al.*, 1997). Larval prawns, therefore, may require more AA in their diet than the levels generally accepted as necessary for fish larvae. If provision of AA and other fatty acids in larval diets is seen as necessary, then organisms such as ACEM 000A could well be used as a reliable source. Further manipulation of absolute and relative PUFA levels

in enriched rotifers to provide optimum fatty acid nutrition can be achieved, as required, through the use of enrichment diets consisting of single or mixed microheterotrophs with different PUFA profiles.

Changes in the levels of AA, EPA and DHA in rotifers between T24 and T48 reveal an interesting trend. The ratio of DHA to both AA and EPA decreased during this starvation period, especially in rotifers containing higher levels of DHA at T24. This indicates that DHA was being preferentially catabolised by these rotifers. Results from earlier studies (Olsen *et al.*, 1993; Rainuzzo *et al.*, 1994b; Barclay and Zeller, 1996) also indicated that rotifers may preferentially catabolise DHA before EPA or AA, and that rotifers may be able to retroconvert some longer chain PUFA to their shorter chain counterparts. Similar data has also been reported for a number of strains of *Artemia* sp. – another live-feed species used by aquaculturists (Han *et al.*, 2000). This ability could have significant implications in cases where a low proportion of AA is required and when rotifers are provided with diets containing high DHA levels for several days prior to being fed to larvae. For short-term enrichments, however, it is unlikely that significant retroconversion would occur.

An important consideration when evaluating the potential of any new rotifer enrichment diet is its influence on rotifer health. Although the experimental system used in this study did not support high rotifer survival, comparison against standard rotifer diets (yeast, T. Iso) allows some assessment of the influence of the enrichment diets on rotifer health. The data indicate that feeding ACEM 000A at 100 mg L^{-1} did not unduly influence rotifer survival during the first 6 h enrichment period. Longer enrichment periods resulted in more variable results, with rotifer survival in Trial 2 being lower than in Trial 1. The reason for this difference could be related to initial rotifer health and/or differences in the ACEM 000A biomass used. The fatty acid levels in ACEM 000A were very different in both trials (~85 mg g^{-1} in Trial 1; ~550 mg g^{-1} in Trial 2), and there could also have been differences in other properties of the biomass. In Trial 2, rotifers fed only ACEM 000A and the ACEM 000A-ACEM 6063 and ACEM 000A-T. Iso mixtures all showed poor survival after T6. This suggests that the ACEM 000A biomass used in Trial 2 may have adversely influenced rotifer health. Survival amongst rotifers fed ACEM 6063 at all sample times was essentially the same as that for rotifers fed yeast and T. Iso, indicating that

the ACEM 6063 biomass did not influence rotifer health during short term enrichments.

Many countries appear to be increasing quarantine restrictions on the importation and use of exotic microorganisms (for example the two Antarctic bacterial strains used to enrich rotifers in Chapter 6) in aquaculture diets. It is likely that such restrictions will be tightened further, making it important for affected countries to have access to native organisms with which to supply the needs of their local industries. In this Chapter, it has been shown that the new Australian thraustochytrid strains ACEM 000A and ACEM 6063 can be used to enrich rotifers with nutritionally significant levels of PUFA. Indeed, enrichment with these thraustochytrid strains increased rotifers PUFA content to levels higher than were achieved using the PUFA-producing bacteria discussed in Chapter 6. It also appears that fatty acids from these two thraustochytrids can be assimilated by rotifers, and therefore should be available to any larvae that subsequently eat rotifers enriched with these thraustochytrids.

The predominant lipid class produced by specific microheterotrophs could be an important consideration when selecting a strain to use for rotifer enrichment. Incorporation of dietary n3 PUFA has been found to be more efficient in some aquaculture species if the PUFA are contained in phospholipid rather than triacylglycerol (Izquierdo *et al.*, 2000). Bacterial lipids are predominantly phospholipid (Harwood and Russell, 1984), whereas the predominant lipid class of some thraustochytrids is triacylglycerol (Chapter 4, Bowles *et al.*, 1999). This indicates that, while thraustochytrids may generally have the potential to enrich rotifers to higher PUFA levels than bacteria, bacterial PUFA may be more nutritionally available to some aquaculture species.

The observation that fatty acid content of thraustochytrids can be modified by manipulation of culture conditions (Chapter 4) potentially adds further flexibility to rotifer enrichment systems. Mixtures of enrichment diets can be used to tailor fatty acid content of rotifers, which may then be used to meet specific absolute and relative PUFA requirements of different larvae. The fatty acid profiles exhibited by PUFA-producing thraustochytrids reported to date are quite diverse (Bowles *et al.*, 1999; Chapter 2). This indicates the significant potential of these organisms to be used as a new source of PUFA for aquaculture (amongst other potential uses), providing a potential alternative to the more traditional sources of PUFA. As

additional strains are isolated, and their production of PUFA optimised, it seems likely that the PUFA requirements of many aquaculture species could be met by thraustochytrid production systems.

Chapter 8 Summary, conclusions and projections

The aim of this study was to isolate PUFA-producing thraustochytrids from Australian habitats, to characterise these strains, and to evaluate the potential application of selected strains for use in aquaculture live-feeds. Initially, 74 thraustochytrid strains were isolated from marine environments in south-eastern Tasmania (Australia). Whole cell fatty acid profiles were used to separate the strains into 10 chemotaxonomic groups. Differences in the relative proportion of DHA in the fatty acid profiles were primarily responsible for the initial separation of the strains into two groups. Subsequent separation of strains within these major groups was largely determined by fatty acids that constituted minor proportions (≤ 10%) of the total fatty acids. Nucleotide sequence data were obtained for the 18S ribosomal DNA gene (18S rDNA) from 15 of the Australian strains and from one non-viable sample of a *Schizochytrium* sp.-based aquaculture feed (Algamac-2000). There was some correlation between the groups formed by fatty acid and 18S rDNA data. The inherent variability of thraustochytrid fatty acid production, in response to environmental conditions, suggests that fine resolution between thraustochytrid strains based on fatty acid or PUFA profiles will be difficult.

Phylogenetic analysis of 18S rDNA data showed that the Australian strains were represented in a wide cross-section of known thraustochytrid diversity. Australian strain ACEM 6063 clustered closely with two *Schizochytrium* strains that are considered to have potential as commercial sources of DHA. As ACEM 6063 has also been shown to produce substantial quantities of DHA-rich biomass, this lineage represents a relatively rich source of DHA-producing thraustochytrids.

Before trials to investigate the influence of specific culture parameters on lipid production could be undertaken, different methods for analysing the lipid and fatty acid content of thraustochytrid biomass were evaluated. Two Australian thraustochytrid strains were examined. One strain (ACEM 6063) was closely related to the highly productive DHA producer, *Schizochytrium limacinum* SR21. The other strain (ACEM 000A) was more distantly related. The choice of extraction technique was found to influence slightly the relative proportion of monounsaturated fatty acids recovered, but did not significantly influence the relative proportions of saturated fatty acids or polyunsaturated fatty acids recovered. There was a marked difference

in fatty acid recovery between the least and the most efficient extraction techniques for the two strains. Total fatty acid recovery from both strains was highest using direct transesterification of the fatty acids in the biomass (i.e. without prior extraction of the fatty acids), and by allowing the transesterification reaction to proceed for at least 60 minutes. This data highlights the importance of carefully selecting and validating analysis methodology before attempting to interpret and compare data for the lipid content of thraustochytrids.

Factors influencing biomass, lipid and fatty acid production by strain ACEM 6063 were examined in detail. Variations in culture conditions were found to substantially influence biomass and lipid production by ACEM 6063. Conditions were identified under which 7 day old ACEM 6063 cultures achieved up to 23 g dry weight L^{-1}, of which up to 45% and 10% (w/w) were total lipid and DHA respectively. In general, biomass production rates (g dry weight L^{-1} d^{-1}) were higher in fermenter cultures than in flask cultures. Biomass production increased with initial glucose concentration (to 100 g L^{-1}), initial sea salt concentration (to 32 g L^{-1}), culture temperature (to 25°C), and fermenter impeller speed (to 200 rpm). Lipid content increased with culture age until cultures reached peak biomass. The dominant lipid classes in ACEM 6063 oil were triacylglycerol and phospholipid. The proportion of triacylglycerol increased to about 90% of the total lipid as cultures reached peak biomass. Increasing DO levels from < 5% to > 40% saturation substantially decreased the proportion of saturated fatty acids and substantially increased the proportion of monounsaturated fatty acids in ACEM 6063. The proportion of DHA in the total fatty acids of ACEM 6063 fermenter cultures decreased with culture age, from about 60-70 %TFA in 2-5 day old cultures to about 20 %TFA at peak biomass. The levels of biomass, lipid and DHA production by ACEM 6063 are amongst the highest for thraustochytrids reported to date. ACEM 6063 is therefore worthy of further research and development to evaluate its commercial potential.

Culture conditions also influenced the squalene and sterol content of ACEM 6063 biomass. Twenty sterols, 13 of which were identified, were detected. Dominant sterols were cholest-5-en-3-ol, 24-ethylcholesta-5,22E-dien-3-ol, 24-methylcholesta-5,22E-dien-3-ol, and two co-eluting sterols – one of which was 24-ethylcholesta-5,7,22-trien-3-ol. These sterols comprised 50-90% of total sterols. Cultures grown at high DO (> 40% saturation) contained relatively simpler sterol profiles than those

grown at low DO (< 5% saturation). In high DO cultures, only the sterols mentioned above were present at levels of > 3% of total sterols. In low DO cultures, up to 6 additional sterols were present at > 3% of total sterols. Total sterol levels (% total lipids) decreased with increasing culture age. Absolute sterol content (mg g^{-1} dry weight) was higher in cultures grown at 20°C than at 15°C. In cultures grown at low DO levels, absolute squalene content decreased with increasing culture temperature. There was a clear inverse relationship between the squalene concentration in ACEM 6063 cultures and the DO level at which those cultures were grown. It is proposed that the efficiency of the conversion of squalene into sterols by ACEM 6063 was reduced at low DO levels. This is the first report relating sterol and squalene content of a thraustochytrid strain to the conditions under which it was cultured. Thraustochytrids contain some sterols common to food plants. However, thraustochytrids also contain low levels of other unusual or unidentified sterols. If thraustochytrids are to be used for commercial production of lipid products for eventual human consumption, the sterols produced by these organisms, and the factors influencing sterol production, will require thorough characterisation.

When two strains of Antarctic bacteria – one EPA-producer and one-DHA producer – were fed to rotifers, the rotifers accumulated only EPA to levels that are considered to be nutritionally significant for many finfish species. Rotifers fed the DHA-producing bacteria alone accumulated only low levels of DHA. However, when the PUFA-rich thraustochytrid strains ACEM 000A and ACEM 6063 were fed to rotifers, the absolute content of AA, EPA and DHA in rotifers exceeded that achieved with the Antarctic bacteria. It has been suggested that some finfish larvae can metabolise dietary PUFA more efficiently if the PUFA is contained in phospholipid, rather than in triacylglycerol. In such circumstances, it may be preferable to enrich rotifers with biomass containing increased proportions of phospholipids (e.g. bacteria, young thraustochytrid cultures). In the majority of cases, however, it is likely that older thraustochytrid cultures (i.e. with a higher absolute PUFA content) would be the favoured choice for rotifer enrichment. This is the first report regarding manipulation of rotifer fatty acid profiles with diets containing different thraustochytrid strains and different proportions of thraustochytrid biomass. These results show that if thraustochytrid strains with appropriate PUFA production characteristics can be cultured commercially, rotifers could be enriched to meet specific PUFA requirements of different aquaculture species. Such tailoring of diets

to meet specific dietary PUFA requirements could result in increased survival, growth and/or production efficiencies for selected aquaculture species and/or life stages.

At present, wild fisheries are able to meet the current demand for cheap PUFA-rich oil. However, demand for PUFA-rich products is considered likely to outstrip supply in the next decade or so. As this scenario develops, prices for PUFA-rich oils are likely to increase to levels capable of supporting a sustainable industry based on microheterotrophic production of PUFA-rich biomass and oils. If such an industry is to develop there will be a number of critical scientific steps to negotiate.

The thraustochytrid strain used as the focus of this study, ACEM 6063, was isolated as a single colony amongst many, from one petri dish amongst many. Although other strains could have been used to examine production and use of thraustochytrid biomass and lipids, it was perhaps a serendipitous occurrence that such a productive strain was isolated. For a viable thraustochytrid-based PUFA production industry to evolve as smoothly as possible, random isolation of useful strains needs to be replaced by more efficient isolation strategies. One such strategy is to identify 18S rDNA oligonucleotide sequences specific to strains with attractive PUFA and biomass production attributes. Environmental samples, or DNA extracted therefrom, could then be examined directly with specific DNA probes. This approach would allow attempts to isolate new strains to be limited to those samples testing positive for the selected marker sequences.

The biomass, lipid and DHA production rates achieved by ACEM 6063 were high by current published standards. However, further culture optimisation work will be required for production costs to approach those required for commercial viability. The culture manipulation studies described in this thesis were based on a small (2 L) fermenter and laboratory-grade nutrients, and have focussed on only a small number of culture variables. One of the biggest challenges to overcome will be achieving similar - if not better - productivity in larger fermenters using cheap and less well-defined culture media. Several studies have shown that undefined ingredients such as corn-steep liquor can be used in thraustochytrid culture media. By-products from other commercial processes should also be tested for their ability to support large-scale production of PUFA-rich biomass.

Little is known about the positional chemistry of thraustochytrid lipids. This gap in our knowledge needs to be addressed. Differences in the positional structure of thraustochytrid oils (as compared to their analogues from traditional food sources) may influence metabolism of these oils by target consumers.

Development of culture technology must be paralleled by development of suitable harvest and down-stream processing technology. As part of this development, thought must also go into efficient reuse and/or disposal of culture by-products. The (assumed) relatively high salt concentration in the culture media may make disposal of used media difficult. Few municipal wastewater treatment facilities are likely to welcome the addition of large quantities of saline effluent to their system.

With the rapid evolution of molecular biotechnology techniques, there is also scope for the development of biotechnological systems based on the introduction and extraction of genes to and from thraustochytrid genomes. It is likely that the genes responsible for PUFA production by thraustochytrids will soon be identified and isolated. When this happens it should then be possible either to genetically manipulate the amount and types of PUFA produced by thraustochytrids, and/or to clone the relevant genes into other, more easily cultured species (including broad-acre crops). Given the relatively high growth rates of some thraustochytrids, the incorporation and expression of exotic genes by these organisms could also be a viable way to produce high value, non-PUFA chemicals.

A number of novel thraustochytrid strains from Australian marine environments were isolated and characterised during this study. Factors influencing biomass and lipid production by one strain - ACEM 6063 - were examined, leading to establishment of culture conditions promoting the laboratory-scale production of substantial quantities of DHA-rich biomass. The potential for two of the new Australian thraustochytrids to be used to enrich live feed organisms with sufficient PUFA to meet currently recognised dietary requirements of some aquaculture species was also demonstrated. It now remains for the commercial potential of these and other thraustochytrid strains to be evaluated in larger scale facilities. If such evaluations show that production of these Australian strains was economically viable, the goal of a sustainable, large-scale PUFA production facility in Australia will be another step closer.

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" He said that knowledge was of little use without wisdom,
and that there was no wisdom without spirituality,
and that true spirituality always included service to others "

Tao Chi'en quoting his *zhong yi* master in:
Allende, I. (1999) *Daughter of Fortune*
Flamingo-HarperCollins, London
(translated by Margaret Sayers Peden)