Isolation of Marine Protists for Production of Polyunsaturated Fatty Acids

by

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“If I have seen further it is by standing on ye shoulders of Giants.”
-Sir Isaac Newton

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ABSTRACT

The aim of this research was to isolate and characterize novel strains of marine protists with potential to commercially produce PUFAs. Twelve trips were made visiting 10 different locations in the Canadian Maritime Provinces. Sixty-nine strains were isolated and screened for biomass and fatty acid production. Those meeting specific criteria were selected for further investigation including characterization by 18S rDNA sequencing. Isolate ONC-KTB-56 produced the greatest amount of biomass (1 807 mg L^{-1}) and fatty acids (24.6% dry weight). Of the total fatty acids, ARA, EPA and DHA comprised 0.89, 1.22 and 4.7 percent, respectively. Isolate ONC-KTB-14 produced 1 704 mg L^{-1} dry biomass with 5.4 percent fatty acids including 1.44, 1.35 and 37.5 percent, ARA, EPA and DHA, respectively.

Through optimization of culture conditions biomass, fatty acid content and the proportions of specific fatty acids can be increased. With such optimization, there is potential for isolates ONC-KTB-14 and ONC-KTB-56 to be grown at a commercial scale for production of PUFAs.

LIST OF ABBREVIATIONS AND SYMBOLS USED

%	Percent
°C	Degrees Celsius
^{14}C	Carbon isotope containing 14 neutrons
A	Agar
A_{600}	Absorbance at 600 nanometres
ACP	Acyl carrier protein
AMPH	Amphotericin B
ARA	Arachidonic acid
ASW	Artificial sea water
AT	Acyl transferase
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
cDNA	Copy deoxyribonucleic acid
cm	Centimetre
CoA	Coenzyme A
Corp.	Corporation
DH	Dehydratase
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPA	Docosapentaenoic acid
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid
ER	Enoyl reductase

et al.	*Et alia*
etc.	Etcetera
FAME	Fatty acid methyl ester
FAS	Fatty acid synthase
FID	Flame ionization detector
g	Gram
g	Gravitational force
G	Glucose
GC	Gas chromatography
hrs	Hours
i.e.	*Id est*
ID	Internal diameter
Inc.	Incorporated
kGy	Kilogray
KMV	Kazama's modified Vishniac's
KR	Ketoreductase
KS	Ketosynthase
L	Litre
Ltd.	Limited
m	Meter
M	Molar
mg	Milligram
min	Minute
mL	Millilitre
mm	Millimetre
mM	Millimolar

MSG	Monosodium glutamate
nm	Nanometre
NS	Nova Scotia
ONC	Ocean Nutrition Canada
P	Peptone
PCR	Polymerase chain reaction
PEI	Prince Edward Island
PKS	Polyketide synthase
PS	Penicillin G and streptomycin sulfate
PUFA	Polyunsaturated fatty acid
rDNA	Ribosomal deoxyribonucleic acid
RPM	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
S	Svedberg unit
s	Second
S1	Primary screen
S2	Secondary screen
S3	Third screen
TFA	Total fatty acid
USA	United States of America
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight
YE	Yeast extract
µL	Microlitre
µm	Micrometre

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CHAPTER 1: INTRODUCTION

Long chain polyunsaturated fatty acids (PUFAs) have been identified as critical components of membranes in most eukaryotic organisms (Lauritzen *et al*., 2001). Their roles extend from cell signalling to acting as precursors for production of hormones. The omega-3 fatty acids eicosapentaenoic acid (EPA, C20:5 (*n*-3)) and docosahexaenoic acid (DHA, C22:6 (*n*-3)) and the omega-6 fatty acid arachidonic acid (ARA, 20:4 (*n*-6)) are examples of such PUFAs. EPA is a precursor to the hormone sub-class prostaglandins of the class eicosanoid. Prostaglandins are important for reducing inflammation in affected tissues. PUFAs have also been found to comprise more than 50% of the total fatty acid content in some mammalian tissues. For example, in humans, DHA accounts for more than 60% of the fatty acids in the photosensitive portion of photoreceptor cells in the retina (Giusto *et al*., 2000). Thus DHA may play a role in photoreceptor functions such as signal transduction processes and rod and cone development. DHA is also a component of the brain membrane and along with ARA comprises the majority of fatty acids in brain tissue (Ratledge, 2004). DHA and ARA are important for normal neurological development making these fatty acids an essential part of infant nutrition, and are also thought important for normal function of the central nervous system (Crawford *et al*., 1997; Sahena *et al.,* 2009). Additionally, omega-3 PUFAs are thought to have the effect of reducing: plasma triacylglycerides, blood pressure, the viscosity of blood, the rate of platelet aggregation, and inflammation related to arthritis, psoriasis and asthma (Simopoulos, 1999; Sahena *et al*., 2009).

The current primary sources of PUFAs in the human diet are fatty fish including sardines, salmon, tuna and herring and oils processed from these organisms (Burja *et al.,* 2006). Fish oils, however, can contain objectionable tastes and odours and when processed require the removal of cholesterol and potentially toxic impurities such as mercury, dioxins and dioxin-like compounds such as polychlorinated biphenyl (Certik *et al*., 1999; Ratledge, 2004). Fish oils are also victim to seasonal variability in their fatty acid profiles. Additionally, concerns have emerged with respect to the sustainability of these marine resources. The total global production of fish oil has reached about one million tonnes annually (Pike *et al.,* 2010). The portion of this oil intended for human consumption was about 13% with the majority (approximately 81%) being used in the aquaculture industry. The production of fish oil is believed unlikely to increase from current levels; however, the trend of human consumption of fish oils has been increasing and is expected to continue to increase as more people become aware of their positive health effects. Furthermore, it has been estimated that if omega-3 PUFAs become widely used as prophylactic drugs the total annual production of marine fish oil would be insufficient to meet the world wide demand (Certik *et al*., 1999).

Microbial oils provide a sustainable alternative to fish oils and do not contain the potentially toxic impurities inherent to fish oils (Hauvermale, 2006). Furthermore, due to the controlled nature of cultivation of microorganisms, very little to no variability is found in the quantity and composition of their oils. DHA is a major component of lipids in some oleaginous microorganisms. The dinoflagellate, *Crypthecodinium cohnii*, is currently grown via fermentation as a source of DHA (Cohen *et al*., 2005). Likewise, the

micro-algae *Schizochytrium* spp. and its relative *Thraustochytrium* spp. (collectively referred to as thraustochytrids) have been shown to produce large amounts of fatty acids – up to 80% of the cell mass – in which DHA comprises more than 50% (Singh *et* al., 1996a; Burja *et al*., 2006). A strain belonging to the genus *Schizochytrium* is currently used for production of DHA-rich oil by DSM-Martek in the USA. Six different PUFA profiles from thraustochytrids have been reported including profiles rich in one or more of EPA, DHA and ARA (Huang *et al*., 2003; Burja *et al*., 2006). ARA has also been shown to occur as a major lipid component in microorganisms particularly in strains of the fungus *Mortierella alpina* which is currently grown commercially in separate processes in Europe and China (Cohen *et al*., 2005).

A successful campaign to isolate PUFA-producing microorganisms has already been conducted by Ocean Nutrition Canada Ltd. (ONC) (Burja *et al.*, 2006). This campaign focused on the isolation of thraustochytrid species with potential to produce DHA at a commercial scale. Similar isolation campaigns have been undertaken and proven successful by many other research groups. Ho *et al.* (2007) were successful in their efforts to isolate the fungus *Mortierella alpina* from soil samples for the purpose of producing ARA. Many other microorganisms have been isolated for purposes other than PUFA production. Examples include: the marine bacterium *Streptomyces* spp. isolated by Bernan *et al*. (1994) for the production of novel antibiotics; Miki *et al*. (1994) isolated a bacterium *Pseudomonas* spp. for the production of a novel carotenoid, okadaxanthin; Lhullier *et al*. (2010) were successful in isolating the red alga *Laurencia catarinensis*, which is capable of producing cytotoxic halogenated metabolites; and a species of

Pseudomonas were isolated in Antarctica, which is capable of degrading diesel fuel (Shukor *et al*., 2009).

The strain isolated in the first campaign by ONC was *Thraustochytrium* sp. ONC-T18 (Burja *et al.,* 2006). This strain is reportedly capable of producing over 20 g L^{-1} biomass, 81.7% of which is fatty acids. Of the fatty acids, 21% was found as DHA. It would be of benefit to ONC to be able to produce both EPA and ARA in addition to DHA from a microbial source. Dietary supplements of omega-3 fatty acids are most often sold containing combinations of EPA and DHA. As mentioned, ARA plays an important role in infant health and nutrition and is required, along with DHA, in infant formula (Ratledge, 2004).

During the previous ONC study, strains of thraustochytrids, in addition to *Thraustochytrium* sp. ONC-T18, were isolated each with their own unique fatty acid profiles (Burja *et al*., 2006). Some of these profiles showed potential for production of EPA and ARA with percentages of total PUFAs of 50% and 15%, respectively. Huang *et al*. (2001) also reported the growth of a thraustochytrid strain with the capability of producing 14% of its total fatty acids as ARA. With the selection of the proper strain and the development of proper culture conditions it seems possible that commercial production of both EPA and ARA in addition to DHA is feasible.

1.1 Objectives and hypothesis

1.1.1 Objectives

The objective of this research was to isolate novel microorganisms from the family *Thraustochytriaceae* with the potential for large scale production of long chain, polyunsaturated fatty acids (PUFA). The specific PUFAs of interest were eicosapentaenoic acid (EPA, C20:5 (*n*-3)), docosahexaenoic acid (DHA, C22:6 (*n*-3)), and arachidonic acid (ARA, C20:4 (*n*-6)).

1.1.2 Hypothesis

Novel marine protists that produce significant amounts of EPA, DHA and/or ARA can be isolated from Atlantic Canadian waters. Isolates can be cultured at the laboratory scale to produce microbial oil rich in EPA, DHA and/or ARA.

CHAPTER 2: LITERATURE REVIEW

2.1 Overview of Family *Thraustochytriaceae*

Dick (2001) erected the Kingdom *Straminipila* and classified thraustochytrids under the Phylum *Heterokonta*, Class *Labyrinthulomycetes,* Order *Labyrinthulales* and Family *Thraustochytriaceae*. Others had previously classified thrautstochytrids in the family *Thraustochytridae* of the Class *Labyrinthulea*, Subphylum *Labyrinthista* and Phylum *Heterokonta* of the Kingdom *Chromista* (Cavalier-Smith, 1993; Cavalier-Smith *et al.*, 1994). There are currently eleven genera classified in the family *Thraustochytriaceae*. These genera include: *Althornia, Aplanochytrium, Aurantiochytrium, Japanochytrium, Oblongichytrium, Schizochytrium, Thraustochytrium, Ulkenia, Sicyoidochytrium, Parietichytrium, and Botryochytrium* (Yokoyama *et al*., 2007a).

Thraustrochytrids are a diverse group of marine, osmoheterotrophic, fungoid protists. Thraustochytrids are often erroneously referred to as microalgae when discussing their biotechnological applications. They are closely related to brown algae, however, thraustochytrids are not algae and there is no literature which classifies them as such (Armenta *et al*., 2012). They are characterized by reproduction through release of biflagellate zoospores; production of Golgi body-derived, scale-like lamellated cell walls; and, with the exception of the Genus *Althornia*, by production of ectoplasmic net structures which extend from a unique organelle called the bothrosome (also called the sagenogenetosome or sagenogen) (Perkins, 1972; Porter, 1990; Bower, 2005). The ectoplasmic net consists of units of membrane tubes containing no cell organelles. This

net contributes to the increased surface area of the cell and contains hydrolytic enzymes that are surface-bound or secreted into the surrounding medium aiding in the digestion of organic material. Additionally, within the bothrosome, an electron dense plug separates the cell cytoplasm from the ectoplasmic network. The ectoplasmic net also attaches the cells to surfaces and penetrates organic particles. Thraustochytrids mostly do not produce ectoplasmic nets when grown in rich liquid media, particularly in shake cultures. The reasoning for this is that in rich cultures the need for extensive cell wall extensions to increase nutrient uptake is unnecessary, and in the case of shake cultures, the ability of cells to adhere to surfaces is inhibited.

2.1.1 Ecology

Most thraustochytrids are free living and usually associated with organic detrital materials possibly playing a role in decomposition. The first description of a thraustochytrid by Sparrow (1936) was from a moribund alga. Thraustochytrids possess enzymes allowing them to utilize many polysaccharides including: dextran, laminarin, mannan and starch (Bahnweg, 1979). The ectoplasmic network of thraustochytrids has been shown to penetrate the sporopollenin layer of pine pollen to allow access to nutrients within (Perkins, 1973). Sporopollenin is a polymer which is normally highly resistant to microbial breakdown. The role of thraustochytrids in decomposition, however, may be most important in later stages of break down. Raghukumar (2002) showed that when thraustochytrids were inoculated onto brown algae and mangrove leaves in early stages of decomposition growth was poor as compared to when inoculated onto more decomposed material.

A few species of thraustochytrid have been associated with disease in molluscs (Bower, 2005). The first report of a thraustochytrid parasite was by Polgase (1980) who found a progressive ulceration of the skin of the lesser octopus *Eledone cirrhosa*, which was associated with the presence of the protist. In three cases, unidentified thraustochytrids were involved in surface lesions on captive octopuses, nudibranchs and squid in the northern hemisphere (Bower, 1987). In addition, *Labyrinthuloides haliotidis* (now *Aplanochytrium haliotidis*) was determined to be a pathogen in cultured small juvenile abalone in British Columbia, Canada and an unnamed species, commonly called Quahog Parasite Unknown, has been associated with mortalities and lesions in hard clams on the eastern seaboard of North America (Anderson *et al.,* 2003).

2.1.2 Life cycle

Thraustochytrids exhibit simple, asexual life cycles. The morphologies observed in these life cycles closely resemble zoosporic fungi and, in fact, thraustochytrids were originally classified under the Class *Oomycetes* of the Division *Mastigomycotina* among Fungi on the basis of their biflagellate zoospores (Sparrow, 1973). Vegetative states of thraustochytrids consist of single cells which are globose to subglobose measuring 4 to 20 µm in diameter, mostly growing epibiontically on various substrata. Most thrautochytrids reproduce by release of heterokont, biflagellate, straminopilan zoospores which possess a long, anterior, tinsel flagellum and a shorter, posterior, whiplash flagellum. The mode of production of zoospores varies between genera and forms the major taxonomic criterion (Raghukumar, 2002). For example, the genus *Thraustochytrium* produces zoospores by direct division of the cytoplasmic contents of

the mature cell; the sporangium (Figure 2.1). The genus *Schizochytrium* is characterized by successive bipartition of a vegetative cell (Figure 2.1). This results in a cluster of cells each of which develops into a zoosporangium or a sorus which will produce zoospores (Porter, 1990; Yokoyama *et al.*, 2007b). Within genera, species are generally defined by the number of proliferating bodies and the size and shape of the sporangia.

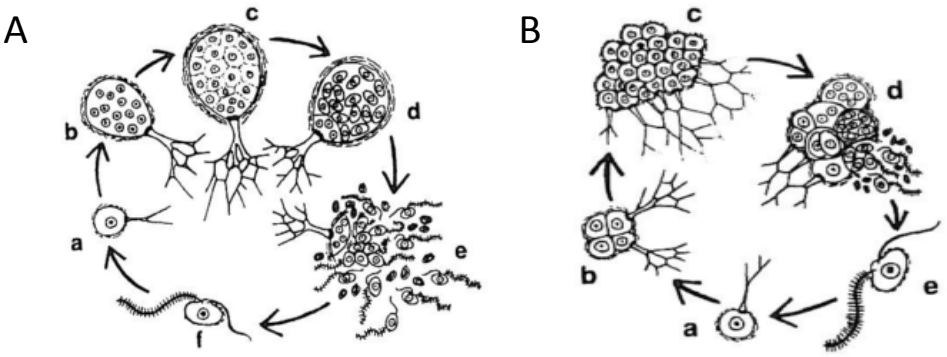

Figure 2.1: (A) Life cycle of *Thraustochytrium* spp. where a thallus (a) grows by enlargement and nuclear division to produce a sporangium (b). Progressive cleavage of the protoplast (c) occurs in the sorus (d). The cell wall then breaks down (e) to produce zoospores (f) that settle to form new thalli. (B) Life cycle of *Schizochytrium* spp. where a thallus (a) divides by two cell divisions to form a tetrad (b) and eventually a cluster of cells (c). Each cell becomes a sorus (d) releasing zoospores (e) which eventually settle to form new thalli (Porter, 1990).

2.2 Production of PUFA

Until recently, it was assumed that all long chain PUFA, including those produced by thraustochytrids, were produced by variations of the same basic fatty acid synthase (FAS) pathway followed by elongation and desaturation. In 2001 an alternative pathway for their synthesis was described by Metz *et al*. called the polyketide synthase pathway (PKS). A significant precedent for this was described in the marine bacteria *Shewanella* sp. SCRC-2738 (Yazawa, 1996; Yu *et al.,* 2000). The large amount of both total fatty acids and PUFA produced by thraustochytrids makes them an ideal model for studying

mechanisms of lipid biosynthesis. In this chapter bio-synthesis of PUFA by both FAS and PKS pathways will be described.

2.2.1 Fatty acid synthase pathway

Prior to 2001, it was thought that thraustochytrids utilized only the FAS pathway followed by desaturation and elongation to produce DHA (Metz *et al*., 2001). The FAS pathway consists of a multi-enzyme protein which catalyzes fatty acid synthesis (Hopwood, 1997). This pathway can be distinguished into two classes: Type I FAS, which is characteristic of fungi and vertebrates, in which the catalytic sites for various steps in lipid biosynthesis are carried as domains along the length of multifunctional proteins; and Type II FAS, characteristic of bacteria and plants, in which each catalytic site is carried on a separate protein subunit. Elongation of fatty acids by FAS stops upon formation of palmitic acid (C16:0); the primary end product of this pathway. Figure 2.2 illustrates the production of a saturated fatty acid by way of the FAS pathway. The process consists of iterative cycles of condensation catalyzed by ketosynthase (KS) to form a ketoacyl-acyl carrier protein (ACP), followed by reduction by ketoreductase (KR) to form hydroxyacyl-ACP, dehydration by dehydratase (DH) to form enoyl-ACP, and further reduction by enoyl reductase (ER) to produce acyl-ACP. When the saturated carbon chain has reached its full length, generally 16 carbons, it is released from the ACP via thioesterase to produce a fatty acid.

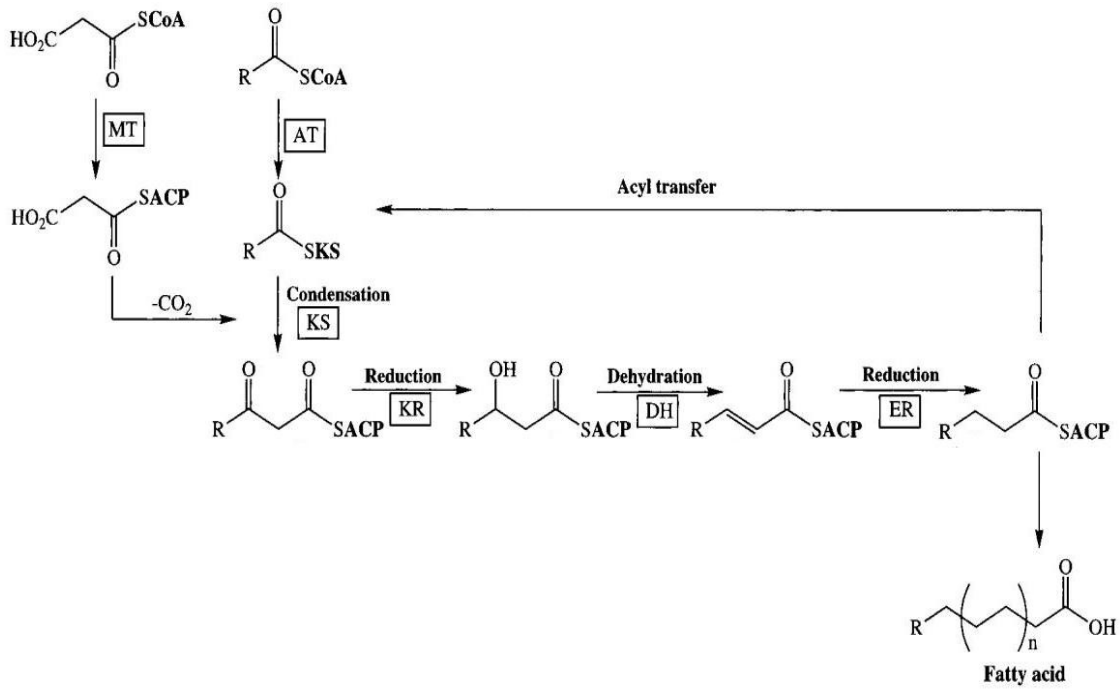

Figure 2.2: Production of a saturated fatty acid via fatty acid synthase pathway. MT = malonyl transferase, AT = acyl transferase, KS = ketosynthase, KR = ketoreductase, DH = dehydratase, ER = enoyl reductase, ACP = acyl carrier protein (Hopwood, 1997).

2.2.1.1 Desaturation and elongation

Palmitic acid alone is not suitable to provide all of the necessary functions of fatty acids. As such, a series of elongation and desaturation steps are employed to create both longer chain and unsaturated fatty acids. Although the specific enzyme types may differ, this elongation and desaturation cascade is responsible for alteration of fatty acids in both terrestrial and marine plants and animals. The pathway involves a series of desaturase and elongase enzymes and is considered aerobic as molecular oxygen is required as a co-factor (Qiu, 2003). Desaturase enzymes are responsible for inserting double bonds at specific points along the carbon chain relative to the carboxylic acid group, whereas elongase enzymes are responsible for lengthening the carbon chain by 2 carbon atoms which are inserted at the carboxylic acid end of the molecule.

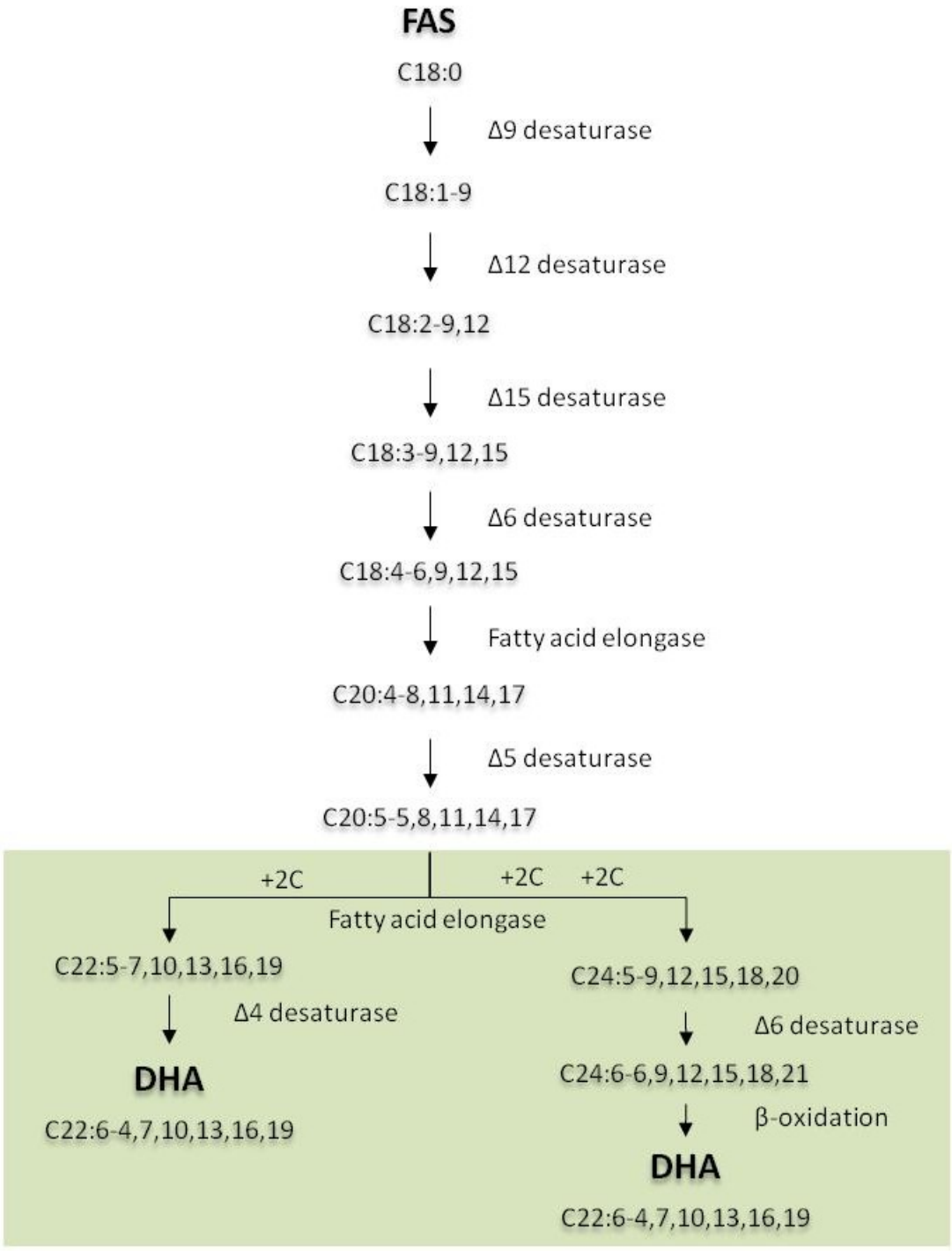

Figure 2.3: Production of DHA by elongation and desaturation. Both the Δ4 desaturase dependent pathway used by thraustochytrids and the Δ4 desaturase independent pathway used by vertebrates are shown (Qiu, 2003).

Figure 2.3 illustrates two pathways for biosynthesis of DHA: Δ4 desaturase dependent and Δ4 desaturase independent. It had long been assumed that all organisms, including mammals, contained genes to produce the Δ4 desaturase enzyme. However, a study by Voss and co-workers (1991) to examine the biosynthesis of the Δ4 desaturase enzymes in mammals using a rat model produced unexpected results. After incubating ^{14}C labelled 22:5 (*n*-3) (docosapentaenoic acid, DPA) doped at the C1 position with rat liver microsomes, they did not observe any detectable labelled DHA products. If a Δ4 desaturase were present it would be expected that the radio labelled DPA would be converted to DHA producing labelled DHA products. What Voss and co-workers did find was labelled C24:5 (*n*-3) and C24:6 (*n*-3). This data led Voss and co-workers to conclude that, in mammals, DHA is produced independent of Δ4 desaturase. Rather, DHA is produced by elongating DPA to C24:5 (*n*-3), desaturating via Δ6 desaturase to C24:6 (*n*-3), and finally oxidation to C22:6 (*n*-3) by β-oxidation. Fatty acid oxidation is the reverse process to elongation in which two carbons are removed from the carboxylic acid end of the fatty acid (Qiu, 2003).

Unlike mammals, thraustochytrid marine protists and many other microorganisms have been found to contain genes which code for Δ4 desaturase enzymes. Evidence for the production of a Δ4 desaturase enzyme and its involvement in DHA synthesis in the thraustochytrid *Thraustochytrium* spp. has been provided (Qiu *et al*., 2001). In their study, Qiu *et al*. (2001) identified a cDNA in *Thraustochytrium* spp. which encodes for a protein similar in structure to Δ4 desaturase enzymes isolated from other organisms. Cloning and expression of this cDNA in both yeast (*Saccharomycese cerevesia*) and

plants (*Brassica juncea*), which do not inherenty produce Δ4 desaturase enzymes, revealed Δ4 double bonds inserted into DPA thereby producing DHA. DHA is not naturally produced in yeast or plants by either the Δ4 dependent or independent pathways. The evidence provided by this study clearly shows thraustochytrids to contain the genes encoding for a Δ4 desaturase enzyme. DHA biosynthesis in thraustochytrids is, therefore, achieved by FAS coupled with elongation and desaturation via a Δ4 desaturase-dependant pathway.

2.2.2 Polyketide synthase pathway

Initial indications of an alternative pathway for long-chain PUFA synthesis came from studies of certain marine bacteria (DeLong *et al*., 1986). Traces of EPA or DHA were found in several strains of psychrophilic marine bacteria. Subsequently, a segment of genomic DNA was identified from an EPA-producing bacterium (*Shewanella* sp. SCRC-2738) that, when transformed into *Escherichia coli,* resulted in the production of EPA (Yazawa, 1996). It was later determined that proteins encoded by five open reading frames were necessary and sufficient for production of EPA (Yu *et al*., 2000). Four of those proteins are subunits of an enzyme complex capable of *de novo* synthesis of EPA. Metz *et al*. (2001) determined that some of the multiple domains of this 'PUFA synthase' enzyme showed homology to those found in FAS systems while others were similar to those of PKS systems.

The PKS pathway has been widely observed in nature. In bacteria and fungi the PKS pathway is involved in synthesis of a large collection of antibiotic and anti-parasite compounds such as tetracycline and erythromycin (Pferfer *et al*., 2001). This pathway

had not previously been observed to produce PUFA. The fifth essential gene required for EPA synthesis in *Escherichia coli* encoded a phosphopantetheinyl transferase which was found to activate the ACP domains present on the enzyme by the attachment of a cofactor (Lambalot *et al*., 1996; Facciotti *et al*., 2000). Genes with homology similar to the EPA gene cluster of *Shewanella* spp. have been identified in other marine bacteria, including one which accumulates DHA (Facciotti *et al*., 2000). Furthermore, it has been hypothesized that most marine bacteria that synthesize long chain PUFA utilize this PKS system (Hauvermale *et al*., 2006).

Until 2001 it was suggested that thraustochytrids utilized only the FAS pathway coupled with elongation and desaturation to produce oils rich in PUFAs. Metz *et al.* (2001) showed the existence of a PKS pathway in the thraustochytrid *Schizochytrium* spp. In their experiment ^{14}C labelled acetate was fed to thraustochytrids. The result showed high level incorporation of the labelled acetate into both DHA and DPA: 31% and 10%, respectively, after just one minute of incubation. This level of incorporation in these fatty acids was consistent over a 24 hour period. Conversely, there was no evidence of incorporation into shorter 16 and 18 carbon fatty acids, indicating no precursor-product relationship between these fatty acids and the 22 carbon fatty acids.

These results are consistent with rapid synthesis of DHA from ^{14}C labelled acetate involving very small pools of intermediates. If we consider this evidence with that of Qiu *et al.* (2001) which showed the existence of genes encoding Δ4 desaturase enzymes in thraustochytrids we can conclude the existence of two distinct pathways for DHA synthesis in some thraustochytrid strains. This compelling evidence for the existence of

a PKS pathway suggests that *in vivo* synthesis of DHA in tested thraustochytrids would be primarily via the PKS pathway.

Polyketides are secondary metabolites containing multiple building blocks of ketide groups (-CH_2-CO-) (Qiu, 2003). Synthesis of polyketides by PKS resembles the enzymatic system of the FAS system and examples have been found of PKS pathways which resemble each of the classical classes of FAS (Hopwood, 1997). Figure 2.4 shows that similar to the FAS pathway, the PKS pathway uses ACP as a covalent attachment for chain synthesis, proceeding with iterative cycles. The full cycle of biosynthesis, like FAS, also includes condensation of an acyl-ACP and a malonyl-ACP to produce a ketoacyl-ACP, ketoreduction to convert ketoacyl-ACP to hydroxyacyl-ACP, dehydration to remove a water molecule from hydroxyacyl-ACP resulting in unsaturated enoyl-ACP, and reduction of enoyl-ACP to a saturated acyl chain. However, unlike fatty acid synthesis by the FAS pathway, polyketide synthesis often omits steps of the full cycle such as dehydration and reduction (Qiu, 2003). As a result, the products of PKS pathways are highly varied in structure and often contain keto and hydroxyl groups and double bonds.

The dashed lines of Figure 2.4 indicate the alternative routes which can be followed in a PKS enzyme system and are in addition to the solid arrows representing the FAS enzymatic system. The reduced polyketide produced shows the result of reduction at different stages of the cycle. Figure 2.4A-D represent reduction of ketoacyl-ACP, hydroxyacyl-ACP, enoyl-ACP and acyl-ACP, and the resulting keto, hydroxyl, enoyl and methylene functionalities in the reduced polyketide molecule, respectively. The PKS pathway also differs in its production of DHA from the traditional elongase/desaturase

system in that it is anaerobic. Although, the pathway is functional in the presence of oxygen, molecular oxygen is not required at any point within the pathway. Double bonds are, therefore, introduced into an existing acyl chain without the requirement of aerobic desaturation.

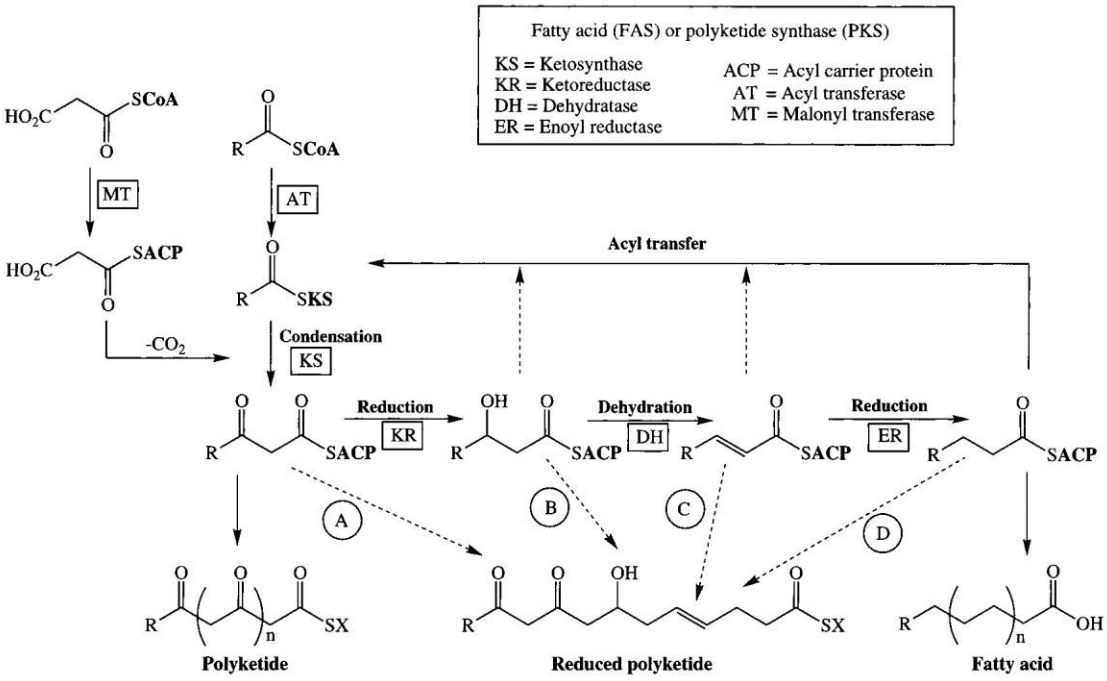

Figure 2.4: The basic pathway of fatty acid and polyketide biosynthesis, showing the various activities carried out by the subunits or domains of the fatty acid or polyketide synthase. The solid arrows represent the path followed by fatty acid synthase. The dashed arrows show the potential path of polyketide synthase and are in addition to the solid arrows. A-D represent the althernative versions of the reductive cycle that lead to keto, hydroxyl, enoyl, or methylene functionality, respectively, as specific β-carbons during the assembly of reduced polyketides (Hopwood, 1997).

The exact mechanism underlying DHA biosynthesis by the PKS pathway remains to be defined (Metz *et al*., 2001). The key features, however, likely include the condensation and isomerisation steps. Ketosynthase, the key enzyme in the pathway, can catalyze the condensation of a wide range of substrates including unsaturated fatty

acids. Thus, selective condensation of enoyl-ACP with malonyl-ACP would result in a fatty acid with double bonds (Qiu, 2003).

In a study by Ren *et al.* (2009), the effect of addition of acetic acid to the culture media of *Schizochytrium* sp. HX-308 was observed. The hypothesis was that by adding acetate to the culture media during the lipid accumulation phase of growth, the supply of acetyl-CoA would be enforced which would lead to increased production of fatty acids and an increase in the percentage of DHA in those fatty acids. Eukaryotes contain the acetyl-CoA synthase enzyme which can directly convert acetate into acetyl-CoA. Results of this study supported the original hypothesis as addition of acetate was shown to cause an increase in both total fatty acid and the percentage of DHA. However, the authors of this study attributed these increases solely to the FAS pathway being reinforced by the addition of acetate. It is likely, however, that a portion of the increase could be attributed to the PKS pathway.

Metz *et al.* (2001) used labelled acetate to corroborate the existence of a PKS pathway in *Schizochytrium* spp. showing incorporation of the labelled acetate into long chain PUFAs. Although, the conclusions of Ren *et al.* (2009) remain sound in terms of feeding with acetic acid causing an increase in total fatty acids and DHA, the reason for some of the increase in DHA may not have been attributed to the appropriate pathway. Nevertheless, this study showed that by understanding the mechanisms of DHA production in thraustochytrids, the biosynthetic pathway can be reinforced through the addition of the right precursors leading to higher levels of production.

2.2.3 Rational for lipid accumulation

The biological role of DHA in human nutrition has been and continues to be investigated. However, the role of DHA in thraustochytrid cells is seldom addressed. DHA is known to play an important role in membrane functions; however, only up to 5% of lipids in thraustochytrids occur as phospholipids, 70-98% of lipids are generally present as triacylglycerols in storage lipids (Nakahara *et al*., 1996; Yaguchi *et al*., 1997; Stillwell *et al*., 2003). It is apparent that accumulation of DHA in the form of triacylglycerols or neutral lipids is important for these protists, although the precise reason for this is not clear. One potential role of DHA and other PUFAs in storage lipids could be that of antioxidants when a cell is subjected to oxidative stress, as during starvation (Mukherjee *et al*., 2004). Another potential role may be as energy for cell motility. During the mobile life stages of thraustochytrids large amounts of energy are required. The lipids stored in triacylglycerols are highly reduced molecules which provide a greater amount of energy per gram than carbohydrates.

Cohen *et al.* (2000) suggested that stored triacylglycerols may serve as sources of fatty acids in cells that undergo rapid changes in environment and require the production of membranes. As discussed previously, thraustochytrids produce a network of membrane extensions called the ectoplasmic net. When grown under nutrient poor conditions this network is more extensively produced to enable them to take up nutrients from the surrounding area. The production of this ectoplasmic net requires phospholipids, and, as Jain *et al*. (2007) showed, these phospholipids are produced at the expense of stored triacylglycerols.

The large amount of DHA produced by thraustochytrids may be particularly useful as a rapid energy source during starvation. Jain *et al*. (2007) showed that not only does DHA decrease during starvation of thraustochytrids, but also the proportion of DHA as compared to other fatty acids also decreases. In contrast, the absolute level of palmitic acid remained consistent throughout starvation. This confirms the notion of DHA being a useful energy source and/or antioxidant during starvation.

In the same study, the importance of lipid content on the specific gravity of cells was also considered. The specific gravity of a cell in an aquatic environment has implications on the buoyancy of the cell with an inverse relationship. It was found that increased lipid content in thraustochytrids increased the specific gravity of the cells, therefore, lowering their buoyancy. There are numerous examples of marine organisms which have evolved the ability to reduce their buoyancy. Among these are, the production of extracellular polymeric substance in diatoms (Decho, 1990), alteration of osmotic balance in some marine copepods and fish (McAllen *et al*., 1998), selective choice of heavier ions in crustaceans (Sanders *et al*., 1988), and a reduction in the amount of gas present in swim bladders of some fishes and invertebrates and storage of low density materials. It is likely that storage of lipids in thraustochytrids, as in several zooplankton, alters their buoyancy (Jain *et al*., 2007).

2.3 Methods of isolation

There are three general methods used to isolate thraustochytrids: baiting, direct plating, and filtration. These methods will be discussed in detail below. Common to

each of these methods are: sample collection (including the types of samples and the environments where they are collected) and growth media used for isolation. With regard to the type of samples, it is possible to use any of the isolation techniques on all sample types, although, some techniques are better suited for specific types of samples. This will be discussed in the descriptions of the techniques.

2.3.1 Sample collection and storage

Thraustochytrids are found throughout the world in estuarine and marine habitats (Burja *et al.,* 2006). They are primary decomposers playing an important role in enriching nutritionally poor mangrove areas by decaying mangrove litter. As such, thraustochytrids are generally associated with benthic algae, marine vascular plants, and detrital sediments including mangrove leaves (Porter, 1990; Bremer, 2000). Fallen tree leaves should be moribund but not senescent as necrotic leaves tend to harbour large amounts of yeasts and moulds which create problems for thraustochytrid isolation. A wide range of marine invertebrate may also be sources of thraustochytrids including: nudibranchs, squid, octopuses and the gut contents of echinoids and sponges (Höhnk *et al.*, 1979; Polgase, 1980; Wagner-Merner *et al.,* 1980; McClean *et al*., 1982; Jones *et al*., 1983). Yet another source of isolates is gills of molluscs (Bremer, 2000; Anderson *et al.,* 2003). Thraustochytrids may also be found in offshore water samples (Porter, 1990).

Samples should be stored on ice or at refrigerator temperature until transported to the laboratory for processing. Processing of samples is preferable at the most immediate point possible following sample collection. Bremer (2000) suggests that samples should be stored at refrigerator temperature for no longer than 24 hrs prior to

processing, others, including Barclay (1994) and Fan *et al.* (2002), take a more immediate approach, storing samples under refrigeration only until return to the laboratory to avoid overgrowth of bacteria, yeast and mould.

2.3.2 Media components

Each of these methods uses agar media to grow isolates. A common nutrient media used in isolation of thraustochytrids is Kazama's Modified Vishniac's media (KMV; 0.1% (w/v) glucose, 0.1% (w/v) gelatin hydrolysate, 0.01% (w/v) yeast extract, 0.1% (w/v) peptone made up in filtered seawater). In general, nutrient media should contain one or two sources of organic nitrogen such as yeast extract, peptone, tryptone, peptonized milk, or gelatin hydrolysate; a carbon source such as glucose or starch (the former is the most common); and sea water be it filtered natural seawater or artificial seawater at strengths ranging from 70% to 100% (Barr, 1990; Bowles, 1999; Bremer, 2000; Quilodran *et al*., 2010).

The addition of a carbon source especially glucose or other mono- or disaccharides, has been disputed by Bremer (2000). It has been suggested that the use of simple sugars will create an environment favourable to the proliferation of yeast. Yeast is the major contamination problem when isolating thraustochytrids as they can rapidly outgrow thraustochytrids. Bacteria are a potential problem, but their growth is normally discouraged through the use of antibiotics, most commonly penicillin G and streptomycin.

2.3.3 Overview of isolation methods

2.3.3.1 Pollen baiting

Baiting to isolate thraustochytrids, first described by Gaertner (1968), is perhaps the most commonly referenced technique. Gaertner's preferred bait was pollen from the genus *Pinus* (i.e. pine pollen). This remains the choice bait of those using this method for isolating thraustochytrids (Raghukumar *et al.,* 1992; Bremer, 1995; Bowles *et al.,* 1999; Bongiorni *et al.,* 2005a; Huang *et al.,* 2008; Quilodran *et al.,* 2010). However, a number of different substrates can be used as bait. These include: pollen from gymnosperms other than *Pinus* spp. and from angiosperms including *Acer* spp. (Burja *et al.,* 2006), apple skin, snake skin, cellophane, bleached grass leaves, insect exoskeleton, and algae (Porter, 1990; Bremer, 2000). We will refer only to pine pollen as bait for the purpose of this review due to its prevalent use in the literature for baiting thraustochytrids.

Pine pollen should be sterilized prior to use as bait, which can be achieved through dry heat, fumigation with propylene dioxide or, preferably, by gamma irradiation (Bremer, 2000). A dosage of 25 kGy has been suggested. Water samples of volumes greater than 20 mL are suitable for baiting, while sediment samples of about 0.1 to 0.2 mL should be suspended in 25 mL sterile seawater prior to baiting. Baiting from small sample pieces of tissue from algae, mangrove leaves, etc., requires these to first be suspended in 20 mL of sterile seawater (Bremer, 2000). In all cases, antibiotics should be added in order to discourage the growth of bacteria. Although this baiting method can be used for all sample types, it is best suited for water and sediment samples.

Following preparation of the sample suspensions, a small amount of sterile pollen is added at levels of about 1 mg per 20 mL suspension. Baited suspensions are then incubated at 18-25°C (Burja *et al.,* 2006; Quilodran *et al.,* 2010). Generally, incubation at room temperature is acceptable (20-25°C), but consideration should be paid to the environmental temperature in which the original sample was collected. After 48 hrs, and daily thereafter, pollen grains should be examined for presence of thraustochytrid thalli (Bremer, 2000). Quilodran *et al.* (2010) suggested samples should be incubated for a 7 day period prior to the pollen grains being collected and examined for thraustochytrids. However, based on our experience, commencing the repeated daily examination after 48 hrs appears to increase the likelihood of isolating thraustochytrids. Longer incubation periods are prone to over-growth with yeast and fungi. During incubation zoospores of thraustochytrids will attach to the pine pollen bait leading to the formation of thraustochytrid thalli found as spheres attached to the pollen. The thalli occur most often in the area between the two large air sacs of the pollen grain. Individual pollen grains with attached thraustochytrid thalli can subsequently be transferred to an isolation media such as that described above (Section 2.3.2). Alternatively, pollen grains can be transferred in mass to agar plates.

The pollen baiting method is effective but does have some inherent difficulties. Contamination of subcultures is common as yeast can be carried over as a contaminant either attached to the pollen or in the sea water (Bremer, 2000). The procedure of transferring pollen grains can be quite difficult, particularly when trying to transfer a single, specific pollen grain. This is best achieved through practice by using sterile

inoculation loops or needles, or a sterile, fine tipped Pasteur pipette. Bongiorni *et al.* (2005a) reported being able to subculture a single pollen grain with an attached thraustochytrid cell using a fine glass loop. An additional concern with the pollen baiting method is the ability of some thraustochytrid strains to colonize pollen grains. For example, *Ulkenia amoeboida* has been found to not readily colonize pollen grains (Bahnweg *et al.*, 1974). This is a particular problem when synoptic collections are desired. This example highlights the importance of using a variety of methods to achieve representation of the thraustochytrid biodiversity of a sampling area.

2.3.3.2 Direct plating

A frequent alternative to the classic baiting method is direct plating of collected material (Porter, 1990; Raghukumar *et al.,* 1995; Bremer, 2000; Fan *et al.,* 2002; Bowles *et al.,* 2009). This method of isolation is preferred when isolating thraustochytrids from plant or animal tissues (Bremer, 2000). Materials should be washed with sterile seawater containing antibiotics to remove sediment particles and loosely attached yeast, mould, bacteria, diatoms and protozoans.

Following this initial washing of the whole sample the sample should be cut into smaller pieces. Porter (1990) suggests cutting pieces to squares of 1 cm^2, Bremer (2000) suggests cutting samples to 0.5 cm^2, and similarly Fan *et al.* (2002) suggests cutting samples into 1.5 cm diameter discs. These smaller pieces are then washed again with sterile sea water containing antibiotics. Bremer (2000) suggests washing these pieces in flasks at 200 RPM for 2-3 hrs, Fan *et al.* (2002) suggests washing three times, while Porter (1990) bypasses this washing step and plates cut samples on agar by dragging

them across the agar surface. It is our experience that a thorough washing will greatly reduce the number of yeast and mould and nearly completely eliminate diatoms from the isolation plates. This will increase the likelihood of a successful isolation and therefore choosing a more extensive washing procedure is advised.

After this secondary wash procedure pieces of tissue can be plated on nutrient agar (Fan *et al.,* 2002). Bremer (2000), however, suggested a further reduction in the size of samples to 2-3 mm^2, followed by yet another washing step in a Petri dish using sterile seawater containing antibiotics. These smaller pieces are then directly plated onto nutrient agar with a total of 6 sample pieces per agar plate followed by overlaying with sterile seawater. Overlaying plates with sterile seawater containing antibiotics is an effective method of inducing release of zoospores. This will increase the number of thraustochytrid thalli on the plates and therefore increase the likelihood of isolation. Incubation of these plates is generally done at temperatures of 18-25°C, however, as with the baiting technique, consideration should be given to the environmental temperature in which the sample was collected.

Plates should be observed using microscopy for the presence of thraustochytrid thalli after 24 hrs of incubation and then daily thereafter until overgrowth with yeasts and moulds renders isolation of thraustochytrids unlikely. Bremer (2000) notes that the use of a transition microscope capable of casting oblique lighting is very useful as it allows one to discern individual sporangia on agar surfaces. Thraustochytrids form colonies similar to yeast but with a different distribution of cell sizes. From mature zoosporangia, the ectoplasmic net structure can be seen, and young zoospores may be

seen actively swimming. Subculture of thalli from static agar surfaces is much easier than trying to select individual or a few pollen grains from a suspension. Agar containing cells can be excised, transferred to fresh plates and overlaid with sterile seawater containing antibiotics to promote growth and zoospore formation.

The direct plating method can be used in a similar manner for sediment samples or gut contents by streaking small quantities onto the surface of agar plates which are then overlaid with sterile seawater containing antibiotics. However the direct plating method is not useful in the case of water samples. Water samples may contain very low levels of thraustochytrids per litre that would require a large quantity of replicates in order to get a positive isolation. Baiting, as described above (Section 2.3.3.1), and filtration, as described below (Section 2.3.3.3) are more effective measures for recovering isolates from water samples.

Raghukumar *et al.* (1995) suggest an alternative direct plating approach in which leaf samples are reduced in size to about 0.5 cm^2 and their surfaces sterilized with 0.5% sodium hypochlorite. Rather than plating these pieces of leaf tissues directly onto nutrient agar they were homogenized in sterile sea water (20 pieces of leaf tissue per 15 mL sea water) and 0.1 mL aliquots of the sea water were plated onto agar plates. This study was performed with the primary aim of enumerating thraustochytrids to determine their role as decomposers of leaf litter. Although this method may be useful for its primary purpose of enumeration it would not be as useful if the intention were to build a library of isolates. This is because this method would require a larger number of agar plate replicates to be representative as the thraustochytrids present on leaf pieces

would be diluted within the sea water with only a small sample portion (0.1 mL, less than 1/100 of the original sample) being plated per agar plate.

2.3.3.3 Filtration

The primary sample type used with the filtration method is water samples (Barclay, 1994; Honda *et al.,* 1998; Bremer, 2000). The problem of low levels of thraustochytrid cells per volume of water can be solved by concentrating samples through filtration. Honda *et al.* (1998) and a method described by Bremer (2000), as performed by Bahnweg (1973), used similar one-step filter approaches to concentrate sea water samples. Honda *et al.* (1998) filtered samples through 0.4 µm membrane filters. Filters were then plated directly onto nutrient agar and incubated at 25°C for 3 days, followed by subculture of visible colonies. It should be noted that in all cases of concentrating by filtration, the entire microflora contained in the sample will be concentrated. This would greatly increase the likelihood of contamination, particularly by yeasts, moulds and protozoa. Growth of bacteria can be discouraged through the use of antibiotics added to the original water sample and to agar plates.

This problem was addressed in a study by Barclay (1994) who used a two step filtration procedure in which 150 mL water samples were first filtered through a sterile Whatman #4 filter (~25 µm pore size), followed by a polycarbonate filter with 1.0 µm pore size. The first filtration served to remove any sediment or particulate matter and also greatly reduced the amount of mould hyphae present in the sample. The second filtration allowed a large portion of bacteria to pass through into the filtrate. Using this method, only organisms in a stage of their life cycle with cell sizes between 1.0 µm and

25 µm would be left on the polycarbonate filter subsequently plated on agar. This method still allows for some concentration of and contamination by yeasts, moulds and protozoa. Barclay (1994) argued this inherent competition to be positive as only the most robust strains of thraustochytrid will be capable of competing with other organisms. In fact, this type of competition would potentially lead to the isolation of strains more suitable for commercial processes.

2.3.3.4 Media manipulation

Additional approaches to increasing isolation efficiency have been taken in which basal media have been manipulated. Taoka *et al.* (2008) used pine pollen to bait suspensions of environmental samples for thraustochytrids and then streaked the pollen onto agar containing a basal media of yeast extract, polypeptone and glucose made in artificial seawater with the addition of polyoxyethylene sorbitan monooleate (Tween 80), phosphorous (KH_2PO_4), and/or tomato juice. This approach is intended to increase the growth of thraustochytrids. Tween 80 is a surfactant which may affect the permeability of cell membranes and enhance the nutritional input from the surrounding. It may also be used as a carbon source.

Phosphorous is generally an important element for growing microorganisms. A study by Bongiorni *et al*. (2002) researched the distribution of thraustochytrids in a coastal area and observed a significant relationship between phosphorous concentration in the water column and thraustochytrid densities. Tomato juice contains large amounts of glutamic acid and pectin. Fan *et al.* (2010) showed that glutamic acid and pectin might be important for signalling the chemotactic response of thraustochytrid

zoospores. Pectin also provides an additional source of carbon for thraustochytrids (Bongiorni *et al*., 2005b). The results of the study by Taoka *et al.* (2008) showed greater numbers of thraustochytrids isolated when media contained phosphorous, Tween 80 or a mixture of phosphorous, Tween 80 and tomato juice as compared to the basal media alone and with the addition of tomato juice alone.

Another approach to increase thraustochytrid isolation efficiency is to inhibit the growth of competing microorganisms. The addition of antibiotics to both resuspension and growth media to discourage the growth of bacteria is essential to isolating thraustochytrids. The eukaryotic fungi, however, are unaffected by antibiotics targeting the prokaryotes, and are the major source of contamination in isolation procedures. Taoka *et al.* (2010) explored the use of antifungal drugs in the baiting isolation procedure.

Three antifungal drugs were investigated: amphotericin B (AMPH), fluconazole and miconazole-nitrate. Firstly, the sensitivity of thraustochytrids to these drugs was tested. Thraustochytrids were capable of growing in the presence of each of the drugs, however, in the presence of AMPH thraustochytrids grew to higher numbers than in media containing no drugs. This increase in growth rate was further illustrated for thraustochytrids growing in liquid culture where cultures containing AMPH grew to higher biomass concentration than cultures without. Secondly, AMPH was added to the media used during isolation procedures. The researchers observed a significant decrease in fungi on plates containing AMPH and a greater number of thraustochytrid colonies being isolated from both leaf and water samples.

CHAPTER 3: MATERIALS AND METHODS

3.1 Sample collection regions

Ten unique locations in the Maritime Provinces were chosen for collecting samples (Figure 3.1). Two of the ten locations (Halifax Harbour and Northwest Arm) were visited on two separate occasions.

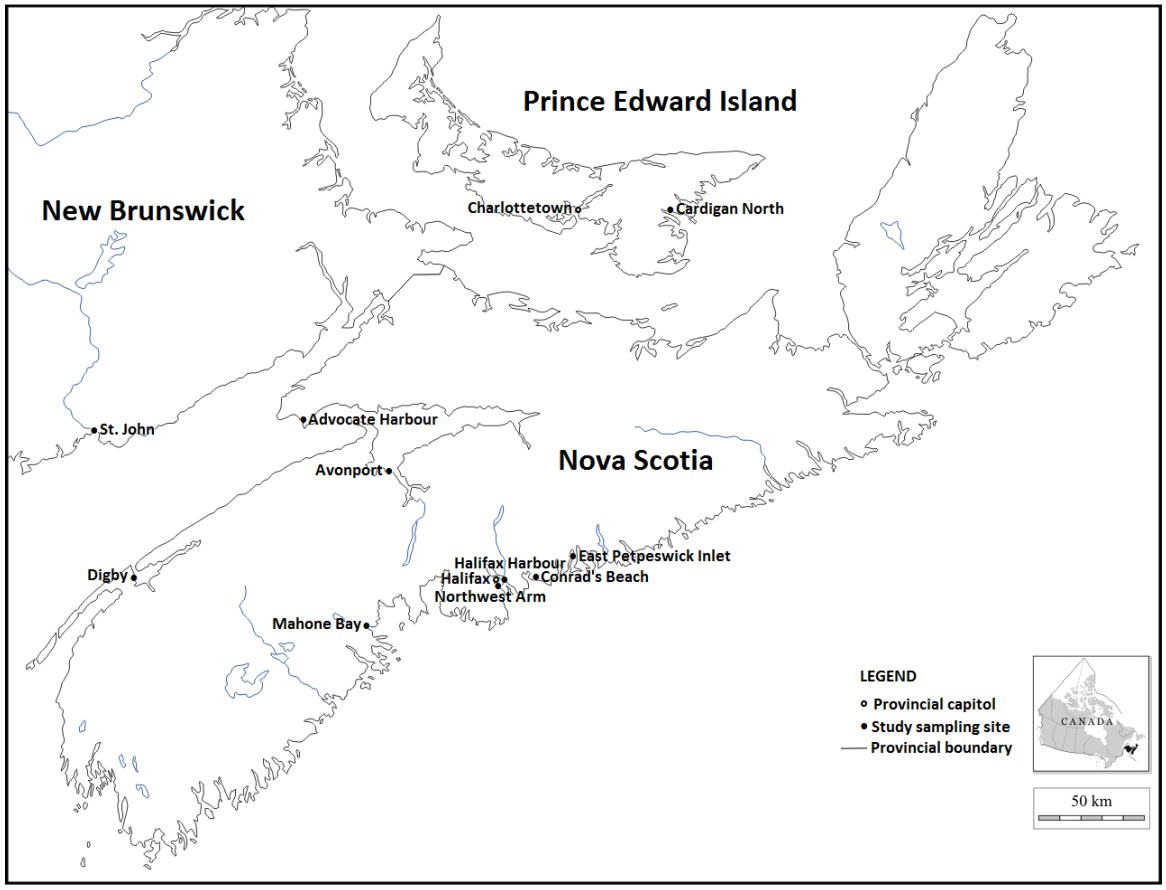

Figure 3.1: The approximate geographic location of the 10 unique collection sites in the Maritime Provinces used in this study from 2010 to 2012.

3.2 Collection procedure

A minimum of four samples were collected at each location. Samples collected were sea weeds or fallen tree leaves found in intertidal areas. Samples were collected using 70% ethanol sterilized forceps, placed into sterile 50 mL tubes and stored on ice

until transported to the laboratory where they were stored at 4°C for a maximum of 24 hrs prior to processing.

3.3 Sample processing for strain isolation

3.3.1 Washing of samples

The procedure used for washing samples was as described by Bremer (2000). Samples were washed with sterile (121˚C for 20 min) artificial sea water (ASW, 35 g L^{-1} sea salts, Sigma-Aldrich, Oakville, Ontario, Canada) containing 1% (v/v) penicillin G and streptomycin solution (PS, 10,000 units penicillin G and 10 mg streptomycin per mL in 0.9% (w/v) NaCl, Sigma-Aldrich). Individual samples were then cut aseptically to 0.5 cm^2 pieces and transferred to sterile 250 mL flasks containing 50 mL of ASW with 1% (v/v) PS. Flasks were incubated at 25°C in an orbital shaker at 250 RPM for 2 hrs. Pieces were then aseptically removed from flasks to a Petri dish containing ASW with 1% (v/v) PS. Sample pieces were further reduced in size to 2-3 mm^2 and placed in yet another Petri dish containing ASW with 1% (v/v) PS.

3.3.2 Plating of samples

Six sample fragments were placed onto nutrient agar plates containing 1 g L^{-1} peptone (P, BD, Franklin Lakes, New Jersey, USA), 1 g L^{-1} yeast extract (YE, BD), and 10 g L^{-1} agar (A, Grade A, BD) made in ASW with 1% (v/v) PS added after sterilization at 121°C for 20 min. Five plates were used per sample collected allowing 30 sample fragments to be plated for each individual sample. Each tissue sample fragment was then overlaid with a few drops of sterile ASW with 1% (v/v) PS. Bremer (2000) suggested overlaying

the entire agar surface with ASW, however, this can cause tissue pieces to lift from the agar surface. Using only a few drops to cover each tissue piece independently will keep the tissue pieces in one location and facilitate systematic microscopy of each fragment over the elongated incubation period directed at identifying thraustochytrid isolates. Plates were incubated in the dark at ambient temperature (~25°C).

3.4 Isolation procedure

Plates were analyzed by microscopy after 24 hrs of incubation and daily thereafter for up to 1 week. The microscope used was an Olympus IX70 microscope (Olympus Canada Inc., Richmond Hill, Ontario, Canada) set to brightfield illumination. A magnification of 200× was used to examine plates and the light source was angled to provide oblique lighting. The area immediately adjacent to sample fragments was most closely analyzed for thraustochytrids. When found, thraustochytrid thalli were subcultured to fresh agar plates and overlaid with sterile ASW containing 1% (v/v) PS. Subculture was done by aseptically excising individual sporangia or colonies and placing them on fresh agar. These plates were then checked for growth and contamination daily. Isolates were subcultured a minimum of three times for purity.

3.5 Isolate maintenance procedures

Isolates were maintained on agar slants (5 g L^{-1} P, 5 g L^{-1} YE, 10 g L^{-1} glucose (G, Sigma-Aldrich), 10 g L^{-1} A, made in ASW with 1% (v/v) PS added after sterilization at 121°C for 20 min overlaid with sterile ASW containing 1% (v/v) PS. Sterile ASW was added in such a way that it covered approximately half of the surface area of the slant.

Slants were subcultured monthly by aseptically transferring 1 mL of ASW containing culture to a fresh agar slant then adding ASW containing 1% (v/v) PS covering approximately half of the surface of the slant.

3.6 Decision tree for selection of thraustochytrid isolates

Figure 3.2 illustrates the decision methodology for selection of isolates moving forward to progressive phases of experimentation. Isolates meeting at least one of the criteria stipulated in the primary screen were submitted to analysis in the secondary screen to determine if productivities could be increased. Isolates showing potential for commercial scale-up were selected for further analysis. Further details regarding the screening process are described in the following sections.

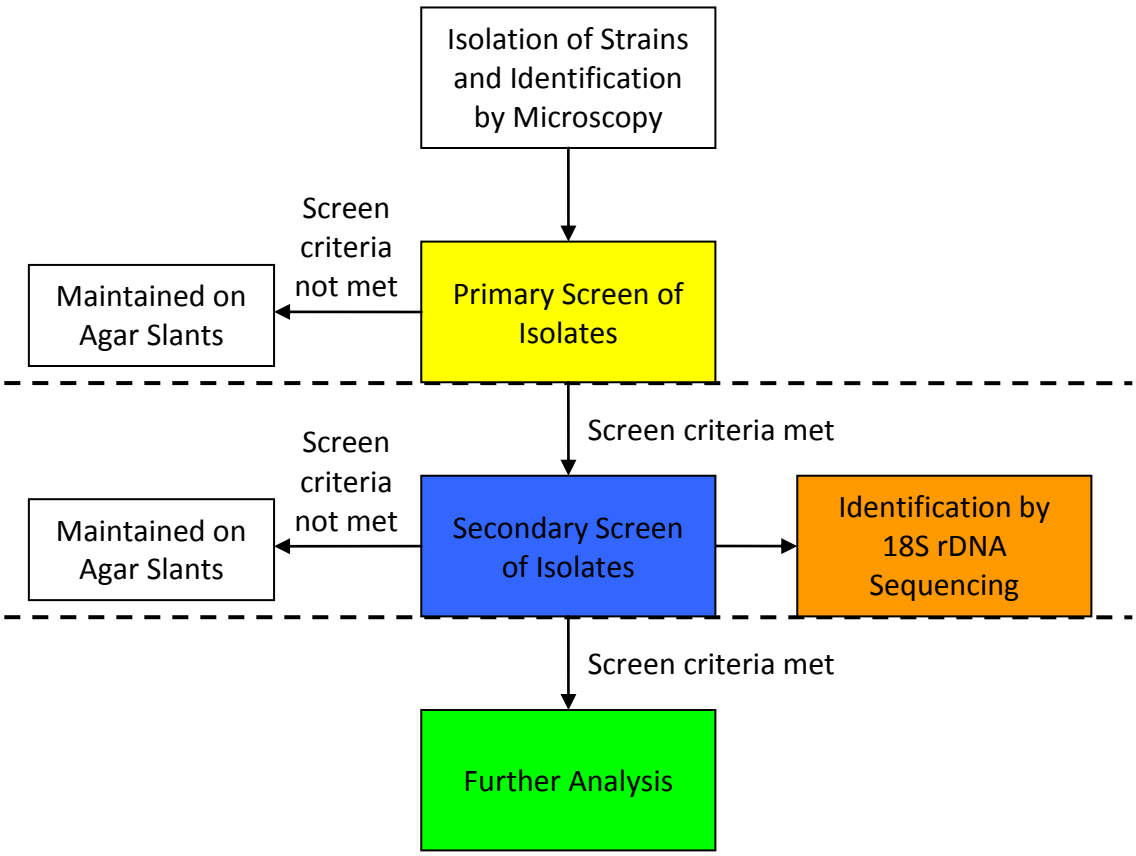

Figure 3.2: Decision tree used for selection of isolates moving to the next phase of experimentation.

3.7 Primary screening of isolates

3.7.1 Culture of isolates and harvesting of cells

Primary screening of isolates was performed as described by Burja *et al.* (2006). Media S1 was used throughout the screen and contained: 2 g L^{-1} P and 2 g L^{-1} YE in ASW sterilized at 121°C for 20 minutes followed by the addition of 5 g L^{-1} G which had been previously sterilized at 121°C for 20 min and 1% (v/v) PS. Erlenmeyer flasks (250 mL) containing 30 mL of media S1 were inoculated with 1 mL of culture from the ASW covering the agar slants. Cultures were incubated at 18°C in an orbital shaker at 100 RPM for 4 days. Five mL of this culture was then used to inoculate 95 mL of media S1 in 500 mL Erlenmeyer flasks. These flasks were then incubated for a further 4 days under the same conditions followed by harvest of cells by centrifugation at 3716 × *g* for 20 min (Sorvall Legend RT+, Thermo Scientific, Asheville, North Carolina, USA). The pellet was rinsed with distilled water and re-centrifuged. Pellets were frozen at -80°C, freeze-dried (FreeZone[18], Labconco, Kansas City, Missouri, USA) weighed, and stored at -20°C prior to fatty acid analysis.

3.7.2 Fatty acid methyl ester preparation and analysis

A direct transesterification method was used for preparation of fatty acid methyl esters (FAMEs) and was performed as modified from Lewis *et al.* (2000) by Armenta *et al.* (2009). Dry biomass was used directly in the transesterification procedure rather than the oil being extracted and subsequently transesterified. Using this method, 20–30 mg of dry biomass was weighed into a glass vial with a screw cap (Fisher Brand

Disposable Culture Tube 16x125 mm, Fisher Scientific, Ottawa, Ontario, Canada). As an internal standard, 100 µl of C19:0 (10 mg mL^{-1}) methyl ester (Nu-Chek Prep Inc., Elysian, Minnesota, USA) was weighed into each tube containing biomass. Weights were measured to 0.1 mg using an analytical balance (M-220D, Denver Instrument, Bohemia, New York, USA).

To each sample, 6 mL of transesterification solution was added (10:1:1 methanol:hydrochloric acid:chloroform (methanol and chloroform - Caledon, Georgetown, Ontario, Canada; hydrochloric acid - Fisher Scientific). Tubes were inverted three times and placed in a 90°C water bath for 2 hrs to produce FAMEs. Samples were then cooled to room temperature followed by the addition of 1 mL of reverse osmosis water to each sample and inversion of tubes 3 times. To extract the FAMEs, 3 × 2 mL aliquots of a hexane:chloroform (4:1; Caledon) mixture was added. After addition of each aliquot, tubes were inverted 3 times to mix and then the organic phase was recovered to a new glass vial with screw cap. After the third aliquot was recovered, 0.5 g of Na_2SO_4 (Sigma-Aldrich) was added to the organic layer to remove traces of water and acid. The organic layer was then transferred to a new glass vial with screw cap prior to evaporation of the solvent with argon to dryness. To protect against oxidation, 5 mL of isooctane (Caledon) containing 0.05 g L^{-1} *tert*-butylhydroquinone (Fluka Analytical, Sigma-Aldrich) was added to each sample. Also, samples were blanketed with argon for storage.

The analysis of FAMEs by gas chromatography was performed as described by Armenta *et al.* (2009). The gas chromatograph (GC) used was an Agilent 6890 equipped

with an autosampler, split injection, 4 mm internal diameter (ID) inlet sleeve with glass wool plug, and flame ionization detector (FID) (Agilent Technologies, Alpharetta, Georgia, USA). The FID was set at 275°C with an inlet temperature of 250°C. The temperature of the oven started at 190°C and increased to 240°C at a rate of 5°C min^{-1}. The column used was a FAMEWAX, 30 m × 0.32 mm ID × 0.25 µm film thickness, with a temperature range of 20-250°C (Chromatographic Specialties, Brockville, Ontario, Canada). Injections of 1 µL of sample were used with a 10:1 split. The hot-needle technique was used in which the syringe was held inside the injector for 5 s, and then the injection was quickly performed. The column was held at 190°C for 1 min after injection following by ramping as described to 240°C. The temperature was held for 1 min at 240°C for a total run-time of 12 min per sample.

3.7.3 Primary screen criteria

Seven criteria were set to determine whether isolates would pass the primary screen. Only one of the seven criteria had to be met by an isolate in order to be considered further. The seven criteria were as follows:

1. Isolate had the highest % total fatty acid (TFA) of EPA, DHA or ARA
2. Isolate had the greatest yield, mg L^{-1}, of EPA, DHA or ARA
3. Isolate had the greatest TFA yield, mg L^{-1}
4. Isolate had the greatest TFA yield, mg L^{-1}, when considering isolates with the greatest % TFA of EPA, DHA or ARA

5. Isolate had the greatest % TFA of EPA, DHA or ARA when considering isolates with the greatest yield of that fatty acid, mg L^{-1}

6. Isolate had the greatest % TFA of EPA, DHA or ARA when considering isolates with the greatest TFA yield, mg L^{-1}

7. Isolate had the greatest biomass yield, mg L^{-1}

3.8 Secondary screening procedure

3.8.1 Culture of isolates and harvesting of cells

The media used for the secondary screen (media S2) was based on those used by Scott *et al.* (2011) and Quilodran *et al.* (2010). Media S2 contained: 2 g L^{-1} YE, 4 g L^{-1} monosodium glutamate (MSG, Sigma-Aldrich) in ASW and sterilized at 121°C for 20 min followed by the addition of 30 g L^{-1} G which had been previously sterilized at 121°C for 20 min and 1% (v/v) PS. Erlenmeyer flasks (250 mL) containing 30 mL of media S2 were inoculated with 1 mL of culture from agar slants and incubated as described by Bowles *et al.* (1999) at 25°C in an orbital shaker at 150 RPM for 4 days. Culture absorbance at 600 nm (A_{600}) was measured using media S2 as a blank for each culture to ensure an equal quantity of cells were inoculated into the next stage of the fermentation. A volume equivalent to $6\times(1/A_{600})$ was used to inoculated 500 mL Erlenmeyer flasks with an appropriate volume of media S2 such that the final culture volume was 100 mL. These cultures were incubated for a further 4 days. Cells were harvested by centrifugation as described above. Pellets were frozen at -80°C, freeze-dried, weighed, and stored at -20°C prior to fatty acid analysis.

3.8.2 Fatty acid methyl ester preparation and analysis

The lipid content of the cells was extracted and derivatized to FAMEs and analyzed by gas chromatography as described in Section 3.7.2.

3.8.3 Secondary screen criteria

The secondary screening procedure was done to identify isolates from the primary screen which showed increases in their desirable attributes as a response to a more nutritionally enriched media. Strains suitable for commercial application should tolerate nutrient rich conditions and also show improved outputs when compared to growth in media which is comparatively nutrient limited. Strains showing increases in biomass, TFA production or production of EPA, DHA or ARA were considered for further evaluation.

3.9 Genetic identification of isolates

3.9.1 Culture of isolates

Based on the results from the secondary screen, isolates ONC-KTB-06, 09, 14, 15, 22, 43, 55, 56, 57, 63, 64, 65 were selected for sequencing of their 18S rRNA genes and grown up in media containing 5 g L^{-1} YE and 5 g L^{-1} P made up in ASW and sterilized at 121°C for 20 min followed by the addition of 10 g L^{-1} sterile G and 1% (v/v) PS. Isolates were cultured at 25°C in a rotary shaker at 150 RPM for 4 days.

3.9.2 Genomic DNA extraction procedure

The method used to extract genomic DNA from isolates was adapted from Tamagnini *et al.* (1997). Strains were harvested via centrifugation at 3716 × *g* for 20 min

and aspiration of the supernatant. A volume of pelleted cells approximately equivalent to 200 µL was recovered and resuspended in 0.5 mL of resuspension buffer (10 mM EDTA, Sigma-Aldrich; 50 mM Tris–HCl, Sigma-Aldrich). Acid-washed glass beads of 0.6 g (425-600 µm, Sigma-Aldrich), 25 µL 10% sodium dodecyl sulfate (Sigma-Aldrich), and 500 µL phenol:chloroform:isoamylalcohol (25:24:1, Sigma-Aldrich) was added to each sample followed by 5 repetitions of vortexing for 1 min followed by incubation on ice for 1 min. Samples were then sonicated in an ice water bath for 10 min. Following sonication, samples were centrifuged at 19,064 × *g* for 10 min at 4°C. The aqueous phase was transferred to a clean 2 mL tube followed by the addition of 500 µL chloroform (Caledon), vortexing for 5 s and then centrifugation under the same conditions for 3 min.

The aqueous phase was again transferred to a clean 2 mL tube. The DNA was precipitated by addition of 1/10 of the sample volume of 3 M sodium acetate (Sigma-Aldrich) and 2.5 volumes of ice cold, 100% ethanol (Sigma-Aldrich). Samples were mixed thoroughly and incubated at -20°C for 1 hour. Samples were centrifuged at 19,064 × *g* for 20 min at 4°C and the supernatant was discarded. To each sample 900 µL of solution MD3 (UltraClean Microbial DNA Isolation Kit, Mobio Laboratories Inc., Carlsbad, California, USA) followed by vortexing for 5 s to dissolve the DNA pellet. Seven hundred µL of each sample was loaded into spin filters and centrifuged at 16,060 × *g* for 30 s at room temperature.

The flow through was discarded and the remaining sample volumes were loaded into their respective filters and centrifuged discarding the flow through. Three hundred

µL of solution MD4 (Mobio Laboratories Inc., UltraClean Microbial DNA Isolation Kit) was added to each sample and centrifuged under the same conditions as above. Flow through was discarded and samples were centrifuged under the same conditions for 1 min. The spin filters were placed in new 2 mL tubes and the DNA was released through the addition of 50 µL of nuclease free water to the centre of the filter membrane followed by centrifugation as before for 30 s. Spin filters were discarded and the DNA stored at -20°C.

3.9.3 Polymerase chain reaction and agarose gel electrophoresis

A master mix solution was prepared containing all constituents for the polymerase chain reaction (PCR) except for DNA. These constituents and volumes required for one 50 µL reaction were as follows: 5 µL 10x OptiBuffer (Bioline, Taunton, MA, USA), 3 µL 50 mM $MgCl_2$ (Bioline), 1 µL 10 mM deoxyribonucleotide triphosphate (dNTP, Bioline), 2 µL 10 mM forward primer (5'-TACCTGGTTGATCTGCCAGTAG-3'; Giribet *et al*., 1996), 2 µl 10 mM reverse primer (5' GAATTACCGCGGCTGCTGG-3'; Giribet *et al*., 1996), 0.5 µL BioXAct DNA polymerase (Bioline), 34.5 µL nuclease free water and 2 µL genomic DNA or, in the case of the negative control, 2 µL nuclease free water. PCR conditions were as follows: (i) initialization at 94°C for 3 min, (ii) denaturation at 94°C for 45 s, (iii) annealing at 64°C for 30 s, (iv) elongation at 72°C for 2 min, steps (ii) through (iv) were repeated 30 times followed by (v) final elongation at 72°C for 10 min. PCR products were stored at 4°C.

To confirm PCR product size gel electrophoresis was performed using 1% agarose (Bioline) in Tris-acitate-EDTA (Sigma-Aldrich) buffer with 0.005% SybrSafe DNA stain (Invitrogen, Burlington, Ontario, Canada). Nine µL PCR products were loaded into

agarose gel wells with 1 µL bluejuice DNA loading dye (Invitrogen). Outside wells were loaded with HyperLadder 1 DNA ladder (Figure 3.3; Bioline). Gel was run at 70 volts for 45 min.

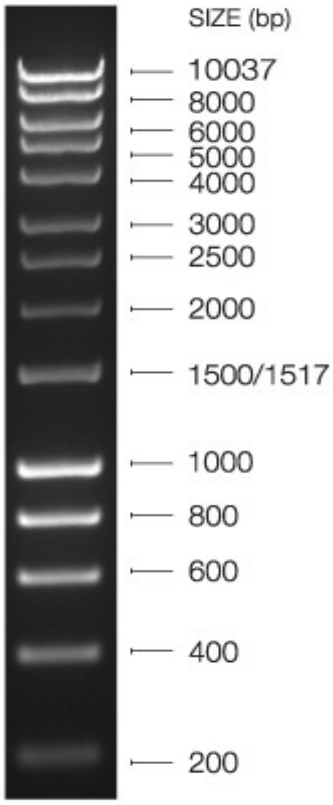

Figure 3.3: HyperLadder 1 DNA size, base pairs (bp).

3.9.4 Sequencing, identification and phylogeny

Sequencing of DNA was performed by Eurofins MWG Operon (Huntsville, Alabama, USA). The resulting sequences were aligned and compared to nucleotide sequences of similar microorganisms stored in GenBank (Benson *et al.,* 2005) using Basic Local Alignment Search Tool (BLAST). A phylogenetic tree was subsequently generated in CLC Main Workbench 6.7.1 (CLC Bio, Cambridge, Massachusetts, USA) by using the

neighbour-joining method (Saitou *et al*., 1987). Bootstrap values were obtained from 1 000 replications of neighbour-joining analyses (Felsenstein, 1985).

3.10 Further evaluation of potential commercial strains

3.10.1 Culture of isolates and harvesting of cells

The production of biomass, TFA and the proportions of ARA, EPA and DHA of isolates ONC-KTB-14 and ONC-KTB-56 were tested at larger volumes at the flask level. Fifty mL seed cultures were grown in 250 mL Erlenmeyer flasks in media S3 containing 2 g L^{-1} YE and 8 g L^{-1} MSG made up in ASW and sterilized at 121°C for 20 min followed by the addition of 60 g L^{-1} sterile G and 1% (v/v) PS. Cultures were incubated for 4 days at 25°C in an orbital shaker at 150 RPM.

In order to standardize inoculums, a haemocytometer was used to estimate the number of cells per mL of culture. Three 1 000 mL flasks were inoculated with each isolate. Flasks contained enough media to give a final volume of 200 mL (fresh medium S3 and inoculums). The media and incubation conditions used in this portion of the fermentation were the same as those for the seed flask. Samples of 1.5 mL were taken from cultures every 12 hrs from time 0 to 96 hrs. A final sample of 100 mL was also taken at 96 hrs for biomass and fatty acid analysis. Each 1.5 mL sample was analyzed for cells per mL through use of a haemocytometer, A_{600}, and for glucose consumption/concentration (g L^{-1}). The appearance of cultures both in the flask and under the microscope was recorded. Cells from the 100 mL samples were harvested by centrifugation at 3716 × *g*. The pellet was rinsed with distilled water and re-centrifuged.

Pellets were frozen at -80°C, freeze-dried, weighed, and stored at -20°C prior to fatty acid analysis.

3.10.2 Fatty acid methyl ester preparation and analysis

The lipid content of the cells was extracted and derivatized to FAMEs and analyzed by gas chromatography as described in Section 3.7.2.

CHAPTER 4: RESULTS

4.1 Strain isolation

Of the 12 isolation trips made, six were successful in recovering isolates. A total of 69 isolates were recovered from six unique locations in the Maritime Provinces (Table 4.1). Samples collected in Avonport, NS were the most abundant in thraustochytrid-like organisms with 40 isolates recovered. Images of thraustochytrid-like thalli and sori can be found in Figure 4.1. The dark portions of these micrographs represent fragments of leaf samples collected in intertidal regions.

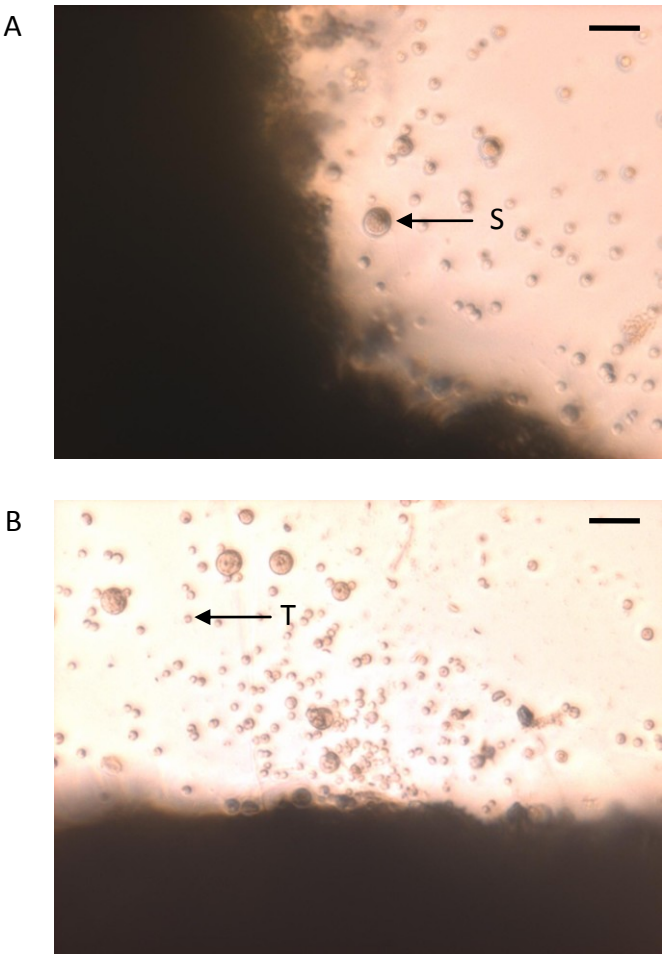

Figure 4.1: A and B show thraustochytrid-like cells growing next to fragments of leaf samples prior to isolation. Thallus (T) and sorus (S) are indicated. Scale bar: 50 µm.

Table 4.1: The six sampling locations in which isolates were recovered and the names assigned to those isolates.

Sampling Locations and Isolates Found					
Halifax Harbour, NS	Northwest Arm, NS	Avonport, NS	Cardigan North, PEI	Digby, NS	Advocate Harbour, NS
ONC-KTB-01	ONC-KTB-13	ONC-KTB-15	ONC-KTB-55	ONC-KTB-60	ONC-KTB-68
ONC-KTB-02	ONC-KTB-14	ONC-KTB-16	ONC-KTB-56	ONC-KTB-61	ONC-KTB-69
ONC-KTB-03		ONC-KTB-17	ONC-KTB-57	ONC-KTB-62	
ONC-KTB-04		ONC-KTB-18	ONC-KTB-58	ONC-KTB-63	
ONC-KTB-05		ONC-KTB-19	ONC-KTB-59	ONC-KTB-64	
ONC-KTB-06		ONC-KTB-20		ONC-KTB-65	
ONC-KTB-07		ONC-KTB-21		ONC-KTB-66	
ONC-KTB-08		ONC-KTB-22		ONC-KTB-67	
ONC-KTB-09		ONC-KTB-23			
ONC-KTB-10		ONC-KTB-24			
ONC-KTB-11		ONC-KTB-25			
ONC-KTB-12		ONC-KTB-26			
		ONC-KTB-27			
		ONC-KTB-28			
		ONC-KTB-29			
		ONC-KTB-30			
		ONC-KTB-31			
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		ONC-KTB-46			
		ONC-KTB-47			
		ONC-KTB-48			
		ONC-KTB-49			
		ONC-KTB-50			
		ONC-KTB-51			
		ONC-KTB-52			
		ONC-KTB-53			
		ONC-KTB-54			

Figure 4.2 presents a micrograph of a colony of thraustochytrid cells growing on an agar plate. Ectoplasmic nets can be seen protruding from the colony as 'vein-like' structures. Figure 4.3 shows micrographs of a thraustochytrid sporangium (A and B) releasing zoospores (C and D) while growing on a freshly inoculated agar plate. Furthermore, Figure 4.4 is a micrograph of a biflagellate zoospore released in liquid culture. The appearance of all strains was similar in regards to colony morphology and zoospores. Spore release, one of the factors determining the genera of thraustochytrids, was not observed for all isolates.

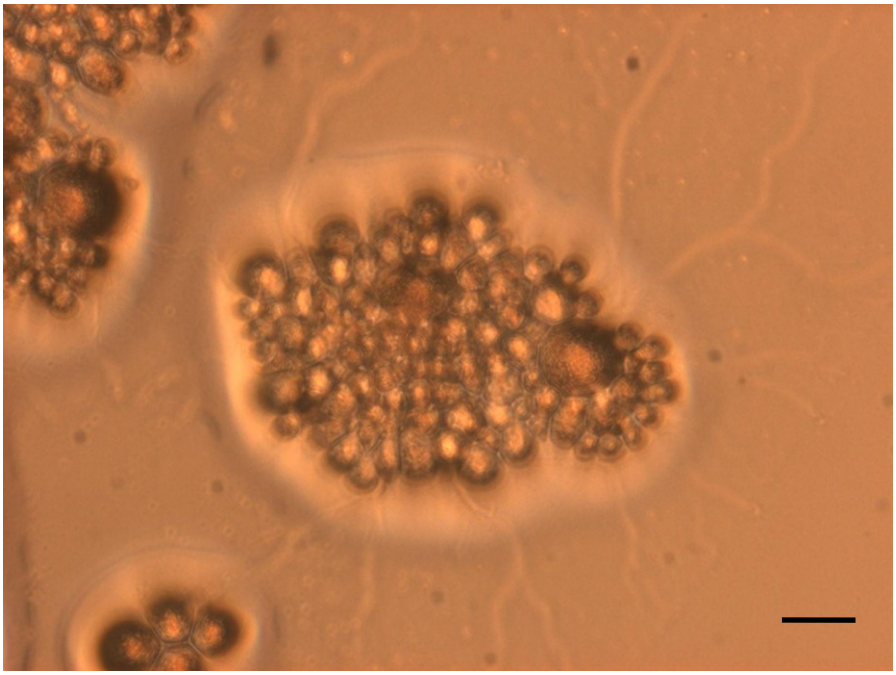

Figure 4.2: A colony typical of a thraustochytrid. Ectoplasmic nets can be viewed as 'vein-like' protrusions from cells growing in the colony. Scale bar: 50 µm.

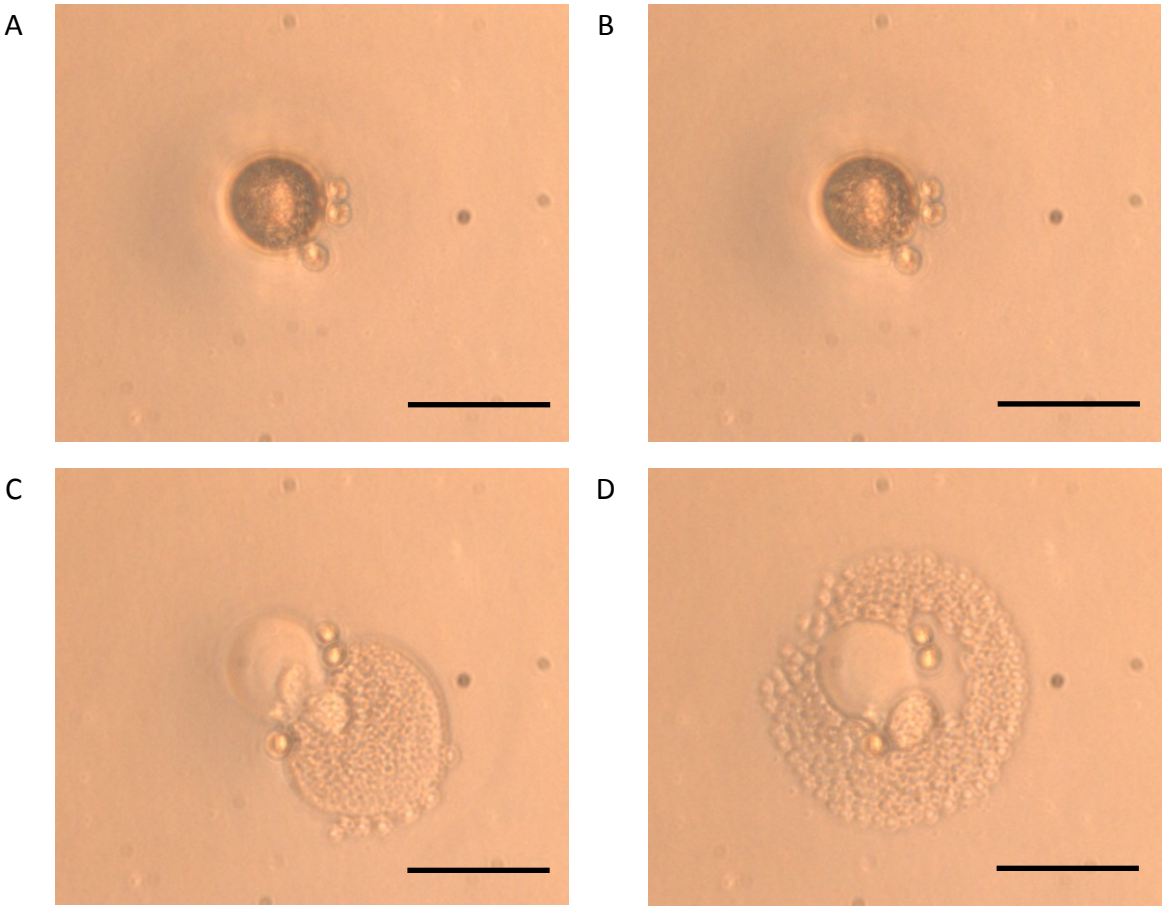

Figure 4.3: Micrographs taken with a one minute interval showing a mature sporangium (A and B) releasing zoospores (C and D) on an agar plate. Scale bar: 50 µm.

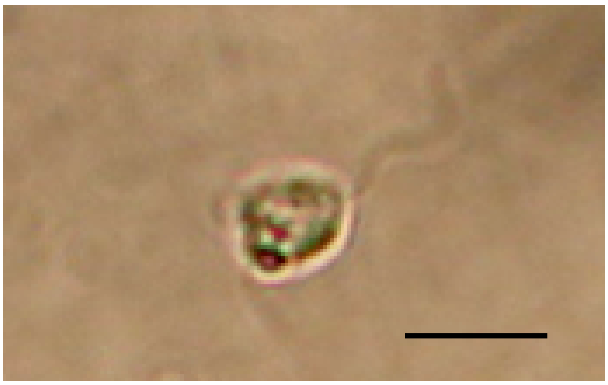

Figure 4.4: Micrograph of the biflagellate zoospore. Scale bar: 5 µm.

4.2 Primary screening of isolates

Figure 4.5 illustrates biomass and TFA yield arising from the initial fermentation for each of the isolates. Raw data for production of biomass, TFA and the specific fatty acids ARA, EPA and DHA can be found in the Appendix in Table A.1. Fatty acid data for isolate ONC-KTB-45 are not shown as this isolate did not produce a sufficient amount of biomass to allow for preparation of FAMEs. Maximum results for biomass and PUFA yields are indicated in Section 4.3, Table 4.2 where isolates passing the primary screen criteria are identified.

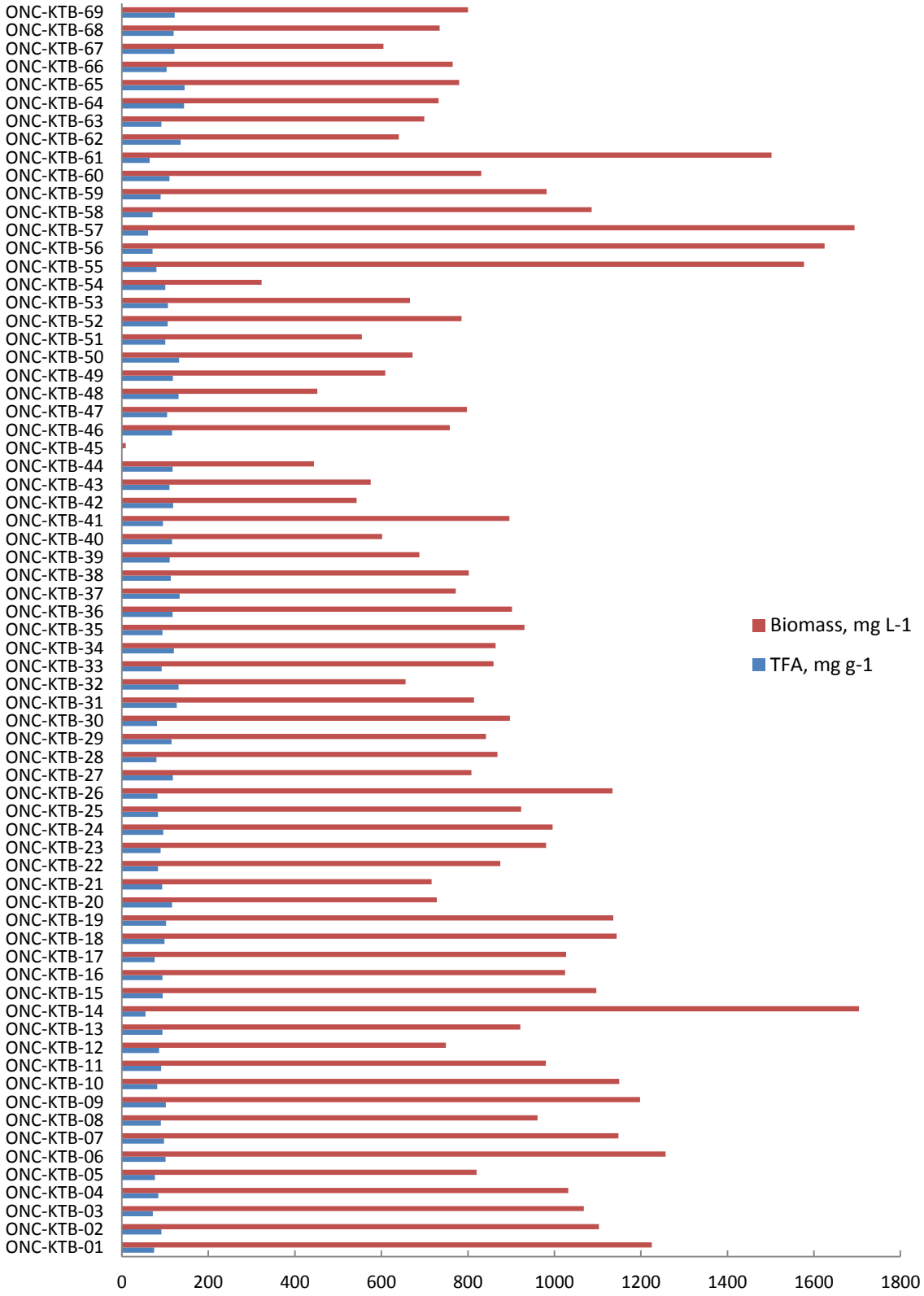

Figure 4.5: Biomass (dry weight) and total fatty acids (TFA) produced by thraustochytrid isolates after incubation in media S1 at 18°C in an orbital shaker at 100 RPM for 96 hrs.

4.3 Primary screen criteria

Table 4.2 shows the strains selected according to each of the criteria considered along with the value of that criteria. As some strains were selected for more than one criterion Table 4.3 shows a summary of the strains selected. Twelve strains were selected for further examination in the secondary screen.

Table 4.2: Summary of the results from the primary screen isolate selection, strains selected and the value of the specific criterion.

Criterion	Strain	Value
Highest ARA as % TFA	ONC-KTB-57	3.93%
Highest EPA as % TFA	ONC-KTB-43	13.20%
Highest DHA as % TFA	ONC-KTB-63	56.84%
Greatest mg L^{-1} ARA	ONC-KTB-56	4.24 mg L^{-1}
Greatest mg L^{-1} EPA	ONC-KTB-15	10.69 mg L^{-1}
Greatest mg L^{-1} DHA	ONC-KTB-65	56.89 mg L^{-1}
Greatest mg L^{-1} TFA	ONC-KTB-06	127.10 mg L^{-1}
Greatest mg L^{-1} TFA, when considering % ARA	ONC-KTB-56	114.89 mg L^{-1}
Greatest mg L^{-1} TFA, when considering % EPA	ONC-KTB-22	73.15 mg L^{-1}
Greatest mg L^{-1} TFA, when considering % DHA	ONC-KTB-63	63.88 mg L^{-1}
Greatest ARA as % TFA, when considering mg L^{-1} ARA	ONC-KTB-56	3.69%
Greatest EPA as % TFA, when considering mg L^{-1} EPA	ONC-KTB-15	10.35%
Greatest DHA as % TFA, when considering mg L^{-1} DHA	ONC-KTB-65	50.36%
Greatest ARA as % TFA, when considering mg L^{-1} TFA	ONC-KTB-55	3.30%
Greatest EPA as % TFA, when considering mg L^{-1} TFA	ONC-KTB-09	5.91%
Greatest DHA as % TFA, when considering mg L^{-1} TFA	ONC-KTB-64	48.93%
Greatest mg L^{-1} biomass	ONC-KTB-14	1 704 mg L^{-1}

Table 4.3: Thraustochytrid isolates selected from primary screen for further examination based on the criteria in Table 4.2.

	Isolates Selected for Further Examination
1	ONC-KTB-06
2	ONC-KTB-09
3	ONC-KTB-14
4	ONC-KTB-15
5	ONC-KTB-22
6	ONC-KTB-43
7	ONC-KTB-55
8	ONC-KTB-56
9	ONC-KTB-57
10	ONC-KTB-63
11	ONC-KTB-64
12	ONC-KTB-65

4.4 Secondary screening procedure

Of the 12 strains selected from the primary screen, 11 were successfully grown in the richer nutrient media (media S2) used in the secondary screen. Isolate ONC-KTB-65 did not grow in either of the duplicate cultures. Figure 4.6 shows the results for biomass and TFA obtained from the fermentation carried out as part of the secondary screen. Also shown are the results of the primary screen for comparative purposes. Figure 4.7 shows the TFA produced in the secondary screen and primary screen broken into component fatty acids.

The purpose of the secondary screening of the 12 isolates selected from the primary screen was to determine (i) the ability of the isolates to grow in a richer media and (ii) if their growth and lipid production would be improved in such a media. Only four strains were found to have significant increases in biomass yield: ONC-KTB-22, 43, 63, 64 (Table 4.4). Two strains, ONC-KTB-55 and ONC-KTB-56 produced biomass results

which did not significantly (p<0.05) differ from those found in the primary screen. Likewise, four strains produced significantly more TFA in the secondary screen: ONC-KTB-14, 55, 56, 57. There was no strain that showed significant improvements in both biomass and TFA yield, however, strains ONC-KTB-55 and ONC-KTB-56 showed significant increases in TFA yield without a corresponding significant decrease in biomass yield.

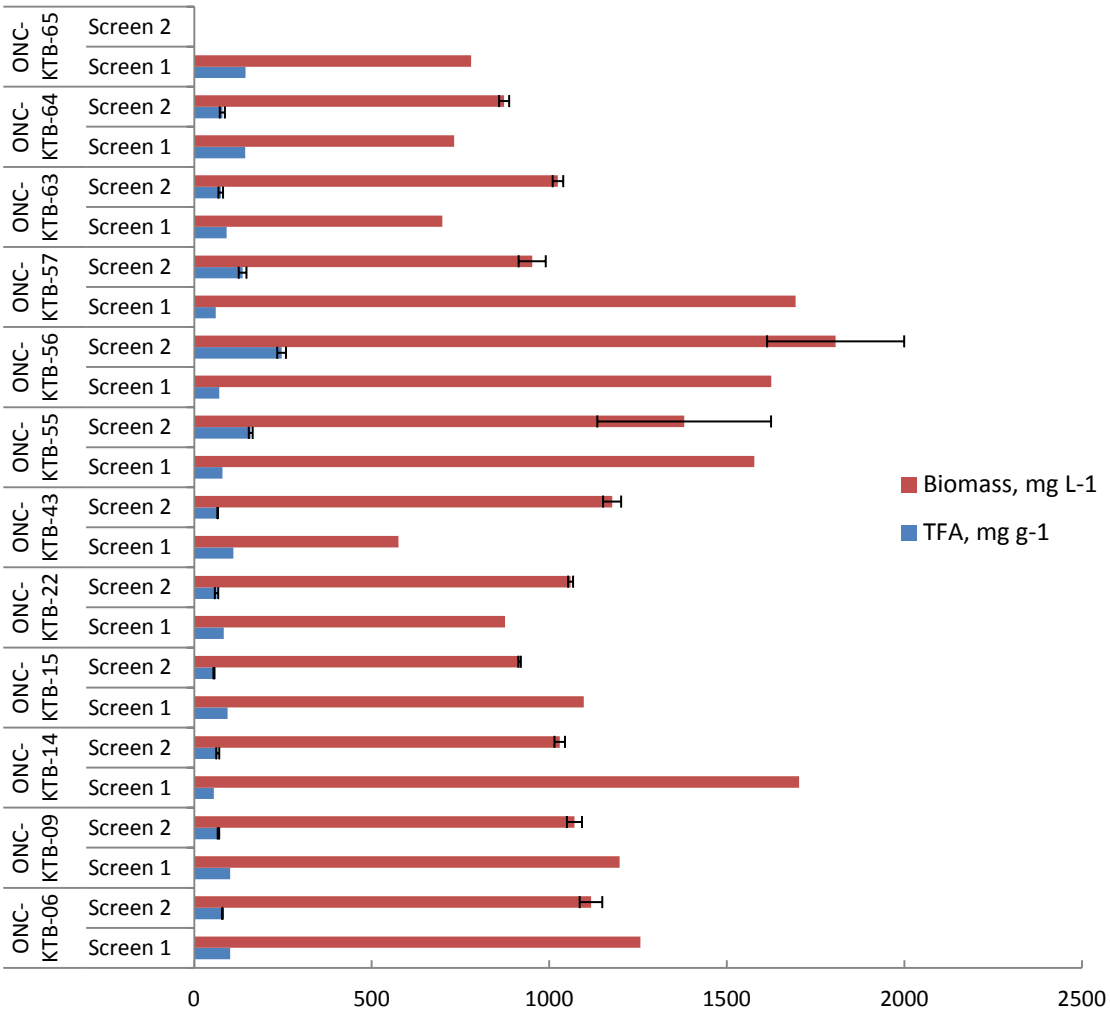

Figure 4.6: Biomass (dry weight) and total fatty acids (TFA) produced in the secondary fermentation carried out in media S2 at 25°C in an orbital shaker at 150 RPM for 96 hrs by thraustochytrid isolates selected from the primary screen. Primary screen results from growth in media S1 are shown for comparison. Screen 2 (n=2).

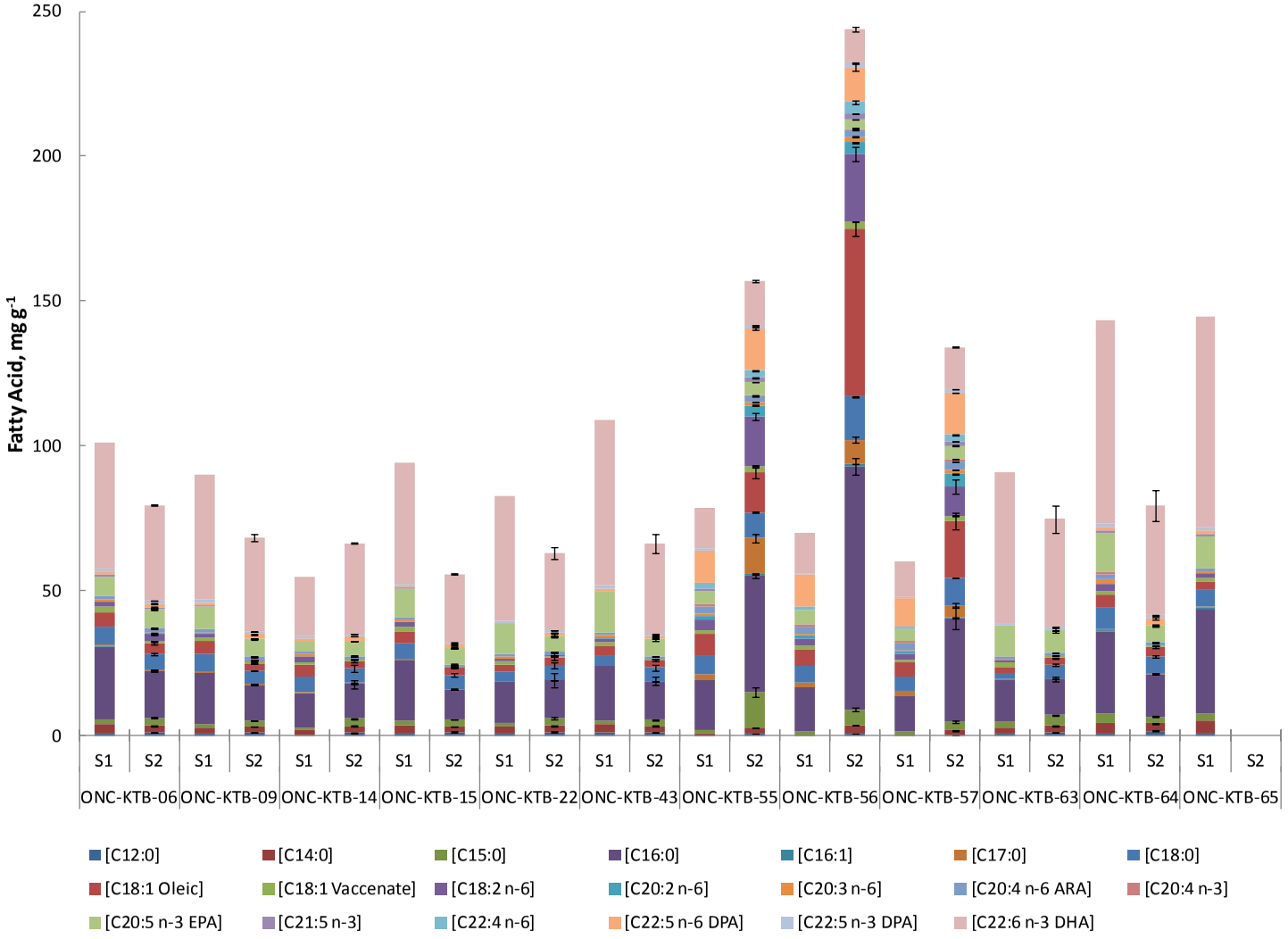

Figure 4.7: Comparison of total fatty acids (mg g^{-1}) of isolates grown in the primary screen (S1) and secondary screen (S2) broken into specific components. Screen 2 (n=2).

Table 4.4: Comparison of primary screen (S1) and secondary screen (S2) data. Green boxes indicate a significant increase in the result of S2 as compared to S1.

Isolate	Biomass, mg L^{-1}		TFA mg g^{-1}		ARA, % TFA		EPA, % TFA		DHA, % TFA		ARA, mg L^{-1}		EPA, mg L^{-1}		DHA, mg L^{-1}		TFA, mg L^{-1}	
	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2
ONC-KTB-06	1257	1117	101.11	79.58	1.36	2.02	6.31	8.35	43.05	41.36	1.73	1.80	8.02	7.43	54.72	36.79	127.10	88.93
ONC-KTB-09	1198	1071	101.76	68.41	0.98	1.68	5.91	8.91	45.47	47.35	1.19	1.23	7.20	6.52	55.43	34.69	121.91	73.26
ONC-KTB-14	1704	1030	54.94	66.50	1.44	1.54	6.88	7.77	37.55	47.05	1.35	1.06	6.44	5.32	35.16	32.21	93.62	68.46
ONC-KTB-15	1097	917	94.16	55.83	1.01	1.41	10.35	10.09	44.30	42.53	1.04	0.72	10.69	5.16	45.76	21.76	103.29	51.17
ONC-KTB-22	875	1060	83.60	63.09	1.13	1.39	12.71	8.91	51.47	42.37	0.82	0.93	9.30	5.96	37.65	28.33	73.15	66.87
ONC-KTB-43	575	1177	110.27	66.26	0.77	0.99	13.20	9.18	51.77	46.96	0.49	0.77	8.37	7.16	32.83	36.62	63.41	77.99
ONC-KTB-55	1577	1380	79.86	159.34	3.30	1.21	5.45	2.95	17.53	9.74	4.16	2.66	6.87	6.49	22.07	21.42	125.94	219.90
ONC-KTB-56	1625	1807	70.70	246.27	3.69	0.89	6.56	1.22	19.59	4.72	4.24	3.94	7.54	5.42	22.50	21.02	114.89	444.89
ONC-KTB-57	1694	952	60.76	136.39	3.93	2.13	6.61	3.56	20.23	10.76	4.05	2.76	6.81	4.62	20.82	13.97	102.92	129.84
ONC-KTB-63	699	1024	91.39	74.90	1.11	0.86	11.70	10.86	56.84	49.84	0.71	0.66	7.47	8.34	36.31	38.24	63.88	76.73
ONC-KTB-64	732	873	143.55	79.40	1.16	1.52	9.53	7.46	48.92	47.60	1.22	1.05	10.01	5.17	51.41	32.99	105.08	69.32

4.5 Genetic identification and phylogeny of isolates

An image of the agarose gel used to confirm the amplification of DNA and the size of the DNA amplified can be found in Figure 4.8. Lanes 1 and 14 contain standard DNA from HyperLadder 1 (see Figure 3.3 for DNA sizes) for reference. Lanes 2 through 11 contain amplified DNA from isolates ONC-KTB-06, 09, 14, 15, 43, 55, 56, 57, 63, and 64; respectively. Isolates ONC-KTB-22 and 65 did not grow and, therefore, DNA was not extracted. Lane 12 shows a negative control and Lane 13 a positive control of amplified DNA from *Thraustochytrium* sp. ONC-T18 (Burja *et al.,* 2006). The amplified PCR product of interest is between HyperLadder 1 standard DNA bands of 400 and 600 base pairs (bp) with an approximately size of 540 bp.

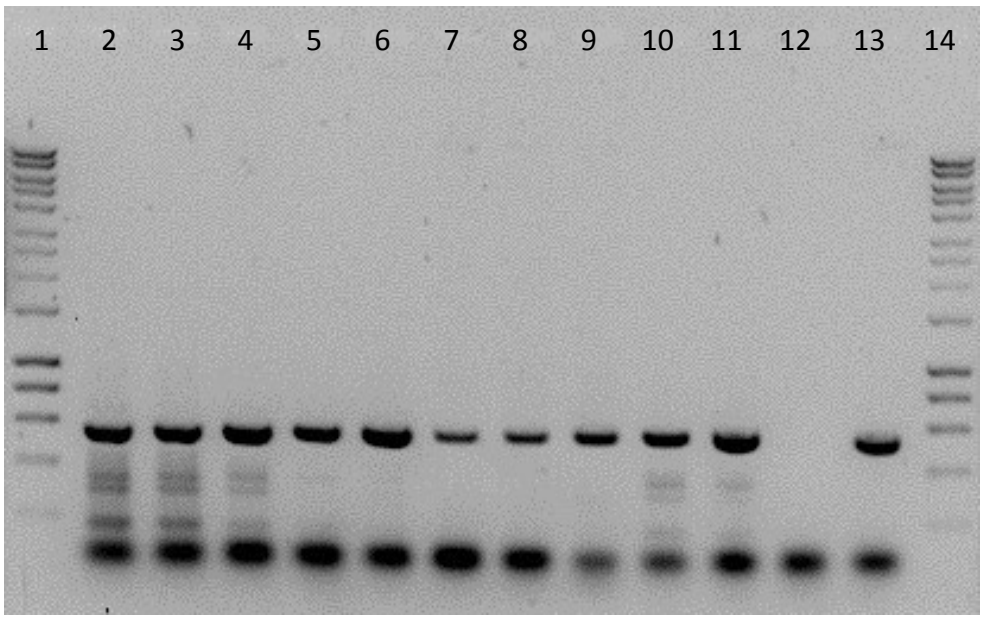

Figure 4.8: Agarose gel image lanes 1 and 14 show the DNA ladder HyperLadder 1. Lanes 2 through 11 contain DNA amplified from isolates ONC-KTB-06, 09, 14, 15, 23, 55, 56, 57, 63, and 64; respectively. Lane 12 contains a negative control and Lane 13 contains positive control DNA from strain *Thraustochytrium* sp. ONC-T18 (Burja *et al.,* 2006).

A phylogenetic tree including isolates ONC-KTB-06, 09, 14, 15, 43, 55, 56, 57, 63, and 64 and other members of the Family *Thraustochytriacea* as well as *Crypthecondinium cohnii* ATCC 30336 can be found in Figure 4.9. Genera of the Family *Thraustochytriacea* included in the tree are: *Parietichytrium, Botryochytrium*, *Ulkenia*, *Thraustochytrium*, *Schizochytrium, Japanochytrium, Aurantiochytrium,* and *Sicyoidochytrium.*

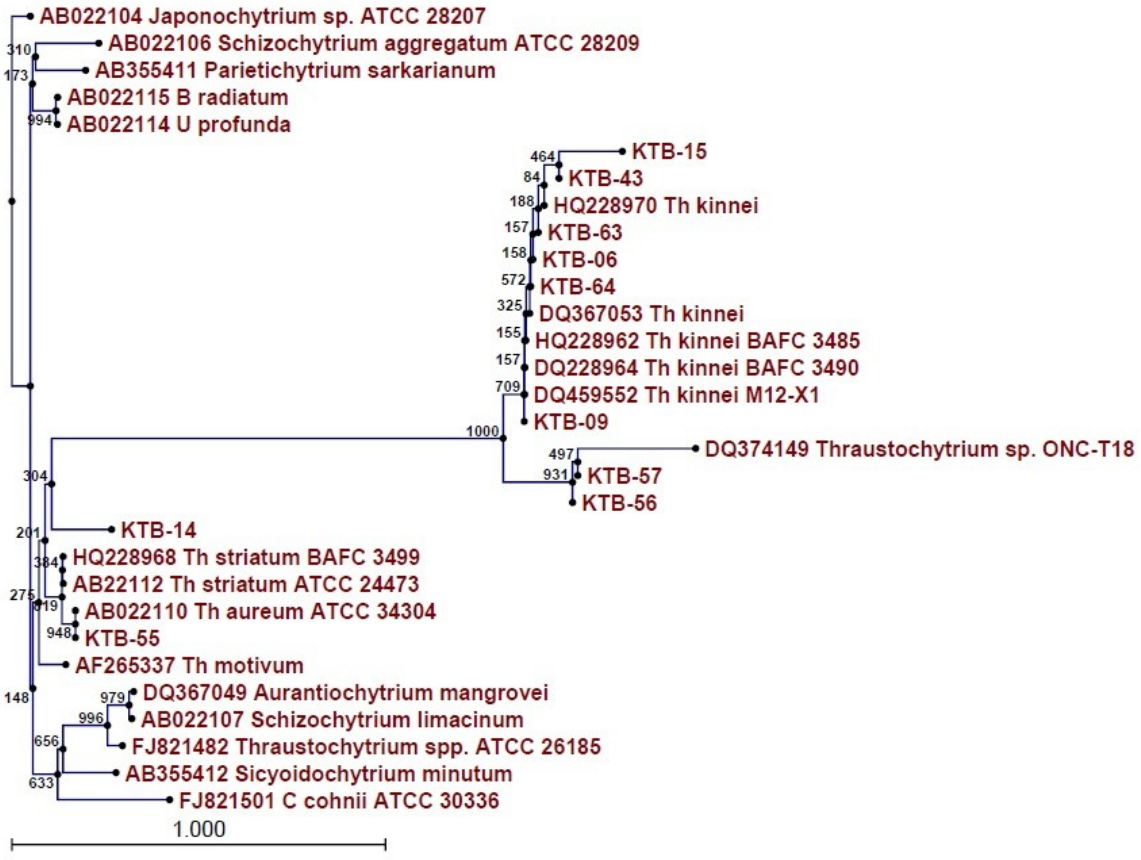

Figure 4.9: Phylogenetic tree (nearest neighbour) showing genetic diversity between the isolates from this study and members of the Phylum *Labyrinthulomycetes* based on 18S rDNA sequences. The genera included are: *Parietichytrium, Botryochytrium* (B), *Ulkenia* (U), *Thraustochytrium* (Th), *Schizochytrium, Japanochytrium, Aurantiochytrium,* and *Sicyoidochytrium*. The dinoflagellate *Crypthecodinium chonii* ATCC 30336 is also included.

4.6 Further evaluation of potential commercial strains

Results of the further evaluation of isolates ONC-KTB-14 and ONC-KTB-56 can be found in Figures 4.10 and 4.11. Figure 4.10 illustrates the biomass and TFA results for these isolates in each of the three levels of evaluation. Figure 4.11 illustrates the TFA produced by ONC-KTB-14 and ONC-KTB-56 in each of the three levels of evaluation broken into component fatty acids. Table 4.5 shows relevant data related to biomass, TFA and ARA, EPA and DHA yields as found in each of the three levels of evaluation for these two isolates. Boxes highlighted in green show results which increased significantly in the third screen as compared to the secondary screen.

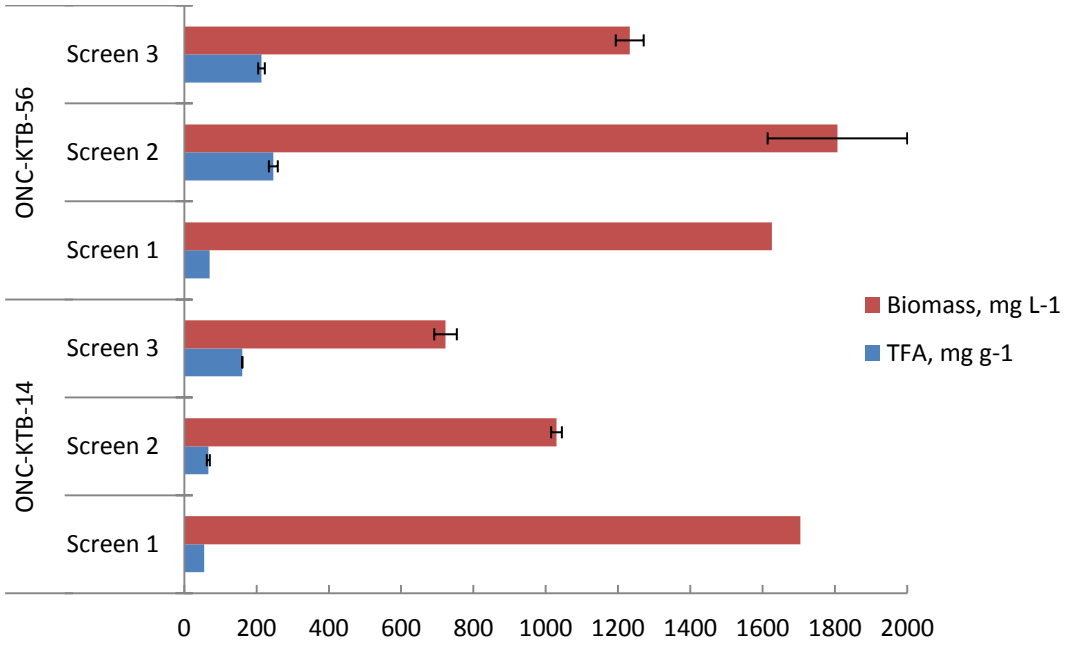

Figure 4.10: Biomass (dry weight) and total fatty acids (TFA) produced in the third screen of ONC-KTB-14 and ONC-KTB-56 as carried out in S3 media at 25°C in an orbital shaker at 150 RPM for 96 hrs. Primary and secondary screen results are shown for comparison. Screen 2 (n=2), Screen 3 (n=3).

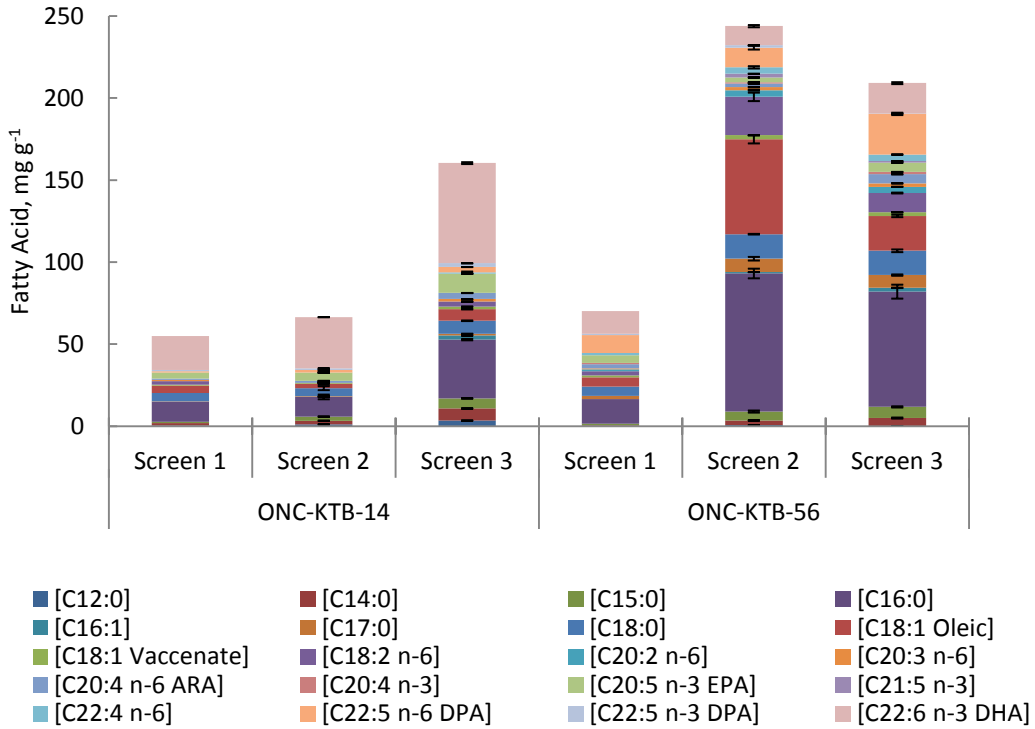

Figure 4.11: Comparison of total fatty acids (mg g^{-1}) of ONC-KTB-14 and ONC-KTB-56 grown in each of the three screens broken into specific components. Screen 2 (n=2) and Screen 3 (n=3).

Table 4.5: Comparison of secondary screen data and data from the further evaluation of isolates ONC-KTB-14 and ONC-KTB-56. Green boxes indicate a significant increase in S3 as compared to S2.

Isolate	Biomass, mg L^{-1}		TFA mg g^{-1}		ARA, % TFA		EPA, % TFA		DHA, % TFA		ARA, mg L^{-1}		EPA, mg L^{-1}		DHA, mg L^{-1}		TFA, mg L^{-1}	
	S2	S3	S2	S3	S2	S3	S2	S3	S2	S3	S2	S3	S2	S3	S2	S3	S2	S3
ONC-KTB-14	1030	723	66.50	160.38	1.54	2.32	7.77	7.31	47.05	38.06	1.06	2.69	5.32	8.47	32.21	44.11	68.46	115.90
ONC-KTB-56	1807	1232	246.27	213.61	0.89	2.59	1.22	2.66	4.72	8.71	3.94	6.82	5.42	6.99	21.02	22.91	444.89	263.17

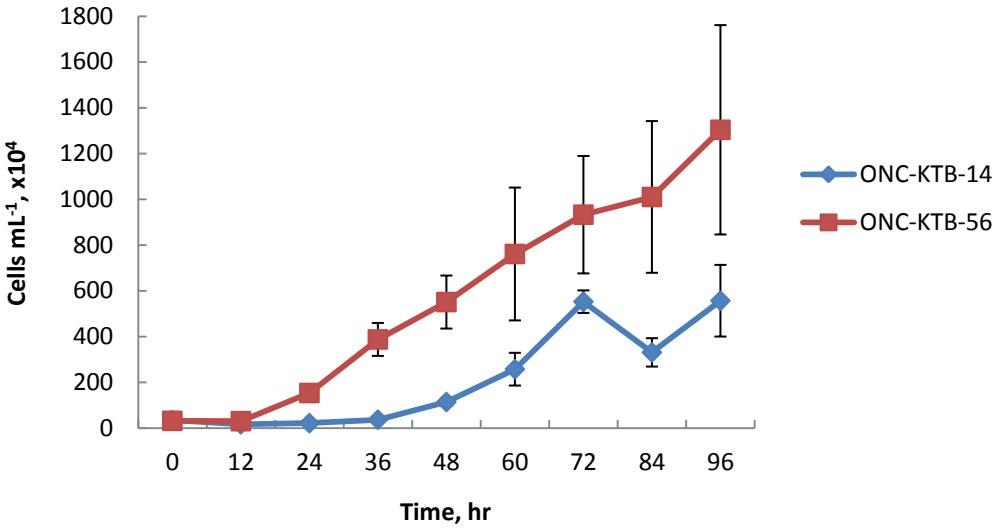

Figure 4.12: Number of cells per mL of culture ($\times 10^4$) for ONC-KTB-14 and ONC-KTB-56 as carried out in media S3 at 25°C in an orbital shaker at 150 RPM for 96 hrs (n=3).

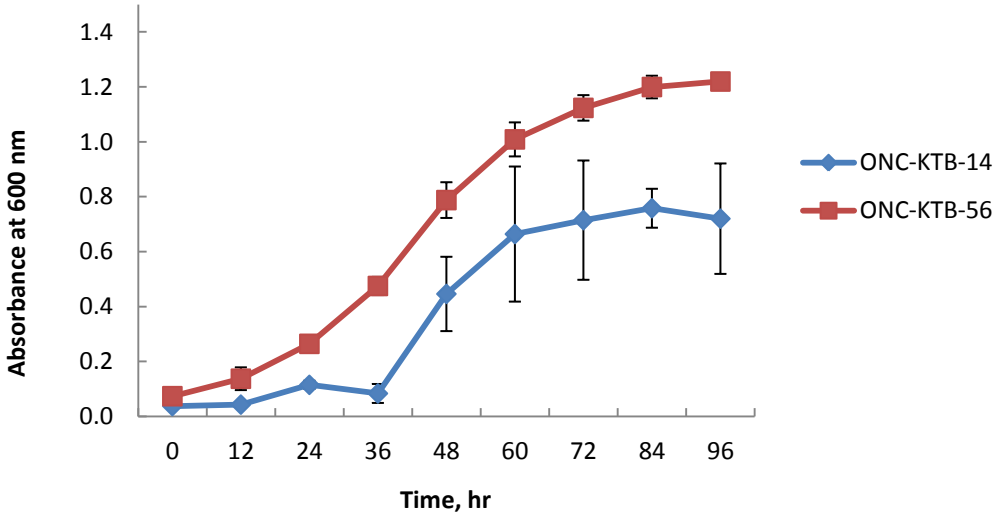

Figure 4.13: Absorbance of cultures at 600 nm for ONC-KTB-14 and ONC-KTB-56 as carried out in media S3 at 25°C in an orbital shaker at 150 RPM for 96 hrs (n=3).

Figures 4.12 and 4.13 above show the cells per mL of culture and Absorbance at 600 nm at 12 hr intervals of isolates ONC-KTB-14 and ONC-KTB-56 growing in media S3 over 96 hrs. Triplicate samples produced large standard deviations for ONC-KTB-56 (Figure 4.13) and ONC-KTB-14 (Figure 4.13). This is due to cells forming tightly bound

clumps in the media, which resulted in inconsistent absorbance readings. ONC-KTB-14 produced larger clumps than ONC-KTB-56. These clumps were large enough prevent them from entering the haemocytometer chamber resulting in smaller standard deviations (Figure 4.12), however, this may also have caused an underestimation of the numbers. The smaller clumps produced by ONC-KTB-56 were large enough to create inconsistent counting using a haemocytometer, however, small enough to remain well dispersed in media while testing absorbance (Figure 4.13). Both cultures reached a seemingly maximum population density during the 96 hrs incubation period

CHAPTER 5: DISCUSSION

5.1 Primary screening of isolates

The primary screen of isolates was performed by the method described by Burja *et al.* (2006). Their interest was largely in thraustochytrids capable of producing DHA. This study can be used as a comparison at the primary screen level because the same media and growth conditions were used. The strain they selected for further examination after screening, *Thraustochytrium* sp. ONC-T18, was shown capable of producing 2 300 mg L^{-1} dry biomass and 321.14 mg g^{-1} TFA or about 32% (w/w) fat in biomass. In the present study the single most comparable strain following the primary screen was ONC-KTB-55 with 1 577 mg L^{-1} dry biomass and 79.86 mg g^{-1} TFA or 7.99% (w/w) fat in biomass. The biomass numbers show some comparability, however, the TFA values differ greatly.

When looking at TFA yield, ONC-T18 produced 738 mg L^{-1} while ONC-KTB-55 produced only 126 mg L^{-1}. In this study, the greatest TFA yield was by ONC-KTB-06 with 127.10 mg L^{-1}. This value is based on biomass of 1 257 mg L^{-1} and 101.11 mg g^{-1} TFA. Additionally, ONC-T18 produced approximately 35% of its TFA as DHA. In the present study ONC-KTB-63 produced the greatest DHA as % TFA in the primary screen with 56.84% (w/w), an exceptionally high percentage, however, the biomass and TFA produced by this isolate were only 699 mg L^{-1} and 91.39 mg g^{-1}; respectively.

In a study by Huang *et al.* (2001), more than 300 thraustochytrids were isolated and screened for PUFA production. Data was reported for 13 strains. Maximum results for ARA, EPA and DHA as %TFA (w/w) were 8.7, 23.4 and 59.7; respectively. In the

present study, the comparable maximum results as presented by different isolates were 3.93, 13.20 and 56.84; respectively. The DHA result obtained in their study and the present one were similar, however, the ARA and EPA results of this study were approximately half that of Huang *et al*. (2001). It should be noted, however, that Huang *et al.* (2001) did not present biomass data for these 13 isolates, so the actual yields of ARA, EPA, DHA and TFA could not be determined. Yields (mg L^{-1}) are extremely important when considering a strain for commercial purposes. Furthermore, different media and growth conditions were used in the two studies. Huang *et al.* (2001) using a much richer growth media, which may have contributed to their results.

In another isolation study by Bowles *et al*. (1999), 57 thraustochytrid isolates were obtained and screened for biomass and DHA production. Their screening results found strains capable of producing 3 140 mg L^{-1} dry biomass, 37.3% (w/w) TFA in this biomass and a content of 7.8% (w/w) DHA in the TFA pool. The present study found strains capable of producing just over half that amount of biomass (1,704 mg L^{-1}), less than half the amount of TFA (12.6% (w/w)), but markedly more DHA in TFA with 56.84% (w/w). Like in the study by Huang *et al.* (2001), the Bowles study did not indicate the yields of the strains. In this study similar growth media were used with differences only in sea salt concentration; however, different conditions (25˚C and 150 RPM for 72 hrs) were used which may affect the comparability of their results to the results of this study.

5.2 Secondary screening of isolates

It is possible that biomass is produced at the expense of fatty acids and vice versa depending on the growth conditions. If conditions are deemed unsuited for biomass accumulation cells may accumulate fatty acids as a coping system as they await more favourable conditions. Likewise, when conditions are favourable, cells may replicate rapidly, however, little lipid is produced in the process**.** When considering the situation in which TFA decreases, it may coincide with a corresponding increase in specific fatty acids. Lipids present as membrane components and not as storage lipids may begin to constitute a greater percentage of TFA. This is illustrated in Table 4.4 where, in many cases, decreases in the absolute amount of TFA (mg g^{-1}) led to increases in the %TFA of ARA, EPA and/or DHA (ONC-KTB-09 & 06). Likewise when mg g^{-1} TFA increased, corresponding decreases were found in the percentage of ARA, EPA and/or DHA in the total amount of TFA (ONC-KTB-55, 56 & 57).

Two isolates were chosen based on secondary screen data for further analysis. The first strain was ONC-KTB-14 which showed an increase in TFA content (mg g^{-1}) and in relative proportions of ARA, EPA and DHA in TFA. However, these increases came at a cost of decreased biomass production. This decrease in biomass may be a contributor toward the apparent increase in TFA, however, it cannot explain the increases in % ARA, EPA and DHA within TFA, which must have proportionally increased within the fatty acid profile. ONC-KTB-14 was also the greatest biomass producer in the primary screen (Table 4.2). Considering the potential for biomass production and the increases in TFA,

and the relevant PUFA components, this strain showed potential for improvement and therefore was chosen for further analysis.

The second strain chosen for further analysis was ONC-KTB-56. Although, the proportion of ARA, EPA and DHA in the TFA produced decreased between the primary and secondary screens, this strain could not be dismissed based on its large TFA production and high biomass output (Table 4.4).

Although these two strains were the only ones chosen for further evaluation in this study, there are others which show potential for PUFA production. ONC-KTB-63 decreased in mg g^{-1} TFA but increased in biomass and had the highest DHA and EPA yields (mg L^{-1}) in the secondary screen showing increases in both as compared to the primary screen. ONC-KTB-43 decreased in mg g^{-1} TFA but increased in biomass (mg L^{-1}) and correspondingly increased in ARA, EPA and TFA yield (mg L^{-1}).

5.3 Genetic identification and phylogeny

Thraustochytrid strains isolated in this study and subsequently chosen for genetic analysis could be grouped into four monophyletic clades (Figure 4.9). The first group contained isolates ONC-KTB-06, 09, 15, 43, 63, and 64 along with five strains of *Thraustochytrium kinnei*. The second monophyletic clade consisted of isolates ONC-KTB-56 and 57 along with *Thraustochytrium* sp. ONC-T18; a strain isolated by ONC which showed high productivity of DHA (Burja *et al.,* 2006). The third group contained isolate ONC-KTB-14 alone. The final group contained ONC-KTB-55 and *Thraustochytrium aureum* ATCC 34304 with 91.8% similarity.

5.4 Further evaluation of potential commercial strains

Production of ectoplasmic nets resulted in large standard deviations when measuring growth as cells per mL (Figure 4.12) by using a haemocytometer or absorbance measurements at 600 nm (Figure 4.13). When ectoplasmic nets are produced during growth in liquid cultures cell clumps can be observed, which can negatively affect growth performance (Barclay *et al*., 2005). Production of ectoplasmic nets requires energy which may otherwise be used in cell replication, therefore, cultures producing more ectoplasmic nets generally produce lower amounts of biomass. It is possible to reduce the extent to which ectoplasmic nets are produced by media optimization. This was achieved by Barclay *et al.* (2005) when they noted a reduction in ectoplasmic net formation and increased growth of a species of *Schizochytrium* when grown in medium containing sodium sulfate.

ONC-KTB-14 decreased in biomass production between secondary screen and the third experiment as well as between the primary and secondary screens (Figure 4.10). This decrease has led to a proportional increase in TFA mg g^{-1}. It is likely that this isolate is sensitive to either an increase in nutrient content or perhaps specifically to an increase in glucose concentration, which is hindering its growth. In an evaluation of *Schizochytrium* sp. SR21 by Yokochi *et al.* (1998), it was shown that biomass and TFA production was increased to glucose concentrations of 9.0 g L^{-1}, however, beyond this concentration rising glucose concentrations appeared to exert an inhibitory effect. A similar effect was found by Burja *et al.* (2006), however, their *Thraustochytrium* sp. ONC-T18 strain showed increases in both biomass and TFA up to glucose concentrations of 40

g L^{-1}. At a concentration of 100 g L^{-1} glucose, however, there was a decline in biomass production but an increase in TFA (mg g^{1}), similar to the effect observed with ONC-KTB-14.

ONC-KTB-56 also showed a decrease in biomass yield between the secondary screen and the further evaluation (Figure 4.11), however, the further evaluation experiment produced results significantly better both in terms of biomass yield and TFA mg g^{-1} than the primary screen. It is likely that nutrient concentration is again playing a role in inhibiting this isolate.

Table 5.1 shows a comparison between the best results obtained for isolates ONC-KTB-14 (primary screen) and ONC-KTB-56 (secondary screen) and some representative results for other thraustochytrid strains found in the literature. It should also be noted that these representative results were achieved by strains grown in optimized growth conditions. Of particular interest are the results of *Schizochytrium limacinum* SR21 and *Schizochytrium* sp. 20888 which are currently used in commercial production of oil rich in DHA (Barclay *et al*., 1994; Yokochi *et al*., 2003). Both ONC-KTB-14 and ONC-KTB-56 yielded lower biomass results as compared to these strains, however, ONC-KTB-56 does appear to produce a competitive %TFA. The DHA yield (g L^{-1}) for both of these isolates was also very low, particularly when compared to *Schizochytrium limacinum* SR21. ONC-KTB-56 produced low levels of DHA as compared to the representative strains. To that point ONC-KTB-56 also had very low yields of ARA and EPA (Table 4.5).

Table 5.1: Comparison of the biomass, lipid and DHA yields in different thraustochytrids and of ARA and EPA in *Mortierella alpina* strains and the thraustochytrids isolated in the present study. Where data is not shown results were not reported in the study.

Organism	Biomass, g L^{-1}	%TFA	DHA, %TFA	DHA, g L^{-1}	ARA, %TFA	ARA, g L^{-1}	EPA, %TFA	EPA, g L^{-1}	Reference
Thraustochytrium aureum ATCC 34304	5.7	8.0	40.0	0.50	-	-	-	-	Iiada *et al.,* 1996
Thraustochytrium aureum ATCC 34304	4.9	20.3	51.0	0.50	-	-	-	-	Bajpai *et al*., 1991
Thraustochytrium sp. ONC-T18	26.0	81.7	21.0	4.47	-	-	-	-	Burja *et al.,* 2006
Thraustochytrium sp. 20892	6.1	15.2	53.1	0.70	-	-	-	-	Singh *et al.,* 1996b
Thraustochytrium sp. G13	7.5	7.3	18.7	0.16	-	-	-	-	Bowles *et al.,* 1999
Schizochytrium limacinum SR21	38.0	50.0	43.1	4.20	-	-	-	-	Yokochi *et al.,* 1998
Schizochytrium sp. ATCC 20888	20	33	32	-	-	-	-	-	Barclay *et al.*, 1994
Mortierella alpina ATCC 32221	41.4	-	-	-	65.1	9.11	-	-	Totani *et al.,* 1992
Mortierella alpina 2O-17	1.35	58.5	-	-	-	-	7.1	0.057	Yazawa *et al.*, 1989
ONC-KTB-14	1.7	5.5	37.6	0.04	1.44	0.001	6.88	0.006	Present study
ONC-KTB-56	1.8	24.6	4.7	0.02	0.89	0.004	1.22	0.005	Present study

The fatty acid constituting the largest proportion of ONC-KTB-56's fatty acid profile was palmitic acid (C16:0), comprising about 30% of the TFA in the secondary screen (Figure 4.9). Medium chain fatty acids including C16:0 can be valuable for production of biofuels (Carmo *et al.,* 2009). This could be another potential applicationof ONC-KTB-56. DHA as % TFA produced by ONC-KTB-14 was similar to the reference data. Optimizing conditions for ONC-KTB-14 to produce greater biomass may potentially enable this

strain to produce DHA in amounts (g L^{-1}) competitive with other commercial DHA strains. There is also potential through optimization for production of EPA with ONC-KTB-14 as it has been shown to produce about 7% of its TFA as EPA (Table 4.4).

Optimization of culture media and conditions can have a significant result on biomass and fatty acid yields. In the initial screening of ONC-T18 by Burja *et al.* (2006), biomass yield was 2.3 g L^{-1} including 32.1% (w/w) TFA. By optimization of carbon concentration (glucose), nitrogen concentration and sources and sea salt concentration as well as growth conditions in a bioreactor, they were able to increase these yields to 26 g L^{-1} biomass including 81.7% (w/w) TFA; and this is only one example. Growth conditions for many thraustochytrids have been optimized with many different nitrogen and carbon sources, salinities, temperatures and pH values being found optimal for different species. Bajpai *et al.* (1991) found *Thraustochytrium aureum* ATCC 34304 to grow best in media containing linseed oil as a carbon source and yeast extract as nitrogen source. Alternatively, Singh *et al.* (1996b) found glucose and glutamic acid to be optimal carbon and nitrogen sources, respectively, for *Thraustochytrium* sp. ATCC 20892, while in a study involving *Thraustochytrium roseum* ATCC 28210 starch was found to be optimal as a carbon source, while a mix of ammonium sulfate, glutamic acid and yeast extract were found optimal as nitrogen source (Singh *et al.,* 1996a).

In a further experiment with *Thraustochytrium roseum* ATCC 28210, medium pH was optimized to 6.0 and temperature was optimized to 25°C (Li *et al*., 1994). Other thraustochytrid species including one which was optimized for biomass and fatty acid production by Perveen *et al*. (2006), grew optimally at a pH of 8.0 and a temperature of

28°C, as well as sea salt concentration of 25% that of natural seawater. Sea water concentration and temperature were also optimized by Zhu *et al.* (2007) for *Schizochytrium limacinum* SR21, where they found a salinity corresponding to 50% of natural sea water and a temperature of 23°C to be optimal for growth and fatty acid production.

An additional factor to optimize is percent dissolved oxygen. Chi *et al.* (2008) suggested a two phase fermentation in which oxygen was shifted from 50% to near 0% at 48 hrs for *Schizochytrium limacinum* SR21. In this method, much of the non-fat biomass is accumulated during fermentation at the higher dissolved oxygen, while in oxygen limited conditions, fatty acids are produced to a greater extent. This technique is also included in patents by Martek Biosciences Corp. for their commercial production strain *Schizochytrium limacinum* ATCC 20888.

ONC-KTB-56, which was originally selected for its yield (g L^{-1}) of ARA (Table 4.2), was unable to retain this level of output. Comparing the secondary screen to the primary screen, the yield of ARA declined when TFA production (mg g^{-1}) increased. When looking at the data from further experiments, the yield of ARA increased, however, this coincided with a decline in biomass and TFA production. This increase in apparent ARA yield appeared to be a result of an increased proportion of ARA in the TFA. Again, this is likely due to membrane lipids becoming proportionally more important as production of storage lipids declines. The results of this study for ARA production by the two strains chosen for further analysis, and of a strain of *Mortierella alpina* ATCC 32221, can be found summarized in Table 5.1. *Mortierella alpina* is a

filamentous fungus known to produce large amounts of ARA, and is grown commercially for this purpose (Totani *et al.,* 1992). ARA yield (g L^{-1}) of ONC-KTB-14 and ONC-KTB-56 were found to be well below that of *Mortierella alpina* ATCC 32221.

Mortierella alpina has also been shown to produce EPA in the concentration indicated in Table 5.1. Comparing the results of ONC-KTB-14 and ONC-KTB-56 to that of *Mortierella alpina* 2O-17 revealed low values for %EPA within TFA and absolute EPA yields (mg L^{-1}). It should be noted that there is no current non-recombinant microbial source of EPA as no environmental isolates have been found to produce it at a level feasible for commercial production (Ratledge, 2004). Some marine bacteria such as SCRC-2738, a new species close in identity to *Shewanella putrefaciens*, have been shown to produce EPA (Yazawa, *et al.,* 1992). However, as with *Mortierella alpina* 2O-17, the yield (g L^{-1}) is low and not suitable for an industrial application.

CHAPTER 6: CONCLUSIONS

Thraustochytrid isolates ONC-KTB-14 and ONC-KTB-56 show potential for production of PUFAs at a commercial scale. ONC-KTB-14 produces a large proportion of its TFA as DHA (37.6%) which is competitive among the commercial strains *Schizochytrium limacinum* SR21 and *Schizochytrium* sp. ATCC 20888 (Table 5.1). Through optimization of growth parameters it is possible that biomass and TFA yields (g L^{-1}) for this isolate can be improved. Isolate ONC-KTB-56 produces a large proportion of its biomass as fatty acids (24.6%). This yield is competitive among the commercial strains of thraustochytrids (Table 5.1). Through optimization of media and culture conditions biomass, fatty acid and specific PUFA yields could be increased. This strain may also be useful in the application of biofuels as much of its fatty acid profile (30%, Figure 4.11) is comprised of palmitic acid (C16:0) which is a medium chain fatty acid important to biofuel production from lipids (Carmo *et al.,* 2009).

Future work should be carried out in two areas. Firstly, more isolation trips should be planned to isolate more novel thraustochytrids with different fatty acid profiles and higher biomass and fatty acid productivities. The second area of future research should be to optimize the growth/fermentation conditions to obtain higher yields for the strains isolated in this experimental work.

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Table A.1: Raw data of isolates from the primary screen after incubation in media S1 at 18°C in an orbital shaker at 100 RPM for 96 hrs.

Isolate	Biomass, mg L^{-1}	EPA, mg g^{-1}	DHA, mg g^{-1}	ARA, mg g^{-1}	TFA, mg g^{-1}	EPA, % TFA	DHA, % TFA	ARA, % TFA	EPA Yield, mg L^{-1}	DHA Yield, mg L^{-1}	ARA Yield, mg L^{-1}	TFA Yield, mg L^{-1}
ONC-KTB-01	1225	7.53	38.11	0.90	74.49	10.10	51.16	1.21	9.22	46.68	1.10	91.25
ONC-KTB-02	1103	5.92	38.23	1.35	91.17	6.50	41.93	1.48	6.53	42.17	1.49	100.56
ONC-KTB-03	1068	6.63	35.27	0.93	71.73	9.25	49.18	1.30	7.08	37.67	0.99	76.61
ONC-KTB-04	1032	6.99	40.07	1.03	83.98	8.33	47.71	1.22	7.22	41.35	1.06	86.67
ONC-KTB-05	820	6.97	33.72	2.09	76.44	9.11	44.11	2.74	5.71	27.65	1.72	62.68
ONC-KTB-06	1257	6.38	43.53	1.38	101.11	6.31	43.05	1.36	8.02	54.72	1.73	127.10
ONC-KTB-07	1148	7.25	42.61	1.16	97.54	7.44	43.69	1.19	8.33	48.92	1.33	111.97
ONC-KTB-08	961	8.00	42.88	1.06	89.96	8.89	47.66	1.18	7.69	41.20	1.02	86.45
ONC-KTB-09	1198	6.01	46.27	1.00	101.76	5.91	45.47	0.98	7.20	55.43	1.19	121.91
ONC-KTB-10	1150	8.05	40.83	0.89	81.92	9.83	49.84	1.09	9.26	46.96	1.03	94.21
ONC-KTB-11	980	6.52	44.98	0.80	90.68	7.19	49.61	0.89	6.39	44.09	0.79	88.86
ONC-KTB-12	749	5.61	43.81	0.72	85.87	6.53	51.02	0.84	4.20	32.82	0.54	64.32
ONC-KTB-13	921	5.80	40.90	1.00	93.69	6.19	43.66	1.07	5.34	37.67	0.92	86.29
ONC-KTB-14	1704	3.78	20.63	0.79	54.94	6.88	37.55	1.44	6.44	35.16	1.35	93.62
ONC-KTB-15	1097	9.75	41.71	0.95	94.16	10.35	44.30	1.01	10.69	45.76	1.04	103.29
ONC-KTB-16	1025	8.92	46.28	0.89	93.75	9.51	49.37	0.95	9.14	47.44	0.91	96.10
ONC-KTB-17	1027	8.10	38.82	1.12	75.48	10.74	51.43	1.48	8.32	39.87	1.15	77.52
ONC-KTB-18	1144	7.28	48.45	1.14	98.52	7.39	49.18	1.16	8.33	55.42	1.30	112.71
ONC-KTB-19	1136	7.58	49.65	1.27	102.45	7.39	48.46	1.24	8.61	56.40	1.44	116.38
ONC-KTB-20	728	12.69	54.58	1.35	116.14	10.92	46.99	1.16	9.23	39.73	0.98	84.55
ONC-KTB-21	716	9.80	43.42	1.12	92.94	10.54	46.72	1.20	7.01	31.09	0.80	66.54
ONC-KTB-22	875	10.62	43.02	0.94	83.60	12.71	51.47	1.13	9.30	37.65	0.82	73.15
ONC-KTB-23	981	7.82	48.42	1.40	89.61	8.72	54.03	1.56	7.67	47.50	1.37	87.91

Isolate	Biomass, mg L^{-1}	EPA, mg g^{-1}	DHA, mg g^{-1}	ARA, mg g^{-1}	TFA, mg g^{-1}	EPA, % TFA	DHA, % TFA	ARA, % TFA	EPA Yield, mg L^{-1}	DHA Yield, mg L^{-1}	ARA Yield, mg L^{-1}	TFA Yield, mg L^{-1}
ONC-KTB-24	996	9.30	47.47	1.01	95.78	9.71	49.56	1.05	9.26	47.28	1.00	95.39
ONC-KTB-25	923	8.76	45.42	0.86	83.86	10.44	54.16	1.02	8.08	41.92	0.79	77.40
ONC-KTB-26	1134	7.88	42.29	0.91	82.68	9.52	51.15	1.10	8.93	47.96	1.03	93.76
ONC-KTB-27	808	10.29	49.91	1.34	117.91	8.73	42.33	1.14	8.32	40.32	1.08	95.27
ONC-KTB-28	868	10.11	41.65	1.09	79.93	12.65	52.10	1.36	8.78	36.15	0.95	69.38
ONC-KTB-29	842	10.93	58.50	1.72	115.16	9.49	50.80	1.50	9.20	49.26	1.45	96.96
ONC-KTB-30	897	9.85	41.92	0.98	80.96	12.16	51.78	1.22	8.83	37.60	0.88	72.62
ONC-KTB-31	814	11.37	63.38	1.57	126.77	8.97	50.00	1.24	9.25	51.59	1.27	103.19
ONC-KTB-32	656	14.00	59.61	1.15	131.22	10.67	45.43	0.88	9.18	39.11	0.76	86.08
ONC-KTB-33	859	10.04	43.54	1.03	91.93	10.93	47.37	1.12	8.63	37.40	0.88	78.97
ONC-KTB-34	864	10.87	59.44	1.56	120.46	9.02	49.35	1.29	9.39	51.36	1.35	104.07
ONC-KTB-35	931	9.45	44.66	1.16	94.09	10.05	47.47	1.23	8.80	41.58	1.08	87.59
ONC-KTB-36	902	9.74	56.95	1.39	117.12	8.32	48.63	1.18	8.79	51.37	1.25	105.64
ONC-KTB-37	772	10.93	65.38	1.81	133.31	8.20	49.04	1.36	8.44	50.47	1.40	102.92
ONC-KTB-38	802	9.79	55.94	1.16	113.14	8.66	49.45	1.03	7.86	44.87	0.93	90.74
ONC-KTB-39	688	12.25	55.13	1.08	110.65	11.07	49.82	0.98	8.43	37.93	0.75	76.13
ONC-KTB-40	602	13.56	57.41	1.52	116.08	11.68	49.46	1.31	8.16	34.56	0.91	69.88
ONC-KTB-41	896	9.99	49.81	1.05	95.19	10.50	52.32	1.10	8.95	44.63	0.94	85.29
ONC-KTB-42	543	14.91	52.24	1.21	118.25	12.61	44.18	1.03	8.10	28.37	0.66	64.21
ONC-KTB-43	575	14.55	57.09	0.85	110.27	13.20	51.77	0.77	8.37	32.83	0.49	63.41
ONC-KTB-44	444	9.63	54.89	1.67	117.52	8.19	46.71	1.42	4.28	24.37	0.74	52.18
ONC-KTB-45	9	-	-	-	-	-	-	-	-	-	-	-
ONC-KTB-46	758	10.04	59.34	1.65	116.32	8.63	51.02	1.42	7.61	44.98	1.25	88.17
ONC-KTB-47	798	10.70	52.15	1.65	104.53	10.24	49.89	1.58	8.54	41.61	1.32	83.41
ONC-KTB-48	452	10.78	65.64	1.80	131.25	8.21	50.01	1.37	4.87	29.67	0.81	59.33
ONC-KTB-49	609	13.05	58.56	1.81	118.11	11.05	49.58	1.53	7.95	35.67	1.10	71.93
ONC-KTB-50	672	10.92	65.27	2.19	132.47	8.24	49.27	1.65	7.34	43.86	1.47	89.02
ONC-KTB-51	555	12.25	47.07	1.18	100.24	12.22	46.96	1.17	6.80	26.13	0.65	55.63
ONC-KTB-52	785	11.14	55.57	1.71	106.05	10.50	52.40	1.61	8.74	43.63	1.34	83.25
ONC-KTB-53	666	12.36	54.46	1.00	106.49	11.61	51.14	0.94	8.23	36.27	0.67	70.92

Isolate	Biomass, mg L^{-1}	EPA, mg g^{-1}	DHA, mg g^{-1}	ARA, mg g^{-1}	TFA, mg g^{-1}	EPA, % TFA	DHA, % TFA	ARA, % TFA	EPA Yield, mg L^{-1}	DHA Yield, mg L^{-1}	ARA Yield, mg L^{-1}	TFA Yield, mg L^{-1}
ONC-KTB-54	323	12.71	44.49	1.84	100.52	12.65	44.26	1.83	4.11	14.37	0.60	32.47
ONC-KTB-55	1577	4.36	14.00	2.64	79.86	5.45	17.53	3.30	6.87	22.07	4.16	125.94
ONC-KTB-56	1625	4.64	13.85	2.61	70.70	6.56	19.59	3.69	7.54	22.50	4.24	114.89
ONC-KTB-57	1694	4.02	12.29	2.39	60.76	6.61	20.23	3.93	6.81	20.82	4.05	102.92
ONC-KTB-58	1086	5.96	36.49	0.83	70.76	8.42	51.56	1.17	6.47	39.62	0.90	76.85
ONC-KTB-59	982	6.30	49.09	1.01	89.82	7.02	54.65	1.13	6.19	48.20	0.99	88.20
ONC-KTB-60	831	10.46	58.35	1.31	110.10	9.50	53.00	1.19	8.69	48.49	1.09	91.49
ONC-KTB-61	1502	5.98	35.10	0.89	64.42	9.28	54.48	1.38	8.98	52.72	1.34	96.77
ONC-KTB-62	640	13.37	65.68	1.83	136.14	9.82	48.24	1.34	8.56	42.03	1.17	87.13
ONC-KTB-63	699	10.69	51.94	1.01	91.39	11.70	56.84	1.11	7.47	36.31	0.71	63.88
ONC-KTB-64	732	13.68	70.23	1.67	143.55	9.53	48.92	1.16	10.01	51.41	1.22	105.08
ONC-KTB-65	780	10.76	72.94	1.42	144.83	7.43	50.36	0.98	8.39	56.89	1.11	112.97
ONC-KTB-66	765	10.13	48.75	1.02	103.42	9.80	47.14	0.98	7.75	37.30	0.78	79.12
ONC-KTB-67	605	12.17	63.71	1.49	121.45	10.02	52.46	1.23	7.37	38.55	0.90	73.48
ONC-KTB-68	735	10.59	55.34	1.43	119.72	8.84	46.23	1.20	7.78	40.68	1.05	87.99
ONC-KTB-69	800	10.62	54.95	1.53	122.09	8.70	45.01	1.25	8.49	43.96	1.22	97.67