Omega 3 Fatty Acids: Identification of Novel Fungal and Chromistal Sources

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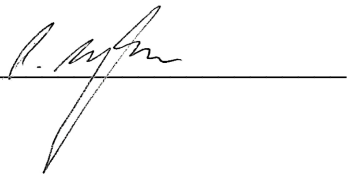
This thesis is submitted for the degree of Doctor of Philosophy at

Royal Holloway, University of London, September 2011

Declaration of Authorship

I, Richard Broughton, hereby declare that this thesis and the work presented in it is entirely my own. Where I have consulted the work of others, this is always clearly stated.

Signed: 

Date: 08/09/2011

Abstract

There is a wealth of scientific evidence associating the dietary intake of omega 3 long-chain polyunsaturated fatty acids with beneficial health properties. In this study, alternative natural sources of these polyunsaturated fatty acids are sought from novel low temperature isolated fungi. Over 100 low temperature isolated fungi were screened for very long chain polyunsaturated fatty acids (VLCPUFAs), such as $C_{20:5}$ n3 and $C_{22:6}$ n3. Of those screened, only ten fungi were capable of VLCPUFA production, with *Mortierella* the predominant VLCPUFA producing species. Four Oomycete species were also capable of VLCPUFA production. It is thought that only basal fungal lineages, such as species from the Chytridiomycota and Zygomycota, are capable of VLCPUFA production. It was also found that VLCPUFAs are not essential for growth at low temperatures, as *Penicillium rugulosum*, capable of producing fatty acids no longer or more unsaturated than $C_{18:3}$ n3, demonstrated over 2 g of biomass per 100 ml of broth when grown at 5°C. This indicates that trienoic fatty acids are sufficient for maintaining membrane fluidity, although other factors may play a role in *P. rugulosum*'s low temperature growth. Comparatively, VLCPUFA producing *Mortierella* species produced 200-250 mg of biomass, whereas the majority of non-VLCPUFA producing isolates produced 106-115 mg of biomass per 100 ml of broth. The total lipid unsaturation indices of nine isolates grown under three temperature regimes showed that the lowest growth temperature, 5°C, produced the highest unsaturation index value in six of the organisms. 15°C produced the highest unsaturation index value in two of the isolates. This suggests that temperature has an effect on fungal lipid composition, and that lower temperatures may increase lipid unsaturation levels. It was also found that the Δ6 elongase, initially identified from *Mortierella alpina*, is indicative for VLCPUFA producing fungi. The genomic conserved sequence found within Δ6 elongases was used to develop primer sets that could be used with a PCR based methodology to screen fungal isolates for VLCPUFA production. The method successfully identified VLCPUFA producing *Mortierella* and *Allomyces* species, and was not found to amplify non-Δ6 elongases. Finally, recombinant *Phaffia rhodozyma* strains were developed using the Δ5 desaturase and Δ6 elongase from *Mortierella alpina*. The fatty acid profiles of the recombinant strains displayed novel fatty acids such as $C_{20:2}$ n6 and $C_{20:3}$ n3, and putatively, $C_{18:2}$ Δ5, 9 and $C_{18:3}$ Δ5, 9, 12 which correlated with the inserted genes.

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Abbreviations

αLA: Alpha linolenic acid

ACP: Acyl carrier protein

AMDIS: Automated mass spectral deconvolution and identification system

ARA: Arachidonic acid

BAS: British Antarctic Survey

bp: Base pair

CABI: Centre for Agriculture and Bioscience International

CBS: Centre for biological sequence analysis

CoA: Coenzyme A

Da: Dalton

DγLA: Dihomo-γ linolenic acid

DHA: Docosahexaenoic acid

dH_20: Distilled water

DNA: Deoxyribonucleic acid

dNTP: Deoxyribonucleotide triphosphate

DPA: Docosapentaenoic acid

DMSO: (Dimethylsulfoxide)

EI: Extracted ion

ELO: Elongase

EMBL-EBI: European Molecular Biology Laboratory- European Bioinformatics Institute

EPA: Eicosapentaenoic acid

ER: Endoplasmic reticulum

FA(s): Fatty acid(s)

FAME: Fatty acid methyl ester

FAS: Fatty acid synthase

FAE: Fatty acid elongation

Fig: Figure

FTIR: Fourier transform infrared spectroscopy

γLA: γ linolenic acid

g: Gravity

GC-FID: Gas chromatogram-flame ionisation detector

GC-MS: Gas chromatogram-mass spectrometry

GLELO: γ linolenic elongase

HDL: High-density lipoprotein

IPTG: Isopropyl-β-D-thiogalactopyranoside

IS: Internal standard

LB: Luria-Bertani

LCPUFA: Long chain polyunsaturated fatty acid

LDL: Low-density lipoprotein

LPLAT: acyl-CoA:lysophospholipid acyltransferase

MA: Malt agar

MAELO: Mortierella alpina elongase

MALCE1: Mortierella alpina long chain elongase 1

OD: Optical density

ORF: Open reading frame

PAGE: Polyacrylamide gel electrophoresis

PC: Phosphatidylcholine

PCA: Principal component analysis

PCR: Polymerase chain reaction

PD: Potato dextrose

PDA: Potato dextrose agar

PE: Phosphatidylethanolamine

PI: Phosphatidylinositol

PLS-DA: Partial least square-discriminant analysis

PS: Phosphatidylserine

PUFA: Polyunsaturated fatty acid

Rf: Retardation factor

RI: Retention indices

RNA: Ribonucleic acid

rpm: revolutions per minute

RRT: Relative retention time

SMART: Simple modular architecture research tool

STA: Stearidonic acid

TAE: Tris acetate EDTA

TAG: Triacylglycerol

TEMED: N, N, N', N'-tetramethylenediamine

TFA: Total fatty acids

TIC: Total ion count

TMHMM: Trans-membrane prediction using hidden Markov models

Tris: Tris-(hydroxymethyl)-aminomethane

UI: Unsaturation index

v/v: Volume by volume

VLCPUFA: Very long chain polyunsaturated fatty acid

WGS: Whole genome shotgun sequence

w/v: Weight by volume

YES: Yeast extract + sucrose

ω3/n3: Omega 3

ω6/n6: Omega 6

Nomenclature used

In regards to nomenclature of fatty acids, C_{20} denotes an acid containing 20 carbons, with $C_{20:5}$ denoting an acid containing 20 carbon atoms and 5 double bonds. The ω and n nomenclature of fatty acids are interchangeable, although the n nomenclature is the widely accepted standard to define the class of a fatty acid. When specific fatty acids are stated, i.e. $C_{18:3}$ n3, the n nomenclature will be used. Within the work, PUFA shall refer to all polyunsaturated fatty acids, LCPUFA shall refer to polyunsaturated fatty acids containing up to 18 carbon atoms i.e. $C_{18:3}$ n3. VLCPUFA shall refer to polyunsaturated fatty acids containing 20 or greater carbon atoms i.e. $C_{20:5}$ n3.

In regards to nomenclature of organisms, the following capitalisations will be used:

Ascomycota (Phylum)

ascomycete/s (colloquial)

Basidiomycota (Phylum)

basidiomycete/s (colloquial)

Chromista (Kingdom)

chromist/s (colloquial)

Chytridiomycota (Phylum)

chytrid/s (colloquial)

Oomycota (Phylum)

Oomycetes (Class)

Zygomycota (Phylum)

zygomycetes (whilst this term is a Class name, it shall be used as a colloquial form of Zygomycota, hence the use of a lowercase z)

Acknowledgements

Firstly, I would like to thank Prof. Peter Bramley and Dr Paul Fraser for their support and for the opportunity to work in such a fantastic lab.

I would also like to thank Dr Matthew Ryan for the support with the mycological aspects of the work and the opportunity to work at CABI.

Many thanks to Dr Matthew Jones and Dr Dana Heldt for your help with the molecular biology aspect of the work.

Many thanks also go to all the staff at CABI who provided assistance during the project, from media preparation to location and growth of cultures, to morphological and molecular identification of isolates.

I would like to thank everyone in the lab at Royal Holloway, for creating a friendly and welcoming atmosphere, and by maintaining my sanity over the four years by having our fair share of laughs.

I would like to thank Prof. Gerhard Sandermann at Goethe University and his group for the transformation of the *Phaffia* isolates.

Thanks goes to CABI for funding the project and providing the cultures, whilst thanks goes to Royal Holloway for providing the lab and equipment for sample analysis.

Finally, many thanks go to my parents who supported me throughout the project, and provided a roof over my head during the final year, of which I'm very grateful.

1 Introduction

A wealth of scientific evidence now exists linking health benefits to the dietary intake of polyunsaturated acids in the human diet to maintain overall good health (Beardsell *et al.*, 2002). The western diet has seen an increase in the levels of saturated fatty acids and *trans*-fatty acids, and a reduction in polyunsaturated fatty acids (Bulliyya, 2002) (Bates *et al.*, 2010). More recently as the consumer has demanded better quality and more nutritious foodstuffs, manufacturers have reduced the content of saturated fatty acids and replaced them with mono- and polyunsaturated fats. This drive to increase the health "credentials" in food has meant that manufacturers are now including polyunsaturated fatty acids, such as omega-3 (ω3/n3) fatty acids in their products. In addition to food, the market for polyunsaturates as health supplements is rapidly growing. Aquaculture is another area where polyunsaturates are utilised to mimic the feed available in the wild. The increasing demand for polyunsaturated fatty acids coupled with the currently declining fish stocks has resulted in the use of microbial based sources. Microbial production of these oils is not a new idea, as algal sources are already available as alternative sources to fish (Doughmann *et al.*, 2007). Fungi are also used to produce certain polyunsaturated oils, such as arachidonic acid. Further, the isolation of fungi from cold environments such as the Antarctic could yield novel fatty acid profiles from these organisms. Whilst the discovery of ω6/n6 fatty acids is commercialy less attractive, the mechanism by which they are produced and induced would advance our understanding of VLCPUFA formation within the fungi. The discovery of VLCPUFAs within fungal species is itself a novel discovery in its own right, as VLCPUFAs are a rarity among true fungi.

The aim of this work is to identify novel VLCPUFA producing fungi isolated from low temperature environments as an alternative source to the non-sustainable oily fish and phototrophic algae. Low temperature isolates will be studied as it is thought temperature plays a key role in microbial fatty acid composition and as such the effects of temperature on the fatty acid profiles of several isolates will also be studied. Finally, a more efficient PCR based screen for VLCPUFA producing fungi will be described which saves time and solvent usage. A key elongase is described which allows the detection of VLCPUFA producing fungi.

1.1 Structure and nomenclature

Polyunsaturated fatty acids (PUFAs), such as those found in fish oils are becoming widely recognised as having a positive impact on human health (Ruxton *et al.*, 2005, Tapiero *et al.*, 2002). These health benefits have stimulated a rapidly growing market for PUFAs as health supplements, additives and feed for aquaculture. There are several important polyunsaturates, which can be divided into the n3 and the n6 families. The long chain n3 polyunsaturates are found predominantly in fish oils, accumulated through a diet of algae. The most important n3 PUFAs with known nutraceutical properties are eicosapentaenoic acid, ($C_{20:5}$ n3) and docosahexaenoic acid, ($C_{22:6}$ n6). The other class, the n6s are typically found in meat, and can be synthesised by plants, with notable fatty acids being γ-linolenic acid, ($C_{18:3}$ n6) and arachidonic acid, ($C_{20:4}$ n6). The polyunsaturated fatty acids are aliphatic, long chain hydrocarbons, containing greater than one carbon-carbon double bond. They are usually 18 or more carbons long, and the position of the first carbon-carbon double bond when counted from the methyl end gives the fatty acid its ω/n- nomenclature (figure 1-1). Therefore the first double bond appears between carbons 3 and 4, counted from the terminal methyl group in an n3 fatty acid, whilst the first double bond appears between carbons 6 and 7 in an n6 fatty acid. The naming schemes for elongases and desaturases however are derived from the carboxylic end and are given the Δ nomenclature (figure 1-2). This indicates the position of a double bond counting from the carboxylic end. Hence, a Δ17 desaturase inserts a double bond between carbons 17 and 18 when counting from the carboxylic end. $C_{20:5}$ n3, when using the common Δ nomenclature becomes *cis*-5, 8, 11, 14, 17-eicosapentaenoic acid.

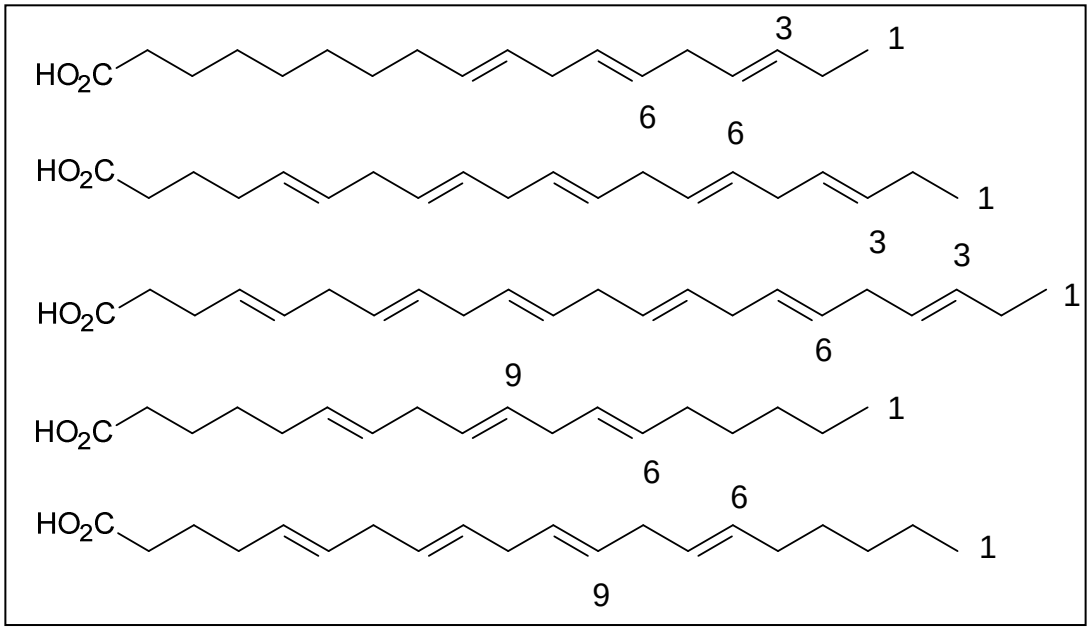


Figure 1-1. The ω/n nomenclature of the fatty acids. The carbon adjacent to the functional group is referred to as the α carbon, whilst the terminal methyl carbon is referred to as the ω carbon. Counting from the ω carbon, if a double bond is found between carbons 3-4 then the n3 nomenclature is given. If a double bond is localised between carbons 6-7 then the n6 nomenclature is given. The examples above from top to bottom are αLA ($C_{18:3}$ n3), EPA ($C_{20:5}$ n3) and DHA ($C_{22:6}$ n3) which are all n3 fatty acids. Next is γLA ($C_{18:3}$ n6) and ARA ($C_{20:4}$ n6) which are both n6 fatty acids. The majority of fatty acids within the fungi are in the *cis* configuration.

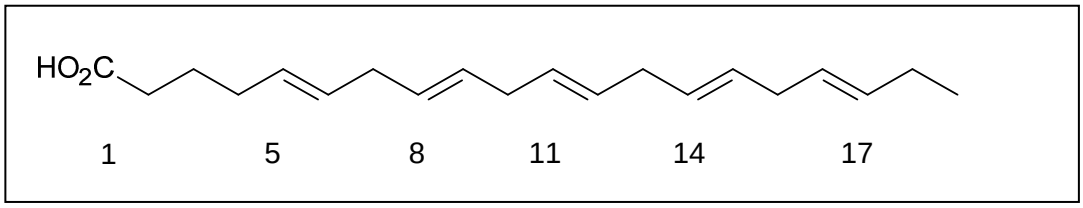


Figure 1-2. The Δ nomenclature of the fatty acids. This method counts from the carboxyl carbon and is the preferred system for naming fatty acids. The example above is $C_{20:5}$ n3 (*cis*- 5, 8, 11, 14, 17- eicosapentaenoic acid). In the case of the desaturases and elongases, the mode of action is usually referenced using the Δ nomenclature.

1.2 The role of PUFAs in health and disease

Polyunsaturated fatty acids play a role in human health primarily due to their close association with the signalling molecules the eicosanoids. The word eicosanoid is derived from the word eicosa- meaning twenty, and it comes as no surprise that two of the major precursors to the eicosanoids are $C_{20:5}$ n3 and $C_{20:4}$ n6 (figure 1-3). The production of eicosanoids is primarily found in complex multi-cellular organisms to instigate inflammatory and anti-inflammatory responses, with the membrane lipids liberated by calcium regulated cytoplasmic phospholipases. Following physical or receptor induced calcium influxes, phospholipases migrate to the phospholipid membrane, whereby they cleave the acyl chains in the sn2 position (Clark *et al.*, 1991).

Using $C_{20:4}$ n6 as an example, the free fatty acids can be converted initially into two products; leukotrienes, named after their three conjugated double bonds, or the prostaglandins, which are modified from the fatty acids by the cyclisation and subsequent formation of a 5 member ring. Prostaglandins can then be further modified by the appropriate synthases to prostacyclins or thromboxanes.

The eicosanoids are produced locally within the cell exerting their effects on the cell that synthesised them and adjacent cells. This is in opposition to hormones where production is usually at a localised site, usually within an organ, and distribution occurs throughout the body targeting various cell types. $C_{20:4}$ n6 is predominantly linked to the inflammatory pathway, with platelets and other cell types converting $C_{20:4}$ n6 to thromboxanes, which have strong vasoconstriction effects and induce platelet activation. In the leukocytes $C_{20:4}$ n6 is converted to leukotrienes, which again exhibit strong vaso- broncho- constriction effects and increase endothelial permeability (De Caterina & Basta, 2001). According to De Caterina *et al*. $C_{20:5}$ n3 in the endothelia is converted to prostaglandins, as is $C_{20:4}$ n6 with the derived compounds strong vasodilators and platelet activation inhibitors, with leukotriene and thromboxane derivatives from $C_{20:5}$ n3 much weaker than those from $C_{20:4}$ n6.

Other modes of action include products derived from DγLA ($C_{20:3}$ n6), such as 15-hydroxyDγLA, which inhibits lipoxygenases, in turn reducing production of pro-inflammatory $C_{20:4}$ n6 based leukotrienes (Zurier, 1993), $C_{22:6}$ n3 regulating cyclooxygenase transcriptionally and the role of $C_{20:5}$ n3 as a poor substrate for $C_{20:4}$ n6 metabolising enzymes, which reduces the net quantity of eicosanoid product (De Caterina & Basta, 2001) are some of the ways the inflammatory pathway is kept in check. Fatty acids can also have a direct effect with $C_{20:3}$ n6 and $C_{20:4}$ n6 inhibiting interleukin-2 production (Santoli & Zurier, 1989). Conditions that are thought to have underlying inflammatory causes are rheumatoid arthritis, with $C_{20:5}$ n3 and $C_{18:3}$ n6 supplementation shown to subjectively ease the condition (Belch *et al.*, 1988), inflammatory bowel disease with $C_{20:5}$ n3 showing in several studies beneficial effects (Belluzzi *et al.*, 2000) and atherosclerosis, with $C_{20:5}$ n3 having been shown to increase plaque stability resulting in decreased incidence of plaque rupturing, possibly leading to myocardial infarction (Cawood *et al.*, 2010). It is therefore thought that many human conditions are linked to eicosanoid biosynthesis, which directly stems from fatty acid consumption and biosynthesis. The imbalance of fatty acids through the change in the Western diet, specifically the increased intake of n6, which can lead to the

overproduction of eicosanoids from the precursor arachidonic acid, is one potential mechanism.

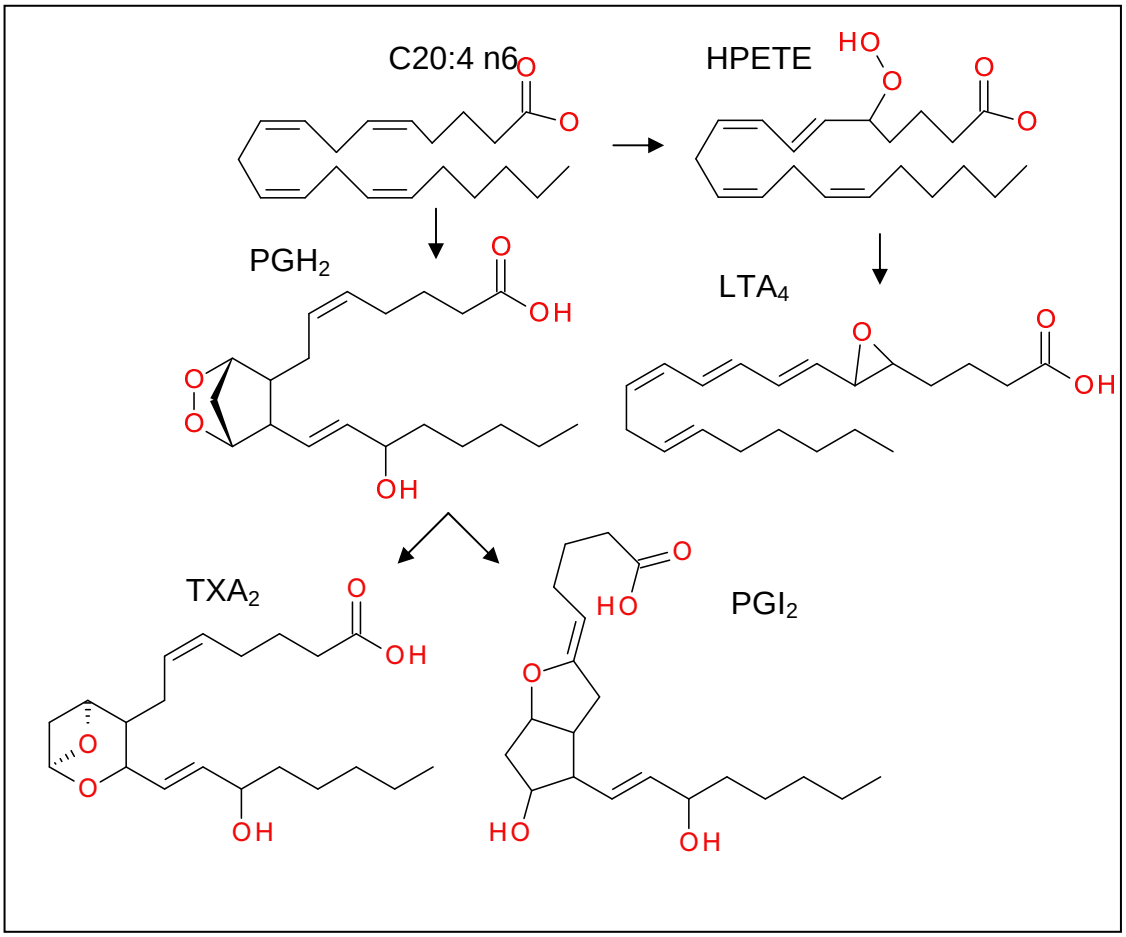


Figure 1-3. The eicosanoids. The n6 fatty acid arachidonic acid ($C_{20:4}$ n6), is converted into several eicosanoid classes which are generally thought to be pro-inflammatory. Arachidonic acid can be converted to the leukotrienes (LTA_4) through hydroperoxyeicosatetraenoic acid (HPETE) through the action of a lipoxygenase. Prostaglandins (PGH_2) are formed through the action of cyclooxygenase followed by subsequent conversion to either the thromboxanes (TXA_2) or the prostacyclins (PGI_2).

The role diet plays in our health is profound, most noticeably in Western societies, with easy access to high calorie and high fat foods. In the case of lipids it is not just the amount of lipid but the composition that is thought to be affecting our health. Precursors to the VLCPUFAs and their uptake from the diet can directly impact the biosynthesis of VLCPUFAs. This in turn has an effect for example on eicosanoid biosynthesis, although not all health related claims may be associated with eicosanoid formation. The relationship between diet and health is more complex than just fuelling eicosanoid biosynthesis directly. As in most organisms, mammalian fatty acid synthesis occurs through a series of elongation and desaturation steps, However, humans along with other mammals lack the ability to desaturate $C_{18:1}$ n9 further, and thus must obtain $C_{18:2}$ n6 and $C_{18:3}$ n3 from their diet (Burr & Burr, 1930). The fatty acids synthesised by

mammalian cells are predominantely C_{16-18} and are synthesised within the cytoplasm (Leonard *et al.*, 2004) utilising the fatty acid synthase (FAS) complex, with $C_{18:0}$ formed within the endoplasmic reticulum (ER) and $C_{18:1}$ formed through a subsequent desaturation. The n3s and n6s are derived from the two aforementioned essential fatty acids, α-linolenic acid (n3 synthesis) and linoleic acid (n6 synthesis). These can then be converted *in vivo* into their long chain counter parts $C_{22:6}$ n3 and $C_{20:4}$ n6 respectively. The conversion of $C_{18:3}$ n3 to $C_{22:6}$ n3 however is inefficient (Ruxton & Reed, 2004) in part due to two steps in the pathway. Firstly, an alternative $C_{20:5}$ n3 to $C_{22:6}$ n3 synthesis route in mammals exists, in which $C_{24:5}$ n3 undergoes β-oxidation to form $C_{22:6}$ n3 in a process known as Sprecher's shunt (Voss *et al.*, 1991). Secondly, C_{18} Δ6 desaturase is required for both n3 and n6 pathways, for the conversion of $C_{18:2}$ n6 to $C_{18:3}$ n6 as well as $C_{18:3}$ n3 to $C_{18:4}$ n3, with increased consumption of linoleic acid ($C_{18:2}$ n6) resulting in the n6 route taking precedence over the n3 pathway reducing the synthesis of n3 VLCPUFAs.

Therefore to counteract this lowered biosynthetic production, n3 VLCPUFAs such as $C_{20:5}$ n3 and $C_{22:6}$ n3 need to be contained in the diet either through fortification of food, consumption of oily fish or diet supplementation. In the western world the ratio of n3:n6 contained within the diet is out of proportion, with the ratio being as low as 1:10 (Horrocks & Yeo, 1999), with the recommended ratio being 1:2.3 (Kris-Etherton *et al.*, 2000). This is quite clearly an issue, with fatty acids such as linoleic acid ($C_{18:2}$ n6) reducing the throughput of n3 biosynthesis and increasing the production of the n6 series, whilst direct consumption of $C_{20:4}$ n6 allows greater $C_{20:4}$ n6 incorporation into membranes followed by modification and utilisation later as pro-inflammatory eicosanoids. $C_{20:4}$ n6 however does have commercial applications as companies such as Martek, using the fungus *Mortierella alpina* have shown by producing this fatty acid for infant replacement milk. However, as $C_{20:4}$ n6 can be produced within the body from the more abundant precursors and is consumed in the diet from animal products, its value is not as great as the n3 class VLCPUFAs. The identification of fungal isolates capable of $C_{20:4}$ n6 production would be useful as the genes responsible for the n6 VLCPUFA biosynthesis would aid our understanding of VLCPUFA formation. The discovery of $C_{20:4}$ n6 producing fungi would also be of interest taxonomically due to the lack of fungi capable of producing this and other VLCPUFAs. Therefore the commercial value of $C_{20:4}$ n6 is lower than that of the n3 VLCPUFAs.

Polyunsaturated fatty acids, especially the long chain n3 class have an integral role in health due to their close association with inflammatory regulation. VLCPUFAs are also associated with other health benefits, which may be independent of the inflammatory response. It has been shown that $C_{22:6}$ n3 and $C_{20:4}$ n6 are required for the development of the infant brain (Horrocks & Yeo, 1999) and infant sight, and that the developing brain is highly influenced by the fatty acids consumed in the diet (Anderson, 1994). VLCPUFAs consumed by the mother benefit the child when *in utero* and whilst lactating. Therefore the mother should endeavour to increase her $C_{22:6}$ n3 intake whilst lactating. However, after a certain point during development, dietary fatty acids do not significantly affect the grey matter fatty acid make up, presumably due to the ability of the organism to synthesise the VLCPUFAs, and due to the decreased production of neurones. Studies suggest infants fed on an omega 3 supplemented diet, of which breast milk is a key source of $C_{22:6}$ n3 (Helland & Saarem, 1998), have a higher IQ than those fed on a $C_{22:6}$ n3-deficient diet (Helland & Smith, 2003). It has been found that children suffering from conditions such as attention-deficit hyperactivity disorder (ADHD) and dyslexia and adults suffering from depression have a lower concentration of VLCPUFAs in their tissues (Ruxton & Reed, 2004). Supplementing the diet of children suffering from dyslexia has in some cases been shown to help alleviate the symptoms (Stordy, 2000). Supplementation with $C_{22:6}$ n3 may also help in the treatment of conditions such as ADHD (Richardson, 2004), cystic fibrosis and depression (Nemets & Stahl, 2002).

It has been shown that consumption of oily fish, first observed in populations whose diet contain primarily fish, which are rich in n3s especially $C_{22:6}$ n3 and $C_{20:5}$ n3 benefit from a lower incidence of chronic heart disease and display lower serum triacylglycerol (TAG) and cholesterol levels (Simopoulos, 2002) and that increased levels of high-density lipoprotein (HDL) cholesterol and a higher ratio to low-density lipoprotein (LDL) may be partially responsible (Bulliyya, 2002). Other factors thought to be jointly responsible for chronic heart disease, of which n3 VLCPUFAs are thought beneficial, is the lowering of blood TAG levels (Simopoulos, 2002), lowering chemoattractants (Sperling *et al.*, 1993), growth factors (Baumann *et al.*, 1999) and adhesion molecules in the elderly (Miles *et al.*, 2001). n3 VLCPUFAs are also thought to have anti-arrhythmic effects (Calder, 2004). Coupled with the previously mentioned anti-inflammatory effects of n3 VLCPUFAs, arteriosclerosis is slowed or prevented, reducing the incidence of chronic heart disease followed by death by myocardial infarction or stroke caused by thrombosis (Mclennan & Howe, 1996). However, the

benefits of fish oils may be reversed, if consumed in large enough quantities by pollutants such as mercury, which may negate the effects of VLCPUFAs (Rissanen & Voutilainen, 2000). It is now recommended to consume less n6, and increase n3 through an estimated weekly intake of 2-4 portions of fish per week for non-pregnant and lactating women (FSA, 2004), which constitutes around 0.45-0.9 g $C_{22:6}$ n3 per day. $C_{22:6}$ n3 has also been shown to correlate with a reduction in the incidence of Alzheimer's disease by up to 60%, by the regular consumption of oily fish or $C_{22:6}$ n3 supplements (Morris & Evans, 2003). It can be seen that these fatty acids have a profound role on our health and that increased consumption of n3 fatty acids through natural or enriched foods may help reduce and alleviate certain conditions.

1.3 Introduction to fungi

Man's exploitation of fungi dates back thousands of years with the process of fermentation yielding ethanol and CO_2, utilised in baking and brewing. The most direct application of the fungi has been as a food source, although many are harmful to humans when consumed due to the presence of mycotoxins. The fungal kingdom has provided us with a wealth of compounds, with Fleming's discovery of antibiotics initially from the genus *Penicillium* propelling medical science forward in the treatment of bacterial infection. Other compounds of use include fatty acids, carotenoids (Frengova & Beshkova, 2009) and organic acids such as citric acid (Tran *et al.*, 1998), and cholesterol-lowering statins (Manzoni & Rollini, 2002). Other uses of fungi include the production of specialist cheeses such as Camembert, using the species *Penicillium camemberti*, the use of organisms such as *Trichoderma sp*. as biocontrol agents (Viniale & Marra, 2006), plant growth enhancers (Naseby & Pascual, 2000) and a source of enzymes (Chand & Aruna, 2005). The wealth of enzymes which can be obtained from fungi is large and diverse, ranging from amylase, cellulase, proteases and lipases. Enzymes involved in the degradation of insect cuticle walls, as found in the fungus *Metarhizium anisopliae* are a key component in its insecticidal role (Smith *et al.*, 2001), which is now used to control locusts. The enzyme itself does not directly kill the insect, rather the colonisation of the host insect by the fungus. Therefore fungi as insect biocontrol agents have been studied to protect agricultural crops from herbivorous insects. The Entomophthorales are one of the most studied fungal classes in regards to insect biocontrol, with fungi such as *Entomophthora* and *Zoophthora* pathogens of

gypsy moths and alfafa aphids for example, which feed on oak trees and legume plants respectively (Shah & Pell, 2003).

It is estimated that there are around 1.5 million different species of fungi (Hawksworth, 2001) although this estimate is conservative, with figures ranging up to several million species. Many of these are classified as saprotrophs, which utilise dead organic matter as a nutrient source, which is digested extracellularly. Others parasitise plants and animals, with fungi forming the majority of plant infections with organisms from the genus *Puccinia* and class *Ustilaginomycete* causing common plant diseases, which are commonly known as rusts and smuts respectively. In fungi, the vegetative cells which propagate throughout the substrate source are referred to as hyphae, which increase the surface area by which secreted digestive enzymes may act upon the complex substrate followed by the subsequent absorption of the digested material. It is these hyphae which form the visible mycelium, the mass of fibrous cells which usually permeate into the growth substrate. The hyphal cell wall is composed of chitin with the hypha itself composed of multiple cells. With the exception of the zygomycetes, the hyphal cells are divided by septa which allow trans-cellular movement of nutrients and organelles through multiple pores present in the septa. The hyphae elongate from the tip through the polymerisation of the cell wall precursor, as well as the expansion of the cell membrane. With respect to reproduction, fungi can propagate though two modes of reproduction; the asexual form (anamorph), or the sexual form (teleomorph). The spore forming structures and the spores themselves are utilised to morphologically characterise the species. A brief overview of each phylum will be given. According to the dictionary of the fungi (Kirk *et al.*, 2008), the Kingdom fungi is split into six phyla; Chytridiomycota, Glomeromycota, Microsporidia, Zygomycota, Ascomycota and Basidiomycota of which the last two are sometimes classified within the sub-Kingdom Dikarya. The Microsporidia are specialised organisms which were once classified as protozoa and are largely parasitic. As they were not studied in the work, they shall not be described further.

1.3.1 Oomycota

The Oomycetes are classified within the Chromista and have several differing biochemical pathways compared with the fungi, however before detailed molecular analysis, species from the Oomycota were classified as fungi due to similar morphological features (Kendrick, 2001). Organisms such as the genus *Thraustochytrium* whilst residing in the Chromista under the phyla Labyrinthista were

not classified by Kendrick and the majority of others as fungi however. The Oomycota are differentiated from the fungi as they contain cellulose in their cell wall as opposed to chitin, and are diploid as oppose to haploid as is the case with most fungi. Like chytrids, the Oomycetes have coenocytic hyphae and produce a sporangium (Link *et al.*). The Oomycota are also known to be parasitic with the infamous *Phytophthora infestans* causing the Irish potato famine and *Saprolegnia* species causing saprolegniasis in fish. The Oomycota produce flagellate zoospores similar to the chytrids and are also capable of VLCPUFA production with *Saprolegnia* and *Pythium* species capable of producing up to $C_{20:5}$ n3.

1.3.2 Chytridiomycota

The Chytridiomycota are thought to be the ancestors of the other phyla of terrestrial fungi (James *et al.*, 2006) (figure 1-4), and are found to be the predominant species in the Devonian period along with the zygomycetes (Taylor & Taylor, 1997). One of the defining features of these organisms is the production of motile zoospores which are flagellate, which allow the organism to sexually reproduce in the aqueous environment in which they are predominantly found. In some instances they have been known to infect hosts such as amphibians, which has been found to be the case with the chytrid *Batrachochytrium dendrobatidis* (Berger *et al.*, 1998). The fact that these organisms posses flagella alludes to the fact that the majority of these organisms spend a portion of their life cycle in an aqueous environment, which suggests that these organisms predate the terrestrial fungi. The Chytridiomycota unlike the other phyla of fungi do not produce hyphae, but produce supportive structures called rhizoids, which may be extensive enough to be called a rhizomycelium (Kendrick, 2001). This in turn supports the differentiated sporangia, which produce the haploid or diploid zoospores. The Chytridiomycota are also known for producing long chain polyunsaturated fatty acids, producing up to $C_{20:4}$ n6 from *Allomyces*, as discussed later.

1.3.3 Zygomycota

The zygomycetes are thought to be the closest relatives of the Chytridioycota (James *et al.*, 2006, van de Peer *et al.*, 1993). This is due to molecular data as well as morphological similarities such as the presence of sporangia in chytrids and zygomycetes as well as zygomycetes being coenocytic (O'Donnell *et al.*, 2001). The Zygomycota can reproduce both sexually and asexually with the asexual form the most prevalent form of reproduction. In this form of reproduction, the hyphae bearing the

reproductive structure are called sporangiophores with the spore producing structures called mitosporangia from which are produced haploid spores called mitospores. Sexual reproduction occurs through the fusion of two compatible hyphae as well as the nuclei, which in turn produce gametangia. This then matures into a diploid zygosporangium which under goes meiosis returning the cells to a haploid state. Germination of the zygosporangium results in the formation of a mitosporangium containing haploid spores formed through mitosis (Kendrick, 2001). It is also known that some of the organisms residing in the Zygomycota, such as *Mortierella* can produce VLCPUFAs up to $C_{20:5}$ n3. Some Zygomycota infect insects, other fungi and nematodes, as well as colonising mammal dung.

1.3.4 Glomeromycota

Species within the Glomeromycota were formerly found within the Zygomycota, however have relatively recently, been elevated to a Phylum. It was shown that those species found within the Glomeromycota were in fact monophyletic and are thought to share common ancestry with the Dikarya, as opposed to the Zygomycota (Schüβler *et al.*, 2001). Species from within the Glomeromycota are predominantly arbuscular mycorrhizal fungi. The fungi form a symbiotic relationship with vascular plants to obtain macronutrients such as carbohydrates, whilst the plant benefits from labilised phosphate ions (Helgason & Fitter, 2009). Fungi from this Phylum typically inhabit soil and form close associations with plant roots. They generally reproduce asexually, producing chlamydospores at hyphal tips. Like the Zygomycota however, they are predominantly coenocytic.

1.3.5 Ascomycota and Basidiomycota; the sub-Kingdom Dikarya

The Dikaryomycota contain two phyla, the Ascomycota and the Basidiomycota. The term Dikaryomycota comes from the fact that both phyla produce dikaryotes, which is a cell containing two individual haploid nuclei. In the Ascomycota this occurs through the fusion of two compatible haploid hyphae in a process called anastomosis. This produces a diploid dikaryotic zygote which then produces many diploid dikaryotic hyphae. After the hyphae have reached specific positions the separate nuclei fuse and meiosis occurs, leading to the formation of ascospores within the ascus. The asci may also be contained within an ascocarp, a fruiting body sometimes diagnostic of the species. A round of mitosis then occurs within the ascus leading to the formation of around eight spores, which are haploid (Deacon, 1997). The spores are then, in a large proportion of

Ascomycota ejected from the top of the ascus at high speed, also leading to the ejection from the ascocarp. In the Basidiomycota the sexual spore forming structure is called the basidium with the meiotically formed haploid spores referred to as basidiospores (Kirk *et al.*, 2008). The sexual form of propagation in the Basidiomycota is similar to that of the Ascomycota, however after the merging of the nuclei and the subsequent meiosis only four basidiospores are formed as opposed to eight ascospores which occurs due to the extra round of mitosis in ascomycetes. Also of note, ascospores are found inside the ascus as opposed to basidiospores which migrate to the exterior of the basidia. The spores of the anamorphic phase of the Ascomycota are called conidia and are mitospores, in reference to their mitotic origin. The spores themselves are produced from hyphal cells and the aggregation of spores can occur on a conidiophore, a hyphal cell which supports the spores. The spores may also be enclosed in a conidioma which typically occurs within a host plant. The Dikaryamycota also constitute the vast majority of extant fungal species, with 64000 species within the Ascomycota known (Kirk *et al.*, 2008).

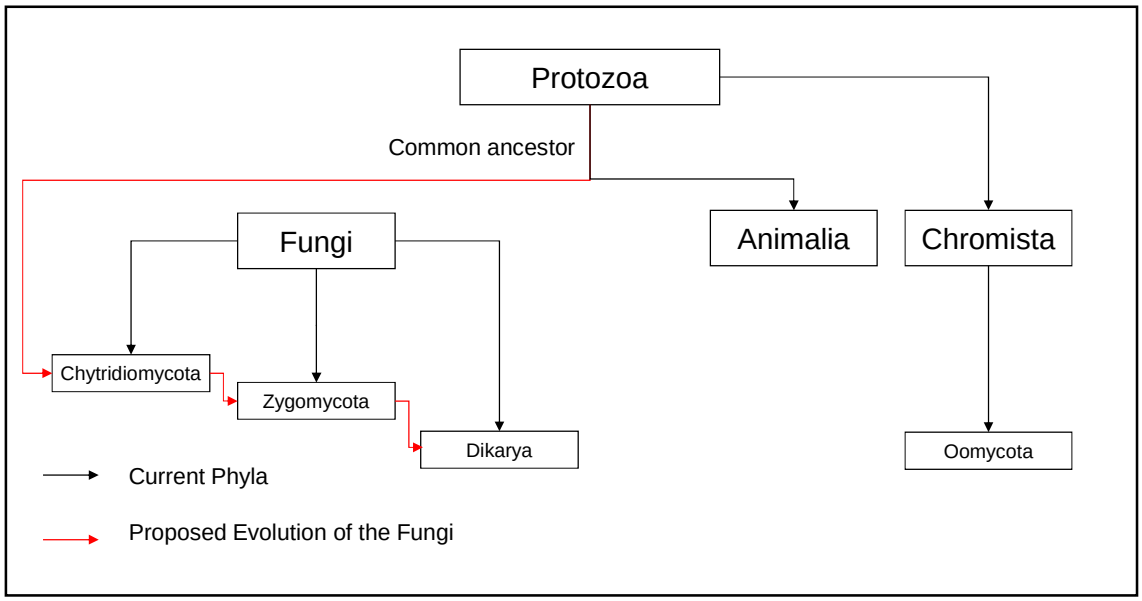


Figure 1-4. The proposed evolution of the fungi from a common ancestor (Carr & Baldauf, 2011, Cavalier-Smith, 2009)**. The fungi are thought to have evolved from the primarily aquatic Chytridiomycota, losing their flagella to become terrestrial Zygomycota followed by morphological and biochemical adaptations to become the Dikarya. The fungi are also thought to be more closely related to the Animalia than the Plantae.**

1.3.6 The role of PUFAs within the fungi

In regard to the role PUFAs play within fungi it is believed that one of their functions may be the maintenance of membrane fluidity in response to temperature. This is because these PUFAs are usually incorporated into phospholipids. Due to their

amphipathic properties they form the cell and organelle membranes, with the fluidity of the membrane regulated by the attached fatty acids. Long chain, saturated fatty acids have a higher melting point than shorter chain saturated fatty acids due to the greater interaction experienced by the larger carbon chains. Two forms of interaction are present within the membrane, adjacent fatty acid interaction and overlapping fatty acid interaction. Adjacent interaction occurs between fatty acids next to one another in the membrane, with long saturated chains providing the maximum area for interaction. Overlapping interaction occurs between fatty acids attached to opposing sides of the membrane with interaction occurring across a proportion of the carbon chain. A long saturated chain, as oppose to a shorter chain, will experience greater interaction due to increased overlap with opposite chains. Interaction with adjacent chains also increases due to the greater charge separation experienced by longer acyl chains, resulting in a higher melting point. This increases order within the membrane creating a more rigid structure (Mykytczuk *et al.*, 2007). The introduction of *cis* double bonds leads to the disruption of membrane fatty acid organisation and results in a less ordered system. This occurs because of the nature of *cis* double bonds, as they add a "kink" to the chain. Each double bond in effect changes the direction of the chain in essence curving it. This leads to less interaction with neighbouring chains due to the multitude of different shaped chains in the membrane, which in turn lowers the melting point. This system of altering the melting point of the fatty acids is a key aspect to the maintenance of membrane fluidity during cold conditions.

Several studies seem to indicate that lower temperatures do indeed induce the formation of a more unsaturated membrane. Maintaining membrane fluidity is essential for organisms such as bacteria (Wada *et al.*, 1990), algae and for the more complex fungi, as well as plants as demonstrated by *Arabidopsis* (Miquel *et al.*, 1993), which demonstrated low temperature induction and the subsequent transcription of a n3 desaturase at 20°C growth (Gibson *et al.*, 1994). Even mammals, whilst capable of maintaining a constant core temperature demonstrate decreased melting points of fats in tissues which are more exposed, such as snouts and hooves (Irving *et al.*, 1957). The fluidity of the membrane is essential for processes such as endo- and exo-cytosis, the formation of vesicles, the turnover of the phospholipids, the movement of protein receptors as well as enzyme activity (Sandermann & Strominger, 1972) and their stabilisation (Dowhan, 1997) as well as conferring a certain amount of flexibility and tolerance to physical and osmotic strains. Therefore, organisms without the ability to regulate their temperature would need a mechanism by which their membrane fluidity

could be controlled when external temperatures fell. This is why organisms isolated from cold locations, such as the Antarctic, are thought to be more likely to produce PUFAs. In the subsequently discussed studies, lowering the growth temperature generally results in an increase in fatty acid unsaturation, and it is thought that organisms adapted to live in temperatures below freezing will display fatty acids with a greater degree of unsaturation, which allow them to survive the harsh climate.

Fungi from the Antarctic are relatively unstudied, with little work having been published on profiling large numbers of fungi from the region. When organisms such as bacteria were isolated and screened from the Antarctic, many were wrongly classified to the species level and grouped with known species, even though they exhibited VLCPUFA production when other species in the genus did not. This led to the re-classification of these organisms, with the discovery of new species within the genus (Nichols *et al.*, 1999). In these bacteria, VLCPUFAs such as $C_{20:5}$ n3 (Nichols & Nichols, 1993) and $C_{22:6}$ n3 were observed. Those organisms isolated from low temperature environments which survive in cold climates are usually designated psychrophilic, i.e. cold loving and therefore have growth maxima <15°C, whereas psychrotolerant organism have the ability to grow at <15°C, however their growth maximum is usually >20°C (Robinson, 2001). In regard to other bacterial studies, when subjected to a temperature change of 42°C to 24°C, *E. coli* responded with the desaturation of the fatty acids in the membrane within 30 seconds, with an increase in *cis*-vaccenic acid ($C_{18:1}$ Δ11) (Garwin & Cronan, 1980). The mesophilic bacterium *Arthobacter* when exposed to cold stress with a decrease in temperature from 37°C to 2°C increased unsaturated fatty acids by 19% (Thieringer *et al.*, 1998).

Several studies have shown the effects of temperature on fatty acid profiles. In certain bacteria such as *Listeria monocytogenes*, low temperature growth promotes the formation of anteiso-$C_{15:0}$ (Annous *et al.*, 1997), a branched chain saturated fatty acid, as well as the formation of short chain saturates. However, increases in unsaturation levels were negligible. Fungal culture temperature experiments have also been conducted. Three Antarctic fungal isolates were subjected to two culture conditions at 5 and 15°C (Weinstein *et al.*, 2000). It was found for all three organisms, *Humicola marvinii*, *Geomyces pannorum* and *Mortierella elongata* that when grown at the lowest temperature of 5°C, the most unsaturated fatty acid, $C_{18:3}$ n3 or $C_{20:5}$ n3 in the case of *Mortierella* were produced at the highest levels. It was also found, with *Mortierella* that the second most highly unsaturated fatty acid, $C_{20:4}$ n6 had the highest abundance at

15°C. The unsaturation indices for both *Geomyces* and the *Humicola* showed increasing unsaturation with the decrease in temperature although *Mortierella* showed a small counter trend, experiencing slightly greater unsaturation at 15°C. The explanation for this was the increased levels of trehalose at 5°C which acted to maintain separation between polar head groups, and maintain the structure of the membrane during desiccation or freezing events. At the other end of the spectrum, thermophilic fungi when compared to their mesophilic counterparts demonstrated a lack of $C_{18:3}$ n3/n6 in a majority of cases (Mumma *et al.*, 1971). Whilst not all the mesophiles demonstrated the capability to produce the trienoic fatty acids, two thirds were able to produce LCPUFAs whilst only 28% of thermophiles demonstrated this capability. When the unsaturation levels were studied, it was found that the mesophiles demonstrated unsaturation indices in the region of 1.01-1.61 for polar lipids, whilst thermophiles showed consistently lower indices in the range of 0.75-1.04, with higher unsaturation indices representing greater levels of unsaturation. Neutral lipids were typically found to be more saturated than the corresponding polar fraction, although several exceptions were noted. In general the neutral fraction was also more unsaturated within the mesophiles than in the thermophiles. However, the study used only one growth temperature for the mesophiles, and a higher temperature for thermophiles. This means that direct comparison of the unsaturation indices is misleading as the profiles of thermophiles may become more unsaturated as the temperature is lowered. In fact thermophiles may not grow at all in colder temperatures, in part due to their inability to modify their fatty acid profile sufficiently.

Several exponentially growing fungi were studied in relation to the effect of temperature on the total fatty acid profile (Suutari, 1995). The temperature range used was 10-35°C. For one of the organisms, *Penicillium chrysogenum*, the lowest culture temperature of 10°C produced the greatest abundance of $C_{18:3}$ (class not known), with *Aspergillus niger* showing elevated levels at 15°C. *Trichoderma reesei* showed that 20°C culture produced the highest levels of $C_{18:3}$, whilst *Neurospora crassa* produced the largest quantity at 20°C. Unsaturation levels demonstrated a large degree of variance, with *A. niger* having the highest unsaturation levels at 15°C, but dropping suddenly with the lowest unsaturation at 10°C. *P. chrysogenum* almost demonstrated a bell shaped curve for unsaturation index when plotted against culture temperature, with 20°C growth yielding the greatest unsaturation. *T. reesei* demonstrated a similar trend, with a gradual rise in unsaturation from 10 to 20°C growth, with the fatty acid profile reaching its maximum unsaturation level at 26°C, followed by a sharp drop in

unsaturation levels when grown at 30 and 35°C. Finally, *N. crassa* demonstrated a relatively constant unsaturation index regardless of temperature. All the organisms studied however were mesophilic, with none demonstrating optimal growth below 20°C. The fact that none of the organisms demonstrated the greatest unsaturation index at 10°C would imply that the organisms are incapable of unsaturation levels beyond that observed at warmer culture temperatures. This may possibly be due to low temperature inactivation of enzymes. Therefore if we assume that mesophiles exposed to low temperature are not adapted sufficiently in other areas, such as protein function at low temperature, we can see that the organisms that demonstrate optimal growth at temperatures in the region of 20-35°C demonstrate increased unsaturation when the culture temperature is lowered from 35°C to 20°C. However, at temperatures below 20°C the organisms seem unable to grow in an optimal manner, with substantially decreased growth rates and declining unsaturation levels.

Whilst little fatty acid profiling of fungi from Antarctica has been carried out, studies looking at lowering growth temperatures of mesophiles to increase VLCPUFA production, lead to the belief that low temperatures induce desaturation. The species *Mortierella verticillata* produces $C_{20:5}$ n3 at around 7.7% TFA (Shinmen & Shimizu, 1989) as well as other *Mortierella* species, which produced lesser amounts. The results also showed that culture at the lower temperature of 12°C induced the formation of $C_{20:5}$ n3, at the expense of $C_{20:4}$ n6. This gives an indication that lower temperatures induce the formation of VLCPUFAs. *Mortierella alpina* was found to produce 1.88g/l of $C_{20:5}$ n3 (Shimizu & Kawashima, 1989), and it was suggested that two routes were used to accumulate $C_{20:5}$ n3. The first was the conversion of $C_{20:4}$ n6 to $C_{20:5}$ n3 when the culture was incubated at low temperatures and the second route was through the supplementation of a precursor to $C_{20:5}$ n3, such as $C_{18:3}$ n3. By combining both these routes it was speculated that the $C_{20:5}$ n3 yield would increase.

The response to cold is an attempt to retain the fluidity of the organism's membrane with the mechanism explored by Mikami *et al.* (Mikami & Murata, 2003). Whilst the mechanism for this response was studied in a prokaryote, the general principle of modifying the membrane to maintain fluidity still holds true. The author suggested that desaturases are switched on at low temperatures, and that decreasing temperatures do in fact cause the membrane to rigidify, as shown by Fourier transform infrared spectroscopy (FTIR). It was also shown that deactivating desaturases resulted in increased rigidification, with the FTIR frequency dropping considerably, demonstrating

the need for PUFAs during cold conditions. This was shown for the cyanobacterium *Synechocystis*, although similar principles are likely to affect fungi as well. Another point noted by Mikami *et al.* is that whilst cold temperatures increased unsaturation of the lipids in the membrane, fatty acid synthesis decreased. This is possibly to stop the introduction of saturated fatty acids into the membrane, as well as the possibility of the cell being metabolically less active at low temperatures. It was also shown how influential temperature is on membrane fluidity, with several examples of reduction in the external temperature leading to the rigidification of the membrane, with the reverse occurring during temperature increases. It is thought that the cold may not directly influence the fatty acid profile, but rather the rigidity of the membrane, through the decrease in temperature. Again, Mikami *et al.* mentions that once a fluidity equilibrium is reached, that is, the membrane reaches an optimum fluidity, the genes transcribing the desaturases become down regulated or cease to be transcribed. Therefore this would indicate that the membrane itself contains some way of detecting fluidity or rigidity. The studies by Mikami also show there to be a protein, Hik33 (histidine kinase 33) that regulates desaturation of the membrane. This is a trans-membrane protein which is speculated to respond to the change in membrane fluidity and regulate several cold induced proteins by dimerising during the rigidification of the membrane. This dimerisation would lead to a phosphorylation reaction, which in turn would instigate a signal cascade, initiating the transcription of cold shock genes, such as desaturases. This again is found in bacteria, although a similar homolog and mechanism may be present in fungi.

1.3.7 Fatty acid biosynthesis within the fungi

1.3.7.1 Elongation

Fatty acid biosynthesis is a highly conserved process in terms of the reactions involved. The specific enzymes vary between prokaryotes and eukaryotes, however their functions remain similar. The process of fatty acid biosynthesis occurs via two sets of reactions, elongation and desaturation. The elongation process up to $C_{16:0}$ is referred to as fatty acid synthesis, whilst the process that elongates fatty acids after this point is referred to as *de novo* elongation. The process of fatty acid synthesis is carried out by the fatty acid synthase complex, whilst elongation is carried out by specific elongases. Fatty acid synthesis in fungi occurs within the cytoplasm whilst fatty acid elongation occurs on the endoplasmic reticulum (Jakobsson *et al.*, 2006). The fatty acid synthesis

pathway is shown in figure 1-5. During the elongation process the acyl chain to be elongated is combined with a malonyl subunit (C_3) in a condensation reaction. This subunit is added to the carboxyl end of the growing acyl chain through the action of either the β-ketoacyl synthase domain or an elongase. Following this is a reduction step followed by a decarboxylation step, which removes CO_2. A final reduction step completes the elongation of the chain by two carbons. More specifically, in fatty acid synthesis the acetyl-CoA and malonyl-CoA both bind to the acyl carrier protein (ACP) domain of the FAS complex forming thioester bonds, although the acetyl/acyl chain attaches to the β-ketoacyl synthase subunit once the malonyl-CoA attaches to the ACP domain. This allows both acyl chains to be attached to the FAS complex permitting the condensation reaction to occur, which results in the acyl group being attached to the malonyl group, with the release of CO_2. At this point the elongated chain is attached to the ACP domain, although during the next round of elongation the growing chain will again be transferred to the β-ketoacyl synthase arm. The fatty acid $C_{16:0}$ is the predominant product of fatty acid synthesis, but shorter chain FAs such as $C_{12:0}$ and $C_{14:0}$ may be released from the FAS complex but require either an acyl-transferase or an acyl-ACP-thioesterase (Bonaventure *et al.,* 2003).

Unlike fatty acid synthesis whereby the reactions are localised to the FAS complex, fatty acid elongation involves four independent enzymes not associated as a complex, which are associated with the ER membrane and utilise acyl-CoA substrates exclusively (Ohno *et al*. 2010), (Domergue *et al.*, 2003). The elongation after fatty acid synthesis is shown in Figure 1-8. When referring to elongases it is important to note that the condensing enzyme, or the β-ketoacyl CoA, the enzyme responsible for the merging of the acyl chain and the malonyl-CoA is in scientific literature referred to as the elongase. This is because the condensing enzyme is the substrate specific step, recognising the chain length as well as the number and position of double bonds, and is also the rate limiting step (Venegas-Calerón *et al.*, 2010). The other three components, when recombinant technology is utilised, are not cloned from the target organism and are produced endogenously by the host cell and can complete the further elongation steps regardless of the condensing enzyme used. It has been found that engineering non-endogenous elongases (condensing enzymes only) of similar (Parker-Barnes *et al.*, 2000) and dissimilar (Paul *et al.,* 2006) classes leads to the initial condensation reaction carried out by the cloned elongase being finished by the native complement of enzymes within the ER. Therefore it is believed that different elongases are synonymous with different condensing enzymes.

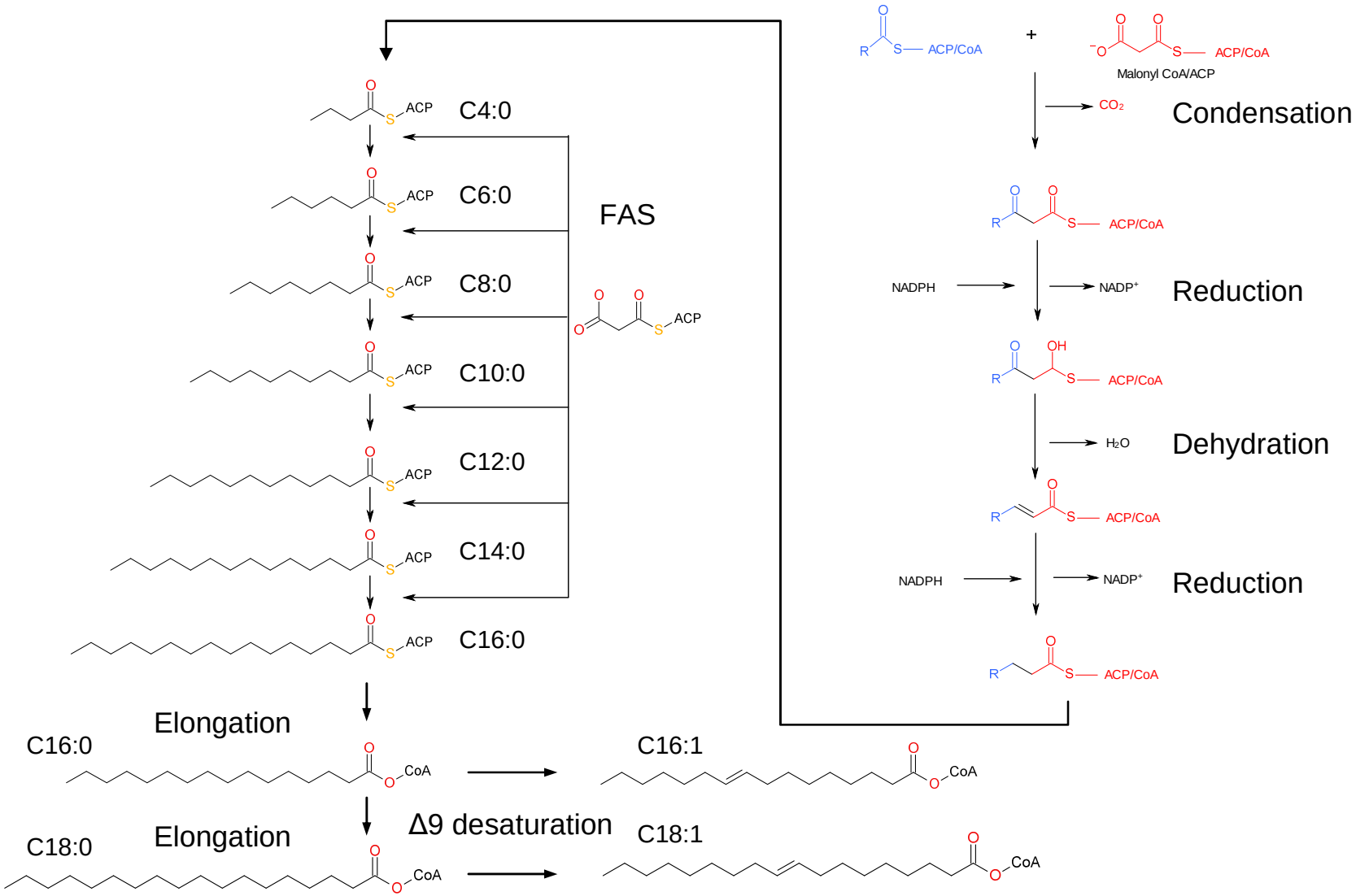


Figure 1-5. The fatty acid synthase pathway which is responsible for generating fatty acids up to $C_{16:0}$. The FAS complex is capable of catalysing all four reactions; condensation, reduction, dehydration and reduction. The FAS complex is found within the cytoplasm and binds to the acyl substrates using an acyl carrier protein (ACP). The elongation itself adds two carbons to the acyl chain at the carboxyl end with the two carbons taken from malonyl-CoA.

As mentioned earlier there are different classes of elongases. In fungi and mammals, the ELO class of elongase is used, found first within the yeast *Saccharomyces cerevisiae*, whilst in plants the FAE class of elongases is present. The ELO class of elongases are also present in plants, although the FAE class is not found within fungi or mammals. In yeast there have been several elongases identified, ELO1, ELO2 and ELO3. It is found that *Saccharomyces cerevisiae* produces mainly saturated fatty acids up to $C_{26:0}$ with the longest unsaturated fatty acid being $C_{18:1}$ as produced by the strain used by Paul *et al.* (Paul *et al.*, 2006). This work concluded that the elongase ELO1 elongates $C_{14:0}$ to $C_{16:0}$, that ELO2 elongates sequentially $C_{16:0}$ to $C_{22:0}$, although elongation to $C_{24:0}$ was possible and that ELO3 elongates again sequentially $C_{20:0}$ to $C_{26:0}$. It has also been shown that ELO2 and ELO3 can elongate the respective monounsaturated fatty acids as well (Oh *et al.*, 1997). Structurally the ELO class contains 5-7 trans-membrane domains, a conserved histidine motif (Pereira *et al.*, 2004,Tan *et al.*, 2011), with the amino acid sequence F-L-H-V-Y-H-H, as well as an ER retrieval motif (Jakobsson *et al.*, 2006). This is in contrast to the evolutionarily distinct FAE class, which contain 1-2 transmembrane domains (Joubès *et al.*, 2008), do not contain the conserved histadine motif, but rather a catalytic triad of amino acids and are are around 500 amino acids long, compared with around 300 amino acids for the ELO class (Venegas-Calerón *et al.*, 2010). The review by Leonard *et al.* (Leonard *et al.*, 2004) states the majority of ELO class elongases discovered so far and it can be seen that the majority of the characterised elongases are found within the mammals.

Within fungi, only the yeast ELO and *Mortierella* γ linolenic acid elongase (GLELO), *Mortierella alpina* elongase (MAELO) and *Mortierella alpina* long chain elongase (MALCE1) have been characterised, although the Δ6 elongase in *Conidiobolus obscurus* has been recently elucidated (Tan *et al.*, 2011). The yeast ELOs and their function have been described previously, though it would seem likely that the majority of fungi contain similar homologs for the production of $C_{18\text{-}24}$ saturates and monounsaturates. The ELO1 elongase's main role appears to complement the FAS complex by elongating fatty acids greater than 8 carbons to $C_{16:0}$, which the FAS cannot utilise (Dittrich *et al.*, 1998). ELO2 as well as the functionally similar elongase MALCE1 from *Mortierella alpina* carry out the predominant formation of $C_{18:0}$, though both elongases have been shown to act on longer saturated fatty acids producing up to $C_{20:0}$ and $C_{22:0}$ from MALCE1 and ELO2 respectively, as well as their equivalent

monounsaturated fatty acids (Sakuradani *et al.,* 2008). MAELO appears functionally equivalent to ELO3 as both catalyse the elongation of saturated fatty acids up to $C_{26:0}$ (Sakuradani *et al.,* 2008). The final rare elongase found within fungi is a Δ6 elongase named GLELO characterised by Parker-Barnes *et al*. (Parker-Barnes *et al.,* 2000). This elongase predominantly catalyses $C_{18:3}$ n6 and STA from the 18 carbon precursors to $C_{20:3}$ n6 and $C_{20:4}$ n3 respectively.

The Thraustochytriaceae, which comprise of organisms capable of producing $C_{20:5}$ n3 and $C_{22:6}$ n3 have been found not to follow the same mechanism of fatty acid synthesis. Polyketide synthesis, which is a bacterial, fungal as well as a chromist process for the production of secondary metabolites can also be used for the production of fatty acids. The process is outlined in Figure 1-6. The process is similar to that of fatty acid synthesis, namely the addition of the acetyl and the malonyl subunits. However, instead of removing the double bond from the intermediates, they are isomerised and the chain continues to grow with the double bond intact (Ratledge, 2004). This process is then speculated to continue with more double bonds being formed and isomerised. The process takes place on a polyketide synthase, similar to the fatty acid synthase in as much as they are both polypeptides containing multiple domains with enzymatic activity.

1.3.7.2 Desaturation

The other major modification of fatty acids is desaturation, the process of introducing double bonds into the chemical structure of the fatty acid through an oxidative process, involving an electron transfer system. The combined action of elongases and desaturases results in the biosynthesis of the long chain unsaturated fatty acids as shown in Figure 1-8. There are two types of desaturases, soluble and membrane associated with three substrate specificities; acyl-ACP, acyl-CoA and acyl-glycerolipid (Pereira *et al.*, 2003). Soluble desaturases can be found within plastids in plants, which catalyse the first desaturation to $C_{18:1}$ (Shanklin & Cahoon, 1998). Membrane bound desaturases then further desaturate FAs within the plastids in plants (Gibson *et al.*, 1994), or in the ER in plants (McCartney *et al.*, 2004) and mammals.

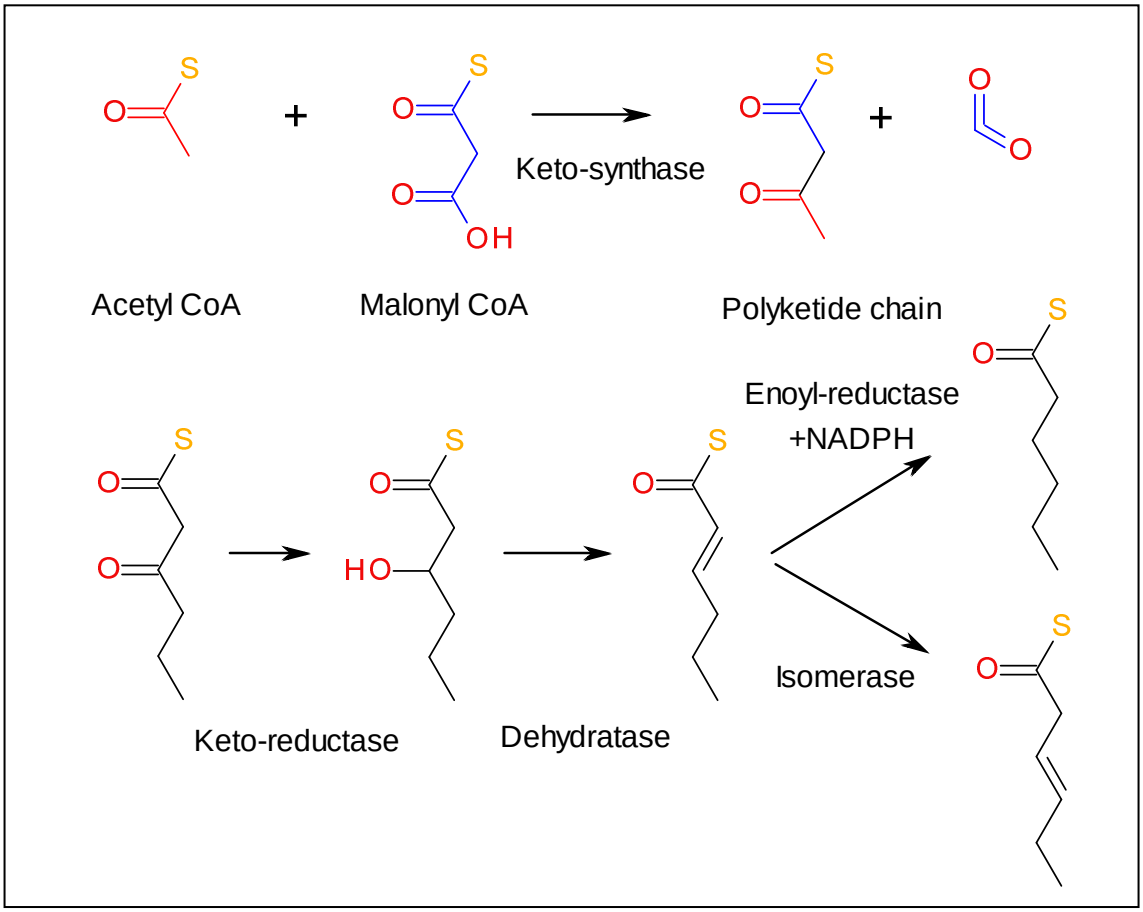


Figure 1-6. The polyketide synthesis pathway. The elongation reaction occurs through the addition of malonyl-CoA to acetyl-CoA or the growing polyketide chain. The condensation reaction is catalysed by a keto-synthase with the elongated polyketide product growing by two carbon units with the loss of CO_2. Continuous elongation results in a carbon chain with keto functional groups every two carbons. In the example above, the first elongated polyketide product (C_4) undergoes reduction, dehydration and hydrogenation by an enoyl reductase followed by an additional elongation to form C_6. The keto-reductase and dehydratase are required to remove the keto group from the elongated chain. The double bond can be removed using a enoyl-reductase and NADPH or the *trans*- double bond can be relocated and converted to a *cis*- bond through the action of an isomerase. This synthesis pathway is thought to be responsible for $C_{22:6}$ n3 production in *Thraustochytrium*.

The desaturases found within the endoplasmic reticulum in animals and plastid membranes in plants utilise acyl-CoA as a substrate, though soluble, plastid localised desaturases utilise ACP substrates. However some ER membrane localised fungal and plant desaturases can use acyl moieties attached to glycerolipids (Kendrick & Ratledge, 1992c), (Talamo *et al.*, 1973), (Domergue *et al.*, 2003) and it was found by Kendrick *et al*. that desaturation within phospholipids is region-specific. It was found that n3 synthesis occurred at position one, whereas n6 synthesis occurred at position two on the glycerol backbone. This means that specific desaturases may only desaturate correctly positioned acyl substrates. Also of note are that some desaturases are referred to as front end desaturases. This is due to the fact that they introduce a double bond between an

existing double bond and the carboxyl group (Pereira *et al.*, 2003). The mechanism by which fatty acids are desaturated is similar between cellular localisations, although there are differences in reducing agent and electron donor. In the ER associated system, the reducing agent is usually NADH. This provides two electrons to the desaturase, as well as two protons for the water formation. The other two electrons and protons are produced from the formation of the double bond in the fatty acid. Hence, NADH oxidises and passes electrons to the FAD containing cytochrome b5 reductase, which then reduces cytochrome b5. The cytochrome b5 domain is found to be fused to the N-terminus of front end desaturases (Sperling & Heinz, 2001). The cytochrome b5 then reduces the desaturase, which in turn removes two hydrogen and two electrons from the fatty acid at a specific carbon number along the FA chain, introducing a carbon-carbon double bond (Shanklin & Cahoon, 1998). The pathway is shown in Figure 1-7.

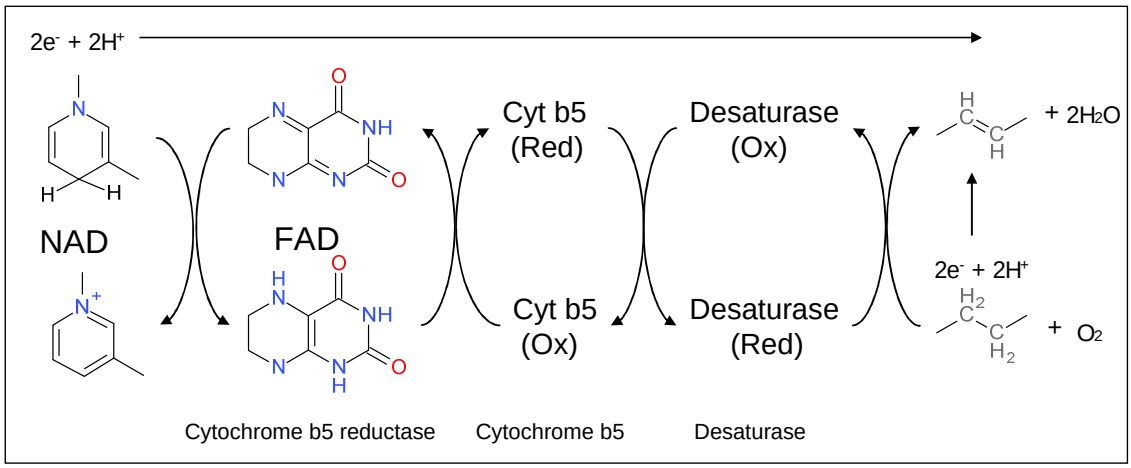


Figure 1-7. The mechanism of desaturation. Desaturases utilise an electron transfer chain to convey electrons from a reducing agent, NADH, to oxygen to remove two hydrogen from the acyl chain to create a double bond and water.

Fatty acids have to undergo several desaturation steps within fungi to become polyunsaturated fatty acids. Firstly within most fungi, $C_{18:0}$ is desaturated by a Δ9 desaturase to $C_{18:1}$. This desaturase was previously thought to have a separate cytochrome b5, although several organisms such as *S. cerevisiae* and *M. alpina* have demonstrated a fused C-terminal cytochrome b5 domain (Sakuradani *et al.*, 1999). Other features typical of this desaturase, as well as the majority of other membrane bound desaturases, is the presence of typical motif features such as three histidine-box motifs and two hydrophobic trans-membrane domains (Pereira *et al.*, 2003). Next a Δ12 desaturase converts $C_{18:1}$ to $C_{18:2}$ n6. This desaturase is lacking within mammals, which explains the necessity of incorporating $C_{18:2}$ n6 into the human diet. This desaturase has been characterised from two *Fusarium* species as well as from *Magneporthe grisea*

(Damude & Zhang, 2006). It was also found that the desaturase had weak substrate affinity for $C_{18:2}$ n6 and could convert it into $C_{18:3}$ n3 and was thought to be the ancestor of the Δ15 desaturase, which is also present within the previously mentioned species. In fact it was shown that the Δ15 desaturase had substrate specificity for $C_{18:1}$ and could form $C_{18:2}$ n6. This overlap in desaturase specificity alluded to the common ancestry of the two enzymes. The role of the Δ15 desaturase is to convert $C_{18:2}$ n6 to $C_{18:3}$ n3, allowing for further fatty acid elongation and desaturation along the n3 route if subsequent enzymes are present.

A further two desaturases are involved in both the n3 and n6 PUFA pathways. The Δ6 desaturase is responsible for the formation of $C_{18:3}$ n6 from $C_{18:2}$ n6, as well as $C_{18:4}$ n3 from $C_{18:3}$ n3. The Δ5 desaturase is responsible for the formation of $C_{20:4}$ n6 and $C_{20:5}$ n3 from $C_{20:3}$ n6 and $C_{20:4}$ n3 respectively (Domergue *et al.*, 2002, Parker-Barnes *et al.*, 2000). The two enzymes have also been characterised in the fungus *Thamnidium elegans* (Δ6) (Wang & Li, 2007) and the oomycete *Phytophthora megasperma* (Δ5) (Hornung *et al.*, 2005). The last desaturase that is known to occur within the fungi is a Δ17 desaturase, which is responsible for the conversion of $C_{20:4}$ n6 to $C_{20:5}$ n3 and has been found within *Mortierella alpina* (Shimizu & Kawashima, 1989) as well as the Oomycete *Saprolegnia diclina* (Pereira & Huang, 2004) with the production of $C_{20:5}$ n3 only occurring in *M. alpina* at low temperature growth. In *S. diclina* it was found that the desaturase only desaturated C_{20} polyunsaturated fatty acids and had no activity on C_{18} fatty acids. The enzyme was found to display the two trans-membrane domains and three histidine motifs thought to be involved with iron binding. It was also shown to be separate from the cytochrome b5 domain as well as being able to utilise acyl-CoA substrate.

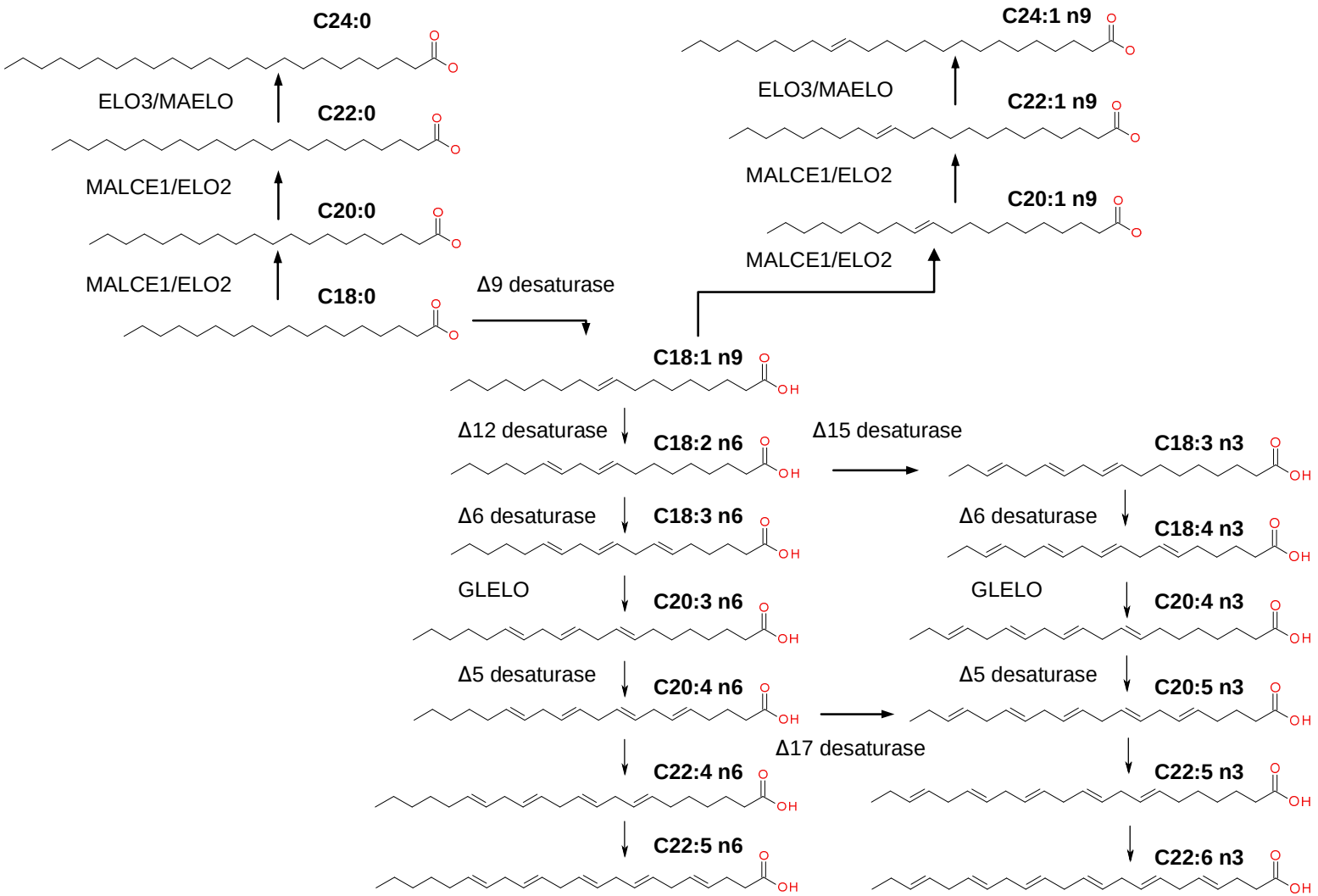


Figure 1-8. The fatty acid elongation and desaturation pathway. Once $C_{16:0}$ has been released from the FAS complex the fatty acid chain may be further elongated and desaturated. Both processes occur at the ER membrane in fungi, however elongation requires four separate enzymes to carry out the individual steps. The rate limiting condensation enzyme is referred to as an elongase.

1.3.8 Oleaginicity

A fungus is described as oleaginous if the lipid accounts for 20% or more of the dry weight, where most of the lipid is stored as triacylglycerides (Zhang & Ratledge, 2007). The synthesis of fatty acids in most fungi requires a sustainable production of acetyl-CoA and NADPH, as both are required for fatty acid elongation. Increased production of these two products can lead to a fungus becoming oleaginous. The method for differentiating oleaginous fungi is to grow them on a high carbon, low nitrogen media, which will induce oleaginous organisms to increase their fatty acid production. The pathway which leads to the accumulation revolves around three key enzymes; the first two are ATP : citrate lyase, and isocitrate dehydrogenase. Isocitrate dehydrogenase is found in the citric acid cycle which converts isocitrate to oxalosuccinate followed by conversion to α-ketoglutarate. A difference between oleaginous and non-oleaginous organisms is the regulation of isocitrate dehydrogenase by adenosine monophosphate (AMP). By restricting nitrogen levels in the media, AMP is degraded by the enzyme AMP deaminase, with its gene up regulated. This in turn releases NH_3 allowing the organism to survive, which also leads to the inactivation of isocitrate dehydrogenase due to it's regulation by AMP, resulting in an accumulation of isocitrate. This is converted back to citrate, which is transported into the cytoplasm via a citrate/malate transporter. Citrate is then acted upon by ATP : citrate lyase converting citrate to acetyl-CoA and oxaloacetate (Ratledge, 2004) as shown in Figure 1-9. This acetyl-CoA can then be used for fatty acid biosynthesis.

The third enzyme responsible for oleaginicity described by Kendrick and Ratledge is malic enzyme (malate dehydrogenase decarboxylating) as it releases NADPH from malate (Kendrick & Ratledge, 1992a), which is also required for fatty acid synthesis as a reducing agent. The cycle was originally thought to occur in the cytoplasm, although a second, membrane bound malic enzyme was also discovered. The cytoplasmic enzyme was postulated to provide NADPH for fatty acid biosynthesis, whilst the membrane bound enzyme was thought to supply NADPH for desaturation and elongation. It was thought that the acyl-lipid Δ6, Δ12 and Δ15 desaturases utilised NADPH for their reducing agent. The mechanism of action involves the conversion of pyruvate to oxaloacetate through the addition of CO_2 and ATP, followed by hydrogenation by NADH to malate. Malate is subsequently converted to pyruvate with the loss of CO_2 and NADPH. The addition of non-endogenous NADPH along with other NADPH producing enzymes does not increase the yield of fatty acids according to Kendrick *et*

al. However, if malic enzyme is expressed constitutively, fatty production can increase by up to two and a half fold (Zhang & Ratledge, 2007). The oleaginicity pathway is shown in Figure 1-9. Therefore oleaginous organisms are defined by their regulation of isocitrate dehydrogenase by AMP, the presence of ATP : citrate lyase and an NADPH generating enzyme such as malic enzyme. Under low nitrogen conditions this results in the accumulation of acetyl-CoA and NADPH, which is fed predominantly to the FAS complex increasing fatty acid biosynthesis. It is also thought by Ratledge that a general NADPH pool does not exist in oleaginous organisms, and that NADPH production is integrated with the fatty acid production machinery (Ratledge, 2004).

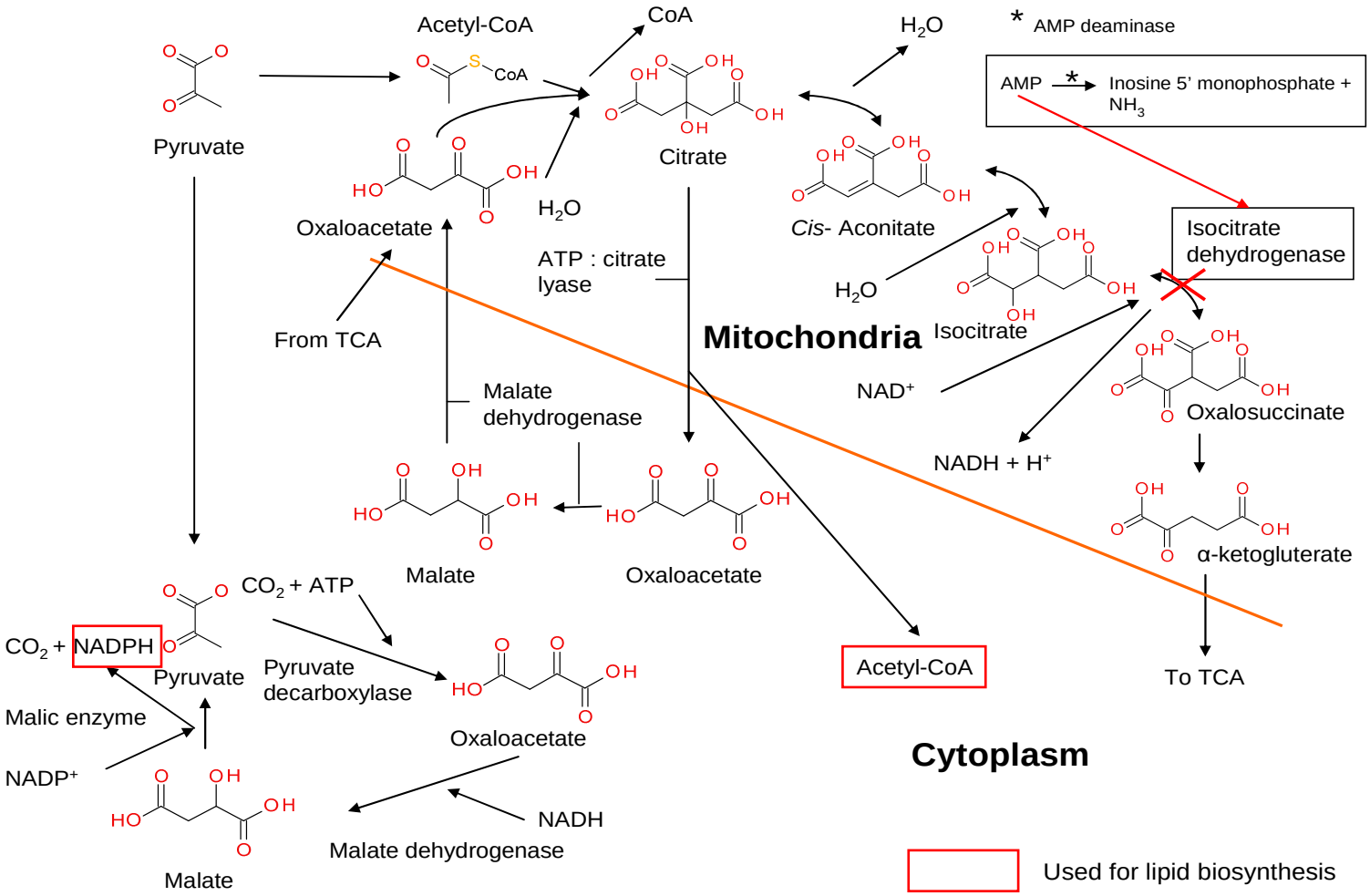


Figure 1-9. The pathways associated with oil accumulation in oleaginous fungi (Ratledge, 2004)**. The regulation of AMP deaminase by AMP and the presence of ATP : citrate lyase and malic enzyme are thought to be indicative of oleaginous organism leading to the accumulation of acetyl-CoA and NADPH, which is funnelled predominantly into the FAS complex.**

1.3.9 Genetic engineering

Several attempts have been made to introduce or increase PUFA levels within plants and fungi, utilising genes from known fungal and algal sources. Initially, to identify and characterise the desaturases and elongases, individual or multiple genes were expressed in organisms which were simple to grow and only produced a limited fatty acid profile, such as *Saccharomyces cerevisiae*. The next step was to express genes of interest in more relevant and commercially viable hosts. As plant oils such as palm, soy and rapeseed account for around 75% of the global vegetable oil production (Damude & Kinney, 2008), and in the case of palm oil, contain a high proportion of saturated fatty acids, creating healthier oils from these plants is desirable. Attempts have been made with *Brassica juncea* (rapeseed) to modify the fatty acid profile. Rapeseed oil contains $C_{18:3}$ n3 as the most unsaturated fatty acid, however with the addition of a Δ6 desaturase from *Pythium irregulare* it was found that $C_{18:3}$ n6, the n6 counterpart of $C_{18:3}$ n3 was produced at levels ranging from 25-40% of the total seed oil (Hong & Datla, 2002). $C_{18:4}$ n3 was also produced in smaller quantities at 2-10% of the total fatty acids (TFA). Tobacco has also been modified with a Δ6 desaturase from *Borago officinalis* (borage) which resulted in the formation of $C_{18:3}$ n6 and $C_{18:4}$ n3, which was formed due to the presence of $C_{18:3}$ n3 in the wild type plant (Sayanova *et al.*, 1997). The presence of both n3 and n6 products was due to the presence of a Δ15 desaturase, which converted $C_{18:2}$ n6 to $C_{18:3}$ n3 which was then acted upon by the inserted Δ6 desaturase. Knocking out enzymes such as the Δ15 desaturase gene can be beneficial if a desired product is required, as it allows for greater availability of specific substrate and prevents competition for enzyme activity, as n3 and n6 pathways compete for desaturases and elongases (Chen *et al.*, 2006).

The next step was the insertion of two desaturases into a host plant, with a Δ6 and a Δ15 desaturase inserted into *Glycine max* (soybean). The host plant was capable of producing $C_{18:3}$ n3 and therefore contained a Δ6 desaturase, however the addition of the Δ6 desaturase from borage and the Δ15 desaturase from *Arabidopsis* led to the formation of $C_{18:4}$ n3, with levels greater than 29% of the TFA (Eckert *et al.*, 2006). It was found that the increased levels of LCPUFAs were primarily incorporated into the TAG fraction, although some were attached to phospholipids. Because of this, levels of $C_{16:0}$ and $C_{18:0}$ were found to increase within the phospholipid fraction, which was thought to compensate for the increased fluidity caused by the increased LCPUFA abundance. Soybean has also been modified with several genes to produce $C_{20:4}$ n6. The

three genes used were all isolated from the fungus *Mortierella alpina* and consisted of a Δ5 and Δ6 desaturase as well as a Δ6 elongase known as GLELO (Chen *et al.*, 2006). The control plant was able to synthesise small quantities of $C_{18:3}$ n3 however, the insertion of the 3 aforementioned genes allowed the production of VLCPUFAs up to $C_{20:4}$ n6, albeit in low abundance with $C_{20:4}$ n6 constituting around 2.1% of the TFA.

The modification of microorganisms is also an important consideration as a large proportion of genes utilised for plant modification are of microbial origin. Several attempts have been made to modify organisms from the fungi. A similar transformation to that undertaken in tobacco and rapeseed was undertaken in the yeast *Hansenula polymorpha*, which normally produces $C_{18:3}$ n3. With the insertion of a Δ6 desaturase, it was capable of producing $C_{18:3}$ n6 as well as $C_{18:3}$ n3 and $C_{18:4}$ n3 (Laoteng & Ruenwai, 2005). The fungus *Aspergillus oryzae* was modified with a Δ9 desaturase from *Mortierella alpina* which resulted in greater quantities of $C_{16:1}$ and $C_{18:1}$ being produced within *A. oryzae* (Sakuradani *et al.*, 1999). As mentioned previously, certain organisms are classified as oleaginous and their potential has not gone unnoticed. The yeast *Yarrowia lipolytica* has been utilised for VLCPUFA production because of its ability to accumulate large quantities of lipid as TAG within its cells. However, many of these modifications have subsequently been covered by patents by companies such as DuPont, with the wild type fatty acid profile of *Y. lipolytica*, with fatty acids up to $C_{18:2}$ n6, being elongated and desaturated further up to $C_{22:6}$ n3 utilising a myriad of genes (Beopoulos *et al.*, 2010).

It is noticeable that transformations utilise genes isolated from the same organisms, one of them being *Mortierella alpina*. One recent method involved transforming *M. alpina* itself using *Agrobacterium tumefaciens*, which is primarily used for plant transformation. This is in contrast to the usual role of *Mortierella* whereby its genes are cloned into other hosts. Ando *et al*. (Ando *et al.*, 2009) showed that the over expression of a Δ17 desaturase in *M. alpina* resulted in a dry weight yield of $C_{20:5}$ n3 of just under six times that of the wild type. The method of *A. tumefaciens* is also applicable to other filamentous fungi as was demonstrated by de Groot *et al*. (de Groot, M. J. A. *et al.*, 1998) and such transformations of VLCPUFA containing species could become more common as recombinant strategies for fungi become more effective and widespread as seen in plant biotechnology. In general, crop plants are a viable, as well as economic source of polyunsaturated fatty acids due in part because of their ubiquitous use in food. Crop modification appears to have predominantly focussed on increasing the yields of

$C_{18:3}$ n3 and $C_{18:4}$ n3 namely because achieving yields of $C_{20:5}$ n3 would require large numbers of genes and many years of optimisation. Whether plants can accumulate large quantities of VLCPUFAs such as $C_{20:4}$ n6 and $C_{20:5}$ n3 is another consideration, as it is possible they could disrupt plant growth making plants unsuitable for large scale VLCPUFA production, as plants try to compensate for the increase in fluidity in their membranes. However if the n3 pathway up to $C_{20:5}$ n3 is achieved commercially in plants, then even small amounts may have a profound impact on our health due to the large number of products these crops find themselves in. However, it would seem likely that microorganism transformation and optimisation will provide a more cost effective and efficient solution to longer chain polyunsaturated fatty acid production. This is due to the greater efficiency with which they are produced and the natural ability of certain species to produce and accumulate them, as well as the developing technology to modify these species. The next section will briefly cover the considerations for industrial production of VLCPUFAs as well as currently known fungal producers of these compounds.

1.4 Fungal PUFA producers

1.4.1 Market

In the past there have been industrial attempts to create microbial oil, from the production of $C_{18:3}$ n6 from *Mucor circinelloides* (Ratledge, 2004) to the production of cocoa butter substitutes (Ward & Singh, 2005). Currently companies such as Martek market $C_{20:4}$ n6 from the organism *Mortierella alpina*. It is estimated that in 2005 the infant formula market, of which companies such as Martek supply VLCPUFA, is worth approximately $10 billion per year (Ward & Singh, 2005). It was suggested by Ward *et al*. that the addition of microbial VLCPUFA to infant formula added an extra 10-20% to the retail price, which is thought to dissuade some consumers from purchase due to the high price. Markets that are expected to show greater growth are the health food (nutraceutical), aquaculture and animal feed industries. Direct inclusion of VLCPUFA into food and liquid products is thought to be problematic due to lipid oxidation, although technologies such as micro-encapsulation may circumnavigate this problem (Jimenez *et al.*, 2004). In regards to industry and lipid production the cost associated with producing these desirable fatty acids determines whether a specific organism is a viable lipid producer. The environmental impact of the lipid producing process is also

worth considering due to the energy intensive processes required. Ideally, VLCPUFA producers need to accumulate large percentages of lipid and produce substantial biomass with a high rate of growth according to Ward *et al*. In oleaginous organisms, the percentage composition of the oil is also important, with oils containing one predominant fatty acid desirable. The VLCPUFA should also be in a TAG form for human consumption.

Algae are one of the currently exploited groups of organisms for VLCPUFA production due to their high accumulation of lipid and relatively large biomass. Algae however are predominantly phototrophic, requiring light and CO_2 to photosynthesise, which in turn produces NADPH from the light dependent reaction and acetyl-CoA from the action of pyruvate dehydrogenase on pyruvate or from β-oxidation of lipids. The requirement for light however, drives costs up as artificial lighting is expensive to continually run, and scaling of algal cultures is difficult and expensive due to this requirement (Li *et al.*, 2008). According to Li *et al*., natural sunlight can be used in conjunction with photo-bioreactors, which capture solar energy in a similar manner to photovoltaic panels to reduce costs. However, natural lighting is not controllable and therefore variations in light level are a consistent problem. Another algal culture method is the raceway pond, which are also naturally lit. However both methods require energy to continually pump algal broth around the system, remove oxygen produced from photosynthesis and harvest the algal culture. Large quantities of water are also required for the circulating algal culture. With increased efficiency, algae could be a viable source of VLCPUFA and in the future may act as a net carbon sink as oppose to a CO_2 emitter. Fungi and several Oomycetes are heterotrophic which reduces the cost of their growth. They can also be grown on waste products such as glycerol i.e. from biodiesel production (Papanikolaou & Aggelis, 2002), food waste (Zhu *et al.*, 2008) (Xue *et al.*, 2006) and sewage (Angerbauer *et al.*, 2008).

Other benefits of microbial production are that there is no requirement for arable land, can be used directly as aquaculture feed and are not generally affected by the season or climate. However, organisms requiring artificially maintained temperatures require increased energy, resulting in increased costs. Microorganisms are also liable to contamination, which would result in the decontamination and destruction of the current batch due to quality control. Plants however are liable to infection, which is remedied by pesticides, but drives up costs and are associated with detrimental environmental effects. This may be remedied by crops genetically modified to resist infection and

insect attack, with the pesticide usage estimated to drop by 14.5 million kg per year if 50% of all maize, oil seed rape, sugar beet and cotton were GM within the EU (Phipps & Park, 2002). Plants are generally not capable of VLCPUFA production, however several mosses such as *Marchantia polymorpha* are capable of $C_{20:4}$ n6 and $C_{20:5}$ n3 production (Shinmen *et al.*, 1991), however production of these VLCPUFAs is usually very low, with slow growth rates and low biomass further reducing their usefulness.

Therefore in crop plants recombinant technology approaches are often used, however VLCPUFA yields are often low (Chen *et al.*, 2006, Damude & Kinney, 2007) which make them uneconomical for VLCPUFA extraction. Bioreactors for microbial growth require a substantial capital investment compared with traditional farming practices. In the long term, the availability of land to grow crops on is the limiting factor, with both food crops and relatively recently, fuel crops competing for arable land. If all soybean and corn production in the USA were reallocated to biodiesel production, it would provide 6% of the country's diesel (Hill *et al.*, 2006). This small percentage demonstrates the required amount of plant material needed to sustain an industry, with VLCPUFA production in direct competition with biodiesel production. Biodiesel according to Hill *et al*. is also much less energy intensive to produce, possibly leading to a reduction of crops grown for bio-ethanol production. Developing a crop solely to produce VLCPUFAs at high levels seems uneconomical as such crops cannot be used for biodiesel and vice versa and reduces the land available for food crops. However, it does seem feasible that food crops will be modified to increase levels of monounsaturated and polyunsaturated fats, although at levels much lower than that found in microorganisms, which most likely will be developed for high purity VLCPUFA containing oils.

One of the targeted markets for microorganism based VLCPUFAs is the aquaculture sector, which has the potential for sustainable and controlled production of fish. Overfishing is foreseen in lowering natural stocks of oily fish such as salmon and tuna, with open water fish stocks being at increased risk of contamination from polychlorinated biphenyls and methyl mercury, which in high enough quantities can be detrimental to health (Yokoo *et al.*, 2003). This puts greater pressure on farm fisheries to produce increased numbers of high quality fish, comparable to their natural open water counterparts. The problem with sustainable farm fisheries is that the feed used usually derives from smaller pelagic fish such as herring and sardine (Pauly *et al.*, 2005) (Hannesson, 2003). This practice is therefore not sustainable and an alternative fish feed

which can supply VLCPUFAs such as $C_{20:5}$ n3 and $C_{22:6}$ n3 is desirable (Harel *et al.*, 2002). The state of the aquaculture industry according to the Food and Agriculture Organization of the UN (FAO) was in 2007 expanding, with levels of fish produced by aquaculture reaching 50.3 million tonnes. Human consumption of fish reached a high of 113.7 million tonnes, with trade in fish again showing growth at $98.1 and $93.5 billion dollars for the world import and export respectively (FAO, 2007). Alternative sources of VLCPUFAs have been found in algal species such as *Crypthecodinium*, *Phaeodactylum tricornutum* and *Tetrahymena pyriformis* (Conner & Stewart, 1976) and some of these are used commercially to produce the valuable VLCPUFA oils. Algae are responsible for the VLCPUFAs found in open water oily fish, as they are consumed by smaller prey fish which leads to the subsequent bio accumulation within larger predatory fish. The aquaculture sector is a viable market for high value VLCPUFA containing oils due to the high nutritional value of oily fish. Aquaculture and supplementation of fish stocks with fungal sources are discussed further in chapter 6.

1.4.2 Currently known producers

In respect to fungal producers of PUFAs, it is found that the majority of fungi have the capability of producing trienoic acids. There is however a distinct divide between the phyla in relation to fatty acid class. Figure 1-10 outlines the trends in fatty acid production within the fungi. It appears that for the most part, the majority of ascomycetes and basidiomycetes produce $C_{18:3}$ n3 as their most unsaturated fatty acid whereas zygomycetes are characterised by their production of $C_{18:3}$ n6 bar the few C_{20} VLCPUFA forming species (Kock & Botha, 1998). There are however a few species capable of further elongation and desaturation, although such known VLCPUFA producers are located within the Zygomycota, Chytridiomycota and the chromistan Oomycota. The majority of fungi however terminate fatty acid production at $C_{18:3}$ n6 and $C_{18:3}$ n3. Therefore in regards to producers of $C_{18:3}$ n6 nearly all literature points to zygomycetes as the primary producers of the fatty acid. The genus *Mucor* contains many species capable of producing $C_{18:3}$ n6. *Mucor circinelloides, M. rouxii* and *M. mucedo* are just some of the species able to produce $C_{18:3}$ n6. *M. circinelloides* has been found to produce 0.25 g/l (Jackson & Fraser, 1998) and 0.8 g/l (Shimizu & Certik, 1999) of $C_{18:3}$ n6. *M. mucedo* was found to have a mycelial $C_{18:3}$ n6 content of 28.4 mg/g (Certik & Sereke, 1993) and *M. rouxii* was found to produce $C_{18:3}$ n6 up to 18.1% of the total fatty acids (Jeennor & Laoteng, 2006). It was also found by Jeennor *et al.*

that different life cycle stages of the organism, the yeast and filamentous form had different fatty acid compositions. These morphologies were induced through the use of culture conditions, such as limiting oxygen or the addition of phenethyl alcohol. The zygomycete *Rhizopus* has also been found to produce $C_{18:3}$ n6, which produced 4.37 mg/g of dry substrate (Conti & Stredansky, 2001).

The Mortierellaceae family contains the genus *Mortierella*, which is well studied for the production of VLCPUFAs. The *Mortierella* genus was once split into two sub-genera, the *Micromucor* and the *Mortierella*, with each sub-genus characterised by their differing fatty acid profiles, with the *Micromucor* only producing up to $C_{18:3}$ n6 and the *Mortierella* sub-genus producing C_{20} VLCPUFAs (Amano & Shinmen, 1992). The *Micromucor* were raised to the genus level (Arx, 1982), which contained the species *Mortierella isabellina* and *Mortierella ramanniana*. However, it was shown that species within the *Micromucor* were more related to the Mucoraceae and these species were transferred to *Umbelopsis* (Meyer & Gams, 2003). Hence *M. isabellina* and *M. ramanniana* are now *Umbelopsis isabellina* and *U. ramanniana* respectively. Literature predating this change in taxonomy uses the *Mortierella* genus and as such will be referred to by that genus name in this work where it applies.

The old *Micromucor* species of *Mortierella* are found to produce up to $C_{18:3}$ n6. *Mortierella isabellina* has been shown to produce 2.44 mg/ml of $C_{18:3}$ n6 (Xian & Yan, 2001) on a media containing 2% hexadecanol. The mycelium was incubated at 5°C for 15 days, which increased the $C_{18:3}$ n6 concentration. This incubation step indicates that low temperatures can increase the level of desaturation. *M. isabellina* strains have also been shown to accumulate up to 22.9% of the TFA as $C_{18:3}$ n6, with the species *M. elongata*, *M. alpina* and *M. ramanniana* having been shown to produce 7.4%, 10.9% and up to 31.4% of the TFA as $C_{18:3}$ n6 respectively (Amano & Shinmen, 1992) in liquid medium. A *M. ramanniana* isolate was found to produce $C_{18:3}$ n6 by Hansson *et al*. (Hansson & Dostalek, 1988) and the levels could be increased by decreasing the culture temperature to 20°C. It was also found that the overall unsaturation index of the fatty acids increased with the decrease in temperature. Total lipid levels however decreased when *M. ramanniana* was subjected to low temperature growth.

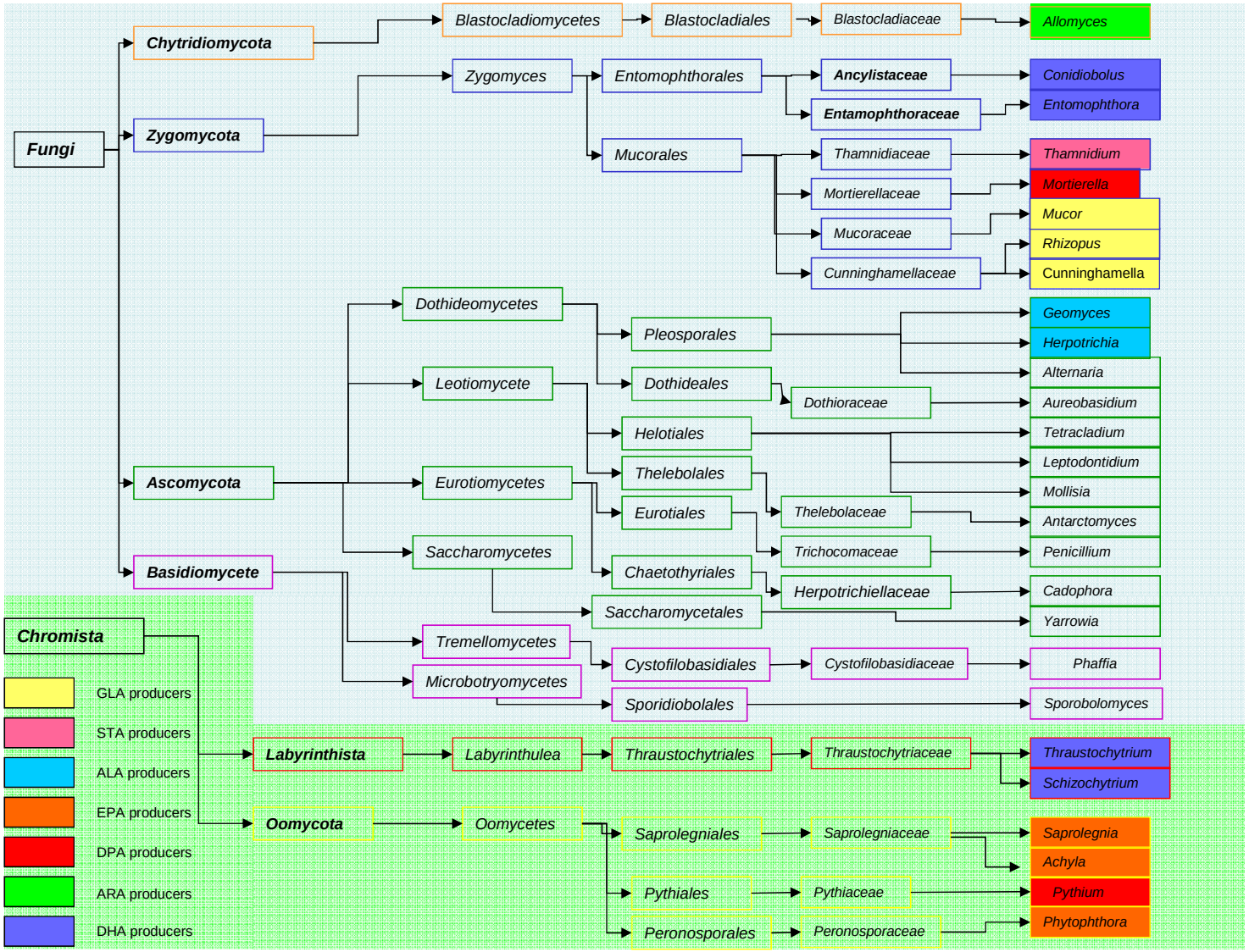


Figure 1-10. Fungal and chromistal producers of polyunsaturated fatty acids. The vast majority of VLCPUFA producers are found within the Chromista, Chytridiomycota and Zygomycota. The Ascomycota and Basidiomycota are thought to be capable of producing up to $C_{18:3}$ n3 only, having acquired a Δ15 desaturase whilst losing a Δ6 elongase and a Δ6 desaturase. Those organisms not in coloured boxes are profiled within this study. Several species in coloured boxes were also profiled within this study.

Another family in the Zygomycota is Cunninghamellaceae. *Cunninghamella echinulata* has been shown to produce 25.1 mg/g of $C_{18:3}$ n6 in the mycelial biomass (Certik & Sereke, 1993), with the species *Cunninghamella elegans* producing 9.26 mg/g of $C_{18:3}$ n6 when grown on dry substrate (Conti & Stredansky, 2001). The cultures were grown directly on varied grains, which could simulate an industrial process for culturing the organisms. It was found that greater yields of $C_{18:3}$ n6 could be produced by lowering culture temperature with the optimum temperature for $C_{18:3}$ n6 being 21°C. Finally, *Thamnidium elegans* was found to contain $C_{18:3}$ n6 at 30% of the total fatty acids (Wang & Li, 2007) when cultured on PDA containing 15% glucose and at 10°C. The strain of *T. elegans* studied by Stredansky *et al*. (Stredansky & Conti, 2000) was found to produce varying quantities of $C_{18:3}$ n6 depending on the culture conditions. The vast majority of fungi within the Ascomycota and Basidiomycota are only capable of trienoic fatty acid production, producing the n3 LCPUFA $C_{18:3}$ n3 (Kock & Botha, 1998). Examples are the fungus *Fusarium moniliforme,* which has been shown to contain the enzyme required for the production of $C_{18:3}$ n3, a Δ15 desaturase (Damude & Zhang, 2006). It was found by Laotang *et al*. (Laoteng & Ruenwai, 2005) that the yeast *Pichia* (*Hansenula polymorpha*) produced $C_{18:3}$ n3 at 10.9% of the total fatty acids (TFA). It would be expected that unknown organisms isolated from cold conditions would produce at least $C_{18:3}$ n6 or $C_{18:3}$ n3. This is due to both fatty acids' 3 double bonds, and their potential fluidising effect within the membrane.

Longer chain C_{20} and C_{22} VLCPUFAs are predominantly produced by organisms within the Zygomycota, Chytridiomycota and the Oomycota. Starting with the zygomycetes, C_{20} VLCPUFAs are produced by the genus *Mortierella*. These *Mortierella* species are found to produce large quantities of the VLCPUFA $C_{20:4}$ n6, which is demonstrated by *M. alliacea*, with the fatty acid comprising 44% of the TFA (Aki *et al.*, 2001). Shimizu *et al*. (Shimizu *et al.*, 1988) demonstrated with several *Mortierella* species the capability of the genus to produce $C_{20:4}$ n6 and $C_{20:5}$ n3. The species studied were *M. alpina, M. hygrophila, M. zychae, M. elongata, M. parvispora* and *M. schmuckeri*. The $C_{20:5}$ n3 and $C_{20:4}$ n6 content was also studied in relation to temperature, with $C_{20:5}$ n3 levels increasing with decreasing temperature and $C_{20:4}$ n6 increasing with increased temperature. $C_{20:5}$ n3 was found not to be produced at the highest temperature of 28°C. Rising levels of $C_{20:4}$ n6 with increased temperature were attributed to the linked biosynthetic pathway of $C_{20:4}$ n6 and $C_{20:5}$ n3, whereby $C_{20:5}$ n3

is formed from the desaturation of $C_{20:4}$ n6. *Mortierella alpina* was found to produce 1.88 g/l of $C_{20:5}$ n3 (Shimizu & Kawashima, 1989), and it was suggested that two routes were used to accumulate $C_{20:5}$ n3. Whilst *Mortierella* does not appear to contain an endogenous Δ15 desaturase, supplementation with $C_{18:3}$ n3 allowed for greater accumulation of $C_{20:5}$ n3. This would suggest that the Δ6/5 desaturases as well as the Δ6 elongase are capable of acting upon n6 as well as n3 substrates. Other documented zygomycetes capable of producing VLCPUFAs are *Entomophthora exitalis* and *Conidiobolus nanodes* which were found to produce up to $C_{22:6}$ n3, albeit in small quantities, up to 3 and 0.8% respectively within the sphingolipids and glycolipids, up to 1.2 and 2.3% within the neutral lipids and up to 1.6% for *C. nanodes* within the phospholipids (Kendrick & Ratledge, 1992b,Kendrick & Ratledge, 1992c). The formation of these VLCPUFAs within these two organisms however, could be the result of already known enzymes acting on $C_{20:5}$ n3 for example, further elongating and desaturating the product as oppose to being the products of novel desaturases and elongases. Within the Chytridiomycota two organisms were identified as producing up to $C_{20:4}$ n6, *Allomyces macrogynus* and *Monoblepharella* sp. with $C_{20:4}$ n6 comprising 13.8% and 9.4% respectively (Southall *et al.*, 1977). Another parasitic chytrid *Zygorhizidium* was found to be capable of producing $C_{20:4}$ n6 within its zoospores at around 8% of the total fatty acids (Kagami *et al.*, 2007).

The chromists contain a large proportion of VLCPUFA producing organisms. Those that were once classified as true fungi are located within the Oomycota. Within this phylum is the genus *Pythium*, which has been shown to produce $C_{20:5}$ n3, with the species *P. ultimum* producing around 0.4 g/l (Shimizu & Certik, 1999). Another *Pythium* strain was shown to elongate $C_{20:5}$ n3 to $C_{22:5}$ n3 (Singh & Ward, 1998), however it is most likely, as with *Conidiobolus* that a non-specific reaction has occurred with a Δ6 elongase predominantly responsible for $C_{18\text{-}20}$ elongation. The paper also showed that *Saprolegnia* species were also capable of $C_{20:4}$ n6 production, although only *Saprolegnia diclina* was capable of $C_{20:5}$ n3 formation at 8.6% of the TFA. *Saprolegnia diclina* has also been noted for its n3 desaturase (Pereira & Huang, 2004). A paper by Aki *et al*. (Aki *et al.*, 1998) shows that *Achlya* is capable of producing up to $C_{20:5}$ n3. The effect temperature had on the fatty acid profile of *Achlya* was also studied. The lowest growth temperature of 15°C promoted the greatest production of $C_{20:5}$ n3 at 14% of the TFA, whilst $C_{20:4}$ n6 levels were found to be greater at the higher temperatures of 20 and 28°C. It was found that when fatty acids were split into neutral and polar lipids, $C_{20:5}$ n3 was in greater abundance in the neutral lipids at the lowest

temperature of 15°C whilst $C_{20:4}$ n6 was found to be greatest at 20°C. In regards to the polar lipids, phosphatidylcholine (PC) was the predominant phospholipid class at all temperatures with the greatest percentage of $C_{20:5}$ n3 found within the phosphatidylethanolamine (PE) fraction at 20°C, although 15°C growth produced the greatest combined total of $C_{20:5}$ n3 within the PE and PC fractions. $C_{20:4}$ n6 was found as the greatest percentage at 20°C growth in the PE fraction. Finally, the organism *Haliphthoros philippinensis* has been found to produce $C_{20:4}$ n6 and $C_{20:5}$ n3, as well as trace quantities of $C_{22:5}$ n3 (Kim *et al.*, 1998).

The final group to produce VLCPUFAs are those organisms found within the Chromista but within the phylum Labyrinthulomycota. Within this phylum reside species such as *Thraustochytrium aureum* which has been found to produce $C_{20:5}$ n3 at levels of 9.1% w/w (Bajpai & Bajpai, 1991). Other thraustochytrid strains have been found to produce $C_{20:5}$ n3 up to 8.19% of the TFA (Bowles & Hunt, 1999), however the percentage of this fatty acid when compared to $C_{22:6}$ n3 was low. It is within this phylum that several $C_{22:6}$ n3 producers reside with the most well studied $C_{22:6}$ n3 producer being the previously mentioned *Thraustochytrium*, with *Thraustochytrium aureum* being shown to produce 0.166 g/l $C_{22:6}$ n3 (Iida & Nakahara, 1996). Other cultivations have produced values of $C_{22:6}$ n3 at 48.5% of the TFA (Bajpai & Bajpai, 1991) and *T. roseum* was found to produce $C_{22:6}$ n3 at 48% of the TFA (Shimizu & Certik, 1999). Another genus related to the *Thraustochytrium* is *Schizochytrium*, with these organisms also having been shown to produce $C_{22:6}$ n3. A similar quantity of $C_{22:6}$ n3 compared with *Thraustochytrium* was obtained from an isolate which resembled *Schizochytrium mangrovei*, producing 2.17 g/l of $C_{22:6}$ n3 (Bowles & Hunt, 1999). Other isolates of *Schizochytrium*, *S. SR21*, were found to produce 15.5 g/l (Shimizu & Certik, 1999), with others shown to have the fatty acid comprising 36.1% of the TFA (Kamlangdee & Fan, 2003).

1.5 Objectives and strategy

1.5.1 Objectives

To identify novel fungal or chromistal producers of VLCPUFA from the CABI and BAS collection.

Over 100 low temperature isolated fungi and several chromist species to be grown using two culture media at 15°C to induce VLCPUFA formation. Fatty acid profiles of isolates to be analysed using GC-FID and GC-MS. The fatty acids extraction, derivitisation and analysis protocols to be confirmed suitable for lipid analysis.

To investigate whether low temperature induces greater lipid unsaturation within fungi.

Several psychrophilic/psychrotolerant fungi, as well as mesophilic isolates are to be grown at 3 temperatures and the total lipid profiles analysed by GC-FID and GC-MS. A preliminary investigation into which glycerolipid fractions primarily experience lipid modification in response to temperature in the fungal isolate *Mortierella alpina* will be explored using TLC and subsequent fatty acid analysis using GC-FID and GC-MS.

To develop a novel PCR based screening methodology to identify VLCPUFA producing fungi. Further to this, to utilise the genes identified for the screen to create a recombinant VLCPUFA producing *Phaffia rhodozyma* strain.

A PCR methodology using primers designed from the Δ6 elongase from *Mortierella alpina* will be developed and tested on both VLCPUFA producing and non-VLCPUFA producing fungi. The Δ6 elongase, as well as the Δ5 and Δ6 desaturases identified as potential PCR targets for the screen, will be cloned and inserted into *Phaffia rhodozyma* to create a $C_{20:5}$ n3 producing strain. The fatty acid profiles of the recombinant strains will be analysed by GC-FID and GC-MS.

1.5.2 Strategy

Table 1-1. The overall strategy of the project, with the priority indicating the order in which the objectives where to be met.

Objectives	Strategy	Priority
Fatty acid analysis using GC-FID and GC-MS **and** refinement of extraction and derivitisation.	GC-FID and GC-MS platform analysis will be carried out prior to fatty acid screening to ascertain suitability. The extraction and derivitisation procedures will be refined based on the results obtained from the FA screen of low temperature isolates.	1
Investigation into the role of temperature on fatty acid profile **and** localisation of lipid modification in response to temperature.	Analysis of several low temperature and mesophilic organisms with respect to temperature will be undertaken first. Localisation studies will be carried out subsequently.	2
The development of a novel PCR based screen **and** the creation of recombinant $C_{20:5}$ n3 producing *Phaffia rhodozyma* strains.	The novel PCR based screen and recombinant *Phaffia* both require shared primer development and gene cloning and will be undertaken in parallel.	3

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals and Kits

The majority of chemicals were sourced from Sigma-Aldrich Ltd, UK. All other compounds were purchased from the following companies:

2.1.1.1 Media

Potato dextrose agar, yeast extract, tryptone and agar no. 3 were from Oxoide, Potato dextrose broth was from Difco.

2.1.1.2 Biochemical reagents

No. 3 filters were from Whatmann, Silica gel 60 plates were from Sigma-Aldrich, 30% acrylamide was from Protogel, Sepharose gel was from Amersham, Comassie blue G250 was from Biorad, antibodies were from Promega, SDS-PAGE running buffer was from National Diagnostics, Molecular rainbow marker for SDS-PAGE was from Amersham.

2.1.1.3 Molecular biology reagents

Plant and fungal DNA extraction kit, blood and tissue DNA extraction kit and plant tissue RNA kit were from Qiagen, DNA loading buffer, ladder and agarose were from Bioline, reverse transcription kit was from Promega, Taq polymerase beads were from GE Healthcare, KOD polymerase was from Novagen, Gel and PCR purification kit and mini-prep kits were from Promega, restriction enzymes were from Promega, TOPO 2.1 vector was from Invitrogen, pET23-b vector was from Novagen, primers were ordered from Operon MWG, TOP10 *E.coli* cells were from Invitrogen, BL21(DE3)pLysis* *E. coli* cells were from Novagen.

2.1.2 Fungal isolates

All isolates used within this work were provided by CABI (Bakeham lane, Egham, Surrey, UK, TW20 9TY) except isolates NCYC 874, 825 and 1464, which were from

the National Collection of Yeast Cultures (Institute of Food Research, Norwich Research Park, Colney, Norwich, UK, NR4 7UA).

Table 2-1. List of organisms used for fatty acid screening with their isolation location and isolation substrate where available.

IMI No.	ID	Location	Isolation substrate
403002	*Herpotrichia* sp.	King George Island, Point Thomas, site 2	*Colobanthus quitensis*
403004	Ascomycota/Sarcosomataceae	King George Island, Potter Cove, site 3	*Colobanthus quitensis*
403008	Ascomycota/Sarcosomataceae	Léonie Island, site 1	*Colobanthus quitensis*
403010	Ascomycota/Sarcosomataceae	Léonie Island, site 1	*Colobanthus quitensis*
403011	*Leptodontidium* sp.	Léonie Island, site 1	*Colobanthus quitensis*
403012	*Leptodontidium* sp.	Léonie Island, site 1	*Colobanthus quitensis*
403014	*Herpotrichia* sp.	South Georgia	*Colobanthus quitensis*
403015	*Herpotrichia* sp.	South Georgia	*Colobanthus quitensis*
403016	*Herpotrichia* sp.	South Georgia	*Colobanthus quitensis*
403017	Ascomycota/Sarcosomataceae	Livingston Island, Hannah Point, site 1	*Deschampsia antarctica*
403019	Ascomycota/Sarcosomataceae	Coronation Island	*Colobanthus quitensis*
403020	Ascomycota/Sarcosomataceae	Coronation Island	*Colobanthus quitensis*
403021	Ascomycota/Sarcosomataceae	Livingston Island, Hannah Point, site 1	*Colobanthus quitensis*
403023	Ascomycota/Sarcosomataceae	Livingston Island, Hurd Peninsula, site 2	*Colobanthus quitensis*
403024	Ascomycota/Sarcosomataceae	Livingston Island, Hurd Peninsula, site 2	*Colobanthus quitensis*
403025	Ascomycota/Sarcosomataceae	Livingston Island, Hurd Peninsula, site 2	*Colobanthus quitensis*
403026	*Pleosporales* sp.	Livingston Island, Hurd Peninsula, site 2	*Colobanthus quitensis*
403027	*Pleosporales* sp.	Livingston Island, Hurd Peninsula, site 2	*Colobanthus quitensis*
403028	*Pleosporales* sp.	Livingston Island, Hurd Peninsula, site 2	*Colobanthus quitensis*
403029	*Pleosporales* sp.	Livingston Island, Hurd Peninsula, site 2	*Colobanthus quitensis*
403030	*Pleosporales* sp.	King George Island, Point Thomas, site 1	*Colobanthus quitensis*

403032	*Pleosporales* sp.	King George Island, Point Thomas, site 1	*Colobanthus quitensis*
403033	*Pleosporales* sp.	King George Island, Point Thomas, site 1	*Colobanthus quitensis*
403037	*Herpotrichia* sp.	King George Island, Point Thomas, site 1	*Colobanthus quitensis*
403038	*Pleosporales* sp.	King George Island, Point Thomas, site 2	*Colobanthus quitensis*
403040	*Pleosporales* sp.	King George Island, Point Thomas, site 2	*Colobanthus quitensis*
403041	*Pleosporales* sp.	King George Island, Point Thomas, site 2	*Colobanthus quitensis*
403042	*Pleosporales* sp.	King George Island, Point Thomas, site 2	*Colobanthus quitensis*
403043	*Pleosporales* sp.	King George Island, Point Thomas, site 2	*Colobanthus quitensis*
403045	*Pleosporales* sp.	King George Island, Point Thomas, site 2	*Colobanthus quitensis*
403046	*Pleosporales* sp.	King George Island, Potter Cove, site 3	*Colobanthus quitensis*
403049	*Pleosporales* sp.	King George Island, Point Thomas, site 2	*Deschampsia antarctica*
403051	*Leptodontidium* sp.	King George Island, Potter Cove, site 3	*Deschampsia antarctica*
403058	*Cadophora/polyscytalum*	South Georgia	*Colobanthus quitensis*
403059	*Cadophora/polyscytalum*	Léonie Island, site 1	*Colobanthus quitensis*
403060	*Leptodontidium* sp.	Léonie Island, site 1	*Colobanthus quitensis*
403061	*Leptodontidium* sp.	South Georgia	*Colobanthus quitensis*
403061	*Leptodontidium* sp.	Lynch Island	*Colobanthus quitensis*
403062	*Leptodontidium* sp.	Lynch Island	*Colobanthus quitensis*
403063	*Leptodontidium* sp.	Lynch Island	*Colobanthus quitensis*
403065	*Leptodontidium* sp.	Lynch Island	*Colobanthus quitensis*
403066	*Leptodontidium* sp.	Lynch Island	*Colobanthus quitensis*
403067	*Leptodontidium* sp.	Lynch Island	*Colobanthus quitensis*
403069	*Leptodontidium* sp.	Lynch Island	*Colobanthus quitensis*
403070	*Leptodontidium* sp.	Léonie Island, site 1	*Deschampsia antarctica*
403072	*Leptodontidium* sp.	King George Island, Potter Cove, site 3	*Colobanthus quitensis*

403073	*Leptodontidium* sp.	King George Island, Potter Cove, site 3	*Colobanthus quitensis*
403074	*Leptodontidium* sp.	King George Island, Potter Cove, site 3	*Colobanthus quitensis*
403075	*Leptodontidium* sp.	King George Island, Potter Cove, site 3	*Colobanthus quitensis*
403077	*Leptodontidium* sp.	Léonie Island, site 2	*Colobanthus quitensis*
403078	*Leptodontidium* sp.	Léonie Island, site 2	*Colobanthus quitensis*
403079	*Leptodontidium* sp.	Léonie Island, site 2	*Colobanthus quitensis*
403080	*Leptodontidium* sp.	Léonie Island, site 2	*Colobanthus quitensis*
403082	*Leptodontidium* sp.	Léonie Island, site 2	*Colobanthus quitensis*
403087	*Mollisia* sp.	Anchorage Island, site 1	*Deschampsia antarctica*
403088	*Mollisia* sp.	Anchorage Island, site 1	*Deschampsia antarctica*
403090	*Mollisia* sp.	Signy Island, site 3	*Deschampsia antarctica*
403091	*Mollisia* sp.	Signy Island, site 3	*Deschampsia antarctica*
403092	*Mollisia* sp.	Léonie Island, site 2	*Deschampsia antarctica*
403093	*Mollisia* sp.	Léonie Island, site 2	*Colobanthus quitensis*
403094	*Mollisia* sp.	Léonie Island, site 2	*Colobanthus quitensis*
403099	*Mollisia* sp.	Signy Island, site 2	*Deschampsia antarctica*
403100	*Mollisia* sp.	Signy Island, site 2	*Deschampsia antarctica*
403102	*Rhizoscyphus ericae* strain	King George Island, Point Thomas, site 1	*Colobanthus quitensis*
403104	*Helotiaceae*	Livingston Island, Hannah Point, site 1	*Deschampsia antarctica*
403109	*Gyoerffyella* sp.	Anchorage Island, site 2	*Deschampsia antarctica*
403110	*Penicillium rugulosum*	Coronation Island, H	*Colobanthus quitensis*
403111	*Gyoerffyella* sp.	Anchorage Island, site 2	*Deschampsia antarctica*
403112	*Gyoerffyella* sp.	Coronation Island, H	*Colobanthus quitensis*
403116		Coronation Island, H	*Deschampsia antarctica*
403119		Coronation Island, H	*Colobanthus quitensis*

403123	*Ascomycota*	King George Island, Point Thomas, site 1	*Deschampsia antarctica*
403124	*Ascomycota*	King George Island, Point Thomas, site 1	*Deschampsia antarctica*
403125	*Ascomycota*	King George Island, Point Thomas, site 1	*Deschampsia antarctica*
403127	*Ascomycota*	King George Island, Point Thomas, site 1	*Deschampsia antarctica*
403129		Signy Island, site 3	*Deschampsia antarctica*
403131		Signy Island, site 3	*Deschampsia antarctica*
403133	*Mollisia* sp.	Signy Island, site 3	*Deschampsia antarctica*
403134	*Mollisia* sp.	Signy Island, site 3	*Deschampsia antarctica*
403135		King George Island, Point Thomas, site 1	*Colobanthus quitensis*
403136		King George Island, Point Thomas, site 1	*Colobanthus quitensis*
403142		Coronation Island, Mansfield point	*Deschampsia antarctica*
403143		Léonie Island, site 2	*Deschampsia antarctica*
403145		Signy Island, site 2	*Deschampsia antarctica*
403147		Livingston Island, Byers Peninsula, site 3	*Colobanthus quitensis*
403151		Livingston Island, Byers Peninsula, site 3	*Colobanthus quitensis*
403158		Livingston Island, Hurd Peninsula, site 2	*Deschampsia antarctica*
403159		Signy Island, site 2	*Deschampsia antarctica*
403177		South Georgia	*Deschampsia antarctica*
403178		South Georgia	*Colobanthus quitensis*
403302	*Geomyces* sp.	Adalaide Island, Rothera Point, Honeybucket	Wood Bait
403303	*Geomyces* sp.	Adalaide Island, Rothera Point, Honeybucket	Wood Bait
403306	*Anarctomyces psychrotrophicus*	Adelaide Island, Rothera, South Cove	Sediment
403307	*Anarctomyces psychrotrophicus*	Adelaide Island, Rothera, South Cove	Sediment
403308	Thelebolaceae	Adalaide Island, Rothera Point, North Cove	Drift Wood

403310		Adalaide Island, Rothera Point, Honeybucket	Ash Bait
403310			
403316	*Cadophora malorum*	Adalaide Island, Rothera Point, Honeybucket	Ash Bait
403318	*Geomyces* sp.	Adalaide Island, Rothera Point, Honeybucket	Pine Bait
403321	*Pleospora/ulocladium*	Adalaide Island, Rothera Point, Honeybucket	Pine Bait
403323	*Tetracladium* sp.	Adalaide Island, Rothera Point, Honeybucket	Beech Bait
403330	*Ascomycota*	Adalaide Island, Rothera Point, Honeybucket	*Eucalyptus* Bark Bait
403332		Adalaide Island, Rothera Point, Honeybucket	Sepele Bait
403333	*Geomyces* sp.	Adalaide Island, Rothera Point, Honeybucket	Sepele Bait
NCYC 874	*Phaffia rhodozyma*	NCYC	Exudate of *Fagus crenata*
NCYC 825	*Yarrowia lipolytica*	NCYC	
NCYC 1464	*Sporobolomyces roseus*	NCYC	*Solanum tuberosum*
Dis 206	*Umbelopsis isabellina*		
Dis 195	*Umbelopsis* sp.		
Dis 169	Clavicipitaceae		
89319	*Trichosphaeria pilosa*	Antarctica, South Orkney	Construction material
396413			
378423	*Alternaria alternata*	Antarctica, Edmonson Point	Soil
377828	*Cladosporium* sp.	Antarctica, Edmonson Point	Soil
369800	*Chrysosporium*	Antarctica	Air
369795	*Thelbolus microsporus*	Antarctica, Signy Island	Air
369790	*Geomyces pannorum*	Antarctica, Signy Island	Air
369788	*Leptodontidium elatius var. elatius*	Antarctica, Signy Island	Air

340093	*Thelebolus*	Antarctic	
312277	*Aureobasidium*	Antarctica	Soil
312275	*Acremonium*	Antarctica	Soil
256351	*Penicillium echinulatum*	Antarctica, South Georgia	*Festuca contracta*
215092	*Embellisia* sp.	Antarctica, Antarctic continent	Soil
82072	*Mortierella alpina*	Great Britain	*Senecio squalidus*
330997	*Mortierella alpina*		*Cucumis sativus*
196057	*Mortierella alpina*	Australia	*Eucalyptus*
403530	*Bjerkandera adusta*	Antarctic	
17313	*Mucor racemosus*	UK	Meat in cold storage
403341	*Penicillium* sp.	Antarctica	
398213	*Mortierella* sp.	Antarctica	
398216	*Mortierella* sp.	Antarctica	
398217	*Mortierella* sp.	Antarctica	
398220	*Mortierella* sp.	Antarctica	
398111	*Mortierella* sp.	Antarctica	
344320	*Achlya americana*	Canada	Soil
340618	*Phytophthora richardiae*	Netherlands	*Zantedeschia*
328662	*Daedaleopsis confragosa*		
332398	*Allomyces macrogynus*	Myanmar	
308259	*Saprolegnia diclina*		
308153	*Pythium irregulare*		
140468	*Mortierella polycephala*	Great Britain	

2.1.3 Media

For sterilisation, all media was autoclaved at 121°C for 20 minutes unless otherwise stated.

2.1.3.1 Potato dextrose (PD) media

Potato dextrose agar (CM0139) from Oxoid was prepared using the manufacturer's instructions. Potato dextrose from Difco (254920) was prepared using the manufacturer's instructions.

2.1.3.2 Malt extract (MA) broth media

Table 2-2. Malt extract broth media composition.

Component	Amount
Amber malt extract (Thomas Coopers, Coopers Brewery, Southern Australia)	20 g
dH_2O	1 litre
pH	6.0 +/- 0.5

2.1.3.3 Yeast extract sucrose (YES) broth media

Table 2-3. Yeast extract sucrose broth media composition.

Component	Amount
Yeast extract, Oxoid (LP0021)	20 g
Sucrose, Fisher (S/8560/64)	150 g
dH2O	1 litre
pH	7.0 +/-0.2

2.1.3.4 Lysogeny broth (LB) media

Table 2-4. Lysogeny broth media composition.

Component	Amount
Yeast extract, Oxoid (LP0021)	5 g
Tryptone	10 g
NaCl	10 g
dH_2O	1 litre
Agar, Oxoid No.3 (for plates)	15 g

2.1.3.5 Antibiotic media additions

Antibiotics were purchased from Sigma-Aldrich and were made up as stock solutions in the appropriate solvent as detailed in Table 2-5. The ampicillin stock solution was filter sterilised using 0.2 μm filters before use.

Table 2-5. List of antibiotics and concentrations used within the study.

Antibiotic	Stock concentration	Media concentration
Ampicillin	50 mg/ml in water	100 μg/ml
Chloramphenicol	34 mg/ml in ethanol	34 μg/ml

2.1.4 Buffers and Solutions

2.1.4.1 DNA gel electrophoresis buffers and solutions

TAE buffer:

Table 2-6. 50x TAE buffer composition

Compound	Amount
Tris base	242 g
Glacial acetic acid	57.1 ml
0.5M EDTA pH 8.0	100 ml
dH_2O	Made up to 1 litre

- Agarose gels were run with a 1x TAE buffer solution, using a 1:50 (v/v) dilution with distilled water.

TBE buffer:

Table 2-7. 5x TBE buffer composition

Compound	Amount
Tris base	54 g
Boric acid	27.5 g
0.5M EDTA pH 8.0	20 ml
dH_2O	Made up to 1 litre

- Agarose gels were run with a 0.5x TBE buffer solution, using a 1:10 (v/v) dilution with distilled water.

Bioline molecular markers:

Table 2-8. The base pair sizes of the components in Hyper ladder 1 and 2 used in this study.

Hyper ladder I	
bp	**bp**
10,000	2,000
8,000	1,500
6,000	1,000
5,000	800
4,000	600
3,000	400
2,500	200
Hyper ladder II	
bp	**bp**
2,000	600
1,800	500
1,600	400
1,400	300
1,200	200
1,000	100
800	50
700	

2.1.4.2 Primers

Table 2-9. Primer names and associated sequences used within the study. Red text indicates a restriction site.

Primer name	Description	Sequence
Uni 1	125 bp Δ6 elongase conserved region amplification, forward	CTACTTCTCCAAGVTCAT
Uni 2	125 bp Δ6 elongase conserved region amplification, reverse	TGACCAACCACCAGATGGT
Uni 3	125 bp Δ6 elongase conserved region amplification, forward	CTACTTCTCCAARVTCAT
Uni 4	125 bp Δ6 elongase conserved region amplification, reverse	TGACVAACCACCAGATGKT
F1	Allomyces elongase amplification, forward	ATGGAGGCGACGACCGACCT
F2	Allomyces elongase amplification, forward	CTACCTGTCTAAGATCCTCG
F3	Allomyces elongase amplification, forward	TTACGACGACTTCTTGGCCAC
R1	Allomyces elongase amplification, reverse	CTCAACTCGGGCATCCAT
1	Δ6 desaturase amplification, forward	ATGGCTGCTGCTCCCAGTGTG
2	Δ6 desaturase amplification, reverse	TTACTGTGCCTTGCCCATC
3	Δ5 desaturase amplification, forward	ATGGGTGCGGACACAGGAAAAAC
4	Δ5 desaturase amplification, reverse	TTACTCTTCCTTGGGACGAAG
5	GLELO amplification, forward	ATGGAGTCGATTGCGCCATTC
6	GLELO amplification, reverse	TTACTGCAACTTCCTTGCC
P1	GLELO amplification, Nde1 restriction, forward	CGGACATATGGAGTCGATTGCGCCATTC
P2	GLELO amplification, Hind III restriction, reverse	CGGAAAGCTTCTGCAACTTCCTTGCC

2.1.4.3 His-tagged protein purification buffers

Table 2-10. Buffers utilised for His-tagged column purification. All buffers are made up in dH2O.

Solution	Compound	Concentration
Charging Buffer	$NiSO_4$	100 mM
Binding Buffer	Tris-HCl pH 7.5	50 mM
	NaCl	0.5 M
	Imidazole	5 mM

Wash Buffer 50 mM	Tris-HCl pH 7.5	50 mM
	NaCl	0.5 M
	Imidazole	50 mM
Wash Buffer 100 mM	Tris-HCl pH 7.5	50 mM
	NaCl	0.5 M
	Imidazole	100 mM
Eluting Buffer	Tris-HCl pH 7.5	50 mM
	NaCl	0.5 M
	Imidazole	400 mM
Strip Buffer	EDTA	100 mM
	Tris-HCl pH 7.5	50 mM
	NaCl	0.5 M
SDS Sample Buffer	0.5 M Tris-HCl pH 6.8	6 ml
	Glycerol	4.8 ml
	10% SDS (w/v)	9.6 ml
	0.05% Bromphenol blue (w/v)	1.2 ml
	dH_2O	24 ml
	β-Mercaptoethanol	14 µl/ml added before use

2.1.4.4 SDS-PAGE buffers and solutions

Table 2-11. Composition of polyacrylamide gels used within the study.

Solution	Compound	Amount
12% separating gel	dH_2O	7.9 ml
24 ml	30% acrylamide	9.6 ml
	1.5 M Tris HCl pH 8.8	6 ml
	10% SDS (w/v)	240 µl

	10% Ammonium persulphate (w/v)	240 µl
	TEMED	24 µl
6% stacking gel	dH_2O	7.9 ml
15 ml	30% acrylamide	3 ml
	0.5 M Tris HCl pH 6.8	3.75 ml
	10% SDS (w/v)	150 µl
	10% Ammonium persulphate (w/v)	150 µl
	TEMED	15 µl

Amersham Rainbow marker:

Table 2-12. Size and colour of components within Amersham polypeptide rainbow markers.

KDa	Colour
225	Blue
150	Pink
102	Green
76	Yellow
52	Purple
38	Blue
31	Orange
24	Beige/green
17	Blue
12	Pink

2.1.4.5 Western blot buffers and solutions

Table 2-13. Composition of buffers used for Western blotting within the study.

Solution	Compound	Amount
Transfer Buffer	Tris	3.03 g (25 mM)
	Glycine	14.41 g (192 mM)
	Methanol	200 ml (20% v/v)
	dH_2O	800 ml
	pH 8.1-8.4	
TBS	Tris	6.055 g (50 mM)
Tris buffered saline	NaCl	8.76 g (150 mM)
	pH 7.4	
TBST	Tris	6.055 g (50 mM)
Tris buffered saline tween	NaCl	8.76 g (150 mM)
	Tween 20	0.05% (v/v)
	pH 7.4	

Table 2-14. Accession numbers of protein sequences used for elongase comparison.

Elongase ID	Accession no.
3 ketoacyl CoA synthase *Arabidopsis*	NP 199189.1
Δ6 elongase *Allomyces macrogynus* WGS	ACDU01000340
Δ6 elongase *Saprolegnia parasitica* WGS	ADCG01000825
ELO1 *Saccharomyces cerevisiae*	NP 012339.1
ELO2 *Saccharomyces cerevisiae*	NP 009963.1
ELO3 *Saccharomyces cerevisiae*	NP 013476.1
ELO2 *Candida dubliniensis*	XP 002417837.1
Δ6 elongase *Phaeodactylum tricornutum*	ABQ18315.1
Δ6 elongase *Pyramimonas cordata*	ACR53359.1
Δ6 elongase *Parietochloris incisa*	ACK99719.1

Δ6 elongase *Marchantia polymorpha*	AAT85662.1
GLELO *Mortierella alpina*	AAF70417
GLELO *Mortierella alpina*	BAF97073.1
MAELO *Mortierella alpina*	AAF71789.1
MALCE1 *Mortierella alpina*	BAI40363.1
Elongase *Physcomitrella patens*	XP 001789388.1
ELOVL1 *Mus musculus*	NP 062295.1
ELOVL2 *Mus musculus*	NP 062296.1
ELOVL3 *Mus musculus*	NP 031729.1
ELOVL4 *Mus musculus*	NP 683743.2
ELOVL5 *Mus musculus*	NP 599016.2
ELOVL6 *Mus musculus*	NP 569717.1
ELOVL5 *Salmo salar*	NP 001130024.1
ELOVL5 *Capra hircus*	BAF49682.1

2.1.4.6 Software used

Table 2-15. Software used within the study.

Program	Source
AMDIS	NIST
ClustalW2	EMBL-EBI
TMHMM	Technical University of Denmark
BLAST	NCBI
CLC sequence viewer	CLC Bio
Genome workbench	NCBI

2.2 Methods

2.2.1 Fungal growth for fatty acid and genomic screening

2.2.1.1 Plate growth

Fungal cultures were resuscitated from cryopreservation, lyophilisation and oil. Cryopreserved samples were immersed in a 30°C water bath, followed by plating the spore suspension or mycelial plugs onto PD plate media. Lyophilised samples were re-hydrated in sterile, distilled water for 30 minutes and the suspension then plated on PD plate media. Oil sample plugs were washed in Tween (0.05% v/v Tween 80), then washed in sterile, distilled water, with the plugs then plated on suitable plate media. Culture plates were inoculated using a tri-point inoculum on suitable media. After a week, the organisms were re-cultured using a tri-point inoculum by taking small portions of mycelia with a sterile needle and plating onto fresh plates. The organisms were grown for a minimum of 1 week prior to broth culture, and were re-cultured at 2-4 week intervals.

2.2.1.2 Broth inoculation for fatty acid screening

Broth culture was carried out in 250 ml Erlemeyer flasks, with a media volume of 100 ml, with organisms being grown in MA, YES and PD broth. All cultures were inoculated into broth using agar plugs. This was done primarily due to the unreliability of propagation when a spore suspension was used. A 5 mm cork borer was used to extract 10 agar/mycelium plugs per broth, which were then inoculated into the broth. Broths were incubated in the dark in a rotary incubator at 15°C, with a rotation speed of 110 rpm, with an incubation period of one to three weeks**,** depending on mycelial growth.

2.2.1.3 Mycelial broth extraction

The mycelial mass was extracted after 1-3 weeks, depending on the growth of the organism. The aqueous extract from the mycelium was separated through a Whatmann No. 3 filter paper, using a Buchner filter vacuum conical flask and a vacuum pump. The mycelium was washed with distilled, sterile water and the mycelium removed and

frozen at -20°C for a period of 24 hours, followed by freeze drying over a period of 24 hours. The dry weight was then taken, and the mycelia frozen until needed at -20°C.

2.2.2 Fungal growth for temperature and its effect on fatty acids

The organisms were grown and maintained on PD plates, followed by culture in triplicate into PD broth for up to 3 weeks at 3 temperatures, 5, 15 and either 20 or 25°C. For two organisms, both of which were *Herpotrichia*, 25°C resulted in no growth. Therefore the organisms were re-grown at 20°C. Because of the slow growth of most organisms at 5°C, all were given an additional 1 week to allow sufficient biomass formation. After harvesting the mycelia from broth, the samples were freeze-dried followed by chloroform/methanol extraction and trans-esterification stated in section 2.2.3.1. Samples were then analysed by GC-FID and GC-MS to confirm the identities of the fatty acids.

Table 2-16. List of organisms grown at three temperatures and their respective length of growth before broth harvest.

Organism	Growth temperature °C	Days of broth growth
Mucor racemosus (17313)	5	14
	15	7
	25	7
Herpotrichia sp. (403016)	5	21
	15	14
	20	14
Herpotrichia sp. (403002)	5	21
	15	14
	20	14
Penicillium rugulosum (403110)	5	7
	15	7
	25	7
Bjerkandera adusta (403530)	5	14
	15	7
	25	7
Mortierella alpina (82072)	5	14

	15	7
	25	7
Mortierella alpina (330997)	5	14
	15	7
	25	7
Umbelopsis isabellina (Dis 206)	5	14
	15	7
	25	7
Umbelopsis sp.(Dis 195)	5	14
	15	7
	25	7

2.2.3 Analytical methods

2.2.3.1 Fatty acid extraction

A 2:1 (v/v) chloroform/methanol (C/M) solution (2ml) was added to 65 mg dry weight of mycelia. The sample was also labelled with 100 µl of 1 mg/ml heptadecanoic acid methyl ester in hexane. The sample was sonicated for 3 minutes and then agitation on a rotary shaker for 30 minutes. The samples were then centrifuged at 3500 rpm to pellet the sediment, and the supernatant removed. A chloroform/methanol re-extraction of the pellet was carried out, with the supernatant pooled with the first extraction. 0.9% (w/v) KCl solution (800 µl) was added to each sample, followed by centrifugation at 3500 rpm to partition phases. The upper methanol layer was removed and the lower chloroform layer dried down under nitrogen. Both fatty acid extraction and trans-methylation were carried out in Pyrex reaction tubes.

2.2.3.2 Fatty acid trans-methylation

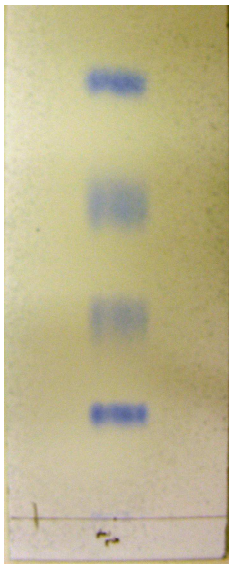
The dried chloroform extract was dissolved in hexane (2 ml) and 1% (v/v) sulphuric acid in methanol (4 ml). The mixture was heated at 85°C for 2 hours, cooled, followed by the addition of hexane (2 ml) and 5% (w/v) KCl solution in water (1 ml). The samples were centrifuged at 3500 rpm to separate the phases. The upper hexane phase was removed using a Pasteur pipette. A further hexane extraction was carried out on the

methanol phase with 2 ml of hexane per sample, with the hexane phases pooled together. The samples were dried under nitrogen and stored at -20°C (Christie, 1989).

2.2.4 TLC purification procedure

2.2.4.1 Separation of phospholipid fractions

Mortierella alpina strain 330997 was grown at 5, 15 and 25°C for 1 week, extracted from broth and then freeze dried. 25 mg of mycelia was extracted using the chloroform/methanol procedure, dried under nitrogen and re-suspended in 100 µl of chloroform. 25 µl (6.25 mg of sample) were loaded onto a preconditioned, glass backed Silica gel (250µl thickness), 20 cm x 20 cm plate. The plate was conditioned and developed using the following solvent system; 58:38.4:2.7:0.9 by volume of chloroform : methanol : acetic acid : water (Kendrick & Ratledge, 1992c), and developed for 1.5 hours. A standard mix of the four phospholipid standards was used to localise the phospholipids. After drying the plate, the phospholipid standards were stained using molybdenum blue stain (Sigma). Areas corresponding to the standards were scraped off from the sample runs.



Rf	Compound
0.71	Phosphatidyl ethanolamine
0.49	Phosphatidyl inositol
0.34	Phosphatidyl serine
0.17	Phosphatidyl choline

Figure 2-1. TLC separation of phospholipid components and their respective Rf values. Visualised using molybdenum blue stain.

2.2.4.2 Elution of TLC fractions

1 ml of 1:1 (v/v) chloroform : methanol solution was added to the scraped silica fractions, and extracted for 30 minutes at room temperature. The samples were then

centrifuged at 3500 rpm and the solvent layer removed. The silica was re-extracted with the same solvent mixture, with the extracts then pooled. The extracts were dried under nitrogen, and then trans-methylated prior to GC-MS.

2.2.5 GC-FID analysis

The samples were analysed on a Varian 8400 GC-FID, with an 1177 injector, a flow rate of 0.8 ml/min of helium, and a 50 m Varian CP-Sil 88 column. Running conditions were as follows; injector set at 250°C, FID at 260°C, with a column oven program of 140°C initially, ramped to 225°C at 4°/min and held for 20 minutes, for a total 45 minutes. The split ratio was 1:50, with a 1 µl injection. Samples were run with a blank (n-hexane) and standard (heptadecanoic acid methyl ester at 1000 ng/µl) preceding the sample runs, with a wash step between each sample. Standards were obtained from Sigma, UK.

Fatty acid identification was carried out using relative retention times, calculated using a 37 FAME component mix spiked with individual FAME standards. Quantitative data analysis was carried out, as response factors were calculated using known quantities of internal standard and fatty acid standards.

2.2.6 GC-MS analysis

The samples were analysed on an Agilent 7890A GC with an Agilent 5975C MS, a flow rate of 1 ml/min of helium and a 30 meter DB-5MS+DG column. The running conditions used were: injector at 250°C, source at 230°C, quadrapole at 150°C. The column oven temperature program was 100°C initially, ramped to 240°C at 4°C/min. The split ratio was 1:50, with a 1 µl injection. The GC-MS was operated using Chem station. Automated mass spectral deconvolution and identification system (AMDIS) and the NIST 05 libraries were used to de-convolute and identify peaks. The AMDIS library was created using the 37 FAME component mix and single FA standards. A retention index and mass spectral database was established to enhance identification of peaks. Quantification of peaks was carried out using AMDIS, with extracted ion areas used to resolve co-eluting compounds and improve quantification.

2.2.7 Molecular biology methods

2.2.7.1 DNA and RNA extraction

DNA was extracted from fungal samples using the Qiagen plant tissue DNA extraction kit, following the manufacturer's instructions. Mouse DNA was extracted using the Qiagen blood and tissue DNA extraction kit, following the manufacturer's instructions. Extracted DNA was stored at -20°C. DNA was quantified using a Nano drop (Thermo Scientific) at 260 and 280 nm. Fungal RNA was extracted using the Qiagen plant tissue RNA extraction kit, following the manufacturer's instructions. RNA was stored at -80°C.

2.2.7.2 Reverse transcription

Reverse transcription of RNA was carried out using the following method, with reagents from Promega:

Attachment of oligo dT primers to RNA:

- RNA (36 ng/µl) = 13 µl
- oligo dT = 1 µl
- water = 1 µl

The reaction was incubated at 70°C for 5 minutes, and then placed on ice.

Reverse Transcription reaction:

- Water = 2.25 µl
- Nucleotides = 1.25 µl
- M-MLV buffer = 5 µl
- RNAase inhibitor = 0.5 µl
- M-MLV Reverse transcriptase = 1 µl

The reaction was incubated at 42°C for 1 hour. 50 µl of sterile water was added after the reaction. The cDNA product was stored at -20°C.

2.2.7.3 PCR

PCRs were carried out using two polymerases, Taq and KOD. The actual PCR varied with the product to be amplified and will be discussed in the relevant sections. All primers were at a stock value of 100 pmol/µl and at a working concentration of 10 pmol/µl.

Taq polymerase:

Taq polymerisation was carried using the Taq bead kit (GE Healthcare). One Taq bead was used for each reaction, which contained the same measure of polymerase per bead. Water, primers and DNA were added to the reaction mix, followed by briefly mixing and centrifuging. The PCR was then thermocycled, the conditions of which depended on the product being amplified. A standard Taq PCR reaction mixture is shown in Table 2-17 with a standard thermocycler program shown in Table 2-18.

Table 2-17. Standard PCR mixture using Taq polymerase beads from GE Healthcare. Values are subject to change depending on the application. However, modifications to the standard procedure are noted.

Reagent	Amount
Taq bead (GE healthcare)	1 bead
Primer 1	1 µl at 10 pmol/µl
Primer 2	1 µl at 10 pmol/µl
DNA template	1 µl
dH_2O	22 µl

Table 2-18. Standard PCR thermocycler temperature program. Any modifications to the procedure are mentioned.

Temperature	Time	Function
95°C	2 minutes	Initial melting step
94°C	30 seconds	Melting step
50-56°C	30 seconds	Annealing step
72°C	30 seconds	Elongation step
72°C	5 minutes	Final elongation step

- Number of cycles =
    - Uni primers 1-4 = 35

- Primers 1-6 and P1 and P2 = 30
- F1-3 and R1 = 35

KOD polymerase:

KOD polymerisation was carried out using the same method throughout the study. The reaction mixture was as follows:

Table 2-19. PCR reaction mix utilising KOD polymerase.

Reagent	Amount
10x buffer	2.5 µl
$MgSO_4$	3 µl
dNTPs	2.5 µl
Primer 1	1 µl at 10 pmol/µl
Primer 2	1 µl at 10 pmol/µl
cDNA	3 µl
KOD polymerase	0.5 µl
dH_2O	22 µl

The thermocycler conditions were as follows:

Table 2-20. PCR thermocycler temperature program for KOD polymerase reaction.

Temperature	Time	Function
95°C	2 minutes	Initial melting step
94°C	20 seconds	Melting step
55°C	1 minute	Annealing step
70°C	30 seconds	Elongation step
70°C	5 minutes	Final elongation step

- 20 cycles

2.2.7.4 Agarose gel electrophoresis

Agarose gels (1 to 3%) (w/v) were prepared depending on the size of the PCR product. 15 ml of agarose solution was used for one 6 or 8 well gel. Agarose was added

to 1x TAE buffer and heated in a commercial microwave for 45 seconds on full power. The solution was allowed to cool slightly before ethidium bromide was added to a final concentration of 0.5 μg/ml. The solution was poured into a mould with a 6 or 8 finger comb and allowed to set. The electrophoresis tank (Embi Tec) was filled with 1x TAE buffer, and the solidified agarose gel placed in the correct orientation.

2 μl of loading buffer was added to 8 μl of PCR reaction, mixed and then centrifuged. All 10 μl of solution was added to one well. 5 μl of DNA ladder was added for reference. The tank was run at 100 V for 20 minutes, followed by UV visualisation and image capture at 312 nm.

2.2.7.5 Gel purification of PCR products

PCR bands were separated using agarose gel electrophoresis. The gel was visualised on an open gel imager, with a UV shield. The band of interest was excised using a clean scalpel, and the weight of the agar piece noted. The PCR and gel purification kit from Promega was used to extract the DNA from the agarose, following the instructions from the manufacturer. The extracted DNA was stored at -20°C.

2.2.7.6 DNA restriction reaction

DNA restriction reactions were carried out using restriction enzymes from Promega. The restriction enzymes used depended on the DNA product. The reaction protocol used for the restriction reaction is as follows:

- DNA = 5 μl
- BSA = 1 μl (0.1 mg/ml)
- HindIII+Nde1 = 0.5 + 0.5 μl
- Multicore buffer = 1 μl
- Water = 2 μl

The reaction was left for 2 hours at 37°C, followed by the reaction mix being purified by the PCR and gel purification kit from Promega. The restricted DNA was stored at -20°C.

2.2.7.7 TOPO-DNA ligation reaction

The TOPO 2.1 kit from Invitrogen was used using the following reaction mix:

Table 2-21. TOPO 2.1 ligation reaction mixture.

Reagent	Amount
PCR insert	4 µl
Salt solution ($MgCl_2$ and NaCl)	1 µl
TOPO 2,1 vector	1 µl

- The ligation reaction was shaken gently and incubated at room temperature for 5 minutes. The reaction was then left on ice for transformation into chemically competent cells.

2.2.7.8 pET-23b ligation reaction

pET-23b ligation calculation:

pET-23b : GLELO = vector to insert is 4.8x larger

Using 100 ng of vector pET-23b gives:

100 ng/4600 bp = 0.022

0.022x950 bp = 20.7 ng GLELO

pET-23b : GLELO ratios of 1:1 and 1:3 were used.

1:1 = 100:20.7 ng

3:1 = 100:62.0 ng

Table 2-22. pET-23b ligation reaction mix.

Reagent	Amount
Vector at 37.4 ng/µl	3.79 µl
Insert at 37.4 ng/µl	0.56 µl
10x buffer (?)	1 µl
Ligase	1 µl
dH_2O	3.65 µl

- The ligation reaction was incubated at 37°C for 3 hours and then kept on ice for transformation into chemically competent cells.

2.2.7.9 Transformation of TOP10 *E. coli* cells

TOPO 2.1 and pET23b vectors were transformed into TOP10 chemically competent *E. coli* cells by heat shock. Chemically competent cells were first thawed on ice, and then 5 µl of ligation reaction mix was added to the cells and incubated on ice for 5-30 minutes. The cells were then heat shocked at 42°C for 30 seconds, then placed on ice for 2 minutes, followed by the addition of SOC media (250 µl) to the cells. The culture was then incubated at 37°C for 1 hour on a rotary incubator, then plated onto ampicillin LB plates with 50 µl and 200 µl of culture used for inoculation. Plates were incubated overnight at 37°C. Four colonies were selected and grown overnight in ampicillin LB broth (5 ml) at 37°C in a rotary incubator. After overnight growth, 1 ml of culture was taken and added to glycerol solution (500 µl), mixed and then stored at -80°C as a glycerol backup. The remaining 4 ml of LB culture was centrifuged and DNA extracted using the mini-prep kit from Promega, following the manufacturer's instructions. The extracted DNA was stored at -20°C.

2.2.7.10 Colony PCR

Colony PCR was used to determine whether *E. coli* colonies had up taken plasmids containing the gene of interest. Transformed colonies were grown overnight on LB agar plates at 37°C. A colony was selected using a sterile Gilson tip, gently removing the colony from the plate. The tip with colony was then introduced into the following PCR reaction mixture:

- Reaction bead (Taq) from GE healthcare
- Water = 23 µl
- Primers = 1+1 µl

The reaction was thermocycled using the following program:

- 95°C initial melting step, 2 minutes
- 94°C melting step, 30 seconds
- 54°C annealing step, 30 seconds
- 72°C elongation step, 30 seconds
- 72°C final elongation step, 5 minutes

- 30 cycles

The tip was then deposited into LB broth (5 ml), with the appropriate antibiotic added and cultured overnight in a rotary incubator at 37°C. The PCR reaction was then run on an agarose gel and visualised as described in section 2.2.7.4. Several colonies from one plate were screened, with those displaying the correct size insert being utilised for further study.

2.2.7.11 Growth of *E. coli* BL21(DE3)pLysis* cells for expression

An initial cell culture of BL21(DE3)pLysis* cells (Novagen) were first streaked out onto LB plates with chloramphenicol at a concentration of 34 μg/ml. The plates were incubated overnight at 37°C. One colony was then picked and inoculated into 5 ml of LB broth with chloramphenicol. The culture was incubated overnight at 37°C in a rotary incubator. 20 ml of fresh LB broth, containing the same concentration of chloramphenicol, was inoculated with 200 μl of the 5 ml starter culture. The broth culture was grown at 37°C in a rotary incubator until an $OD_{578\ nm}$ of 0.3-0.8 was achieved. The culture was then transferred to a 50 ml Falcon tube, where the cells were cooled on ice for 10 minutes. The cells were then centrifuged at 3000 rpm for 10 minutes at 4°C.

The LB media supernatant was decanted and 4 ml ice cold, filter sterilised $CaCl_2$/glycerol (0.1 M/10% v/v) was added to the cells, which were re-suspended and kept on ice for 20-60 minutes. The cells were then centrifuged at 3000 rpm at 4°C for 10 minutes followed by the removal of the supernatant. The cells were again re-suspended in 400 μl ice cold $CaCl_2$/glycerol solution. 25 μl aliquots were taken and dispensed into 1.5 ml Eppendorf tubes, and stored at -80°C.

2.2.7.12 Transformation of BL21(DE3)pLysis* cells with pET23b-GLELO

The pET23b vector containing GLELO (pET23b-GLELO) was transformed into BL21(DE3)pLysis* cells. The transformation procedure was the same as previously mentioned for the transformation of TOP10 cells, however the following changes were made. Agar plates and LB broth media contained both ampicillin and chloramphenicol as selection antibiotics, at the concentrations stated earlier. Glycerol backups of the 5 ml LB media liquid culture colonies were made, and colony PCR of the plated cells was

also performed to check for gene insertion. However, the overnight 5 ml LB broth culture was not harvested the following day for DNA extraction. Instead, fresh LB broth (400 ml) with corresponding amounts of ampicillin and chloramphenicol were made up in 2 litre flasks, and 2 ml of overnight broth starter culture was inoculated into these broths. These broths were cultured at 37°C for around 3 hours in a rotary incubator until an $OD_{578\ nm}$ of 0.6-1.0 was observed. 160 μl (stock 1 M) of the inducer IPTG was added to the culture flasks to a final concentration of 0.4 M. The flasks were then transferred to rotary shakers at 25°C overnight. The broths were then transferred to 500 ml centrifuge tubes, and centrifuged at 4000 rpm for 20 minutes at 4°C. The supernatant broth was decanted off, and the pellet re-suspended in protein binding solution (10 ml). The re-suspended cell suspension was then frozen at -20°C. An aliquot of re-suspended cell pellet was then taken. SDS sample buffer (30 μl) mixed with β-mercaptoethanol (14 μl) was added to 30 μl of cell suspension, and then boiled for 5 minutes in sealed Eppendorf tubes. The samples were then frozen at -20°C.

2.2.8 Protein Analysis

2.2.8.1 Purification of His-tagged GLELO from BL21(DE3)pLysis* cells

BL21(DE3)pLysis* cells suspended in protein binding solution were defrosted and lysed by sonication. The sonicator was 20 - 25 W (20% power). Samples were kept cool by placing them into ice water whilst sonicating. Samples were sonicated 5 times for 30 seconds, with a 30 second break between each sonication. A 30 μl aliquot was taken of the sonicated lysed cells and boiled with SDS buffer (30 μl) (table 2-10). The lysate was aliquoted in 2 ml fractions and centrifuged for 5 minutes at 14000 rpm at 4°C. A 30 μl aliquot of the supernatant from the centrifuged sonicated cell solution was taken, and boiled with SDS buffer (30 μl) (pre-column supernatant).

Columns containing approximately 4 ml of resin (Chelating Sepharose Fast Flow, Amersham) were used to purify His-tag proteins from LB cultures. The resin was first cleaned by running distilled water through the column and resin. The column was then loaded with charge buffer and equilibrated with binding buffer. The supernatant of the sonicated extract was applied to the column, and the eluent collected. 30 μl of eluent was removed and boiled with SDS buffer (30 μl). The cell pellet from the centrifugation of lysed cell material was re-suspended in of binding buffer (2 ml), and a 30 μl aliquot taken and boiled with SDS buffer. The column was then washed with several buffer solutions to elute unbound proteins. The first buffer used was binding buffer (20 ml),

followed by 50 mM (20 ml) wash buffer, finally followed by 100 mM (20 ml) wash buffer. The fractions were collected in falcon tubes, and 30 µl of each eluent was boiled with SDS buffer. The bound protein was eluted in approximately 2 ml fractions in separate Eppendorfs with eluting buffer. Each fraction was tested using comassie blue G250 dye (BioRad). Drops (10 µl) of distilled water were applied to a parafilm strip, with 5µl of comassie added to each drop. A 5 µl aliquot of each eluted fraction was mixed with a comassie spot, with the formation of a distinct blue colour indicating the presence of protein. Any fraction eluted with eluting buffer and found to contain protein had a 30 µl aliquot removed and boiled with SDS buffer (30 µl). The resin was regenerated by applying a 1:5 dilution of strip buffer to the column, and collecting the nickel eluent. Protein containing fractions were analysed by SDS PAGE.

2.2.8.2 SDS PAGE

The separating gel was loaded into the gel apparatus after first adding TEMED (24 µl). A thin layer of water saturated butanol was poured on top of the gel, which was allowed to set. Once set, the butanol was poured off and the top of the gel rinsed with distilled water. The stacking gel was then added, with the addition of TEMED (15 µl) shortly beforehand, to the already set lower separating gel. Combs were then placed into the stacking gel and the gel allowed to set. The gel was then transferred to the running tank, which was filled with 1x running buffer (National Diagnostics). Samples were then loaded onto the gel, with 5 µl of molecular rainbow marker (Amersham) loaded, 10 µl of all other samples except those eluted with elution buffer, of which 20 µl was loaded. The gel was run for 2 hours at 100 V. The gel was then stained using the silver staining kit from Sigma Aldrich, following the manufacturer's instructions.

2.2.8.3 Western blotting

The cooling block used in the Western blotting apparatus was filled with distilled water and frozen overnight. The following day the SDS PAGE gel was run. Whilst the gel was running, a polyvinylidine fluoride (PDVF) membrane was cut to the size of the gel, and then equilibrated in methanol for 10 seconds. The membrane was then rinsed in distilled water, followed by equilibrating in transfer buffer at 4°C. Any visible air bubbles were removed. Two pieces of filter paper with a thickness greater than 3mm were cut to the size of the gel. These as well as the fibre pads were emerged in the cold transfer buffer, with visible air bubbles removed. The blotting equipment was thoroughly washed with distilled water. The buffer tank was filled half way with

transfer buffer, and a magnetic stirrer placed at the bottom. The frozen Bio-ice cooling unit was inserted to cool the buffer. The gel from the SDS PAGE was rinsed in transfer buffer, and the stacking gel removed with a scalpel. The holder cassette was removed from the trans-blot electrode casing. The cassette was opened and membrane and gel loaded. Briefly, a pre-soaked fibre pad was placed on the cathode plate, followed by a piece of saturated filter paper on the top. Around 2 ml of transfer buffer was poured onto the surface and the pad and paper compressed to remove air bubbles from the apparatus. The gel was aligned in the centre of the filter paper and the surface again flooded with transfer buffer. The other pre-soaked membrane was placed on top, with light compression to remove any air bubbles. The surface was flooded with transfer buffer, with the last piece of saturated filter paper followed by the second pre-soaked fibre pad placed on top. Finally, pressure was applied to remove trapped air bubbles. The cassette was carefully closed, and inserted into the electrode housing in the correct orientation. The electrode housing was then inserted into the buffer tank, which was then filled with transfer buffer and run at 100 V for 1 hour.

After the transfer procedure, the membrane was removed and placed into a clean box. The membrane was placed so that the side in contact with the gel was facing upwards. 0.1 ml of blocking solution (TBST with 1% BSA w/v) per cm^2 of membrane was added and incubated for 1-2 hours at room temperature or overnight at 4°C with gentle agitation.

After incubation, the blocking solution was discarded. The membrane was then immediately incubated with the primary antibody (anti-His). The membrane was incubated in 15 ml TBST with a 1:3000 dilution of anti-His antibody, as well as 1% (w/v) BSA. The blot was incubated for 1 hour at room temperature with gentle agitation. After incubation, the solution was discarded and the membrane rinsed 3 times for 10 minutes with TBST. The membrane was then incubated in 15 ml of TBST with a 1:5000 dilution of anti-mouse peroxidase and 1% (w/v) BSA for 1 hour with gentle agitation. The membrane was rinsed 3 times for 10 minutes in TBST buffer, followed by rinsing in TBS twice for a minute each. The membrane was visualised using BCIP/NBT stain (Sigma). Briefly, a tablet was dispensed into 20 ml of distilled water and vortex thoroughly for 2-5 minutes. The solution was then poured over the membrane and the stain allowed to develop for 5-10 minutes. The staining was stopped using copious amounts of distilled water, followed by rinsing the membrane with

distilled water. The membrane was then air dried in a dark, dry environment for visualising.

3 Fatty acid profiling of novel fungal strains isolated from psychrophilic environments

3.1 Introduction

Alternative producers of fatty acids are sought to supplement the primary source of VLCPUFA production, oily fish. Algal fermentation is an alternative source of these polyunsaturates although the high energy cost required to culture algae is a major consideration for any company wishing to exploit this source. Alternative organisms which have been studied derive from the fungi and the Chromista. Fungi offer the attraction that they are not phototrophic, can be grown on waste material due to their saprotrophic nature and can be grown in liquid or solid culture. The main issue is that only a handful of true fungi are capable of producing these VLCPUFAs. Fungi such as *Mortierella* have been studied extensively because of their ability to produce the VLCPUFAs arachidonic acid and eicosapentaenoic acid. Many parameters have been studied in relation to increasing the yield of these fatty acids, and this Fungus is utilised in the industrial production of $C_{20:4}$ n6. The migration from an aqueous to a terrestrial habitat led to the formation of the zygomycetes, which retained several features that highlight their ancestry to the chytrids such as the lack of septate hyphae, the presence of sporangia as well as the prevalence of the n6 over the n3 pathway. The Dikarya evolved predominantly from the zygomycetes but have lost the n6 pathway as well as the Δ6 elongase. Therefore these organisms primarily produce up to $C_{18:3}$ n3. It has been found that low temperatures stimulate the production of unsaturated fatty acids to increase the fluidity of the membrane. It was reasoned that organisms isolated from low temperatures may have adapted to the conditions through selection. Therefore, organisms were studied which were isolated from the Antarctic, which endures constantly low temperatures. It was thought that either organisms such as zygomycetes or chytrids would predominate due to the presence of a Δ6 elongase capable of producing VLCPUFAs, or that if ascomycetes or basidiomycetes were isolated, they would have retained the Δ6 elongase or developed a similar elongation or desaturation mechanism. One of the themes within this chapter with regard to fungi is the role of taxonomy as an indicator of VLCPUFA production and whether temperature is a

selection factor for VLCPUFAs, or whether other factors are responsible for the presence of these novel fatty acids. Organisms from the recently deposited British Antarctic Survey (BAS) collection and those organisms isolated from low temperature environments will be studied utilising a lipid screening methodology using GC-FID and GC-MS as the analysis platforms.

3.1.1 Aim

The aim of this part of the study is the fatty acid profiling of a novel collection of Antarctic and low temperature isolated fungi utilising GC-FID and GC-MS as the analysis platforms, with the initial aim of identifying long chain polyunsaturated fatty acids from novel fungal producers.

3.2 Results

3.2.1 GC-FID and GC-MS fatty acid screening development

3.2.1.1 Extraction efficiency and reproducibility

The extraction and derivitisation procedure was assessed by studying the extraction recovery efficiency and the quantification reproducibility on four isolates using three technical repetitions per sample (table 3-1). The recovery and quantification were carried out using the internal standard (IS) heptadecanoic acid. The recovery was calculated by spiking a known amount of heptadecanoic acid into the sample matrix and comparing it to the value a 100% conversion would give of heptadecanoic acid methyl ester. The majority of instances saw recovery values over 100% indicating that error had been introduced during the procedure. Likely sources of error could come from increased dilution of the heptadecanoic acid methyl ester stock solution or from pipette error when spiking samples. Slight sample concentration may have occurred prior to autosampler injection. The high values of IS spike compared with the control would indicate an efficient method for the analysis of fatty acids even though the current data cannot be quantified. The reproducibility data favoured using the IS to eliminate technical repetitions as standard error between technical replicates was low. The high error associated with sample 398111 was due to an outlier, although for the purposes of screening only one technical repetition was deemed necessary as the internal standard

compensates for the majority of error associated with the extraction, derivitisation and analysis methodology.

Table 3-1. Reproducibility between three technical repetitions using GC-FID as the analysis platform. The use of the internal standard heptadecanoic acid when used for fatty acid extraction and derivitisation negates the need to perform technical repetitions due to the proportionally low standard error of the mean (SE). The error associated with isolate 398111 was due to an outlier. Fatty acid values are in mg/g of biomass.

FA	398111	SE	NCYC 825	SE	330997	SE	82072	SE
$C_{12:0}$	0.84	0.08	0.00	0.00	0.00	0.00	0.00	0.00
$C_{14:0}$	6.54	0.59	0.19	0.01	1.27	0.01	0.88	0.03
$C_{15:0}$	0.00	0.00	0.18	0.00	0.60	0.02	0.64	0.00
$C_{16:0}$	32.85	3.39	23.87	0.51	21.23	0.21	8.12	0.11
$C_{16:1}$	0.28	0.03	11.69	0.11	0.00	0.00	0.00	0.00
$C_{17:1}$	0.00	0.00	1.77	0.03	0.11	0.01	0.30	0.01
$C_{18:0}$	24.09	2.90	16.97	0.45	13.04	0.24	4.17	0.06
$C_{18:1}$ cis	76.65	9.78	107.63	1.71	4.79	0.58	6.11	0.05
$C_{18:2}$ cis	10.28	0.76	44.70	0.25	0.84	0.24	0.82	0.07
$C_{20:0}$	0.86	0.10	1.07	0.04	0.97	0.05	0.23	0.01
$C_{18:3}$ n6	8.22	0.67	0.00	0.00	0.51	0.15	0.51	0.02
$C_{20:1}$	1.97	0.27	0.25	0.01	0.40	0.03	0.40	0.00
$C_{21:0}$	0.00	0.00	0.00	0.00	0.14	0.02	0.00	0.00
$C_{20:2}$ n6	0.26	0.04	0.24	0.04	0.31	0.05	0.33	0.04
$C_{22:0}$	1.48	0.21	1.08	0.04	2.35	0.05	0.54	0.02
$C_{20:3}$ n6	11.09	1.33	0.00	0.00	1.13	0.45	0.30	0.01
$C_{22:1}$ n9	0.24	0.03	0.00	0.00	0.00	0.00	0.00	0.00
$C_{20:4}$ n6	8.71	0.84	0.00	0.00	1.42	0.52	0.51	0.03
$C_{24:0}$	1.61	0.22	2.61	0.10	2.77	0.06	0.45	0.07
$C_{20:5}$	0.95	0.07	0.00	0.00	0.32	0.03	0.00	0.00
$C_{24:1}$ n9	2.07	0.29	0.00	0.00	0.42	0.04	0.22	0.01

3.2.1.2 GC-FID and GC-MS parameters

Fatty acids were identified using a 37 fatty acid methyl ester (FAME) standard to calculate the relative retention time compared with the internal standard heptadecanoic acid methyl ester on the GC-FID as shown in figure 3-1 The 37 FAME standard was also utilised for creating a fatty acid mass spectral library with associated retention indices for more accurate identification using the GC-MS. Response factors for both GC-FID and GC-MS in relation to heptadecanoic acid methyl ester were also calculated using the 37 FAME standard. The response factor is a corrective factor to account for the differing response of the detector to different compounds relative to the internal standard, i.e. the same amount of internal standard and compound of interest may result in different integrated areas. The resolution of the CP-Sil 88 column used in the GC-FID is sufficient for resolving *cis-trans* isomerism as well as $C_{18:3}$ n3 from $C_{18:3}$ n6. This column is more polar in nature and hence elutes fatty acids with a greater numbers of double bonds after those that are more saturated.

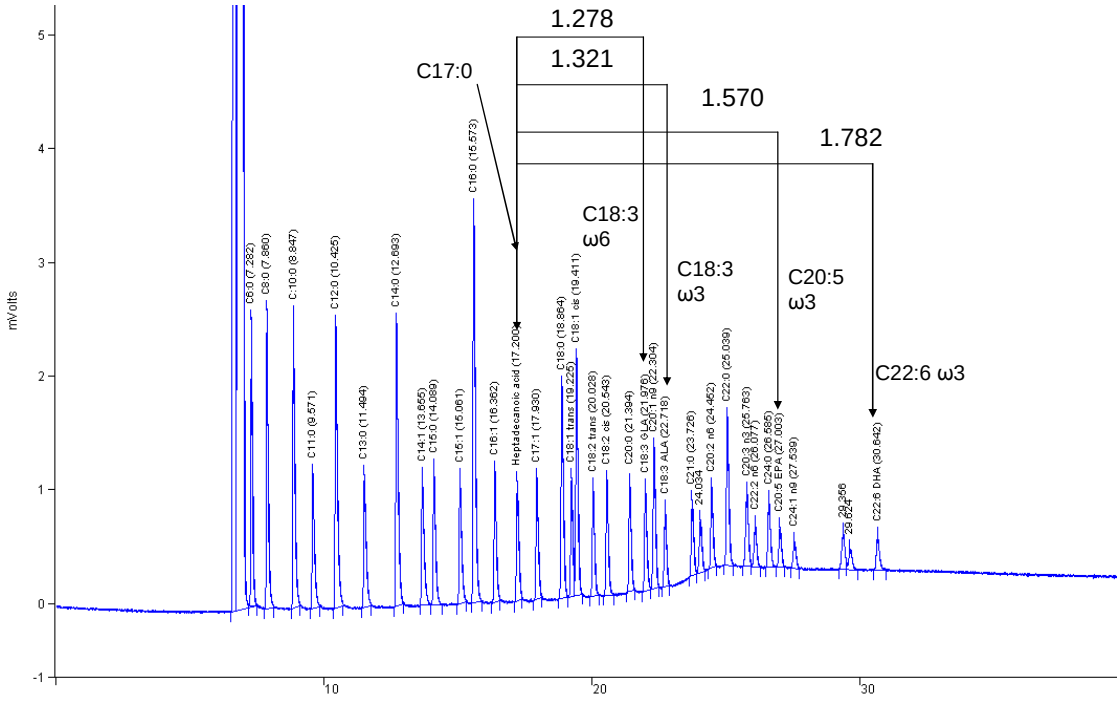


Figure 3-1. GC-FID trace of 37 FAME standard using the CP-Sil 88 column. Good resolution is achieved using the column and oven program specified in the materials and methods section. For compound identification, the most accurate method is to calculate relative retention times (RRT) to the internal standard, methylated heptadecanoic acid. The RRTs are calculated as the ratio of the eluted compound's retention time over the retention time of the internal standard. Absolute retention times can lead to false identifications as compounds may elute slightly earlier or later although RRTs can help minimise this.

Table 3-2. Table showing the parameters used for identification and quantification using the GC-FID. RRT = relative retention time, MQ = minimum quantifiable limit in ng, RF = response factor, SE = standard error of the response factor. The minimal quantifiable amount for $C_{4:0}$, $C_{6:0}$ and $C_{8:0}$ resulted in values that were negative due to the shallow slope of the response vs. amount in ng plot.

FA	RRT	MQ/ng	RF	SE	FA	RRT	MQ/ng	RF	SE
$C_{4:0}$	0.31	N/A	0.21	0.02	$C_{18:2}$ cis	1.218	0.86	1.06	0.02
$C_{6:0}$	0.34	N/A	0.57	0.09	$C_{20:0}$	1.248	0.91	1.08	0.03
$C_{8:0}$	0.38	N/A	0.76	0.06	$C_{18:3}$ n6	1.277	0.97	1.04	0.03
$C_{10:0}$	0.459	0.65	0.87	0.04	$C_{20:1}$	1.295	0.85	1.08	0.03
$C_{11:0}$	0.514	0.86	0.90	0.02	$C_{18:3}$ n3	1.311	0.88	1.05	0.02
$C_{12:0}$	0.58	0.89	0.94	0.03	$C_{21:0}$	1.33	0.87	1.09	0.03
$C_{13:0}$	0.656	0.89	0.99	0.05	$C_{20:2}$ n6	1.378	0.82	1.05	0.04
$C_{14:0}$	0.739	0.91	1.01	0.03	$C_{22:0}$	1.408	0.89	1.08	0.03
$C_{14:1}$	0.812	0.84	1.00	0.03	$C_{20:3}$ n6	1.44	0.92	1.04	0.03
$C_{15:0}$	0.825	0.89	1.02	0.02	$C_{22:1}$ n9	1.46	0.58	1.07	0.03
$C_{15:1}$	0.903	0.93	1.00	0.03	$C_{20:3}$ n3	1.474	0.92	0.99	0.03
$C_{16:0}$	0.914	0.92	1.05	0.03	$C_{20:4}$ n6	1.486	0.96	1.46	0.04
$C_{16:1}$	0.976	0.85	0.99	0.03	$C_{23:0}$	1.497	1.17	0.64	0.10
$C_{17:0}$	1	1.01	1.00	0	$C_{22:2}$ n6	1.55	0.78	0.99	0.05
$C_{17:1}$	1.062	0.95	1.04	0.02	$C_{24:0}$	1.583	0.84	1.04	0.04
$C_{18:0}$	1.086	1.00	1.06	0.02	$C_{20:5}$ n3	1.603	0.97	1.03	0.02
$C_{18:1}$ trans	1.123	0.85	1.12	0.02	$C_{24:1}$ n9	1.648	0.78	1.12	0.04
$C_{18:1}$ cis	1.142	0.98	1.06	0.02	$C_{22:5}$ n3	1.841	N/A	1.00	N/A
$C_{18:2}$ trans	1.183	0.93	1.03	0.02	$C_{22:6}$ n3	1.91	0.77	0.92	0.05

For the GC-FID the response factors for most fatty acids are around 1, as shown in table 3-2, indicating that the fatty acids produce the same area as the IS when the same amount is introduced into the detector. The response factors were calculated over five concentrations and the standard error of these responses was taken. The generally low standard error indicates that the response factors vary little over the concentration range. The minimum quantifiable amount was calculated by plotting amount against integrated

area and using y = mx + c for each compound, where y is the integrated area and x is the amount. The GC-FID would not integrate any compound with an area less than 1000, so x was calculated when y = 1000. In general the minimal quantifiable amount by the GC-FID was just under 1 ng. The plots (figures 3-2 to 3-4) were also used to determine the detector linearity over several concentrations. In general the R^2 value for most of the compounds was around 0.99 indicating that there was a strong linear correlation between area and amount. The saturated fatty acids were in higher abundance within the standard mixture and still maintained good linearity over the concentration range.

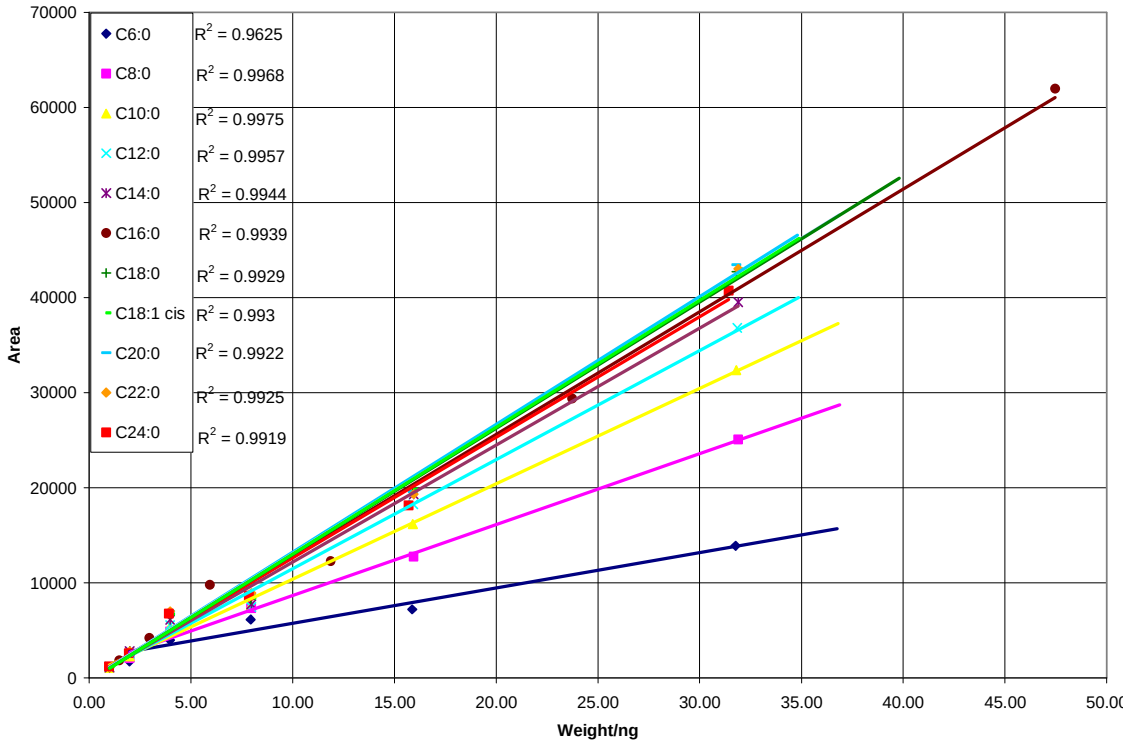


Figure 3-2. GC-FID detector linearity of saturates. The detector linearity with regards to area and concentration with the saturated fatty acids is high with the exception of $C_{6:0}$, although $C_{8:0}$ and $C_{10:0}$ also exhibit lower responses when compared to the other saturated fatty acids. The saturated fatty acids were in higher abundance within the standard mixture, up to twice the concentration of the remaining fatty acids although good linearity was maintained throughout the range.

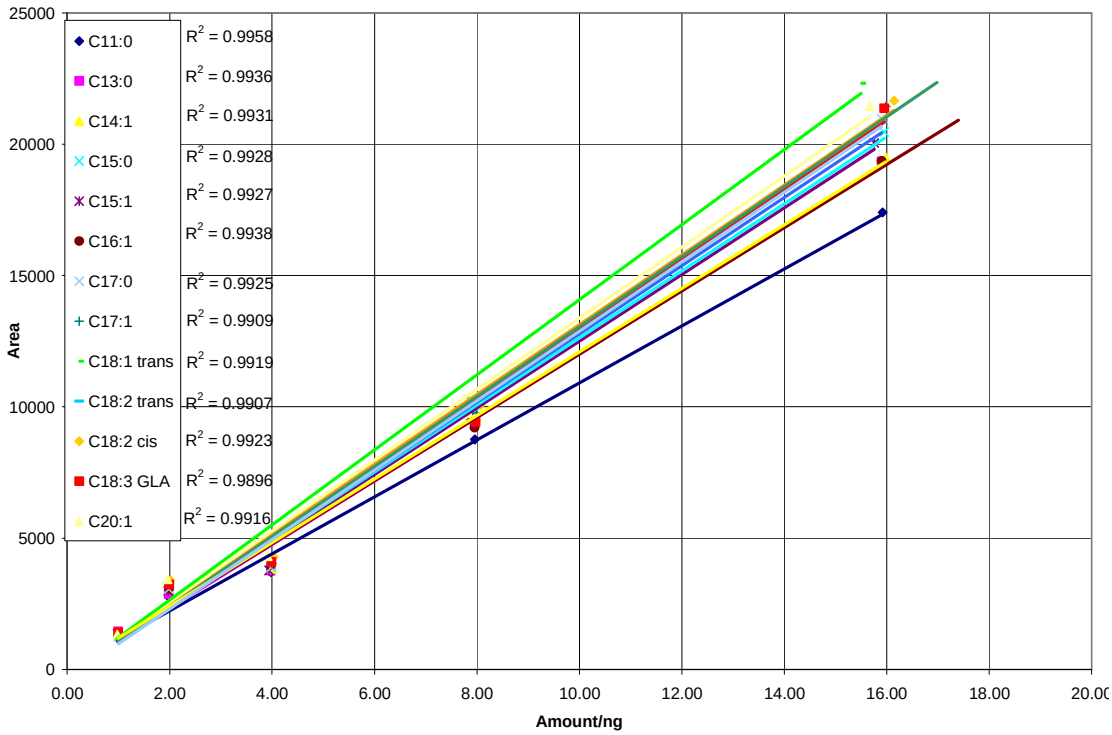


Figure 3-3. GC-FID detector linearity of unsaturates. Lower abundance saturates and unsaturates also demonstrated good detector linearity with all values excluding $C_{18:3}$ n6 having R^2 values above 0.99. The deviation in the points at 4 and 8ng is possibly due to error in sample preparation.

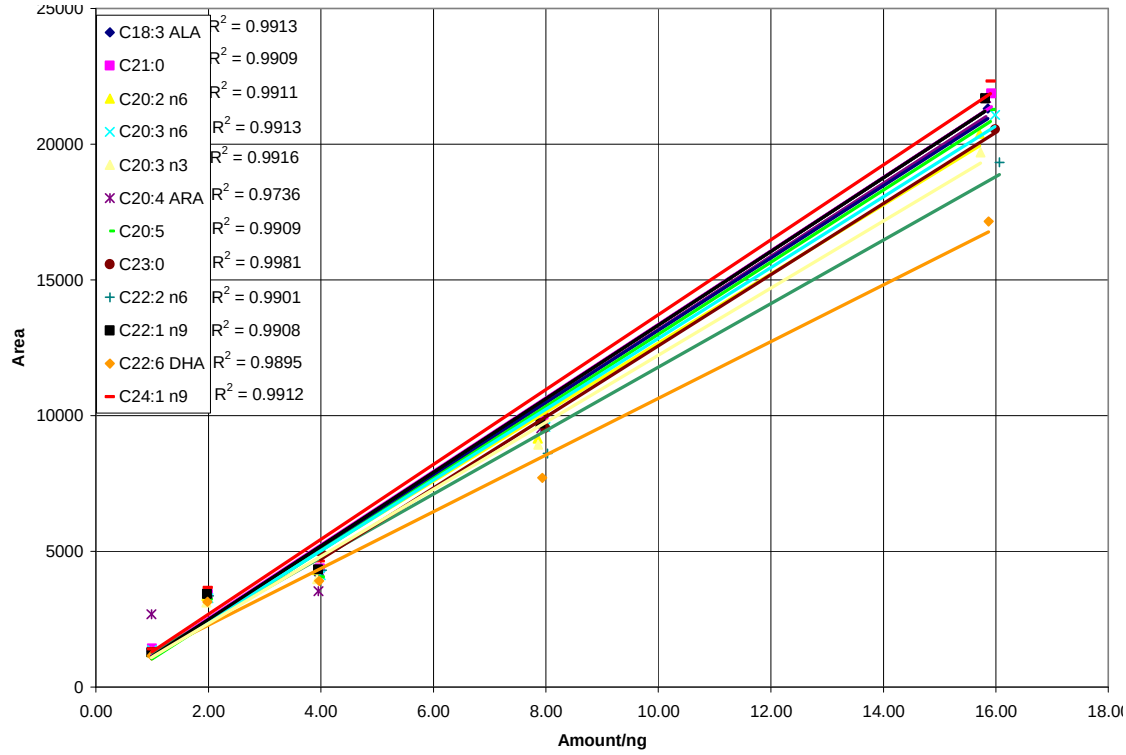


Figure 3-4. GC-FID detector linearity of PUFAs. The GC-FID detector linearity is again high for predominantly VLCPUFAs with the majority of compounds displaying R^2 values around 0.99. $C_{20:4}$ n6 demonstrates an erroneous point at 1ng although was found to partially co-elute with $C_{23:0}$, which could explain the larger than average area.

The GC-MS was equipped with a DB-5MS+DG column (figure 3-5) which is more non-polar than the CP-Sil 88 column, which resulted in the elution of unsaturated fatty acids prior to saturates. The column had adequate resolution for fatty acid separation, however the fatty acids $C_{18:3}$ n3, $C_{18:1}$ and *cis*-$C_{18:2}$ co-eluted. This meant that total ion count (TIC) integrated areas were inaccurate for these fatty acids if in high abundance. Therefore extracted ions (EI) as oppose to TIC were used to quantify fatty acids, as they allow single ions which distinguish between co-eluting fatty acids to be selected and provide greater accuracy. The fatty acid mass spectrums were added to a custom AMDIS library to allow accurate identification of the compounds as were retention indices (RI). Retention indices allow for more accurate identification of compounds by attributing an index value to that compound. The index values are based on a homologous series such as the n-alkanes, or in this case the saturated fatty acids C_{10}-$C_{24:0}$ with values of 1000-2400 given to the homologous series. These can be plotted against retention time and the remaining compounds can be assigned RIs. If the column or running conditions are changed, a calibration can be performed to readjust the RI vs. retention time plot. The EIs and RIs are shown in table 3-3.

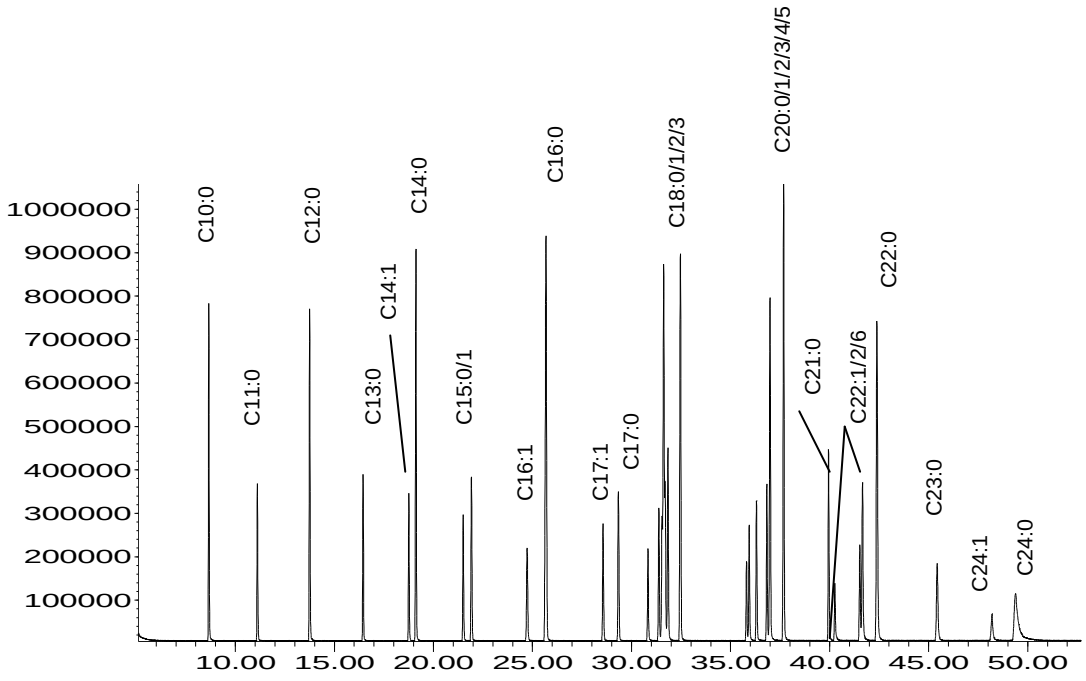


Figure 3-5. GC-MS trace using the DB-5MS+DG column and the 37 FAME standard. Resolution between most fatty acids was high however the C_{18} series suffers from co-elution due to the large abundance of these compounds within fungi. Extracted ions were used to quantify all compounds and this increased the accuracy of quantification of co-eluting fatty acids. The saturated fatty acid series were used to calculate the RI values due to the strong correlation of fatty acid length and retention time.

Table 3-3. GC-MS parameters used throughout the work. m/z = ion selected for EI quantification, EI RF = extracted ion response factor, SE = standard error of EI RF, RI = retention index. The EI RF are more variable than with the GC-FID as individual ions are used. To prevent co-elution from other peaks influencing quantification, unique ions were selected which where not present in the co-eluting compound. These ions in some instances are in low abundance, resulting in a large correction factor needing to be used when compared to an abundant ion, as found in the internal standard. The response factors are slightly more variable than with the GC-FID as is seen by the standard error over the range of concentrations.

FA	m/z	EI RF	SE	RI	FA	m/z	EI RF	SE	RI
$C_{10:0}$	74	1.05	0.07	1000	$C_{20:0}$	74	1.06	0.06	2000
$C_{11:0}$	74	1.07	0.05	1100	$C_{18:3}$ n6	79	0.25	0.03	1750.4
$C_{12:0}$	74	1.15	0.06	1200	$C_{20:1}$	74	0.14	0.01	1974.8
$C_{13:0}$	74	1.12	0.03	1300	$C_{18:3}$ n3	79	0.35	0.05	1776.8
$C_{14:0}$	74	1.26	0.08	1400	$C_{21:0}$	67	0.76	0.04	2100
$C_{14:1}$	55	0.43	0.01	1386.6	$C_{20:2}$ n6	98	0.31	0.02	1967.9
$C_{15:0}$	74	1.19	0.05	1500	$C_{22:0}$	74	0.74	0.04	2200
$C_{15:1}$	55	0.45	0.02	1487.7	$C_{20:3}$ n6	81	0.21	0.01	1949.1
$C_{16:0}$	74	1.17	0.07	1600	$C_{22:1}$ n9	74	0.33	0.02	2172.6
$C_{16:1}$	55	0.31	0.02	1577.2	$C_{20:4}$ n6	119	0.03	0.01	1930.3
$C_{17:0}$	74	1	0	1700	$C_{23:0}$	74	0.47	0.01	2300
$C_{17:1}$	55	0.35	0.02	1678.5	$C_{22:2}$ n6	74	0.26	0.02	2167.9
$C_{18:0}$	74	1.14	0.06	1800	$C_{24:0}$	74	0.13	0.02	2400
$C_{18:1}$ trans	55	0.43	0.04	1781.1	$C_{20:5}$ n3	80	0.07	0.02	1936.8
$C_{18:1}$ cis	84	0.20	0.05	1775.5	$C_{24:1}$ n9	74	0.17	0.03	2367.5
$C_{18:2}$ trans	67	0.34	0.04	1777.2	$C_{22:5}$ n3	TIC	0.47	N/A	2137.4
$C_{18:2}$ cis	67	0.38	0.02	1766.8	$C_{22:6}$ n3	55	0.20	0.04	2121.9

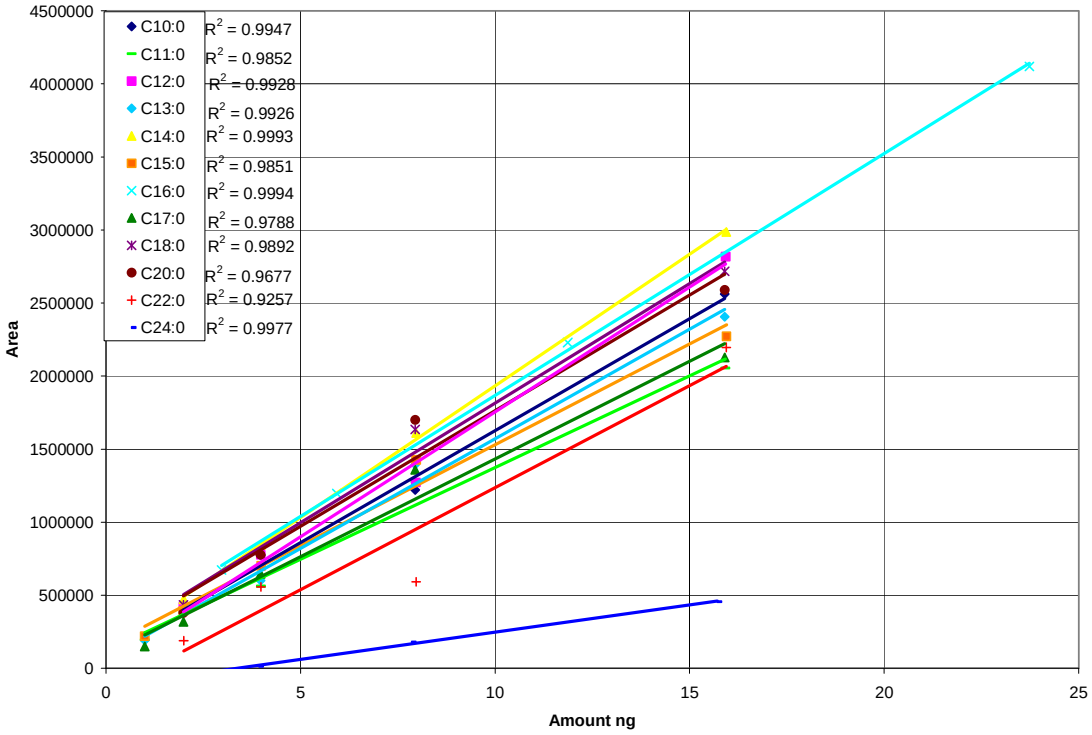


Figure 3-6. GC-MS detector linearity of saturates. The saturates show good detector linearity up to an area of $4x10^6$, however beyond this point detector linearity is lost. Saturates were quantified using the major ion m/z 74 which accounts for the large detector response with low amounts of compound. The selection of a less abundant ion may allow for greater linearity over a greater concentration range. The R^2 scores of $C_{11:0}$, $C_{17:0}$, $C_{18:0}$, $C_{20:0}$ and $C_{22:0}$ are lower then that found for the GC-FID scores.

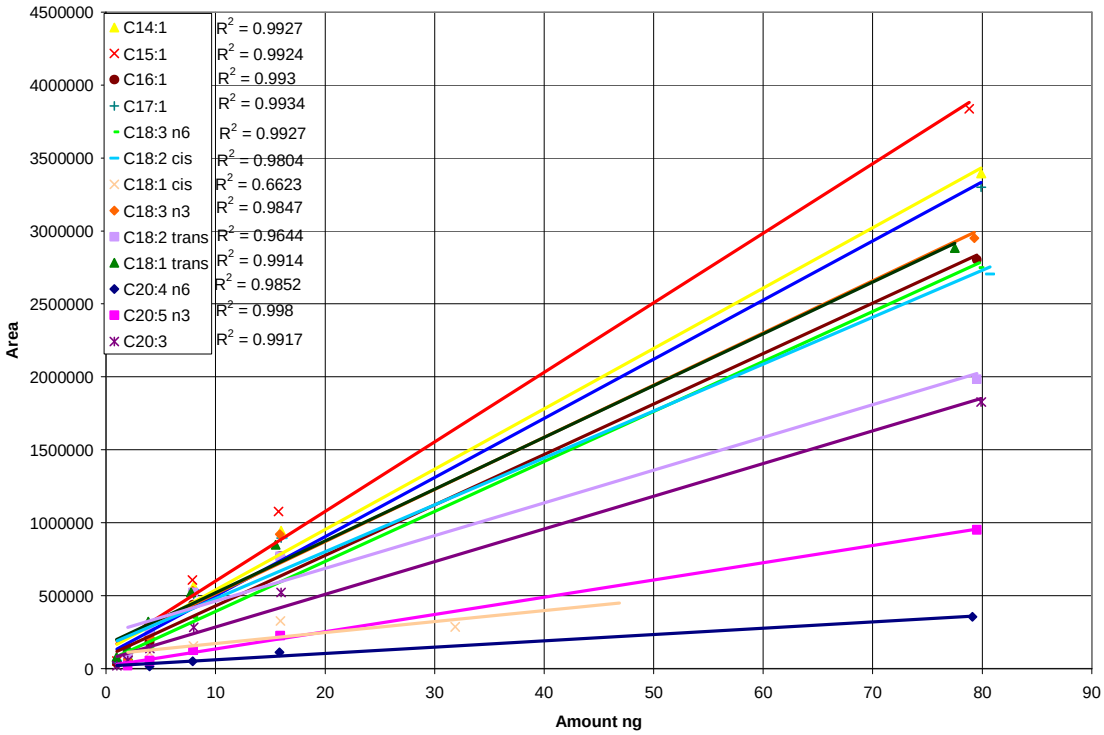


Figure 3-7. GC-MS detector linearity for mono, di and polyunsaturates. In general the linearity for most fatty acids was high, although $C_{18:1}$ *cis* showed an unusually low R^2 score. Comparable quantities of fatty acid resulted in lower area values compared to the saturates due to less abundant ions being selected for quantification. $C_{18:2}$ cis, $C_{18:1}$ cis, $C_{18:3}$ n3 and $C_{18:2}$ *trans* were found to have a higher linearity when using the GC-FID.

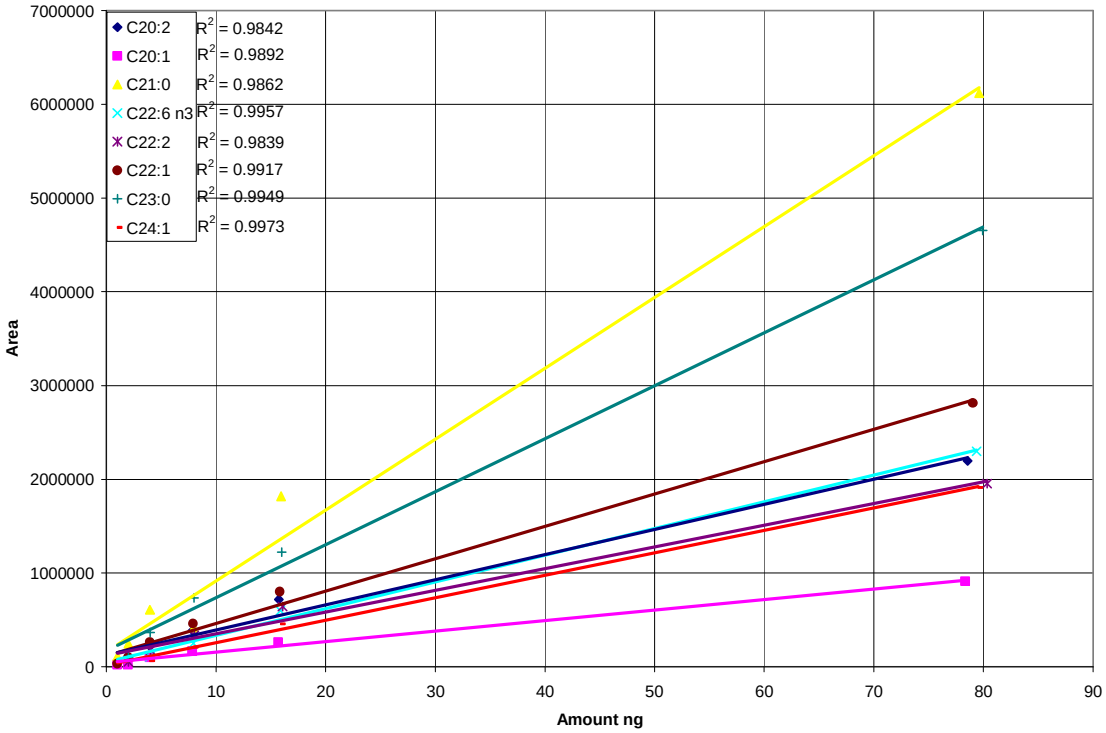


Figure 3-8. GC-MS detector linearity. The linearity of the remaining compounds is relatively high however R^2 scores for the GC-FID for $C_{20:1}$, $C_{20:2}$, $C_{21:0}$ and $C_{22:2}$ are slightly higher. It is to be noted that higher concentrations of fatty acids are used on the GC-MS, and this is due to the final concentration being achieved through decreasing the split value. The GC-FID was not capable of this and so the highest concentration was not achieved.

3.2.2 Screening of BAS and low temperature isolated fungi.

In total 142 organisms were screened for novel VLCPUFAs. All were initially screened using GC-FID, and those that demonstrated retention times in accordance with fatty acids of interest were run on GC-MS for confirmation. All BAS isolates were screened utilising two media, PD and YES or MA at 15°C. Cultures such as *Mortierella* were only grown on PD after it became apparent that this media was sufficient for production of VLCPUFAs and that different media were unlikely to result in the production of VLCPUFAs from ascomycetes and basidiomycetes. Cork bores of plate mycelia were utilised for broth culture throughout the entire study as broth growth using spore suspensions of several isolates resulted in poor or no growth. Organism identification remains putative due to the lack of spore forming structures which

prevented morphological identification. The lack of spore forming structures may partially explain the difficulty in broth culture using spore suspensions. Initial molecular identification was obtained from BAS and CABI, however in a large proportion of cases identities were made only to the phylum, order or genus levels. In multiple instances no identity was achieved at all. More detailed molecular analysis is currently underway to determine isolates to genus or species levels. Organisms were grown at 15°C as a compromise between mycelial growth and possible VLCPUFA production, with temperatures above 15°C inhibiting $C_{20:5}$ n3 production in *Mortierella alpina* as will be shown in the next chapter.

In all but 13 organisms the primary long chain polyunsaturated fatty acids detected were $C_{18:3}$ n3 or $C_{18:3}$ n6. In general the fatty acid profiles of the organisms contained $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$ and in the majority of cases $C_{18:3}$ n3. $C_{18:3}$ n6 was detected exclusively in organisms found within the Zygomycota, Chytridiomycota and Oomycota whilst the n3 C_{18} LCPUFA was found within organisms distributed within the Ascomycota and Basidiomycota. Table 3-4 shows the majority of fatty acid profiles, many of which are BAS isolates as well as several non-BAS organisms. All these organisms however share the inability to elongate past $C_{18:3}$. Small amounts of $C_{20:0}$ and $C_{22:0}$ as well as the monounsaturated forms where also found within isolates. It is probable that more samples contain these longer chain saturates and monounsaturates, although due to a period of poor column resolution owing to column degradation on the GC-FID, the shift in retention times resulted in unidentified compounds or were merely hidden within the much larger C_{18} peaks due to poor resolution.

The initial hypothesis that stated low temperature environments would promote organisms to produce a greater complement of unsaturated fatty acids appears not to be the case within the ascomycetes and basidiomycetes. Several samples though were found to produce VLCPUFAs up to $C_{22:5}$ n3. These are identified in table 3-5. A large number of these are Antarctic *Mortierella* species. Other organisms capable of producing VLCPUFAs are those located within the Oomycota such as *Saprolegnia diclina*, *Pythium irregulare* and *Achlya americana*. The chytrid *Allomyces macrogynus* is also capable of producing the VLCPUFA $C_{20:4}$ n6. Several organisms were studied which were found to produce uncharacteristic fatty acid profiles and on isolate sequencing, it was found that three organisms were not the initially isolated organisms. The first was identified when two strains of *Phytophthora richardiae* demonstrated

strikingly different fatty acid profiles which appeared linked to their isolation location, with one isolated from Antarctica whilst the other from a warmer climate (figure 3-9). The Antarctic strain showed the ability to produce VLCPUFAs such as $C_{20:4}$ n6 and $C_{20:5}$ n3 whereas the mesophilic strain clearly showed the absence of these long chain unsaturated fatty acids. The Antarctic strain also produced elevated quantities of $C_{14:0}$. It was also noted that the Antarctic isolate was capable of producing both $C_{18:3}$ n3 and $C_{18:3}$ n6, with this n6 LCPUFA the most likely precursor for the C_{20} VLCPUFAs. The mesophilic strain however lacked $C_{18:3}$ n6, instead producing $C_{18:3}$ n3 as the most unsaturated fatty acid. The lack of VLCPUFAs and the sole presence of $C_{18:3}$ n3 indicated that the mesophilic isolate may not be an Oomycete and sequencing confirmed the isolate as *Daedaleopsis confragosa*. The other two identified contaminants will be discussed shortly.

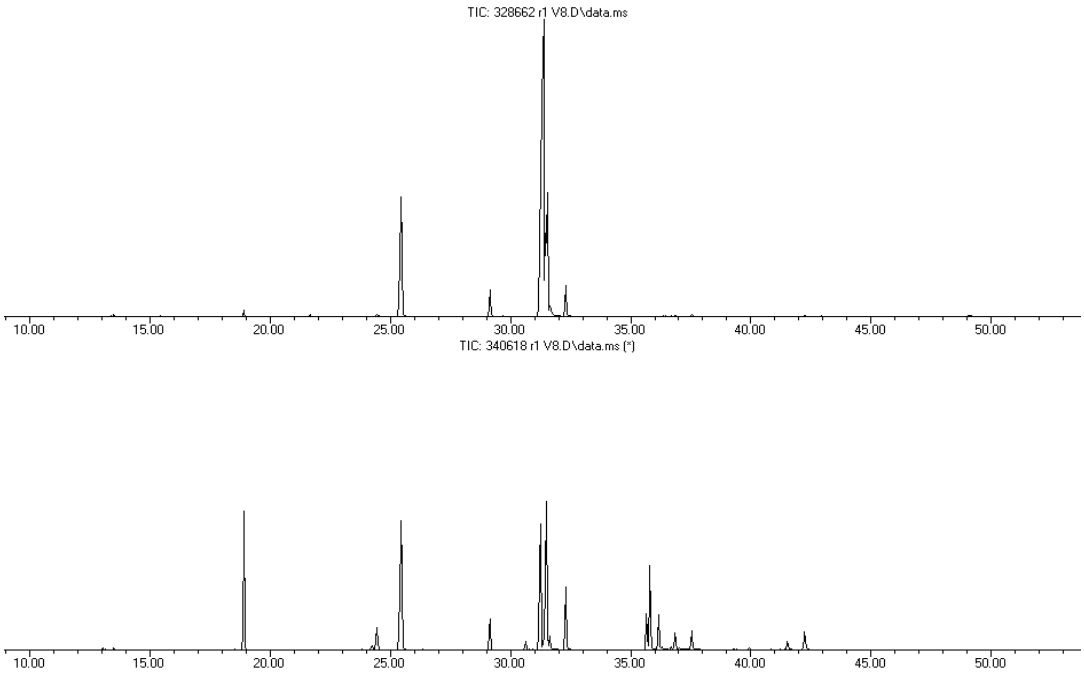


Figure 3-9. Comparison of two strains, 328662 and340618, thought to be *Phytophthora richardiae*. The upper trace shows a strain thought to be a mesophilic isolate whilst the lower trace shows a strain isolated from a psychrophilic environment. The low temperature isolated strain (bottom) clearly demonstrates the ability to produce fatty acids up to $C_{20:5}$ n3 (35-38 minutes) whereas the contaminant organism does not produce PUFAs beyond $C_{18:3}$ n3.

The genus Mortierella provided some notable results, not least its ability to produce long chain polyunsaturates. It was found that *Mortierella alpina* strain 330997 was

capable of producing trace amounts of C_{22} VLCPUFA such as $C_{22:5}$ n3 (up to 0.1 mg/g of dry biomass, 0.18% of total lipid) shown in figure 3-10. Due to the low levels of these fatty acids, it was thought that their formation was a side reaction of an already known elongase enzyme utilising C_{20} VLCPUFAs such as $C_{20:4}$ n6 and $C_{20:5}$ n3. However the discovery of this side reaction prompted further investigation into the biosynthesis of VLCPUFAs within the fungi, which will be elaborated upon in the discussion. Two of the *Umbelopsis* isolates demonstrated $C_{18:3}$ n3 production as oppose to the commonly observed $C_{18:3}$ n6 and it was these isolates which were also considered to not be the original identified organism. The isolates that demonstrated this preference were Dis 169, a mesophilic organism, and 403341 an Antarctic isolated organism. On returning to the original cultures it was found that they produced the same fatty acid profiles. This indicated that during the initial isolation or transfer to the culture collection contamination had occurred, or the isolate had been outgrown by a co-isolate which now replaced the original strain. These isolates were molecularly identified and found to be a *Penicillium* isolate (403341) and an unknown species from within the Clavicipitaceae (Dis 169). The analysis of the fatty acid profiles, as will be described shortly, also acted as a quality control screen indicating that in this case contamination had occurred. The other *Mortierella* species studied all contained the fatty acid $C_{18:3}$ n6 either as the most unsaturated fatty acid or as a precursor for the synthesis of $C_{20:4}$ n6 and $C_{20:5}$ n3. In regards to the production of VLCPUFAs such as $C_{20:4}$ n6, *Mortierella* strain 398216 accumulated the fatty acid at 67.84% of its total lipid, with the second highest accumulation being 34.92% from *Mortierella alpina* strain 196057. $C_{20:4}$ n6 levels were subsequently between 8-14% of total lipid for the other strains of *Mortierella*. Production of $C_{20:5}$ n3 was at a much lower level, ranging from 1.40-5.04% of the total lipid with production in the high $C_{20:4}$ n6 strain reaching 3.18%. The greatest proportional producer of $C_{20:5}$ n3 was *Saprolegnia diclina* at 19.9% of the total lipid, with the majority of the Oomycetes producing $C_{20:5}$ n3 in the range of 12.09-14.3% of the total lipid. In absolute value terms, *Pythium irregulare* produced the greatest $C_{20:5}$ n3 quantity at 20 mg/g of dry biomass, *Achlya americana* at 12 mg/g and *Saprolegnia* at 4 mg/g. Comparatively *Mortierella alpina*, strain 196057, produced $C_{20:5}$ n3 at 6.9 mg/g of the dry biomass. The sole chytrid, *Allomyces macrogynus* was capable of producing $C_{20:4}$ n6 at 11.2% of the total lipid although was incapable of further desaturation to $C_{20:5}$ n3.

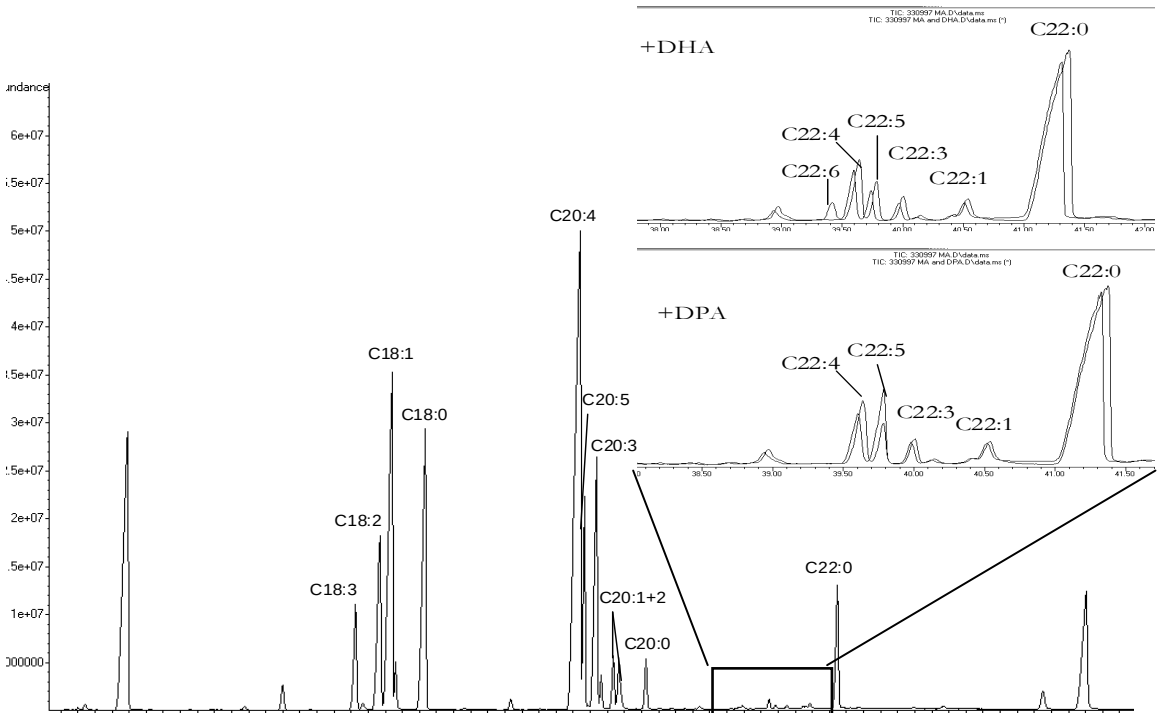


Figure 3-10. Mortierella alpina strain 330997 is capable of producing C_{22} VLCPUFAs up to $C_{22:5}$ n3. However it can be clearly seen that they are trace fatty acids. $C_{22:6}$ n3 and $C_{22:5}$ n3 were spiked to confirm the identity of the fatty acid.

Table 3-4. Fatty acid profiles of 128 low temperature isolated fungi incapable of elongation beyond $C_{18:3}$. The majority of the organisms are ascomycetes, with several zygomycetes such as *Mortierella* and *Mucor* species and a few basidiomycetes, such as *Phaffia* and *Sporobolomyces*. Those isolates with IMI numbers starting 403xxx are from the BAS collection. Fatty acid values are stated as the % (w/w) of the total lipid fraction.

Organism	IMI	C14:0	C15:0	C16:0	C16:1	C17:1	C18:0	C18:1	C18:2	C20:0	C18:3 n6	C20:1	C18:3 n3	C22:0	C22:1	C24:0	C24:1	Total lipid mg/g
Mucor racemosus	17313	6.7	0.0	21.2	0.0	0.0	13.3	18.7	13.7	0.4	17.5	0.0	0.0	0.0	0.0	1.9	0.0	52.8
Mucor hiemalis	17363	1.7	0.0	16.7	3.0	0.0	9.4	25.0	26.4	0.0	14.1	0.0	0.0	0.0	0.6	0.0	1.1	48.3
Trichosphaeria pilosa	89319	0.0	0.0	20.5	1.0	0.0	6.8	37.0	26.1	0.5	0.0	0.0	7.5	0.3	0.0	0.4	0.0	194.0
Curvularia inaequalis	114060	0.0	0.0	18.3	0.0	0.0	10.7	12.5	35.8	0.0	0.0	0.0	17.9	0.0	1.0	0.0	1.7	9.5
Thielavia peruviana	135024	0.0	0.0	15.1	1.2	0.0	3.0	8.2	65.3	0.0	0.0	0.0	7.2	0.0	0.0	0.0	0.0	34.4
Mucor hiemalis	138261	3.7	0.3	18.9	3.7	0.2	5.3	23.2	18.7	0.0	20.4	0.0	0.4	0.0	0.5	0.0	1.2	68.7
Embellisia sp.	215092	0.0	0.0	20.6	0.5	0.0	7.1	31.3	31.8	0.0	0.0	0.0	8.3	0.0	0.0	0.3	0.0	218.2
Penicillium echinulatum	256351	0.0	0.0	12.3	0.9	0.0	5.6	10.0	56.6	0.0	0.0	0.0	13.6	0.0	0.0	1.0	0.0	50.5
Acremonium sp.	312275	0.7	0.0	26.7	3.9	0.0	1.9	40.0	25.1	0.0	0.0	0.0	1.5	0.0	0.0	0.2	0.0	361.6
Aureobasidium sp.	312277	0.3	0.0	0.0	2.8	0.1	1.2	54.4	25.0	0.1	0.0	0.2	0.5	0.1	0.0	0.0	0.0	695.1
Thelebolus sp.	340093	0.4	0.0	20.6	1.1	0.0	5.8	45.1	18.6	0.5	0.0	0.0	6.2	0.6	0.0	1.2	0.0	192.0
Leptodontidium elatius var. elatius	369788	0.0	0.0	23.5	0.0	0.0	4.9	20.5	30.7	0.0	0.0	0.0	18.7	0.0	0.0	0.9	0.0	122.7
Thelebolus microsporus	369795	0.5	0.0	23.5	3.5	0.0	2.8	35.9	28.6	0.0	0.0	0.0	4.4	0.0	0.0	0.5	0.3	191.4
Chrysosporium sp.	369800	0.3	0.3	18.8	0.6	0.0	5.4	36.0	35.0	0.4	0.0	0.0	2.9	0.3	0.0	0.0	0.0	288.6
Cladosporium cladosporioides	377828	0.0	0.0	15.0	1.0	0.0	5.7	42.7	32.5	0.3	0.0	0.0	2.7	0.0	0.0	0.0	0.0	171.2
Alternaria alternata	378423	0.5	0.0	20.7	0.6	0.0	18.1	12.9	42.8	0.5	0.0	0.0	3.7	0.0	0.0	0.0	0.0	209.7
	396413	0.0	0.0	16.3	0.8	0.0	8.1	19.4	31.9	0.4	0.5	0.0	21.9	0.0	0.0	0.0	0.0	211.3
Cladosporium cladosporioides	396505	0.0	0.0	19.8	0.6	0.3	5.6	18.9	42.4	0.0	0.0	0.0	12.2	0.0	0.0	0.0	0.0	29.4
Herpotrichia sp.	403002	0.0	0.0	12.2	0.7	0.2	4.5	13.3	38.7	0.2	0.0	0.1	14.3	1.1	0.0	0.2	0.4	42.1
Ascomycota	403004	0.0	0.0	16.3	1.6	0.0	5.4	21.3	49.9	0.0	0.0	0.0	4.2	0.7	0.4	0.0	0.0	34.9
Ascomycota	403008	0.0	0.0	16.5	1.8	0.0	7.8	26.8	39.7	0.0	0.0	0.0	6.2	0.7	0.0	0.0	0.0	49.9
Ascomycota	403010	0.0	0.0	14.1	1.1	0.0	6.1	18.7	42.7	0.0	0.0	0.0	11.8	1.1	0.6	0.6	0.2	35.0
Leptodontidium sp.	403011	0.4	0.3	16.8	0.9	0.3	3.5	33.0	32.0	0.0	0.0	0.1	10.1	0.0	0.4	0.0	0.2	59.2
Leptodontidium sp.	403012	0.0	0.0	13.8	0.5	0.0	0.4	19.9	28.1	0.0	0.0	0.6	33.6	0.0	0.3	0.0	0.4	38.0
Herpotrichia	403014	0.0	0.0	14.2	0.5	0.0	4.7	16.8	50.9	0.0	0.0	0.0	9.8	1.2	0.0	0.0	0.0	37.3
Herpotrichia	403015	0.0	0.0	14.2	0.8	0.0	3.8	15.9	55.1	0.0	0.0	0.0	10.1	0.0	0.0	0.0	0.0	23.9
Herpotrichia	403016	0.0	0.0	15.5	0.3	0.1	8.4	22.6	42.0	0.1	0.0	0.0	9.9	0.5	0.0	0.0	0.0	39.3
Ascomycota	403017	0.0	0.0	17.8	3.3	0.2	4.8	22.0	43.3	0.0	0.0	0.0	7.8	0.0	0.0	0.0	0.0	38.5
Ascomycota	403019	0.0	0.0	14.4	1.4	0.3	7.6	17.8	46.2	0.0	0.0	0.0	11.1	0.0	0.5	0.0	0.0	32.9
Ascomycota	403020	0.3	0.0	20.9	0.8	0.0	4.1	31.9	32.9	0.4	0.0	0.0	7.6	0.5	0.0	0.0	0.0	87.2
Ascomycota	403021	0.0	0.0	18.3	0.9	0.0	6.9	36.5	31.6	0.3	0.0	0.0	5.2	0.2	0.0	0.0	0.0	76.6
Ascomycota	403023	0.0	0.0	18.8	1.0	0.0	7.0	40.9	30.0	0.0	0.0	0.0	1.8	0.4	0.0	0.0	0.0	44.2
Ascomycota	403024	0.0	0.0	13.2	0.6	0.0	6.4	32.9	37.9	0.3	0.0	0.0	8.1	0.4	0.0	0.0	0.0	61.2
Ascomycota	403025	0.0	0.0	13.9	0.4	0.0	7.7	32.7	38.2	0.0	0.0	0.0	7.1	0.0	0.0	0.0	0.0	52.4
Pleosporales	403026	0.0	0.0	17.5	1.4	0.0	7.6	23.2	41.3	0.0	0.0	0.0	8.0	0.6	0.0	0.0	0.5	49.9
Pleosporales	403027	0.0	0.0	14.1	1.0	0.0	2.5	25.6	52.2	0.0	0.0	0.0	4.5	0.0	0.0	0.0	0.0	38.1
Pleosporales	403028	0.0	0.7	15.3	0.8	0.9	4.6	24.4	36.1	0.0	0.0	0.7	16.0	0.0	0.0	0.0	0.0	15.7
Pleosporales	403029	0.0	0.0	13.8	0.6	0.0	6.0	43.5	31.9	0.0	0.0	0.0	3.7	0.2	0.0	0.0	0.0	74.0
Pleosporales	403030	0.0	0.0	16.1	1.0	0.0	5.2	38.8	34.5	0.2	0.0	0.0	4.0	0.3	0.0	0.0	0.0	82.4

Organism	IMI	C14:0	C15:0	C16:0	C16:1	C17:1	C18:0	C18:1	C18:2	C20:0	C18:3 n6	C20:1	C18:3 n3	C22:0	C22:1	C24:0	C24:1	Total lipid mg/g
Pleosporales	403032	0.0	0.0	13.4	1.2	0.0	4.3	39.0	38.3	0.0	0.0	0.0	3.4	0.0	0.0	0.0	0.0	27.8
Pleosporales	403033	0.0	0.0	13.9	0.7	0.0	8.0	31.0	42.2	0.0	0.0	0.0	3.4	0.0	0.4	0.0	0.0	31.1
Herpotrichia sp.	403037	0.0	0.0	15.3	1.3	0.0	3.8	25.0	45.0	0.0	0.0	0.0	8.8	0.3	0.0	0.0	0.0	57.2
Pleosporales	403038	0.0	0.0	13.4	1.0	0.0	4.2	23.9	51.1	0.0	0.0	0.0	5.3	0.0	0.0	0.0	0.0	25.4
Pleosporales	403040	0.0	0.0	16.2	0.5	0.0	7.3	21.0	47.0	0.0	0.0	0.0	6.2	0.7	0.0	0.0	0.0	34.5
Pleosporales	403041	0.4	0.6	19.0	0.6	0.4	3.1	14.6	38.9	0.0	0.0	0.0	14.7	2.9	1.5	0.0	0.0	22.1
Pleosporales	403042	0.0	0.0	12.5	1.7	0.3	2.7	43.3	36.5	0.0	0.0	0.0	1.8	0.0	0.0	0.0	0.0	26.2
Herpotrichia juniperi	403043	0.0	0.0	13.9	1.2	0.9	6.0	22.3	49.8	0.0	0.0	0.0	5.9	0.0	0.0	0.0	0.0	29.5
Pleosporales	403045	0.0	0.0	13.3	2.4	0.4	5.6	22.1	36.5	0.0	0.0	0.0	14.0	1.4	0.8	0.6	0.0	59.7
Pleosporales	403046	0.0	0.0	16.6	2.5	0.0	6.5	25.3	44.5	0.0	0.0	0.0	4.6	0.0	0.0	0.0	0.0	33.2
Pleosporales	403049	0.0	0.6	13.3	0.7	0.0	4.1	15.6	49.0	0.8	0.0	0.0	10.7	0.7	0.0	0.0	0.0	36.8
Leptodontidium sp.	403051	0.4	0.3	24.1	1.1	0.1	6.8	34.5	26.5	0.0	0.0	0.3	3.9	0.0	0.0	0.0	0.0	99.6
Cadophora/polyscytalum	403058	0.0	0.0	19.8	1.1	0.0	3.6	30.9	34.7	0.0	0.0	0.0	9.2	0.0	0.0	0.0	0.0	20.3
Cadophora/polyscytalum	403059	0.3	0.0	18.2	0.8	0.5	4.9	41.0	21.5	0.0	0.0	0.0	11.7	0.6	0.0	0.0	0.0	76.2
Leptodontidium sp.	403060	0.0	0.3	19.8	1.0	0.3	7.5	25.4	36.1	0.0	0.0	0.0	8.1	0.5	0.0	0.0	0.0	6.7
Leptodontidium sp.	403061	0.0	0.3	14.8	0.4	0.4	3.8	34.7	36.9	0.0	0.0	0.0	5.9	0.0	0.5	0.0	0.0	32.2
Leptodontidium sp.	403061	0.3	0.0	20.7	0.5	0.4	8.4	37.0	24.2	0.0	0.0	0.0	6.5	0.9	0.0	0.0	0.0	53.9
Leptodontidium sp.	403062	0.0	0.0	15.7	0.0	0.0	4.1	36.4	35.4	0.0	0.0	0.0	8.5	0.0	0.0	0.0	0.0	29.3
Leptodontidium sp.	403063	0.0	0.3	22.0	0.7	0.3	5.6	35.6	21.3	0.0	0.0	0.0	10.8	0.0	0.0	0.0	0.0	69.5
Leptodontidium sp.	403065	0.0	0.0	15.9	0.6	0.4	3.8	36.1	31.4	0.0	0.0	0.0	9.7	0.0	0.5	0.0	1.0	29.7
Leptodontidium sp.	403066	0.0	0.0	19.7	0.0	2.3	4.5	33.4	28.8	0.0	0.0	0.0	11.3	0.0	0.0	0.0	0.0	10.6
Leptodontidium sp.	403067	0.0	0.0	16.1	0.4	0.0	4.8	36.7	27.9	0.0	0.0	0.0	12.3	0.0	0.0	0.0	0.0	48.9
Leptodontidium sp.	403069	0.0	0.3	16.2	0.6	0.6	4.4	34.7	31.1	0.0	0.0	0.0	10.2	0.0	0.5	0.0	0.3	31.9
Leptodontidium sp.	403070	0.0	0.2	15.9	0.6	0.2	1.9	16.2	38.7	0.0	0.0	0.7	22.9	0.0	0.4	0.0	0.3	41.1
Leptodontidium sp.	403072	0.0	0.0	19.2	0.0	0.0	4.9	31.1	32.3	0.0	0.0	0.0	12.5	0.0	0.0	0.0	0.0	30.9
Leptodontidium sp.	403073	0.0	0.0	24.7	0.9	0.0	4.6	40.7	27.5	0.0	0.0	0.0	1.6	0.0	0.0	0.0	0.0	54.4
Leptodontidium sp.	403074	0.0	0.0	23.8	0.7	0.0	4.9	39.8	29.0	0.0	0.0	0.0	1.8	0.0	0.0	0.0	0.0	48.0
Leptodontidium sp.	403075	0.0	0.0	16.1	0.0	0.0	3.2	22.7	32.2	0.0	0.0	0.0	25.9	0.0	0.0	0.0	0.0	17.2
Leptodontidium sp.	403077	0.0	0.0	22.3	0.0	0.0	6.7	14.3	45.5	0.0	0.0	0.0	11.2	0.0	0.0	0.0	0.0	17.1
Leptodontidium sp.	403078	0.0	0.0	16.0	0.0	0.0	3.3	16.1	57.9	0.0	0.0	0.0	6.8	0.0	0.0	0.0	0.0	24.4
Leptodontidium sp.	403079	0.3	0.0	22.0	0.6	0.0	5.5	36.1	26.2	0.3	0.0	0.0	8.7	0.0	0.0	0.0	0.0	68.2
Rhexocercosporidium sp.	403080	0.0	0.0	17.8	0.0	0.0	2.6	31.5	31.7	0.0	0.0	0.0	16.5	0.0	0.0	0.0	0.0	23.1
Thelebolus microsporus	403082	0.0	0.0	15.4	0.0	0.0	2.9	22.5	31.1	0.0	0.0	0.0	28.1	0.0	0.0	0.0	0.0	15.7
Mollisia sp.	403087	0.0	0.0	19.1	0.6	0.0	3.9	16.7	36.9	0.0	0.0	0.0	22.7	0.0	0.0	0.0	0.0	20.9
Geomyces sp.	403088	0.5	0.0	26.2	0.6	0.0	5.7	30.8	31.2	0.0	0.0	0.0	4.7	0.0	0.0	0.0	0.0	63.6
Mollisia sp.	403090	0.0	0.0	28.2	0.0	0.0	6.1	21.4	36.0	0.0	0.0	0.0	4.5	0.0	0.0	0.0	0.0	18.8
Mollisia sp.	403091	0.5	0.0	29.1	0.6	0.0	5.2	29.0	30.6	0.0	0.0	0.0	5.1	0.0	0.0	0.0	0.0	37.0
Mollisia sp.	403092	0.8	0.0	32.0	0.5	0.0	5.3	26.2	29.7	0.0	0.0	0.0	4.8	0.0	0.0	0.0	0.0	38.9
Mollisia sp.	403093	0.0	0.0	20.4	2.3	0.3	9.0	27.5	32.5	0.0	0.0	0.0	6.5	0.0	0.0	0.0	0.0	63.9
Ophiostoma stenoceras	403094	0.0	0.0	14.7	6.2	0.8	2.1	27.0	36.3	0.0	0.0	0.0	6.2	0.5	0.3	0.0	0.0	28.5
Mollisia sp.	403099	0.3	0.0	23.0	0.4	0.0	4.4	28.8	34.8	0.0	0.0	0.0	6.7	0.0	0.4	0.0	0.4	40.4
Mollisia sp.	403100	0.5	0.0	28.5	0.4	0.0	7.5	33.1	24.6	0.0	0.0	0.0	4.9	0.0	0.0	0.0	0.0	102.6

Organism	IMI	C14:0	C15:0	C16:0	C16:1	C17:1	C18:0	C18:1	C18:2	C20:0	C18:3 n6	C20:1	C18:3 n3	C22:0	C22:1	C24:0	C24:1	Total lipid mg/g
Rhizoscyphus ericae strain	403102	0.0	0.0	18.3	0.0	0.0	3.8	26.3	43.6	0.0	0.0	0.0	6.6	0.0	0.0	0.0	0.0	28.9
Helotiaceae	403104	0.0	0.0	19.3	1.0	0.0	3.3	17.8	36.1	0.0	0.0	0.0	22.0	0.0	0.0	0.0	0.0	46.0
Gyoerffyella sp.	403109	0.0	0.0	18.4	0.7	0.6	3.4	27.0	44.2	0.0	0.9	0.0	1.8	0.0	0.0	0.0	0.0	31.4
Penicillium rugulosum	403110	0.0	0.0	13.6	0.9	1.4	5.2	24.3	29.3	0.0	0.0	0.0	23.5	0.0	0.0	0.0	0.8	19.6
Gyoerffyella sp.	403111	0.0	0.0	18.4	0.6	0.0	5.8	22.1	38.5	0.4	0.0	0.0	8.3	0.6	0.0	0.0	0.0	40.6
Gyoerffyella sp.	403112	0.0	0.0	14.9	0.9	0.5	7.7	8.7	45.1	0.6	0.0	0.6	21.0	0.0	0.0	0.0	0.0	17.2
	403116	0.0	0.0	0.0	2.0	0.0	9.6	30.6	27.7	0.5	0.0	0.0	2.2	0.0	0.0	0.0	0.4	62.1
	403119	0.0	1.0	12.9	0.8	0.0	10.4	9.7	47.5	1.8	0.0	0.0	11.8	0.0	0.7	0.0	1.2	11.7
Ascomycota	403123	0.0	0.0	21.2	0.4	0.0	0.6	14.5	41.3	0.0	0.0	0.0	17.4	0.0	0.5	0.0	0.0	22.6
Ascomycota	403124	0.0	0.4	15.8	0.0	1.0	4.1	28.1	33.1	0.0	0.0	0.0	9.4	0.0	0.5	0.0	0.0	19.8
Ascomycota	403125	0.0	0.0	21.1	0.4	0.0	0.5	11.9	39.8	0.0	0.0	0.4	20.0	0.0	0.6	0.0	0.0	24.2
Ascomycota	403127	0.0	0.7	18.9	0.5	0.9	5.0	21.7	40.7	0.0	0.0	0.0	7.9	0.0	0.5	0.0	0.0	20.9
	403129	0.6	0.0	27.2	0.6	0.0	5.4	28.4	32.1	0.0	0.0	0.0	5.3	0.0	0.0	0.0	0.0	45.0
	403131	0.4	0.0	23.6	0.4	0.0	5.9	29.6	32.2	0.0	0.0	0.0	6.8	0.4	0.0	0.7	0.0	50.2
Mollisia sp.	403133	0.4	0.0	22.5	0.0	0.0	6.8	20.6	27.5	0.0	0.0	0.0	9.9	0.6	0.0	0.9	0.0	53.8
Mollisia sp.	403134	0.3	0.0	19.5	0.3	0.0	5.9	22.6	27.3	0.3	0.0	0.0	12.0	0.5	0.0	1.1	0.0	62.7
	403135	0.0	0.0	19.3	0.0	0.0	6.1	21.8	27.3	0.3	0.0	0.0	12.3	0.5	0.0	1.1	0.0	53.9
	403136	0.0	0.0	21.9	0.0	0.0	5.9	31.3	30.6	0.0	0.0	0.0	6.6	0.0	0.0	0.0	0.0	12.5
	403142	0.0	0.0	12.5	0.0	0.0	1.9	9.4	26.0	0.0	0.0	0.0	11.6	0.0	0.0	0.0	0.0	41.1
	403143	0.0	0.0	19.7	0.3	0.0	5.4	23.2	36.9	0.0	0.0	0.0	12.7	0.5	0.0	1.4	0.0	52.7
	403145	0.0	0.0	17.3	0.5	0.0	3.9	25.4	29.3	0.3	0.0	0.0	2.6	0.4	0.2	1.0	0.0	77.9
	403147	0.0	0.0	4.6	0.0	0.0	0.0	7.1	7.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	12.9
	403151	0.0	0.0	10.4	0.0	1.0	3.7	11.9	27.8	0.0	0.0	0.0	12.7	0.0	0.0	0.0	0.0	20.0
	403158	0.0	0.0	11.0	0.0	1.0	3.5	11.6	27.0	0.0	0.0	0.0	19.7	0.0	0.0	0.0	0.0	19.8
	403159	0.0	0.0	15.8	0.0	0.9	3.8	23.2	31.7	0.0	0.0	0.0	24.6	0.0	0.0	0.0	0.0	17.9
	403177	0.0	0.0	21.0	1.3	0.0	4.9	20.3	36.4	0.0	0.0	0.0	16.1	0.0	0.0	0.0	0.0	34.1
	403178	0.0	0.0	11.2	0.0	0.0	2.8	14.3	23.7	0.0	0.0	0.0	14.1	0.0	0.0	0.0	0.0	41.3
Geomyces sp.	403302	0.3	0.2	22.7	1.3	0.0	4.9	52.3	16.5	0.0	0.0	0.6	0.5	0.0	0.0	0.0	0.0	101.6
Geomyces sp.	403303	0.0	0.2	21.6	1.4	0.0	3.5	53.4	17.8	0.0	0.0	0.5	0.6	0.0	0.0	0.0	0.0	67.2
Anarctomyces psychrotrophicus	403306	0.4	0.0	18.1	1.1	0.3	3.7	40.7	23.5	0.0	0.0	0.2	10.1	0.0	0.6	0.0	0.0	47.5
Anarctomyces psychrotrophicus	403307	0.5	0.0	21.7	0.9	0.8	4.0	39.7	23.8	0.0	0.0	0.0	7.7	0.0	0.0	0.0	0.0	32.8
Thelebolus microsporus	403308	0.0	0.0	17.4	2.3	0.0	3.3	31.1	34.7	0.0	0.0	0.0	10.2	0.0	0.0	0.0	0.4	20.7
	403310	0.0	0.0	14.3	0.4	0.2	10.9	11.4	43.2	0.0	0.0	0.0	18.4	0.0	0.3	0.0	0.0	36.4
Cadophora malorum	403316	0.2	0.1	21.2	0.4	0.0	10.8	41.5	19.9	0.0	0.0	0.7	1.7	0.0	0.0	0.0	0.0	77.0
Geomyces sp.	403318	0.2	0.2	19.0	0.7	0.1	6.5	44.2	26.9	0.0	0.0	0.0	0.9	0.0	0.0	0.0	0.0	111.9
Pleospora/ulocladium	403321	0.3	0.0	26.5	0.8	0.2	8.8	37.3	21.8	0.0	0.0	0.0	3.5	0.3	0.0	0.0	0.0	55.0
Tetracladium sp.	403323	0.0	0.0	19.9	1.0	0.0	5.3	25.8	31.6	0.0	0.0	0.0	16.5	0.0	0.0	0.0	0.0	22.7
Ascomycota	403330	0.3	0.2	22.0	1.2	0.0	5.5	51.6	17.1	0.0	0.0	0.0	1.0	0.6	0.0	0.0	0.0	176.8
Geomyces sp.	403333	0.0	0.0	20.2	1.4	0.0	3.4	44.4	28.6	0.0	0.0	0.0	1.3	0.0	0.0	0.0	0.0	70.9

Organism	IMI	C14:0	C15:0	C16:0	C16:1	C17:1	C18:0	C18:1	C18:2	C20:0	C18:3 n6	C20:1	C18:3 n3	C22:0	C22:1	C24:0	C24:1	Total lipid mg/g
Penicillium sp.	403341	0.0	0.0	11.7	0.5	0.0	8.9	8.9	40.8	0.0	0.0	0.0	27.1	0.0	0.0	0.5	0.0	25.8
Bjerkandera adusta	403530	0.0	0.0	20.0	0.0	0.0	1.3	3.5	69.5	0.0	0.0	1.0	0.0	0.0	0.0	1.0	0.3	9.7
Clavicipitaceae	dis 169	0.2	0.0	20.4	1.8	0.2	6.4	24.5	34.1	0.9	0.0	0.0	8.7	0.2	0.0	0.6	0.0	84.7
Umbelopsis isabellina	dis 195	0.6	0.0	19.1	1.9	0.0	3.9	52.1	11.2	0.5	10.3	0.0	0.0	0.0	0.0	0.4	0.0	135.3
Umbelopsis sp.	dis 206	0.7	0.0	22.3	2.4	0.0	3.8	46.6	10.8	1.0	11.0	0.3	0.0	0.3	0.0	0.3	0.3	224.0
Sporobolomyces roseus	ncyc 1464	0.9	0.0	13.5	0.8	0.0	3.5	43.5	20.9	0.0	0.0	0.0	14.7	0.0	0.0	0.9	0.0	39.5
Yarrowia lipolytica	ncyc 825	0.0	0.0	16.8	11.3	0.9	5.6	34.6	27.7	0.0	0.0	0.0	0.0	0.0	0.0	1.9	0.0	46.3
Phaffia rhodozyma	ncyc 874	0.0	0.0	14.0	0.8	0.0	2.6	46.0	30.5	0.4	0.0	0.0	4.5	0.5	0.0	0.7	0.0	140.9
Daedaleopsis confragosa	328662	0.40	0.19	12.46	0.27	0.00	1.91	7.81	68.83	0.23	0.00	0.00	5.71	0.25	0.0	0.51	0.0	54.3

Table 3-5. The fatty acid profiles of 14 isolates, from both the fungi and Oomycetes capable of VLCPUFA production. In general *Mortierella* species produce the greatest proportion of $C_{20:4}$ n6 whilst species from the Oomycetes produce the greatest proportion of $C_{20:5}$ n3.

Organism	IMI	C14:0	C15:0	C16:0	C16:1	C17:1	C18:0	C18:1	C18:2	C20:0	C18:3 n6	C20:1	C18:3 n3
Mortierella alpina	82072	1.43	0.00	17.61	0.08	0.05	11.47	23.55	7.59	1.94	4.03	1.67	0.00
Mortierella polycephala	140468	1.45	0.68	15.96	0.40	0.75	5.69	42.57	3.73	0.75	3.31	1.22	0.26
Mortierella alpina	196057	1.42	0.15	15.21	0.14	0.00	7.34	11.75	4.65	0.40	7.60	0.41	0.16
Pythium irregulare	308153	5.47	0.00	13.39	11.51	0.58	1.97	23.45	9.15	0.55	1.38	4.60	0.00
Saprolegnia diclina	308259	11.20	0.65	15.86	3.64	0.00	5.10	11.97	10.04	0.99	2.28	0.00	0.00
Mortierella alpina	330997	0.69	0.00	21.47	0.30	0.26	9.50	20.48	6.60	1.73	3.64	0.97	0.00
Allomyces macrogynus	332398	1.17	0.00	17.83	0.00	0.00	3.78	5.81	13.87	2.33	19.13	1.57	0.00
Phytophthora richardiae	340618	11.41	0.00	17.71	2.79	0.00	5.15	16.24	17.05	1.38	1.31	1.31	1.14
Achlya americana	344320	13.55	0.00	21.11	2.58	0.00	5.36	15.92	6.59	1.21	1.82	0.00	0.00
Mortierella sp.	398213	1.99	1.11	23.63	0.85	1.14	4.12	48.18	3.42	0.29	1.84	1.26	0.00
Mortierella sp.	398216	0.32	0.08	4.02	0.05	0.00	4.16	2.94	3.36	0.52	4.33	0.53	0.00
Mortierella sp.	398217	4.34	0.48	17.21	0.45	0.30	8.42	37.03	3.08	0.30	5.17	0.98	0.00
Mortierella sp.	398220	1.46	0.59	14.52	0.36	0.25	7.93	47.70	2.12	0.55	3.17	2.06	0.00
Mortierella sp.	398111	3.38	0.00	16.76	0.15	0.00	12.00	37.80	5.53	0.39	4.40	0.97	0.00

Organism	IMI	C21:0	C20:2 n6	C22:0	C20:3 n6	C22:1	C20:4 n6	C22:2 n6	C24:0	C20:5 n3	C24:1	C22:5 n3	Total lipid mg/g
Mortierella alpina	82072	0.00	0.00	2.58	8.46	0.34	13.00	0.00	2.13	1.40	0.86	0.00	133.9
Mortierella polycephala	140468	0.09	0.16	0.83	5.18	0.12	6.81	0.05	0.64	4.11	0.78	0.00	57.0
Mortierella alpina	196057	0.00	0.33	0.88	5.98	0.00	34.92	0.27	1.79	5.04	0.64	0.00	137.8
Pythium irregulare	308153	0.00	0.55	1.19	1.36	0.80	3.81	0.00	0.00	14.30	0.00	0.15	144.5
Saprolegnia diclina	308259	0.00	0.00	0.00	2.26	0.00	9.87	0.00	0.00	19.90	0.00	0.00	41.4
Mortierella alpina	330997	0.00	0.00	1.86	9.84	0.00	14.43	0.00	2.77	1.55	0.42	0.18	53.8
Allomyces macrogynus	332398	0.00	2.24	0.00	16.60	0.00	11.20	0.00	0.00	0.00	0.00	0.00	34.4
Phytophthora richardiae	340618	0.00	0.33	1.93	4.17	0.92	2.71	0.00	0.00	12.09	0.00	0.00	36.3
Achlya americana	344320	0.00	0.00	0.00	3.43	0.00	8.51	0.00	0.00	13.35	0.00	0.00	93.6
Mortierella sp.	398213	0.08	0.08	0.57	1.40	0.19	6.16	0.06	0.52	1.14	1.01	0.00	72.8
Mortierella sp.	398216	0.00	0.80	0.00	3.57	0.00	67.84	0.05	2.85	3.18	0.33	0.00	231.0
Mortierella sp.	398217	0.00	0.08	0.62	3.81	0.00	13.57	0.08	0.90	1.51	1.20	0.00	106.4
Mortierella sp.	398220	0.03	0.18	0.80	3.25	0.13	7.93	0.04	0.72	4.34	0.76	0.00	135.7
Mortierella sp.	398111	0.00	0.10	0.72	5.59	0.11	4.60	0.00	0.78	0.50	1.01	0.00	173.7

The above data are presented as the percentage of the total lipid as a means of normalisation, as well as allowing for direct comparison between isolates. Other fatty acid profiling studies of fungi have also utilised the same methodology (Stahl & Klug, 1996). The aim of the work by Stahl & Klug was not to identify fungi, but rather to characterise and differentiate them. In the case of this study, the data collected was not intended for taxonomic classification, but the resulting data were used to identify trends within groups of isolates, e.g. within genera for example. The analysis of large sets of data containing multiple variables can be more readily studied in a visual format, whereby the multiple variances in variables can be condensed into several variables which express the majority of the variance within the data. The analysis of multiple variables such as metabolites can be visualised using a technique called principle component analysis (PCA). A similar study was carried out in the fungus *Histoplasma capsulatum* (Zarnowski *et al.*, 2007), whereby strains were grouped by their fatty acid profiles. By utilising this type of analysis, samples displaying similar trends within their multiple variables will be clustered together. Such a technique can be used in an exploratory or predictive fashion, with exploration allowing for example, the identification of the fatty acids which contribute to the clustering of organisms. In the predictive sense it can be used as a taxonomic tool to place organisms based on their metabolite profile. Due to the relatively unspecific classification of isolates coupled with the lack of replication, PCA failed to provide any clear separation of isolates into clear groupings. However, a related technique, partial least squares discriminant analysis (PLS-DA) was used which provided much clearer separation between the groups of organisms. The technique effectively sharpens the separation between groups of observations by utilizing class information, however the ability of this method to accurately predict further classifications based on unknown data has been called into question (Westerhuis *et al.*, 2008). Due to this limitation and the lack of replication and detailed taxonomic information regarding the BAS isolates the presented cluster analysis cannot be utilised for taxonomic identification, but rather the differentiation of a large number of isolates into similar groupings based upon characteristic trends.

A PLS-DA was performed utilising the major known groupings of organisms using 6 fatty acid components, $C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ n3 shown in figure 3-11. $C_{18:3}$ n6 wasn't used for the initial PLS-DA as the majority of organisms contained $C_{18:3}$ n3 and excluding $C_{18:3}$ n6 resulted in clearer separation. The organisms were split into

Pleosporales, *Leptodontidium* sp., *Mollisia* sp., Oomycota, zygomycetes capable of producing $C_{18:3}$ n6 only and *Mortierella* species. Longer chain fatty acids were not utilised for the analysis due to the spurious and trace quantities present in the majority of organisms. The cluster analysis discriminates between the VLCPUFA producing organisms and those incapable of elongating past $C_{18:3}$ n3. *Mortierella*, Oomycete and the majority of zygomycete isolates effectively form an out group from what is effectively an Ascomycota cluster, with the fatty acids $C_{18:0}$, $C_{18:2}$ and $C_{18:3}$ n3 contributing largely to component 1, which predominantly segregate the two groupings. The PLS-DA loading plots are found in the appendix, section 7.3.

The $C_{18:3}$ n3 producing contaminant isolate 328662, initially thought to be a *Phytophthora* isolate, was removed to improve the clustering of the groups. It was shown to lie outside both clusters although appeared to be an outlier of the Ascomycota cluster rather than the Oomycete/*Mortierella* cluster. The two *Umbelopsis* isolates Dis 206 and 195, from the zygomycetes were shown to cluster toward the fringes of the Ascomycota grouping separated primarily by the first component. The *Umbelopsis* isolates do group in proximity to the *Mucor hiemalis* isolates, suggesting that similar fatty acid profiles are shared by zygomycetes. *Mucor racemosus* and *M. hiemalis* however group more definitively within the *Mortierella*/Chromista cluster, with the two *M. hiemalis* isolates grouping closer together than to the *M. racemosus* isolate. The inter-species and intra-species separation based on fatty acid profiles highlights the potentially large variation found in fatty acid composition between organisms. This inter/intra-species variation is found within the *Mortierella*, as the species *Mortierella alpina* shows some intra-species variation with isolates 330997 and 82072 clustering together, whilst 196057 groups away. The relatively loose clustering of the *Mortierella* indicates differing species, especially for isolate 398216, which produces high $C_{20:4}$ n6 levels. This large degree of variation is also found within the ascomycete isolates studied, as most species level identities are not known. It is this level of variation between multiple species which prevents PCA from clustering these organisms. The $C_{18:3}$ n3 producing isolates Dis 169 and 403341, initially identified as *Umbelopsis* isolates are seen embedded in the centre of the Ascomycota cluster. This correlates with the DNA identification of the isolates, as an isolate from the Clavicipitaceae and a *Penicillium* sp. which confirms their grouping within this cluster. The Pleosporales can be seen to form a distinct grouping away from the intermingled *Leptodontidium* and *Mollisia* species suggesting a set of fatty acid profiles unique to the Pleosporales. The

intermingled nature of the *Leptodontidium* and *Mollisia* species indicates that they share similar fatty acid profiles.

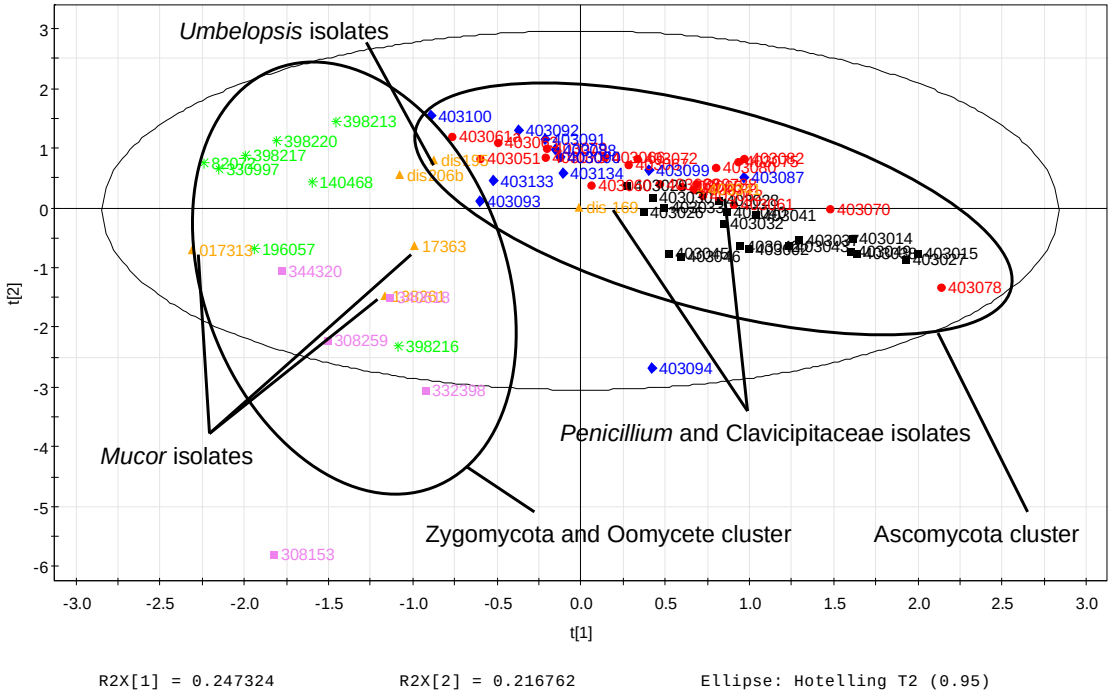


Figure 3-11. PLS-DA of fatty acid profiles grown in PD media. ■ = Pleosporales ● = *Leptodontidium sp.* ◆ = *Mollisia* sp. ■ = Oomycetes ▲= *Umbelopsis and Mucor* sp. ✦ = *Mortierella*. The PLS-DA generated 3 components which explained 60% of the variance and had a cumulative fit to the Y-data (R2Y) of 0.34.

A second PLS-DA was run using only the three most abundant organism classes identified from BAS, the Pleosporales, *Leptodontidium* and *Mollisia* species as shown in figure 3-12. The analysis was run using only 6 components as $C_{18:3}$ n6 is not present in any of the isolates. It was found that several samples, particularly *Leptodontidium* isolates overlapped or in some cases were significantly removed from their grouping. This is possibly due to the fact that multiple species may have been studied as identification was made only to the genus level. It may also suggest that several organisms may be incorrectly identified, as their fatty acid profiles more closely resemble that of the other species. Variation amongst different strains may also account for some of the variability. The *Pleosporales* group which contained several identified *Herpotrichia* sp. isolates formed the most cohesive group, whilst *Mollisia* isolates predominantly clustered together. The fatty acids $C_{16:0}$ and $C_{18:2}$ primarily explained the separation along component 1, whilst $C_{16:1}$, $C_{18:0}$, $C_{18:1}$ and $C_{18:3}$ n3 explain the majority of the separation along component 2. *Mollisia* segregation was found to be associated with $C_{16:0}$ levels, Pleosporales with $C_{18:2}$ levels and *Leptodontidium* isolates with $C_{18:1}$

and $C_{18:3}$ n3 levels. However, the variation within these fatty acids was exemplified by class information which allowed for greater segregation than PCA provided alone.

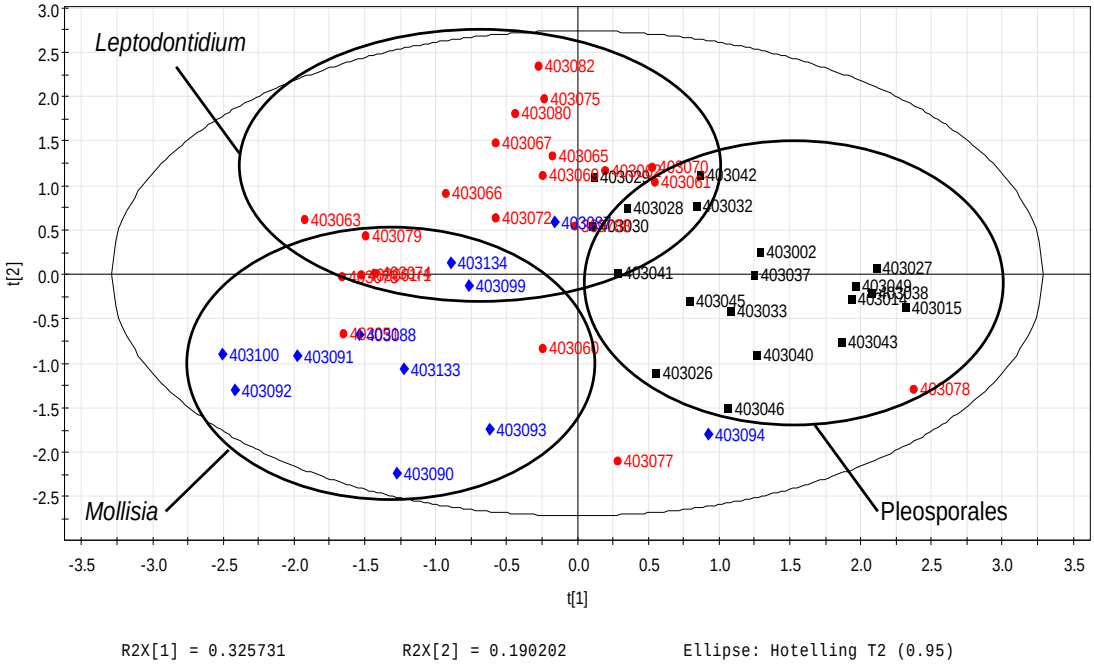


Figure 3-12. PLS-DA of BAS *Leptodontidium*, *Mollisia* and Pleosporales isolates grown in PD broth. ■ = Pleosporales ● = *Leptodontidium sp.* ◆ = *Mollisia sp.* The PLD-DA generated 3 components which accounted for 73% of the variation and had a cumulative fit to the Y-data (R2Y) of 0.46.

A further analysis was undertaken using the fatty acid profiles from isolates grown on YES media shown in figure 3-13. The table of MA and YES fatty acid profiles is given in the appendix, section 7.3. Fewer organisms were utilised for this analysis as several were grown in MA media and weren't included in the PLS-DA. The *Mollisia* clustering is relatively tight separated by $C_{16:0}$ levels, whereas Pleosporales isolates form a distinct but looser cluster separated by $C_{16:1}$ and $C_{18:2}$ levels. The *Leptodontidium* isolates again coalesce to form a grouping separated by $C_{18:0}$, $C_{18:1}$ and $C_{18:3}$ n3. Their wide spread however indicates variation within the fatty acid profiles, and several isolates lay within the Pleosporales and *Mollisia* clusters indicating that these isolates share greater similarity in fatty acid profiles to these organisms than to their actual class.

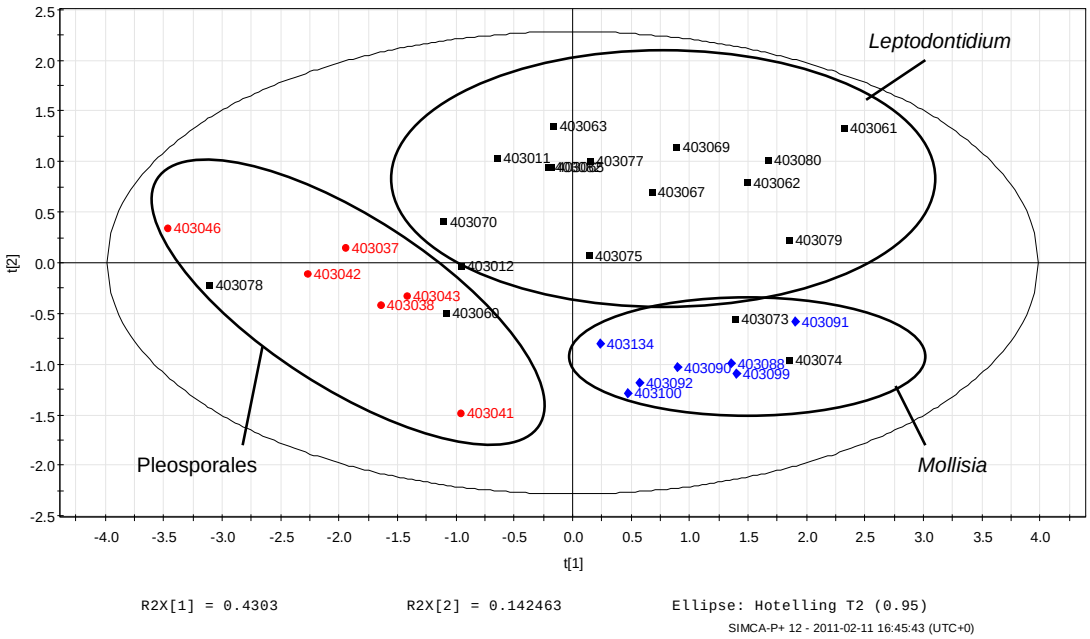


Figure 3-13. PLS-DA of BAS *Leptodontidium, Mollisia* and Pleosporales isolates grown in YES broth. ■ = *Leptodontidium* sp. ● = Pleosporales ◆ = *Mollisia sp*. The PLS-DA generated 2 components which explained 57% of the variance and had a cumulative fit to the Y-data (R2Y) of 0.49.

Finally, a PLS-DA was run using Oomycete, *Mortierella* and representative zygomycete isolate fatty acid profiles shown in figure -3-14. The number of fatty acids components was increased to 16 including the VLCPUFAs such as $C_{20:4}$ n6 and $C_{20:5}$ n3, as the majority of compounds were common to a greater proportion of organisms and in higher abundance. The Oomycetes clustered loosely together, predominantly because the isolates were of different species but appear to contain enough similarity within their fatty acid profiles to distinguish them from the other groupings. The *Mortierella* form a tight grouping indicating their similar fatty acid profiles, with the non-VLCPUFA producing zygomycete isolates clustered away based on $C_{18:2}$ and $C_{18:3}$ n6 levels. Within the zygomycete cluster, in contrast to the initial PLS-DA, fatty acid profiles between *Umbelopsis* and *Mucor hiemalis* appear to be substantially different with *Mucor racemosus* displaying much greater similarity to the *Umbelopsis* isolates. The separation between the two *Mucor* species is again due to $C_{18:2}$ levels as well as $C_{16:1}$. The Oomycetes cluster due to their levels of $C_{20:1}$, $C_{20:2}$ n6, $C_{16:1}$, $C_{24:1}$ and $C_{20:5}$ n3 with the genus *Mortierella* forming a cluster based upon the levels of $C_{20:3}$ n6 and $C_{20:4}$ n6. It comes as little surprise that *Mortierella* species segregate due to their $C_{20:4}$ n6 levels as values as high as 67.8% have been observed within these isolates. Oomycete isolates also display a higher percentage of $C_{20:5}$ n3 than that produced by *Mortierella* strains justifying their segregation by this fatty acid.

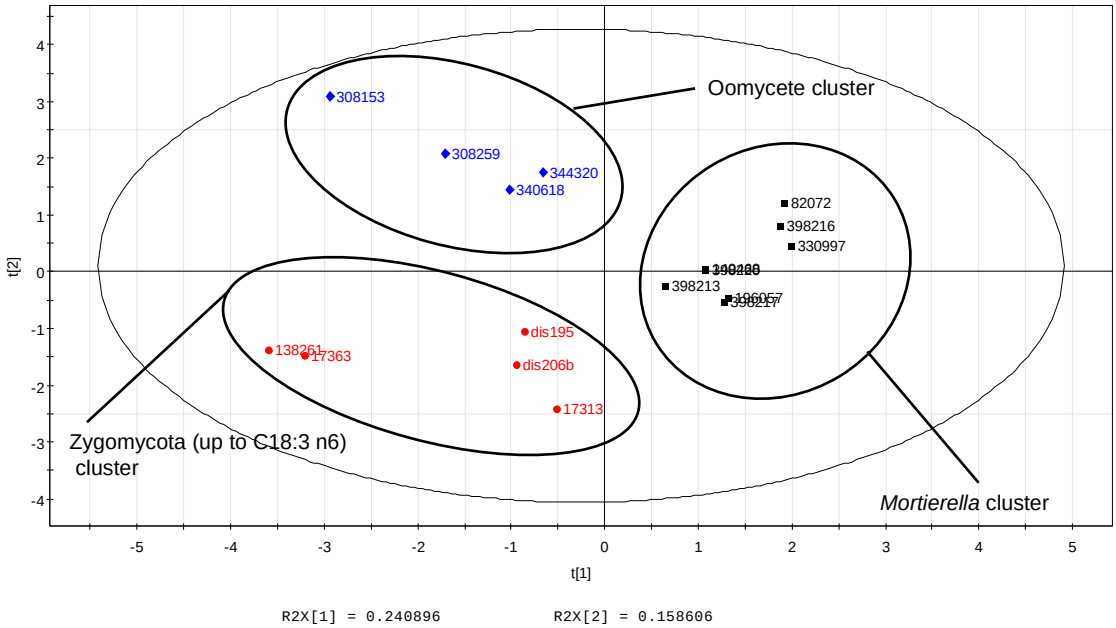


Figure 3-14. PLS-DA of VLCPUFA producing organisms. ■ = *Mortierella* sp. ● = *Umbelopsis* and *Mucor* sp. ◆ = Oomycetes. The PLS-DA generated 2 components which explained 40% of the variance and had a cumulative fit to the Y-data (R2Y) of 0.79.

The majority of BAS isolates were grown in two media, PD and YES, with organisms thought to be of the same species analysed together to give an indication of the effect media plays on the major fatty acid components. Averages were taken between different isolates of the same species due to the lack of replication. Organisms from the Pleosporales were found to produce on average larger proportions of $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ when grown on PD media (Figure 3-15) whereas YES media on average induced a slight increase in $C_{18:2}$ production and a more significant increase in $C_{18:3}$ n3.

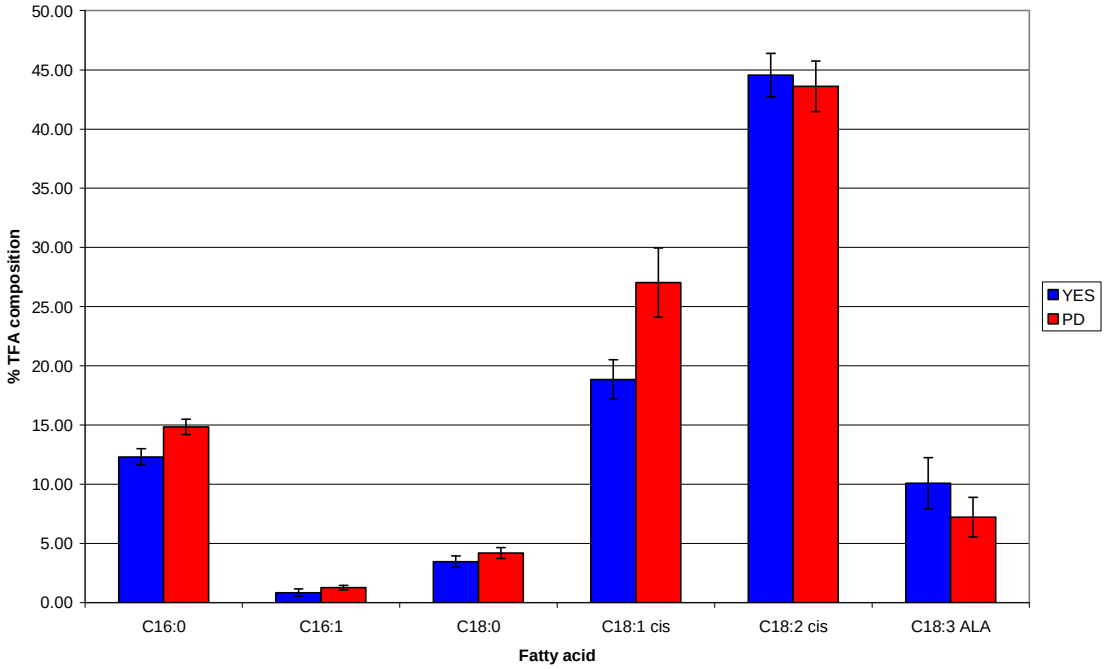


Figure 3-15. The major fatty acid components of 9 fungi from within the Pleosporales, most likely *Herpotrichia* species grown in two media YES and PD. The averages are calculated between different isolates.

Isolates from the species *Leptodontidium* showed increased production of $C_{16:0}$, $C_{18:2}$ and $C_{18:3}$ n3 when grown in PD broth, whilst YES media in general increased $C_{18:0}$ and $C_{18:1}$ levels (figure 3-16). *Leptodontidium* appears to demonstrate opposite FA trends compared with the Pleosporales isolates in regards to culture media, with the only consistency between the two groups of organisms being the elevated level of $C_{16:0}$ when grown in PD media.

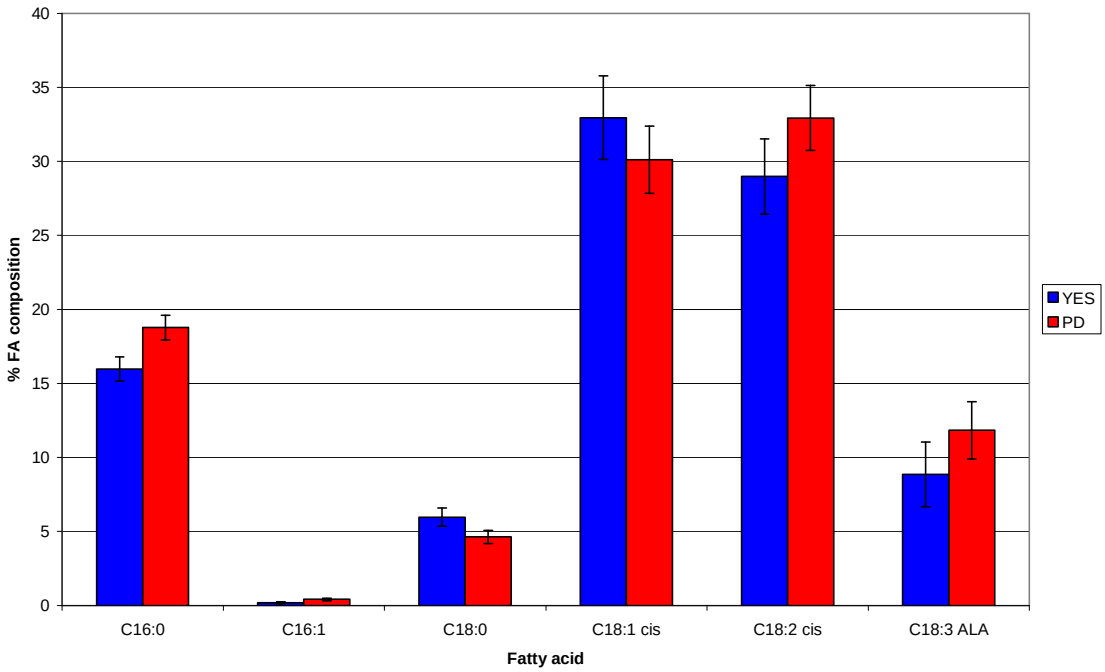


Figure 3-16. The major fatty acid components in 16 *Leptodontidium* species grown in two media YES and PD.

Mollisia species demonstrated lower percentages for all the major components when grown in YES media (Figure 3-17) although $C_{18:0}$ and $C_{18:3}$ n3 level differences were much smaller. The reason for this was a peak which eluted around the same time point as $C_{17:1}$ which comprised up to 16.8% of the TFAs in several of the isolates. This peak was not found when the organisms were grown in PD media, and explains the overall lower values found with YES media. The identity of the peak was not pursued. In general Pleosporales cultures demonstrated the greatest proportion of $C_{18:2}$, which is in contrast to the lowest levels found on average within *Mollisia* isolates. $C_{16:0}$ levels conversely were highest in *Mollisia* species but found to be lowest in Pleosporales, with these two fatty acids primarily segregating these two clusters in PLS-DA when grown in PD media. *Leptodontidium* isolates are found to group away from *Mollisia* and Pleosporales isolates based on $C_{18:1}$ and $C_{18:3}$ n3 levels, which are found to be highest within *Leptodontidium* isolates. Lower levels of $C_{16:1}$ and $C_{18:0}$ although small, are also responsible for the grouping of *Mollisia* species away from *Leptodontidium* species along the second PLS-DA component, with *Mollisia* isolates containing higher proportions of both fatty acids compared with Leptodontidium. *Leptodontidium* and *Mollisia* cultures produce roughly equal amounts of both $C_{18:1}$ and $C_{18:2}$, which in *Leptodontidium* are the predominant FAs. *Mollisia* cultures also produced equal amounts of $C_{16:0}$, making $C_{16:0}$, $C_{18:1}$ and $C_{18:2}$ the three most abundant FAs. $C_{16:0}$ was found to be the third most abundant FA within both Pleosporales and *Leptodontidium* cultures. Levels of $C_{18:3}$ n3 and $C_{18:0}$ are consistently lower in level, usually comprising the 4^{th} and the 5^{th} most abundant FAs within the studied groups of organisms.

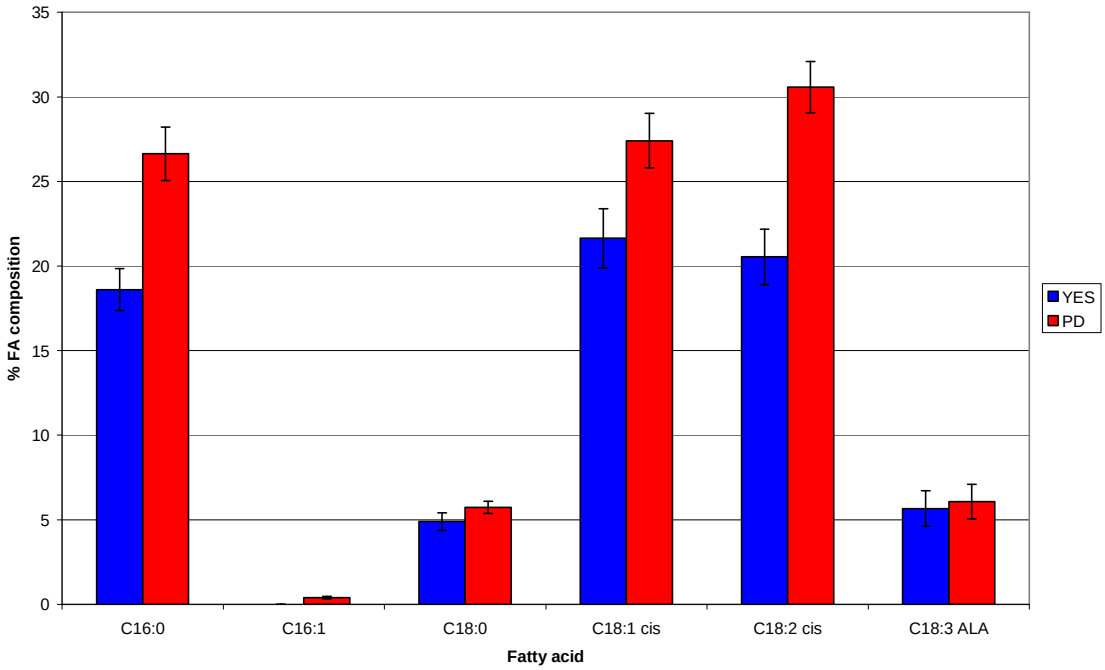


Figure 3-17. The major fatty acid components in 7 *Mollisia* species grown in two media YES and PD.

3.3 Discussion

3.3.1 GC-FID and GC-MS conditions

The methodology used for fatty acid analysis was studied to ascertain whether the analysis platform was suitable. The extraction recovery values whilst higher than 100%, which are most likely attributed to dilution error or pipetting error, still indicate a high recovery rate. It is to be noted that the internal standard is likely easier to extract and derivatise as it is not highly associated with a complex matrix such as fatty acids within the membrane or within the cell. Whilst the recovery figure could not be quantified the introduction of an appropriate internal standard, $C_{17:0}$ was used to account for loss during the procedure. It follows that as the IS is a fatty acid which resides roughly in the middle of the fatty acid series any loss affecting the sample will equally affect the IS. Any loss occurring through extraction, derivitisation and machine error should be reflected by the internal standard, in essence reducing the need for technical repetition. Quantification without an internal standard requires either fold difference as compared to a control or calibration curves. Fluctuations within the integrated area however cannot be normalised and hence lead to intrinsic error, which would be shown between technical repetitions. The reproducibility between technical repetitions when spiked with an IS, demonstrates that only one technical repetition needs to be used for fatty acid analysis. The benefits of this include less sample preparation and less solvent use.

The removal of technical replicates means that greater numbers of biological replicates could be used, where the greater extent of compound variation exists. Factors which enable technical replicates to be removed in this work also include total homogenisation of samples which allows for fatty acid homogeneity throughout the sample. Another factor to consider is that only one compound class is being studied as oppose to a complete metabolomics approach, whereby several compound classes are studied. In such a case, each compound class may experience loss and differential derivitisation leading to fluctuation within technical repetitions. In such a scenario multiple internal standards may be utilised to compensate, which may allow for the removal of technical replicates. The majority of the work was also focussed as a screening approach for the identification of VLCPUFAs and as such did not warrant technical replication.

The GC-FID and GC-MS response factors show little variation between different concentrations however, response factors on the GC-MS using TIC were more variable and subsequently showed greater standard error. Because of this EI was used for GC-MS quantification. Linearity for both machines was high; however the linearity of the GC-MS response was reduced and started to plateau above $5x10^6$ area units. TIC measurements showed the least linearity predominantly because they had the greatest area and hence detector saturation was noticed at lower concentrations. As EI only focuses on one ion, saturation occurred at higher concentrations leading to a more linear response. GC-FID provided a linear response over the entire range tested. However, the GC-FID was only tested with lower concentrations due to the lack of the ability to change the split ratio, whereas the GC-MS was capable of allowing more analyte into the detector increasing the concentration. Due to the high abundance of saturates within the FAME standards, these compounds were usually the most affected by detector saturation. Whilst a greater range was looked at using the GC-MS, when comparable quantities of saturates were studied between the GC-MS and GC-FID, the GC-FID maintained linearity up to 50 ng whereas equivalent amounts run on the GC-MS resulted in areas greater than $5x10^6$ and resulted in detector saturation. It is reasonable to assume that all the fatty acids on the GC-FID would display high linearity up to 35-45 ng as shown with the saturates. Saturation of the detector and the subsequent decline in detector response to analyte at higher concentrations, can to a degree, be mitigated by selecting less prominent ions to quantify, which in turn leads to lower area counts and subsequently greater linearity. Selecting an appropriate ion depends largely on whether many closely co-eluting compounds are present as is demonstrated by the $C_{18:0}$ fatty acid series. An ion needs to be selected which is unique to each compound and allows

for reliable quantification. In summary, GC-MS linearity is lost above a certain integrated area which makes TIC the most inaccurate quantification method and EI the most accurate, with the ion selection dependent on co-elution and the concentration range being studied. The GC-FID however showed no such saturation effects at the concentrations studied. GC-FID however lacks definitive identification of compounds due to time being the only identifying measure, whereas a custom mass spectrum library provides reliable identification.

3.3.2 Fatty acid profiling of novel low temperature fungal isolates.

After screening over 100 organisms isolated from low temperature environments it was found that only zygomycetes, Oomycetes and chytrids were capable of producing VLCPUFAs, with those within the Ascomycota found only to produce up to $C_{18:3}$ n3. It has to be noted that a large proportion of the organisms studied were from several distinct groups and although 102 BAS isolates were studied, several hundred isolates remain unstudied. A large proportion of the BAS collection are not identified to the species or genus level, though it is thought new species may be present within this collection. Whilst the use of uncharacterised and potentially novel samples for screening can provide unexpected results, it can however come with consequences. The lack of highly characterised organisms for example leads to a broad and non-targeted approach to organism selection as well as the possibility of unintentional redundancy. If a broader range of Antarctic ascomycetes and basidiomycetes were studied and it was found that none were capable of VLCPUFA production then one would assume that the taxonomic ranking of the organism would play a greater role as a marker for VLCPUFA production as oppose to environment alone. Hence, if further screening were to occur utilising a fatty acid screening technique then organism selection from the collection would focus predominantly on Oomycetes, chytrids and zygomycetes due to the increased prevalence of VLCPUFA production within these organisms. Even though the screen itself did not describe any new species capable of producing VLCPUFAs, it does raise some interesting questions. Firstly why did the higher fungi lose their ability to produce VLCPUFAs and switch to the n3 route, and parallel to that what function do long chain n3/6 fatty acids play in the basal lineages of fungi? It also highlights that in low temperature environments, specifically those in contact with air, $C_{18:3}$ fatty acids are sufficient for maintaining membrane fluidity and allowing growth at 15°C, and in some cases growth at 5°C, although the growth of the majority of organisms was slow

requiring 2 to 3 weeks before sufficient mycelial biomass was produced. Whilst no growth rate studies were carried out some incidental data would indicate that several of these organisms are psychrophilic, which shall be highlighted in the next chapter.

The use of two media was to ensure growth of the organisms as well as ascertaining whether cultures were capable of VLCPUFA production. It appears that the BAS isolated organisms studied, which were predominantly ascomycetes, are incapable of producing fatty acids longer and more unsaturated than $C_{18:3}$ n3. There were some fatty acid compositional changes when different media were utilised, with organisms from the Pleosporales showing greater production of $C_{16:0}$ and $C_{18:1}$ on PD media, whilst YES stimulated a slight increase in $C_{18:2}$ and a more substantial increase in $C_{18:3}$ n3. The elevated levels of $C_{18:2}$ are most likely responsible for the increase in $C_{18:3}$ n3 due to its role as a substrate, as well as depleting levels of $C_{18:1}$ which itself is a substrate for $C_{18:2}$. *Leptodontidium* species demonstrated higher production of more unsaturated fatty acids with PD media with elevated levels of $C_{18:2}$ and $C_{18:3}$ n3, in contrast to Pleosporales. Levels of $C_{16:0}$ and $C_{18:0}$ remain around 15% and 5% respectively between both groups, however the FAs $C_{18:1}$ and $C_{18:2}$ are the main components which distinguish the two groups. This is confirmed by PLS-DA, figure 3-12, which clusters the Pleosporales and *Leptodontidium* by $C_{18:1}$ and $C_{18:2}$, with the Pleosporales demonstrating on average just under 27.0% and 43.6% $C_{18:1}$ and $C_{18:2}$ respectively whereas *Leptodontidium* species show $C_{18:1}$ and $C_{18:2}$ levels of 30.1% and 32.9% respectively, with the values used from PD media. The *Mollisia* species were found to produce an unknown peak which comprised a large proportion of the fatty acid profile when grown on YES media, however only a few of these isolates demonstrated this high abundance peak. However this was one of the principle reasons why the other major peaks were lower than their PD media counterparts. Comparison of *Mollisia* species with the other two groups shows increased production of $C_{16:0}$ at 26.6% of the TFA compared with 18.8% and 14.9% for *Leptodontidium* and Pleosporales respectively. Again, PLS-DA clustered the *Mollisia* species away from the other two groupings based on this character. $C_{18:3}$ n3 levels are also noticeably lower in *Mollisia* species when grown on both media with *Leptodontidium* species producing the highest TFA percentage at 11.8% although this value drops to 8.9% when grown on YES media. The high $C_{18:3}$ n3 production on PD media therefore distinguishes *Leptodontidium* species from the other two groups. However, values are much closer when grown on YES media where Pleosporales shows the highest production at 10.1%. The differences in fatty acid composition are predominantly due to the carbon source used in the media. All three media

predominantly use monosaccharides (glucose) or disaccharides (sucrose) as the carbon source with little lipid present within the media. If fatty acids are present within the media, they may be transferred to the acyl-CoA pool whereby they are acted upon by elongases or desaturases. As sugars and starch, in the case of PD media, are the primary components, it can be assumed that lipid production is predominantly *de novo*. It was found that in *Mortierella ramanniana* glucose produced the lowest $C_{18:3}$ n6 value (% w/w) whilst sucrose resulted in both higher $C_{18:3}$ n6 levels and overall increased unsaturation levels of lipid. Starch however resulted in the highest values for both $C_{18:3}$ n6 and total lipid unsaturation levels (Hansson & Dostalek, 1988). Another study by Jang *et al.* (Jang *et al.*, 2005) found that varying the concentration of both glucose and starch in the media resulted in differing compositional fatty acid profiles. It was found that the concentration of the carbon source greatly affected the absolute values of fatty acids, however this is likely due to the oleaginous nature of the *Mortierella* species. Yeast extract was found to lower the overall unsaturation of the lipids when compared with media lacking this component.

Whilst the hypothesis of low temperature environments inducing VLCPUFA formation seems to be untrue in light of the present data, subsequent data discussed in the next chapter reinforces the validity of the hypothesis that low temperatures increase FA unsaturation levels. What can be stated according to the presented data is that ascomycetes and basidiomycetes are no more likely to contain fatty acids longer than C_{18} and with more than three double bonds when isolated from low temperature environments than if they were isolated from warmer climates. However what was not taken into account in this study are the numerous other modifications that the organisms may have undergone such as protein modification to allow for enzyme function at low temperatures, which play an equally large role in organism survival.

During the analysis several isolates demonstrated curious fatty acid profiles, which if taken with their taxonomy would have illustrated an unknown fatty acid biosynthesis pathway within the zygomycetes. However on DNA sequence analysis the identifications of the organisms were found to lie within the Ascomycota cluster, which correlates with the observed fatty acid profiles. Whilst the PLS-DA was not intended for taxonomic inference, the analysis highlighted the grouping to which the contaminant organisms belonged. Isolates 403341 and Dis 169, *Penicillium* sp. and an organism from the Clavicipitaceae respectively, were found to cluster centrally within the Ascomycota cluster alluding to their actual identities. Isolate 328662, found to be

Daedaleopsis confragosa, whilst not shown on the PLS-DA due to it being an outlier and its removal improved clustering lay further towards the Ascomycota cluster than to the Zygomycota and Oomycete cluster. The fact that all three contaminant organisms produced $C_{18:3}$ n3 as oppose to $C_{18:3}$ n6 was one of the key issues which highlighted the possibility that these organisms were in fact contaminants, although the presence of $C_{18:3}$ n3 within a *Mortierella* species has been described albeit in low levels and was co-produced with $C_{18:3}$ n6 in *Mortierella polycephala* (Weete & Gandhi, 1999). It also highlights that fatty acid profiles may still be used to taxonomically identify fungi, with basic yet fundamental checks such as the preference for $C_{18:3}$ n3 or $C_{18:3}$ n6 and the presence or absence of VLCPUFAs confirming or raising doubt on the initial taxonomic classification. Going further, by creating an accurate fatty acid database coupled to proven taxonomic identities would allow for multivariate techniques such as PCA to classify unknown isolates. Such a model however would require multiple biological replications as well as a model for each media type due to the variation which can occur when grown on various media. It also highlights the dangers of accepting the taxonomy of isolates at face value, even from well established culture collections, especially if one is unfamiliar with the organism, or if multiple varied isolates are being studied.

Of the organisms capable of producing VLCPUFAs, the genus *Mortierella* was one of the few fungal species capable of producing such fatty acids. This genus whilst being well studied does present some interesting findings. Firstly, the production of small quantities of $C_{22:5}$ n3 and associated C_{22} VLCPUFAs hinted at an unspecific reaction, with the most likely target being a Δ6 elongase referred to as γ-linolenic elongase (GLELO). The role of this elongase is to elongate C_{18} polyunsaturated fatty acids to C_{20} VLCPUFAs. The subsequent elongation of C_{20} to C_{22} VLCPUFAs was therefore thought to be a small unspecific reaction. The only other organism to produce $C_{22:5}$ n3 was *Pythium irregulare*, which again was most likely a secondary reaction of a Δ6 elongase. Whilst the C_{22} VLCPUFA production of this enzyme is relatively negligible, its role as a recombinant gene has already started to be appreciated, with its recombination into *Glycine max (Damude & Kinney, 2008)*. Its use as a staple recombinant gene is not its only further use however, as the gene itself is most likely indicative of the presence of VLCPUFAs such as $C_{20:4}$ n6 and $C_{20:5}$ n3. Hence designing a specific genomic screen for this gene to predict VLCPUFA formation is yet another way in which this gene may assist in discovering novel VLCPUFA producing organisms, as well as the characterisation of these potential homologs. Both these strategies will be pursued in the following chapters. Of the *Mortierella* isolates, 398216

is notable for its sizeable production of $C_{20:4}$ n6, which comprises 67.84% of the total fatty acids. In fact, all *Mortierella* isolates, regardless of isolation location were capable of VLCPUFA production. It was found for the majority of *Mortierella* isolates that proportionally, $C_{20:4}$ n6 and $C_{20:5}$ n3 levels varied little in respect to isolation location. The Antarctic isolate 398216 however was found to produce the greatest proportion of $C_{20:4}$ n6 by a substantial degree, although isolate 196057 isolated from Australia, still produced $C_{20:4}$ n6 at 34.9%. Whilst temperature did not appear to influence fatty acid complement within the Ascomycota and Basidiomycota, the fact that no other BAS zygomycetes were studied, other than *Mortierella* may still partially validate the hypothesis that low temperatures select for VLCPUFA producers, due to the more basal nature of the Zygomycota. Isolates capable of high percentages of VLCPUFAs are valuable in industrial applications due to the decreased cost associated with producing marketable oil. Modification of such a high $C_{20:4}$ n6 producing organism could yield substantial quantities of $C_{20:5}$ n3 through the addition of more efficient or additional Δ17 desaturases. However, it appears only select zygomycete species such as *Mortierella*, the Chytridiomycota and Oomycetes are capable of producing these VLCPUFAs. The majority of the zygomycetes, as represented by *Umbelopsis* and *Mucor* species are incapable of producing fatty acids beyond $C_{18:3}$ n6.

The retention of, or gain of a functional elongase capable of elongating C_{18} polyunsaturated fatty acids does not appear to have occured within low temperature isolated ascomycetes and basidiomycetes, although it appears the loss of the VLCPUFA pathway occurred during the evolution of the zygomycetes. The majority of zygomycetes are capable of elongation only up to $C_{18:3}$ n6, as demonstrated by the *Umbelopsis* and *Mucor* isolates. When plotted on a PLS-DA the *Umbelopsis* isolates are found to sit on the outside of the Ascomycota grouping indicating that they share similarities in fatty acid profile with both the ascomycetes and zygomycetes/Oomycetes. The *Mucor* isolates sit further in the zygomycete/Oomycete cluster, with the two *Mucor hiemalis* isolates grouping closer together. *Mucor racemosus* segregates based on its heightened $C_{18:0}$ levels and lowered $C_{18:2}$ levels compared with the other *Mucor* species. The separation of the *Umbelopsis* and *Mucor* isolates from zygomycetes and Oomycetes is explained by their lack of VLCPUFAs, which in turn leads to greater percentages of other fatty acids such as $C_{18:2}$ and $C_{18:3}$ n6, which are used as substrate in *Mortierella* and Oomycete species. The lack of VLCPUFAs within the Ascomycota also results in increased $C_{18:2}$ levels when compared with the zygomycetes and Oomycetes. The Oomycetes and *Mortierella* segregate into separate clusters based on $C_{20:5}$ n3 for the

Oomycetes and $C_{20:4}$ n6 for the *Mortierella*. Fatty acids such as $C_{16:1}$ and $C_{20:2}$ n6 help define the Oomycetes with $C_{20:3}$ n6 levels shaping the *Mortierella* clustering as well.

The production of fatty acids longer and more unsaturated than $C_{18:3}$ appears to be a trait of the basal fungal lineages, such as *Allomyces macrogynus* from the most basal lineage the Chytridiomycota. *Mortierella*, *Conidiobolus* and *Entomophthora (Kendrick & Ratledge, 1992b)* are from the second divergence from the fungal lineage, the Zygomycota. In the transition from the Chytridiomycota to the Zygomycota the loss of the flagella is thought to have occurred several times (James *et al.*, 2006) indicating that several ancestors may have existed for the Zygomycota. Those zygomycetes capable of VLCPUFA production could be considered basal to the rest of the phyla due to their shared trait with the ancestral Chytridiomycota. They may represent an intermediary stage where certain morphological and biochemical processes are retained but others are lost, such as flagella. The unique fatty acid profile of *Mortierella* species appears to support this, with the loss of VLCPUFA production separating them from the vast majority of the phyla. The fatty acid profile therefore appears to follow the evolution of the fungi, with the Chytridiomycota and early zygomycetes capable of C_{20} VLCPUFA production. The establishment of the zygomycetes resulted in the loss of a Δ6 elongase, with the majority of organisms capable of producing only up to $C_{18:3}$ n6. The next step was the divergence to the Dikarya (Gehrig *et al.*, 1996), which resulted in the shift to the n3 pathway. This was most likely through the change in function of a desaturase, which enabled the insertion of double bond between carbons 15 and 16. The ancestral protein to the Δ15 desaturase may have been the Δ12 desaturase (Damude & Zhang, 2006), as it was found that the Δ12 desaturase could catalyse the formation of $C_{18:3}$ n3 from $C_{18:2}$ n6, and that the opposite was true, that the Δ15 desaturase could catalyse the formation of $C_{18:2}$ n6 from $C_{18:1}$. Therefore, it is possible through gene duplication, that the Δ12 gradually changed function and became the Δ15 desaturase found in the Dikarya. During the evolution of the Dikarya, the Δ6 desaturase responsible for $C_{18:3}$ n6 formation appears to have also been lost. The Dikarya further demonstrate this trend of fatty acid loss with multiple species incapable of trienoic and even dienoic fatty acid production (Kock & Botha, 1998).

It is quite clear from the acquired data as well as existing literature regarding higher fungi and their lack of VLCPUFAs that at some point the basal fungal lineages lost key enzymes involved with the biosynthesis of fatty acids longer and more unsaturated than $C_{18:3}$. The loss of enzymes and change in function over the course of evolution suggests

that changes in fatty acid composition must have been driven by, or lack of external pressures. This returns then to the question of the true function of VLCPUFAs within fungi, and possibly within the earlier ancestors of all these organisms. Several roles of VLCPUFAs have been put forward although the majority of them have been demonstrated within organisms other than true fungi. Several of them rely on the key property of highly unsaturated fatty acids and their contribution to the fluidity of the membrane. One suggestion is that flagella require fluid membranes (Thomashow & Rittenberg, 1985) and that incorporation of VLCPUFAs such as $C_{20:5}$ n3 would maintain the fluidity of the membrane to allow for the most efficient flagella operation. Zoospores have also been documented to contain VLCPUFAs such as $C_{20:4}$ n6 (Kagami *et al.*, 2007) as have Human spermatozoa which have been demonstrated to show a greater fatty acid unsaturation at later developmental stages, which in turn is thought to enable sperm motility (Haidl & Opper, 1997). Sperm early in development which had lower levels of unsaturation were immobile. Spermatozoa show similar fatty acid profiles with that found within the Chytridiomycota and Oomycetes, with chytrids and spermatozoa sharing striking similarities physiologically. It was therefore thought that fungi and the Animalia were more closely related than once thought and have been grouped together as the Opisthokonts, literally meaning posterior flagellum. It is also thought that both the fungi and Animalia shared a common ancestor with the motile sperm cells the remnants of our ancient evolutionary ancestors (Cavalier-Smith, 1998).

The correlation between flagellated cells and VLCPUFA production is also seen within the plant Kingdom. The Kingdom Plantae is split into two, the Chlorophyta and the Charophyta (Lewis & McCourt, 2004), with the former comprising the majority of the green algae and the latter leading to the evolution of the terrestrial embryophytes (Kenrick & Crane, 1997). The most basal terrestrial plants are those from the Liverworts, followed by the Mosses with the higher vascular plants evolving last (Qiu *et al.*, 2006). Like the chytrids, the most basal terrestrial plants still produce flagellated reproductive cells (Renzaglia *et al.*, 2000,Shimamura *et al.*, 2008) indicating their algal ancestry. The ability of the Liverwort *Marchantia polymorpha* to produce up to $C_{20:5}$ n3 (Kajikawa *et al.*, 2004) reinforces the idea that VLCPUFA production is linked to flagellated cell production. As is the case in fungi, the higher vascular plants, to our knowledge, are incapable of VLCPUFA production and do not produce a flagellated cell type during their lifecycle. Fungi do however appear to demonstrate an intermediary stage, as seen in *Mortierella* species, the production of VLCPUFAs without producing a flagellated cell type.

Flagella are not the only mechanism by which zoospores disperse, with amoeboid spores having been documented from the phylum Blastocladiomycota (Hoffman *et al.*, 2008). It is thought that locomotion involving crawling or gliding also requires a fluid membrane to permit efficient movement (Johns & Perry, 1977) with the bacterium *Flexibacter polymorphus* being documented as producing $C_{20:5}$ n3. Chytrid zoospores demonstrate a varied degree of cell coat material but ultimately they are not thought of as rigid structures (Powell, 1994). Because of this, the plasma membrane is thought to regulate osmotic control through its permeability. High salinity solutions have been shown to lower the fluidity of membranes (Fontana & Haug, 1982) as well as increase levels of saturated and mono-unsaturated fatty acids whilst lowering levels of polyunsaturated fatty acids (Xu & Beardall, 1997). This mechanism of lowering fluidity is thought to rigidify the membrane and reduce its permeability to Na^+ ions. The expansion and shrinkage of the membrane due to osmotic pressure may also act as a turgor sensor and in some cases increase intracellular levels of glycerol to counter the elevated external solute levels. In regards to the Chytrids, the majority of them are found within fresh water, low salinity environments (Gleason *et al.*, 2008) indicating that the VLCPUFAs may play a role in these environments. As it can be seen, the aqueous environment in which the zoospores exist most likely contributes to the presence of VLCPUFAs. These fatty acids therefore appear essential for the motility and survival of the spore stage. In regards to algae and some Chromista, it is well known that these organisms thrive in saline sea water which would appear to contradict the previous statement that high salinity conditions impair unsaturated fatty acid production. There are two possible reasons for algae production of VLCPUFAs that will be briefly mentioned. The first is the production of algal pheromones, whereby the fatty acids $C_{20:4}$ n6 and $C_{20:5}$ n3 are utilised for the formation of C_{11} signalling molecules (Pohnert & Boland, 2002) utilised in part for male gamete chemotaxis. Shorter chain fatty acids such as $C_{18:3}$ n3 were found not to be utilised in the formation of these pheromone molecules suggesting the importance VLCPUFAs play in algal reproduction. Another role VLCPUFAs may play within photosynthesising marine microorganisms is the protection of photosystem II. It is thought that VLCPUFAs provide protection by acting directly in the thylakoid membrane to protect the oxygen evolving machinery, as well as providing sufficient fluidity for the Na^+/H^+ anti-porter pump which reduces cytoplasmic Na^+ levels (Allakhverdiev *et al.*, 1999).

It is most likely then that several of these explanations account for the differentiation in fatty acid profile between the higher fungi, Oomycetes, Zygomycota and

Chytridiomycota. The shift from an aqueous habitat to a terrestrial one is a probable cause for the shift in fatty acid profile. Once on land other factors may have compounded the loss of VLCPUFAs from the newly established terrestrial fungi. It is known that the fatty acid $C_{20:4}$ n6 is a plant elicitor found within the plant pathogen *Phytophthora*. An elicitor is a compound which triggers a response within the plant, with the aim of the response to prevent microbial propagation and host cell damage. In the case of the *Phytophthora*-potato interaction, $C_{20:4}$ n6 is converted by potato lipoxygenase (LOX) into compounds such as hydroperoxy-eicosatetraenoic acid (Ricker & Bostock, 1994) which are thought to mediate the plant response to the pathogen. The loss of $C_{20:4}$ n6 from plant pathogens would have most likely been an advantage, however $C_{18:3}$ n3 has been shown to increase transcription of lipoxygenase in tobacco and is also converted by LOX and several subsequent reactions to jasmonic acid, which is also involved in plant wounding and defence responses (Veronesi *et al.*, 1996). The source of $C_{18:3}$ n3 however could come from either plant membranes or the fungal pathogen themselves, as the large majority of fungal species studied contain this fatty acid. As to why fungi contain $C_{18:3}$ n3 is most likely due to its role as the precursor to the fungal oxylipins, which modulate diverse functions such as cell growth and proliferation, apoptosis and spore differentiation (Christensen & Kolomiets, 2010). Whilst the majority of terrestrial fungi have lost the ability to produce VLCPUFAs it hasn't stopped them from benefitting from them, by utilising host VLCPUFAs instead to biosynthesise oxylipin mediators. This ironic mechanism by which compounds intended to provide resistance against pathogenic attack are turned against the host by modulating and increasing the growth of the pathogenic invader, would provide another reason for the redundancy of VLCPUFA responsible genes. This mechanism by which fungi utilise host fatty acids is observed within the Human pathogen *Candida albicans*, with the Fungus utilising host $C_{20:4}$ n6 to produce carbohydrates and fungal eicosanoids such as 3,18-dihydroxy-5,8,11,14-eicosatetranoic acid, with 3(R)-hydroxy-oxylipins thought to regulate morphogenesis and reproduction as well as modulating cell signalling in neutrophils (Deva *et al.*, 2000).

3.4 Conclusion

Low temperatures do not appear to increase the likelihood of finding VLCPUFA producing ascomycetes, as it appears that trienoic fatty acids are sufficient for maintaining membrane fluidity under low temperature conditions. Whilst temperature was not shown to be a sufficient driving factor to retain key genes for VLCPUFA

synthesis it is likely that temperature will lead to the modulation of the fatty acid profile. Organisms capable of producing VLCPUFAs were *Mortierella* species, *Allomyces macrogynus* and organisms from the Oomycetes. Both Antarctic and mesophilic isolated *Mortierella* species were all found to produce $C_{20:4}$ n6 and $C_{20:5}$ n3. Fatty acid composition between the isolates was similar, however the Antarctic isolate 398216 was found to contain $C_{20:4}$ n6 at 67.8% of the total lipid profile whereas the mesophilic isolate 196057 was found to produce the fatty acid at 34.9% of the total lipid profile. The fatty acid $C_{20:4}$ n6 was proportionally higher in *Mortierella* species, which distinguishes them from the Oomycetes which contain the highest proportional $C_{20:5}$ n3 levels. The *Mucor* and *Umbelopsis* isolates were distinguished from the *Mortierella* isolates based partially on higher $C_{18:3}$ n6 levels as well as $C_{16:1}$ and $C_{18:2}$ levels. The ascomycetes primarily group together away from the zygomycetes and Oomycetes based on high $C_{18:2}$ levels.

As it can be seen, there are many roles that PUFAs play within fungi as well as other microorganisms. The fact that the higher fungi lost the ability to produce VLCPUFAs can possibly be attributed to the transition from the aqueous to terrestrial environment. The loss of flagella and reliance on aerial spore dispersal, as well as the lack of osmotic potentials most likely resulted in the gradual loss in key enzymes responsible for VLCPUFA production. To date, the vast majority of VLCPUFA producing organisms such as algae, plants, chromists and fungi are usually associated with water and produce a flagellated cell type. Therefore VLCPUFA production appears to be correlated with flagella production, which to a large degree correlates with the organism's habitat. This generalisation however does include exceptions such as the zygomycete *Mortierella* and VLCPUFA producing plants. *Mortierella* does not produce a flagellated form and neither is intrinsically linked to an aqueous environment however, both reside in basal phyla which evolved from a flagellated ancestor. It is these conditions which seem to be most indicative in regards to VLCPUFAs formation and therefore any further screening of novel fungi should focus on the Zygomycota and Chytridiomycota initially. The taxonomy of the organism in regards to VLCPUFA production appears to be crucial in screening for novel producers of these high value fatty acids.

4 Fatty acid analysis of total lipid under three temperature regimes

4.1 Introduction

Maintaining a constant membrane fluidity in response to differing external conditions is referred to as homeoviscous adaptation (Sinensky, 1974). The role of fatty acids and their modification within the membrane are essential to maintaining the viscosity of the membrane. Cell damage may be experienced when the plasma membrane undergoes the transition from the liquid crystalline state to the gel state, which alters the permeability of the membrane, allowing osmotic induced damage to occur (Crowe *et al.*, 1987). The membrane is also required for other roles including protein function, of which a large proportion are membrane bound. It is generally believed that to maintain membrane viscosity, several modifications can be made to the fatty acid composition including incorporation of short chain saturates, branched chain saturates or the desaturation of fatty acids (Suutari & Laakso, 1994). In addition, other compounds are responsible for the resilience against low temperatures. Polyalcohol sugars such as trehalose have been shown to act as cryoprotectants (Weinstein *et al.*, 2000) as well as glycerol, which are routinely used in the process of cryopreservation to prevent osmotic and freeze related damage (Smith *et al.*, 1986). Other membrane associated compounds such as ergosterol, in the case of the fungi, or the lack of it may be beneficial to the survival of the organism at low temperature, due to the rigidifying effect of the sterol on the membrane (Weinstein *et al.*, 2000).

Due to previous studies suggesting that low temperature can lead to the production of elevated levels of the most unsaturated fatty acid (section 1.3.5), as well as increasing the overall unsaturation index of the cell, it was decided that several psychrophilic fungi were to be studied and their fatty acid response to temperature analysed. Psychrophilic organisms were studied for two reasons; firstly little research has been carried out on psychrophilic fungi in relation to fatty acid modification in response to temperature. Secondly, it was thought that culture at 15°C may not have induced the formation of VLCPUFAs such as $C_{20:5}$ n3 within these psychrophilic organisms. Therefore culture at 5°C was hoped to induce expression of polyunsaturate elongases and desaturases. This

was be done by analysing the total extractable fatty acids under three temperature growth regimes. In total nine organisms were studied with regard to the temperature effects on fatty acid profiles. Fungi from the genus *Herpotrichia*, *Mucor*, *Penicillium* and *Mortierella* were studied, predominantly as these organisms were associated with low temperature environments, except two *Mortierella* isolates which were mesophilic. Fatty acid unsaturation indices were calculated using the following formula:

$\Delta mol^{-1} = \Sigma$(% monoene + (2 x % diene) + (3 x % triene) + (4 x % tetraene) + (5 x % pentene))/100 (Suutari, 1995)

The unsaturation index takes into account the fatty acid composition and attributes a higher value the greater the level of unsaturation. Fatty acid such as $C_{18:3}$, for example, are more heavily weighted than a monoene such as $C_{18:1}$. Hence, higher levels of polyunsaturates and lower levels of monounsaturates will lead to an increased index score. Saturates are accounted for as the percentage of each fatty acid is used to calculate the index, so high saturate levels will result in decreased unsaturate percentages, which in turn give a lower index score. A list of organisms studied, culture temperature and period of growth are given in the materials and methods section, table 2-18.

4.1.1 Aim

The aim of this study was to explore the effect of temperature on the fatty acid complement of seven Antarctic or low temperature isolated fungi and two mesophilic fungi. The hypothesis was that low temperature growth induces greater unsaturation within the fatty acids. The experiment set out to confirm this postulation. Further to this, *Mortierella alpina* strain 330997 was analysed further with individual phospholipid components studied to determine whether membrane associated lipids showed greater unsaturation under low temperature conditions.

4.2 Results

4.2.1 Total fatty acid analysis

4.2.1.1 *Mucor racemosus*

Mucor racemosus (17313) was capable of producing a variety of fatty acids (figure 4-1). At 5°C it was apparent that all fatty acids increased in content with the exception of $C_{24:0}$. This is clear when the total fatty acid as a percentage of dry weight is plotted (Figure 4-11), with the total fatty acid production levels highest at 5°C, although total dry biomass at this temperature was not noticeably different from that observed at the two other temperatures (Figure 4-12). The lowest biomass level was found at 15°C, with 25°C producing the greatest biomass. Upon increasing the temperature to 15°C, TFA production declined from 8.4% to 4.6% of the dry weight, with 25°C demonstrating a marginally higher value of 5.5%. The majority of the compounds were produced at the lowest concentrations at 15°C with the exception of $C_{18:3}$ n6. The levels of $C_{18:3}$ n6 showed a negative correlation, with increasing temperature resulting in decreased abundance of this LCPUFA, with growth at 25°C producing 8.3 mg/g compared with the highest value at 5°C of 14.9 mg/g.

The majority of the other fatty acids, which are mainly saturated FAs, decreased initially with the lowering of the temperature from 25-15°C, but then showed a subsequent rise at 5°C. $C_{18:2}$, the second most unsaturated FA within *Mucor*, produced a similar quantity at 15°C to that at 25°C, with 5°C growth producing almost a two fold increase from 5.4 mg/g to 10 mg/g. It is also worth noting that short chain saturates can confer fluidity due to their lower melting point. $C_{10:0}$ levels were highest at 5°C (0.6 mg/g) as observed with all fatty acids at this temperature, although levels appear to stay constant between 15 and 25°C. The negative correlation seen with $C_{18:3}$ n6 was also observed with $C_{12:0}$, with decreasing FA level seen with increasing temperature. $C_{14:0}$ showed a similar trend to $C_{10:0}$, with the highest levels at 5°C but with 15 and 25°C cultures showing similarly lower levels. By applying the unsaturation index calculation to the data (figure 4-10), growth at 15°C produced the greatest unsaturation levels followed by 5°C growth. This elevated unsaturation at 15°C is due to the high $C_{18:3}$ n6 TFA percentage and lower saturate and monounsaturated TFA percentage. Even though 5°C growth produced a higher total level of $C_{18:3}$ n6, increased levels of all the FAs were also observed with $C_{18:3}$ n6 representing 17.6% of the total fatty acids compared

with 22.6% at 15°C. Results were subjected to a single factor analysis of variance (ANOVA) statistical test to ascertain whether individual fatty acid components were significantly different from one another. A p value of $p \leq 0.05$ was considered statistically significant.

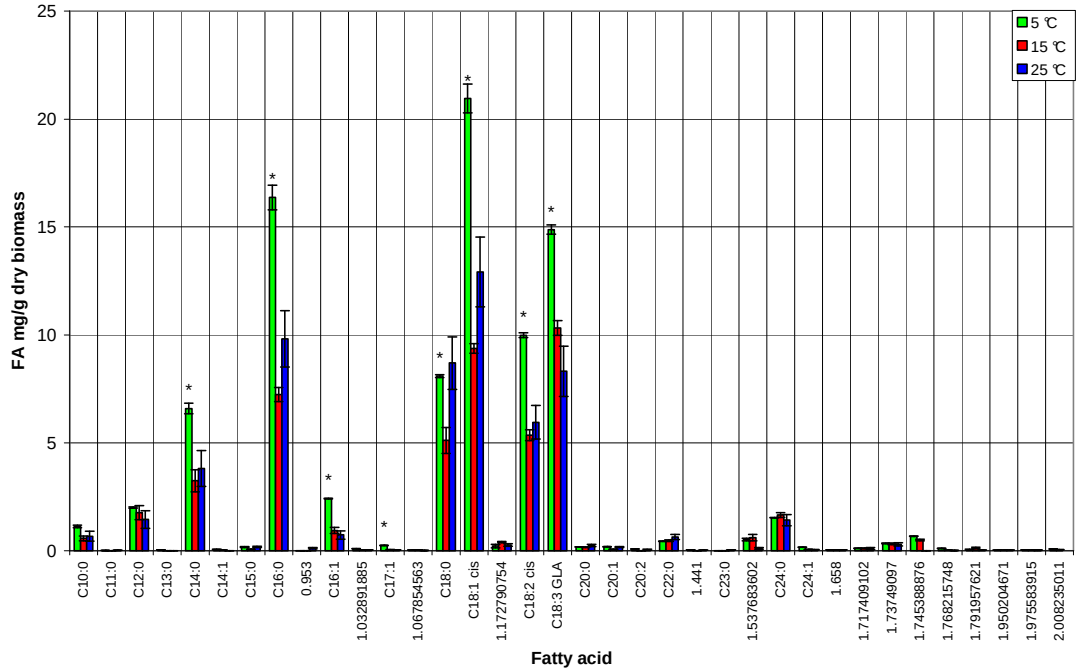


Figure 4-1. The fatty acid profiles of *Mucor racemosus* grown under three culture temperatures. * = $p \leq 0.05$, n = 3 and error bars are standard error of the mean.

4.2.1.2 *Penicillium rugulosum*

Penicillium rugulosum produced the greatest biomass of any of the samples (figure 4-12); with a mycelial dry weight of 2.4 g per 100 ml at 15°C. With the definition of a psychrophile being an organism displaying maximum growth below 20°C, this *Penicillium* strain appears to be classified as a true psychrophile. Growth at 5°C promoted the greatest production of $C_{18:3}$ n3 comprising 47.6% of the fatty acid profile (figure 4-2) making it the predominant FA with levels of other unsaturates such as $C_{18:1}$ and $C_{18:2}$ and saturates such as $C_{16:0}$ at their lowest levels. Culture at 15°C produced elevated levels of $C_{18:1}$ and $C_{18:2}$, as well as significant amounts of $C_{18:3}$ n3, at 24.4% of the total fatty acids. The levels of saturates stay relatively unchanged with the increase in temperature, although saturates within *Penicillium* make up a small fraction of the total FAs. At 25°C, a marked increase in $C_{18:2}$ is observed although the lowest levels of $C_{18:3}$ n3 at 8.3% and the highest levels of $C_{16:0}$ are also found, with $C_{18:1}$ levels also

slightly increased. The unsaturation index showed the highest value at 5°C (figure 4-10), which can be attributed to the high $C_{18:3}$ n3 levels relative to the lower saturate, mono and di-unsaturate levels. As the temperature increased, a negative correlation between the unsaturation index and temperature is observed, with increased temperature leading to decreased unsaturation within the fatty acids. Although total FA levels in this organism are comparatively low, a positive correlation is seen with the increase in temperature leading to an increase in lipid content (figure 4-11).

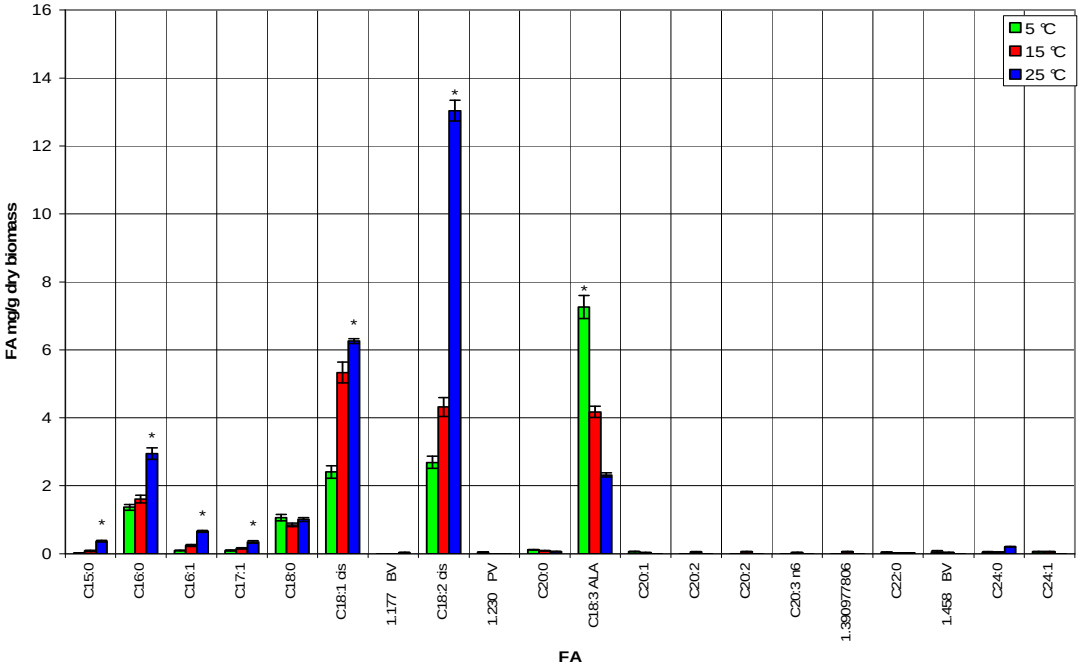


Figure 4-2. The fatty acid profiles of *Penicillium rugulosum* grown under three culture temperatures. * = $p \leq 0.05$, n = 3 and error bars are standard error of the mean.

4.2.1.3 *Herpotrichia* sp.

Both *Herpotrichia* species contained small quantities of trace compounds, which were thought to be sterols. These low abundance compounds appear to be characteristic of these two organisms, although have little affect on regulation in response to temperature. Biomass levels for 403016 showed increasing biomass with increased temperature although 403002 produced its maximum biomass at 15°C. Strain 403016 produced elevated levels of $C_{18:3}$ n3 at 5°C, producing more than twice the absolute value as that found at 15°C, which corresponds to 20.1% and 11.4% of the TFA respectively (figure 4-3). With the increase in temperature all the fatty acids decreased except $C_{18:2}$, which maintained a relatively constant level regardless of temperature. $C_{18:3}$ n3 experienced the sharpest drop with the increase in culture temperature. When

the unsaturation index was calculated as shown in figure 4-10, culture at 20°C showed the highest unsaturation level, due partly to the high level of $C_{18:2}$ compared to the relatively low levels of other fatty acids. Culture at 5°C showed the second most unsaturated index, bolstered by the high levels of $C_{18:3}$ n3. Levels of saturates were highest at 5°C, with $C_{16:0}$ and $C_{18:0}$ peaking at this low temperature. *Herpotrichia* strain 403002 also showed a temperature driven trend for $C_{18:3}$ n3. $C_{18:3}$ n3 comprised the greatest percentage of the total fatty acids at 5°C at 19.1%, whilst making up 15.6% of the TFA at 15°C (figure 4-4). The two species of *Herpotrichia* do differ in their fatty acid profiles in regards to temperature. Strain 403002 produced the greatest saturate, mono- and di-unsaturate levels at 20°C, with the second highest levels produced at 5°C. This is in contrast to strain 403016 which displayed the greatest fatty acid production at 5°C. 15°C produced the lowest fatty acid levels except for $C_{18:3}$ n3 in 403002 whereas this temperature promoted the second highest levels in strain 403016. The unsaturation index for strain 403002 reveals that 5 and 15°C culture produce similar unsaturation indices, due to high levels of $C_{18:3}$ n3 and $C_{18:2}$ with reduced levels of saturates. The total FA content in strain 403016 shows a counter trend in relation to biomass, with increasing temperature resulting in increased biomass production, however this also results in a decrease in total fatty acid production. Strain 403002 does not display a clear trend, although the highest biomass production at 15°C produced the lowest total fatty acid level.

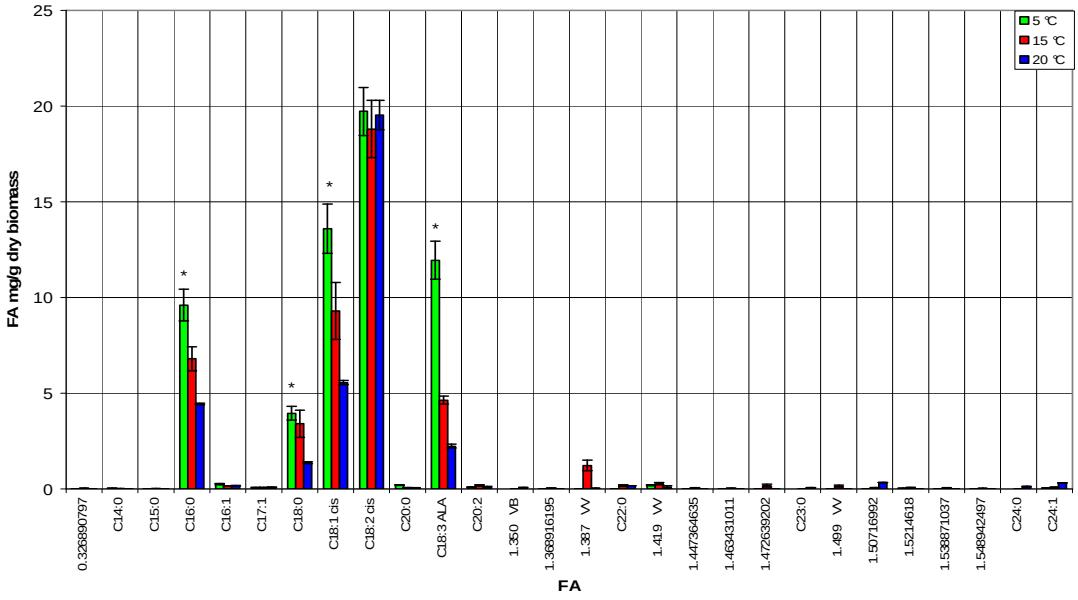


Figure 4-3. The fatty acid profiles of *Herpotrichia* sp. (403016) grown under three culture temperatures. * = $p \leq 0.05$, n = 3 and error bars are standard error of the mean.

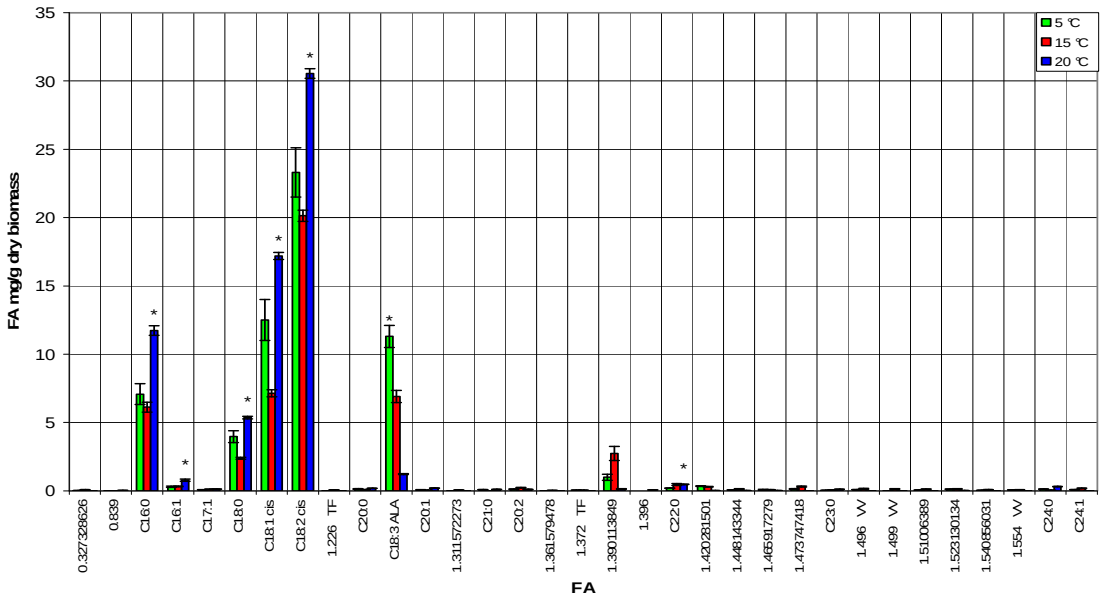


Figure 4-4. The fatty acid profiles of *Herpotrichia* sp. (403002) grown under three culture temperatures. * = p ≤ 0.05, n = 3 and error bars are standard error of the mean.

4.2.1.4 *Bjerkandera adusta*

Bjerkandera adusta produced only two measurable fatty acids, $C_{16:0}$ and $C_{18:2}$, although $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, $C_{20:1}$ and $C_{22:0}$ are present although as minor compounds (figure 4-5). No trienoic acids were detected. Growth at 5°C resulted in the highest levels of $C_{18:2}$, comprising 79.6% of the TFA compared with 71.6% at 15°C. Levels of $C_{16:0}$ and $C_{18:1}$ also decreased slightly with the increase in temperature. Total FA levels showed an increase with the decrease in temperature, with the unsaturation index highest at 5°C growth presumably because of the limited repertoire of fatty acids at the organism's disposal, forcing an increase in the only substantial unsaturated fatty acid produced by this organism. The unsaturation indices between 15 and 25°C are marginal, in part because so few fatty acids constitute the profile of this organism. The biomass was found to be at its lowest at 5°C with a steady increase observed with the increase in temperature.

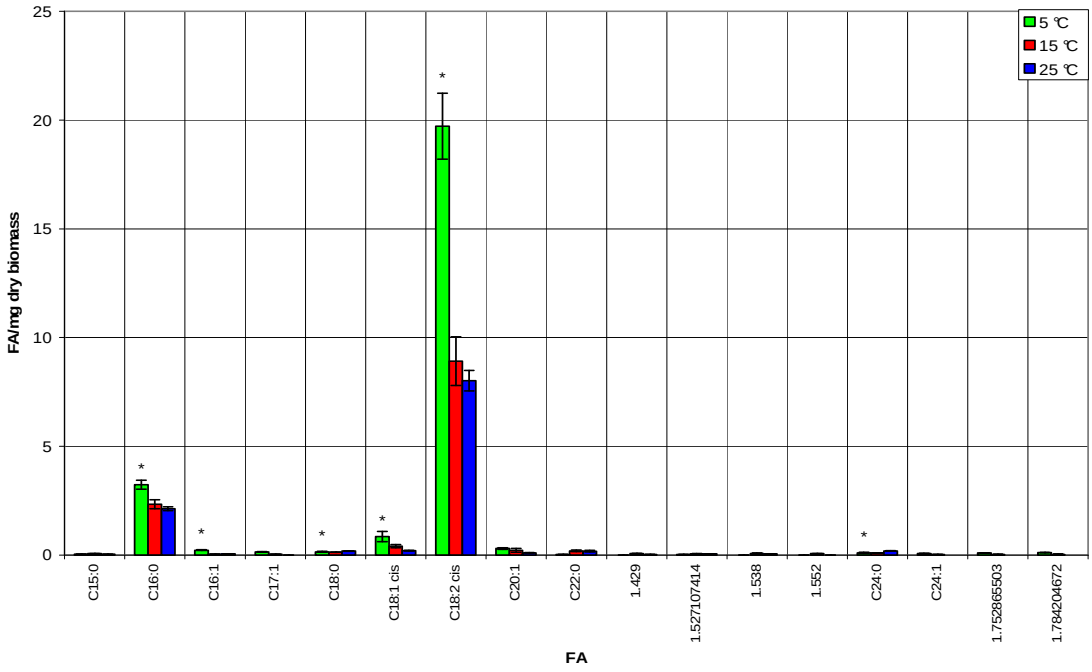


Figure 4-5. The fatty acid profiles of *Bjerkandera adusta* grown under three culture temperatures. * = $p \leq 0.05$, n = 3 and error bars are standard error of the mean.

4.2.1.5 *Mortierella* spp.

Two organisms from the genus *Mortierella*, were studied, both species of *M. alpina*. The *Mortierella alpina* species were studied as they are one of the few true fungi capable of producing VLCPUFAs such as $C_{20:5}$ n3 (section 1.4.2). Strains Dis 195 and 206 are from the former sub-genus *Micromucor*, now classified as *Umbelopsis* and are therefore incapable of producing VLCPUFAs. They were initially studied as a comparison to the VLCPUFA producing *Mortierella* species as both are capable of $C_{18:3}$ n6 production. Two *Umbelopsis* species were studied in respect to culture temperature and fatty acid profile. In *Mortierella alpina* strain 82072 the most unsaturated fatty acid $C_{20:5}$ n3 is present at both 5 and 15°C growth (figure 4-6). Growth at 15°C produced increased levels at 3.1 mg/g compared with 2.9 mg/g at 5°C growth, with $C_{20:5}$ n3 production at 25°C resulting in trace levels of the fatty acid. Growth at 5°C resulted in $C_{20:5}$ n3 TFA levels of 4.7% whereas at 15°C it represented 2.6%. In absolute value terms, $C_{20:5}$ n3 values remain relatively constant although proportionally, levels decreased. Due to the presence of 5 *cis* double bonds it would be expected that this FA would have a substantial role in fluidising the membrane. The second most unsaturated fatty acid, $C_{20:4}$ n6, was at its lowest level at 5°C at 5.3 mg/g, with 20.1 mg/g produced

at 15°C growth and 20.3 mg/g produced at 25°C. Levels of $C_{18:3}$ n6 and $C_{18:2}$ were highest at 15 and 25°C growth, with marked decreases at 5°C. The saturate FA $C_{16:0}$ is at the lowest level at 5°C, as is the FA $C_{18:0}$. $C_{16:0}$ is produced to the greatest extent at 15°C, although $C_{18:0}$ shows an intermediary value. $C_{16:0}$ levels drop slightly at 25°C whereas $C_{18:0}$ levels increased with the subsequent rise in temperature. The two saturated FAs $C_{16:0}$ and $C_{18:0}$ are the predominant saturates within this strain, with $C_{20:0}$, $C_{21:0}$, $C_{22:0}$ and $C_{24:0}$ forming much smaller fractions. All the saturated FAs bar $C_{21:0}$ showed an increase with temperature, with $C_{21:0}$ not produced at 5°C. The increase in saturates as well as mono-unsaturates should prevent membrane fluidity from increasing with the elevation in temperature. The most appreciable monounsaturated fatty acid $C_{18:1}$ is produced in the highest quantity at 25°C with declining production observed with declining temperature. $C_{20:1}$ production increases with the increase in temperature although to a much lesser extent. $C_{18:2}$ levels peak at 15°C, though 25°C growth results in slightly lower levels, with 5°C significantly reducing $C_{18:2}$ production. Total fatty acid levels show 25°C culture followed by 15°C culture produced the highest accumulation of lipid, with levels higher than that found within strain 330997. The unsaturation index at 15°C showed the fatty acid composition of the cell was at its most fluid, with 5 and 25°C growth demonstrating nearly equal unsaturation indices. The increased unsaturation at 15°C is likely the cause of high levels of $C_{20:4}$ n6, $C_{20:3}$ n6 and $C_{18:3}$ n6, and lower levels of $C_{18:1}$ at this temperature. It would seem therefore that 5°C growth is not optimal for this strain of *Mortierella alpina* due to the low fatty acid content and low biomass production.

Mortierella strain 330997 demonstrates a nearly identical fatty acid complement, although the levels between strains 330997 and 82072 are distinct. The most unsaturated fatty acid $C_{20:5}$ n3 is only produced at the lower two temperatures of 5 and 15°C, with 5°C growth promoting the greatest production at 2.2 mg/g (8.1% of the TFA) and 15°C producing 1.1 mg/g (2.1% of the TFA) (figure 4-7). Unlike strain 82072, both absolute and proportional values decrease with the decrease in temperature although both strains show negligible production at 25°C. The FA $C_{20:4}$ n6 is produced at elevated levels at 25°C followed by a marked decrease with the drop in temperature which is not observed within strain 82072. This trend occurs with all the fatty acids except $C_{20:5}$ n3, with levels of all FAs decreasing sharply with the drop in temperature. When compared to strain 82072, it is noticeable that the previous strain produces a similar profile at both 15 and 25°C, with exception to $C_{18:0}$ and $C_{18:1}$, whereas strain

330997 produces distinctive profiles at both these temperatures. The total fatty acid levels are highest at 25°C, followed by the expected decline with the drop in culture temperature. The unsaturation indices for strain 330997 are highest at 5°C followed by 15 and 25°C growth respectively. The high index observed at 5°C is in part due to the relatively high $C_{20:5}$ n3 level. The biomass for strain 330997 showed a steady increase with the increase in temperature, which was inline with the total fatty acid level, with both values lowest at 5°C.

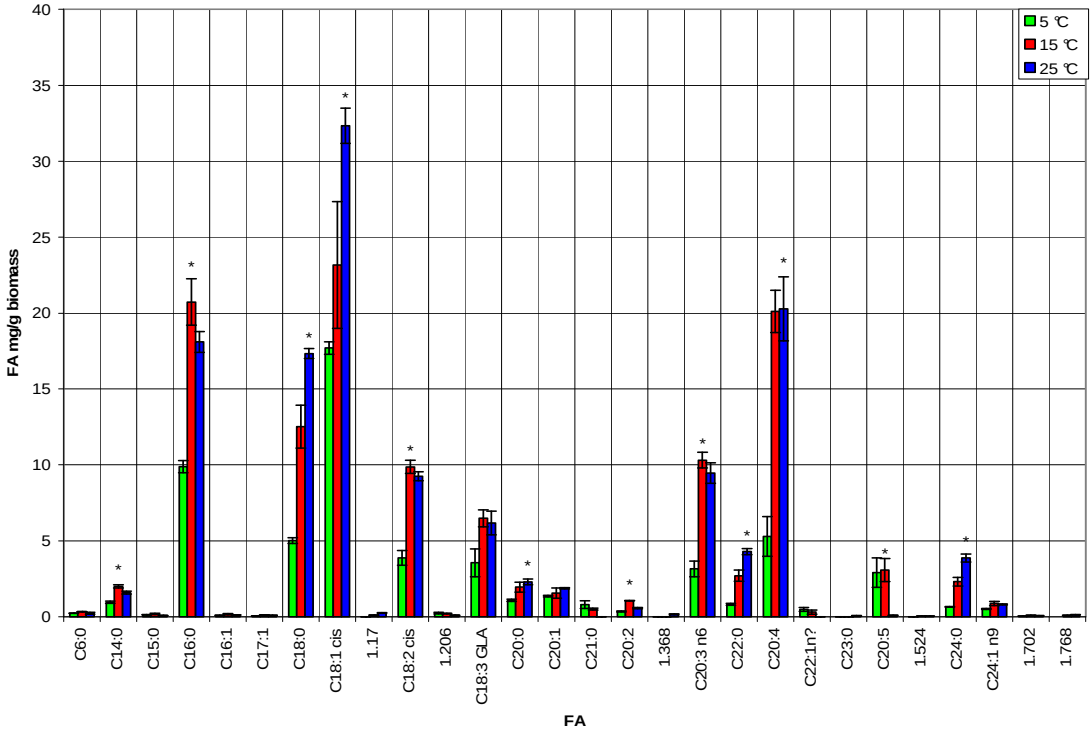


Figure 4-6. The fatty acid profiles of *Mortierella alpina* (82072) grown under three culture temperatures. * = $p \leq 0.05$, n = 3 and error bars are standard error of the mean.

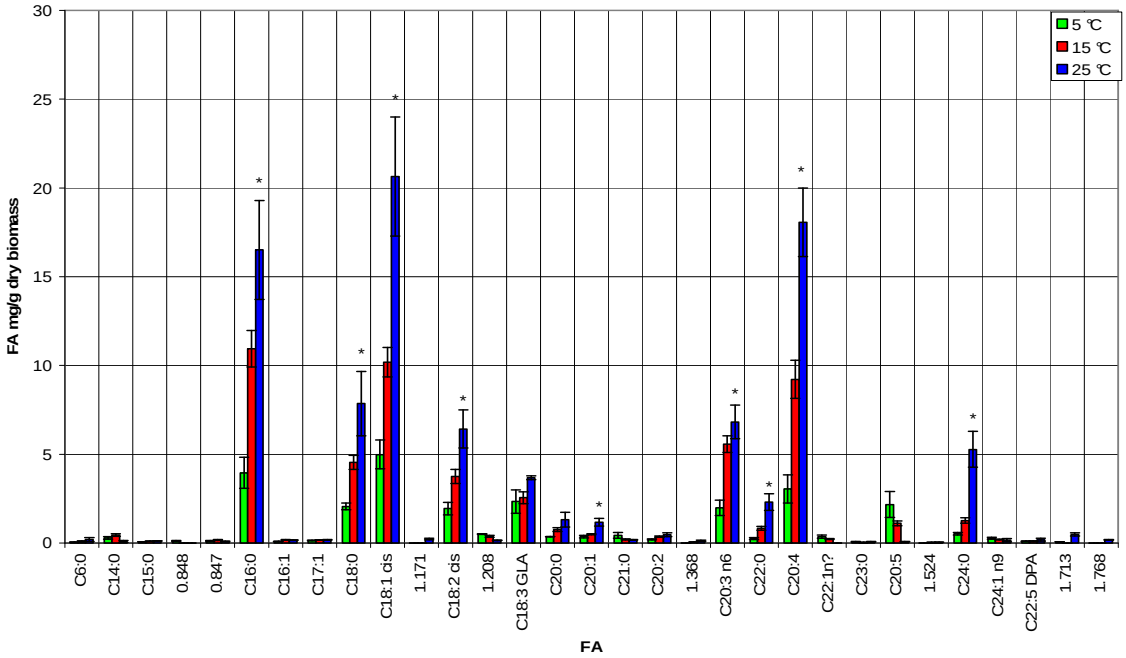


Figure 4-7. The fatty acid profiles of *Mortierella alpina* (330997) grown under three culture temperatures. * = $p \leq 0.05$, n = 3 and error bars are standard error of the mean.

4.2.1.6 *Umbelopsis* spp.

The *Umbelopsis* isolates were studied as representatives of the zygomycetes, which are typically non-VLCPUFA producing and characteristically produced $C_{18:3}$ n6 as oppose to $C_{18:3}$ n3. Several of the *Umbelopsis* isolates were until relatively recently classified within the *Mortierella* genus. Therefore, these two isolates were used to compare mesophiles with psychrophiles, and to also compare true *Mortierella* species with representatives of the majority of the zygomycetes. The two *Umbelopsis* species did not produce polyunsaturates beyond $C_{18:3}$ n6. These organisms were also isolated from mesophilic environments, so initial predictions would point to these organisms being ill suited to low temperature growth. Strain Dis 206 (*Umbelopsis isabellina*) produced the greatest level of $C_{18:3}$ n6 when cultured at 5°C, with an absolute value of 31.4 mg/g (13.9% of the TFA) which subsequently decreased with increasing temperature to 13.2 mg/g and 8.2 mg/g at 15 and 25°C respectively (figure 4-8). Levels of the $C_{18:1}$ unsaturate also increased with the decrease in temperature, although $C_{18:2}$ levels showed little change when grown under the three temperature conditions. The other major fatty acid present was $C_{16:0}$ and this showed an increase in abundance with the decrease in temperature, as did $C_{16:1.}$ The unsaturation index showed a negative correlation, with decreasing temperature leading to an increase in FA unsaturation, due to the elevated $C_{18:1}$ and $C_{18:3}$ n6 levels. The total fatty acid level showed this organism

to contain the highest content of lipid of any of the organisms studied reaching 22.4% lipid content at 5°C culture.

Isolate Dis 195 (*Umbelopsis* sp.) demonstrated the same complement of fatty acids, although different ratios were observed. However, high levels of $C_{18:3}$ n6 at 5°C, 15.6 mg/g (21.4% of the TFA) were similar, followed by reduced quantities at 15 and 25°C with 7.2 mg/g (10.0% of the TFA) and 7.4 mg/g (7.2% of the TFA) respectively (figure 4-9). The negative correlation between $C_{18:3}$ n6 and temperature was not as evident here, as levels between 15 and 25°C remained relatively consistent. This strain demonstrated a reversal in $C_{18:1}$ and $C_{16:0}$ levels, with the lowest quantities produced at 5°C growth and the highest at 25°C. $C_{18:2}$ displayed a similar pattern to strain Dis 260, with elevated levels at 5 and 25°C compared with 15°C culture. The unsaturation index shows clearly that 5°C growth results in the most unsaturated fatty acid production, predominantly due to the relatively low $C_{18:1}$ levels and high $C_{18:3}$ n6 level. The differences between 15 and 25°C growth in regards to fatty acid unsaturation however are minimal. Whilst $C_{18:3}$ n6 at 15°C has the same absolute concentration, it represents a greater percentage of the TFA, which in turn offsets the increased $C_{18:1}$ in the 25°C samples.

The differing strategies to coping with temperature variation would lead to the conclusion that these two *Umbelopsis* isolates are in fact differing species. It is surprising to see these mesophilic organisms cope with these low temperatures, as previous studies which looked at several mesophilic organism such as *Penicillium* and *Aspergillus*, found that they had difficulty adapting to the low temperature with fluctuating unsaturation indices and low growth rates. The biomass of both *Umbelopsis* isolates show a clear response to temperature, with low growth experienced at 5°C whilst subsequent increases in temperature see marked increases in biomass. Strain Dis 195 showed an increase from 164.3 mg at 5°C to 406.3 mg at 15°C, peaking at 626.0 mg at 25°C. Strain Dis 206 produced 111.3 mg of biomass at 5°C, with an increase to 340.3 mg at 15°C with a maximum biomass of 654.6 mg at 25°C. The response of growth to temperature is more substantial with *Umbelopsis* than with *Mortierella*.

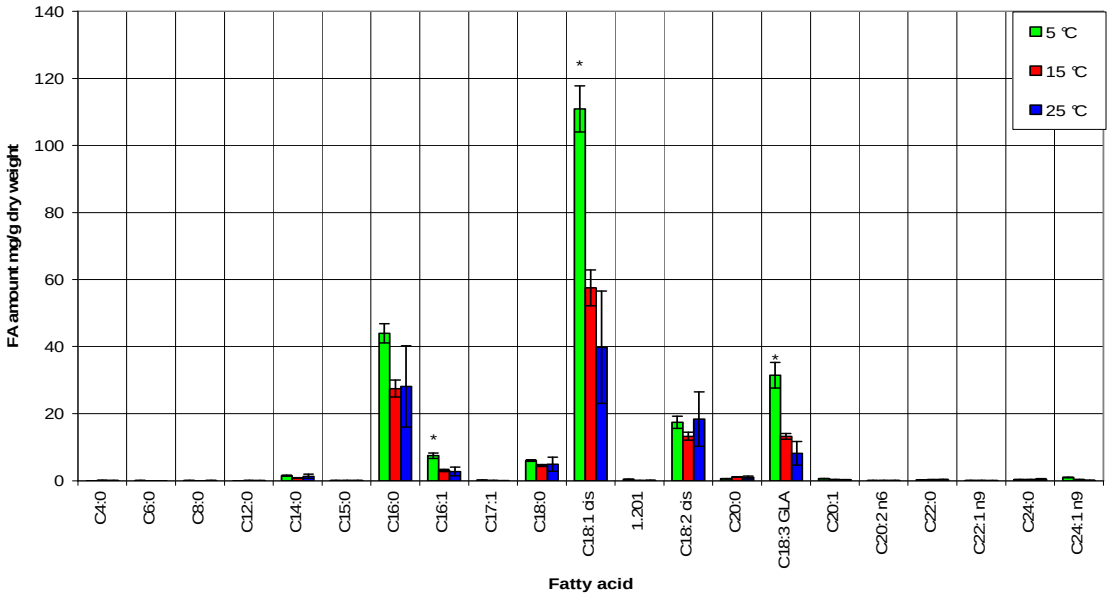


Figure 4-8. The fatty acid profiles of *Umbelopsis isabellina* (Dis 206) grown under three culture temperatures. * = $p \leq 0.05$, n = 3 and error bars are standard error of the mean.

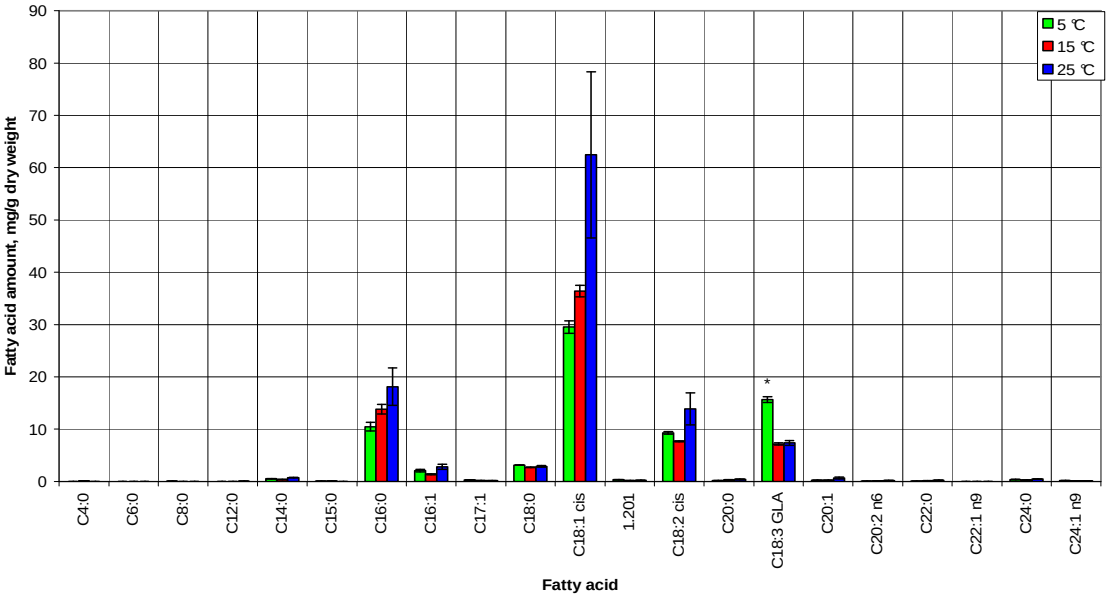


Figure 4-9. The fatty acid profiles of *Umbelopsis* sp. (Dis 195) grown under three culture temperatures. $p \leq 0.05$, n = 3 and error bars are standard error of the mean.

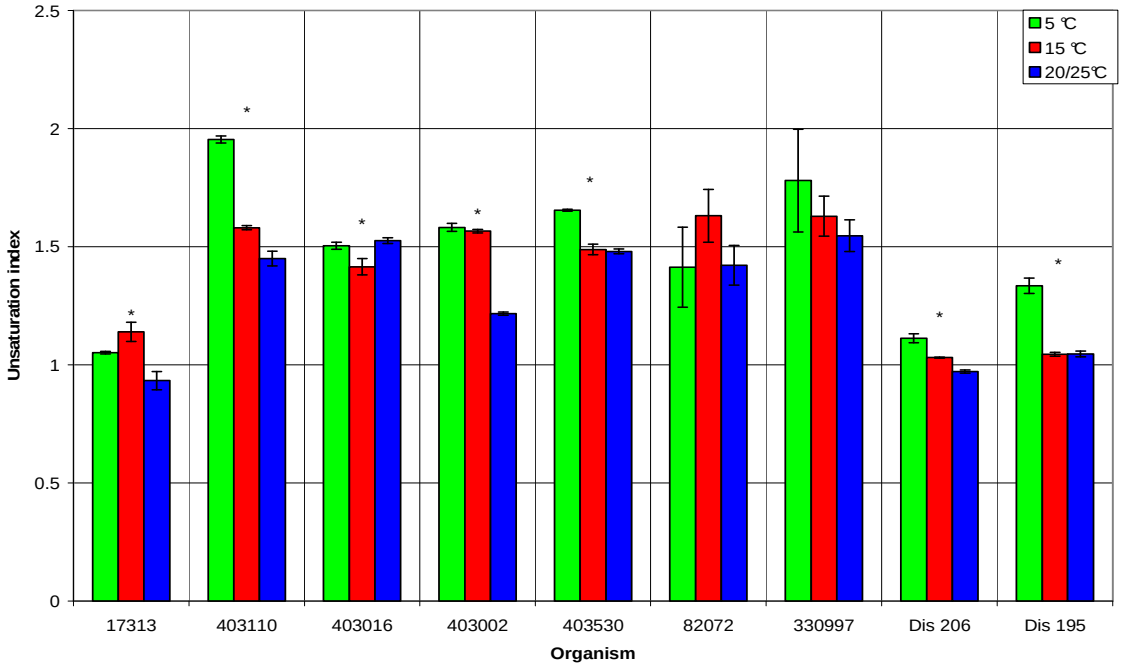


Figure 4-10. Fatty acid unsaturation indices of isolates when cultured under three temperatures. In general 5 or 15°C growth produces the greatest unsaturation, with several isolates 403110, 403002, 403530, 330997, Dis 206 and Dis 195 producing the most unsaturated fatty acid profile at 5°C. Isolates 17313 and 82072 produced the greatest degree of unsaturation at 15°C although *Herpotrichia sp*. isolate 403016 was found to produce the greatest unsaturation at 20°C. The isolates 17313 and 82072 which show 15°C as producing the highest unsaturation levels are also known to produce large quantities of storage lipid which may result in lower UIs due to the unknown localisation of the fatty acids. * = $p \leq 0.05$, n = 3 and error bars are standard error of the mean.

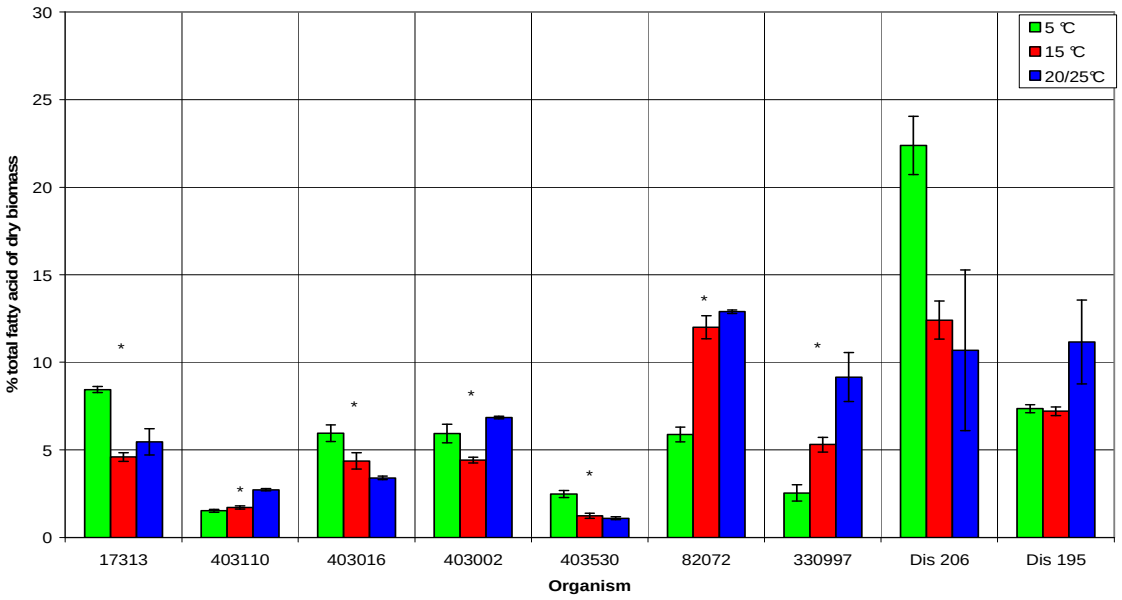


Figure 4-11. The percentage total lipid produced by isolates when grown under three temperature regimes. Two main trends are seen with the percentage composition of lipid in regards to temperature. Several isolates such as 17313, 403016, 403530 and Dis 206 produce the highest percentage of lipid at 5°C. Isolates 403110, 403002, 82072, 330997 and

Dis 195 produce an opposite trend by showing elevated lipid levels at 25°C. * = p ≤ 0.05, n = 3 and error bars are standard error of the mean.

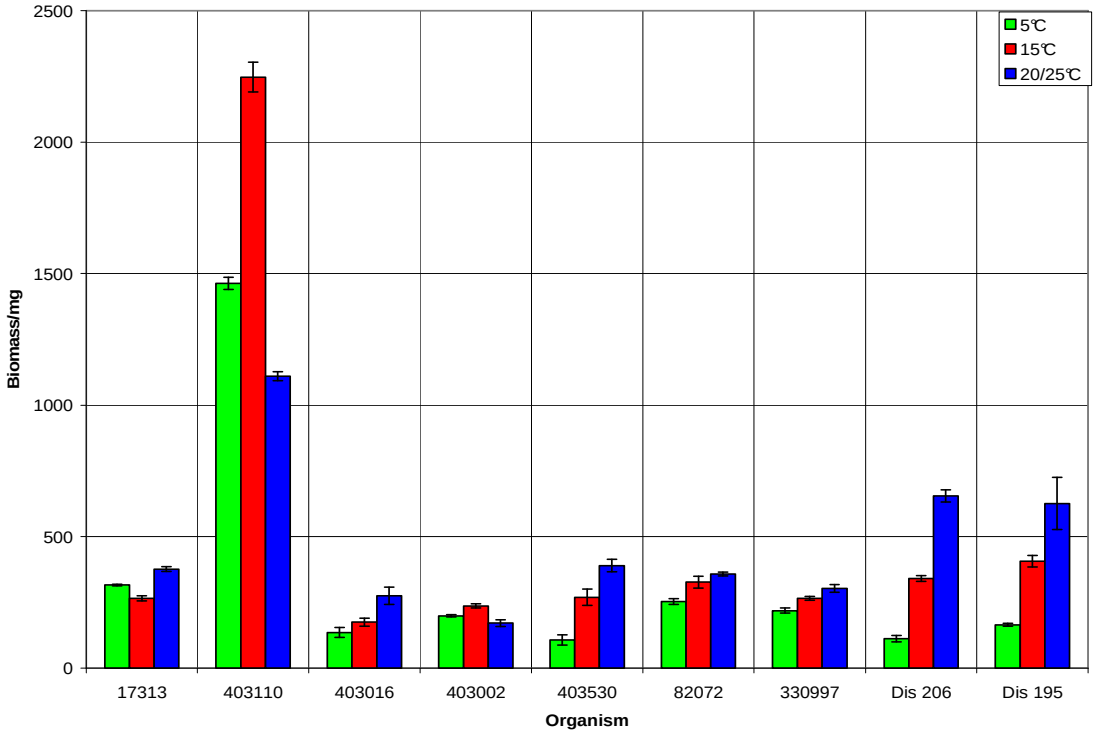


Figure 4-12. Biomass production of isolates when grown under three temperature regimes. Biomass production is found to be highest in the majority of isolates at 25°C. Biomass was highest at 15°C for 403110 and significantly higher than that found in any other isolate indicating that *P. rugulosum* is a true psychrophile. p ≤ 0.05, n = 3 and error bars are standard error of the mean.

4.2.2 Fatty acid analysis of phospholipid fractions under different growth temperature regimes in *Mortierella alpina* strain 330997

4.2.2.1 Phosphatidylcholine fraction

The most unsaturated fatty acid $C_{20:5}$ n3 was produced in the greatest quantity at 5°C in the phosphatidylcholine (PC) fraction (figure 4-13), as was seen with the total fatty acid extract (Figure 4-7). Again the $C_{20:5}$ n3 abundance decreased with the increase in temperature, a trend observed in the total FA extract. Levels of the second most unsaturated fatty acid, $C_{20:4}$ n6 were found to be highest at 25°C, following a positive correlation with the rise in temperature. Levels of the third most unsaturated fatty acid, $C_{18:3}$ n6 were found to be highest at 5°C followed by the second greatest production at

25°C. The fatty acid $C_{18:1}$ was found to be highest at 25°C, with lower but almost equal levels found at 15 and 5°C. $C_{18:2}$ levels were found to be relatively even between all three temperatures. The trends encountered in the TFA profile are represented in the PC fraction for several of the compounds such as $C_{16:0}$, $C_{18:0}$, $C_{20:4}$ and $C_{20:5}$ however most of the trends are somewhat subdued, as level differences between temperatures are far less prominent. The PC fraction contains a balanced profile of saturates and unsaturates, with the majority of compounds found in the TFA profile present in the PC fraction. Temperature variation seems to a have a less drastic effect on this phospholipid fraction, with the majority of FAs experiencing a moderate change in level with change in temperature. Saturates experience an increase in level with rise in temperature, possibly due to their rigidifying effect on the membrane. The VLCPUFA $C_{20:5}$ n3 experiences a large level increase when temperatures reach 5°C, although the levels of $C_{20:4}$ n6 experience a similarly large increase when 25°C is reached. When the unsaturation index is calculated (figure 4-19), 5°C culture growth promotes the most unsaturated membrane fatty acid composition, although 25°C demonstrates the second greatest unsaturation index in part due to the elevated $C_{20:4}$ n6 and $C_{18:1}$ levels.

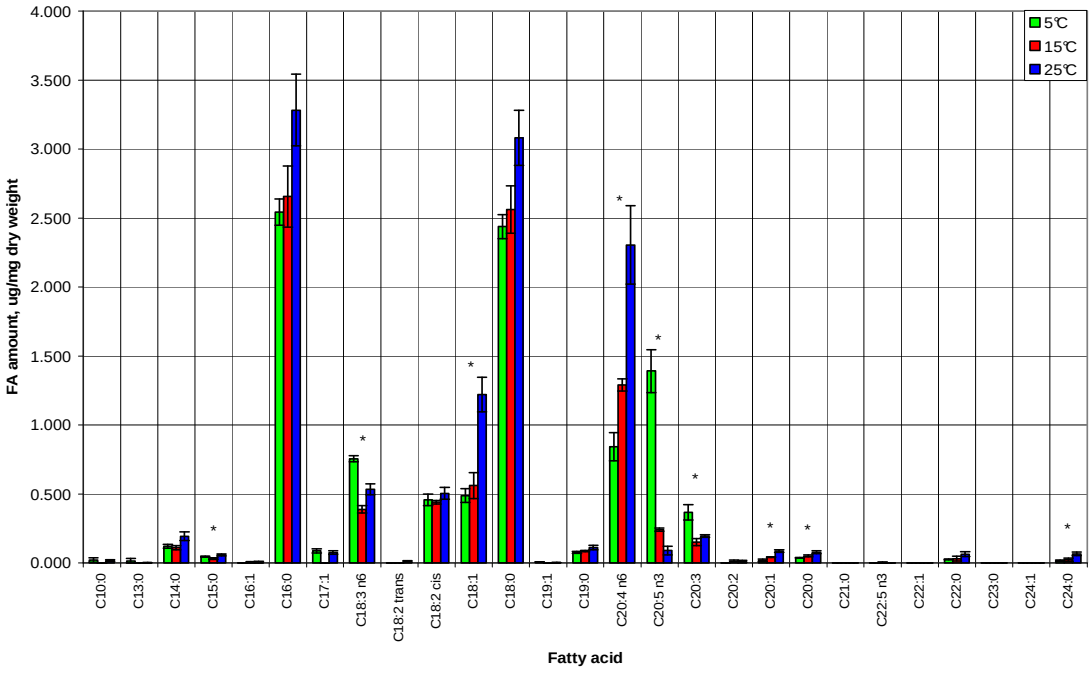


Figure 4-13. The PC fraction of *Mortierella alpina* grown under three temperatures. The PC fraction contains a similar fatty acid profile to that seen within the total lipid fraction. * = $p \leq 0.05$, n = 3 and error bars are standard error of the mean.

4.2.2.2 Phosphatidylinositol fraction

The phosphatidylinositol fraction (PI) contained very few fatty acids, and at relatively low levels (figure 4-14). The predominant fatty acids present were $C_{16:0}$ and $C_{18:0}$ and they showed a slight increase in abundance with the increase from 5 to 15°C. $C_{16:0}$ experienced a large decrease at 25°C growth with $C_{18:0}$ experiencing a smaller decrease with the increase in temperature. Minor components of this PL fraction were $C_{14:0}$ and $C_{18:1}$, with $C_{18:1}$ showing little significant change with temperature though $C_{14:0}$ levels decrease at 25°C. The fraction lacks the majority of the VLCPUFAs as $C_{18:3}$ n6 and the majority of the C_{20} VLCPUFAs are either absent or in trace quantities. The fatty acid $C_{20:4}$ n6 is present however, albeit at low quantities at 15°C, showing an increase in level with the increase to 25°C. When the unsaturation index was calculated for the fraction it was found that 25°C displayed the greatest unsaturation value (figure 4-19). However, the values calculated were comparatively low, with the increased level of $C_{20:4}$ n6 and substantially decreased $C_{16:0}$ in the 25°C culture contributing to the higher UI. It would seem that this fraction contributes little to the fluidity of the membrane due to the limited fatty acid complement, of which the main components are saturated. The relatively small profile changes, excluding $C_{16:0}$ in response to temperature also appear to confirm this idea, although the rise in $C_{20:4}$ n6 will be discussed shortly.

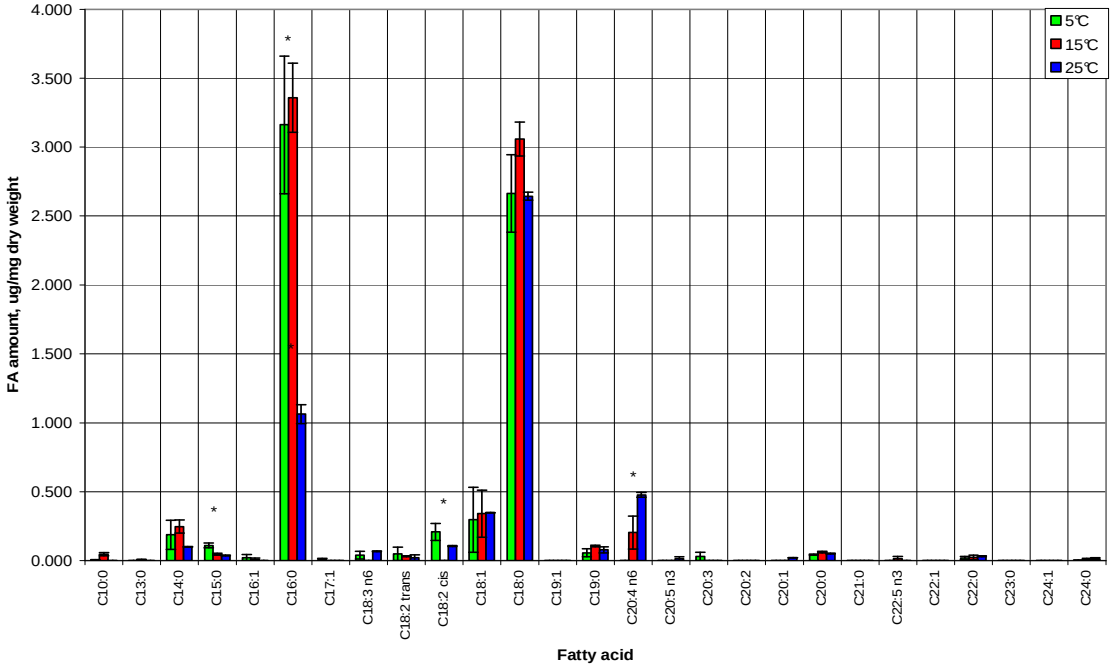


Figure 4-14. The PI fraction of *Mortierella alpina* grown under three temperatures. * = $p \leq 0.05$, n = 3 and error bars are standard error of the mean.

4.2.2.3 Phosphatidylserine fraction

The phosphatidylserine fraction (PS) is similar to the PI fraction, due to its large percentage of $C_{16:0}$ and $C_{18:0}$ and limited unsaturate content (figure 4-15). Both the aforementioned saturate levels increase with the increase in temperature from 5 to 15°C, however decrease with the further rise to 25°C, although levels remain above that observed at 5°C. All forms of PUFA within this fraction at 5°C are absent, although small traces of $C_{18:1}$ are present. Levels of $C_{18:3}$ n6 and $C_{20:3/5}$ are found in small quantities although levels stay relatively constant at 15 and 25°C growth. $C_{20:4}$ n6 is the predominant PUFA within this fraction, although levels are relatively low, they experience a small increase with the rise in temperature from 15 to 25°C. When the unsaturation indices are calculated, 5°C demonstrates the lowest UI. This is due to the absence of any major unsaturates within this PL fraction at this temperature. A modest increase in unsaturation index is seen with the increase in temperature, due in part to the $C_{18:3}$ n6 and $C_{20:4}$ n6 fatty acids. This fraction seems to resemble that of the previous PI fraction, in that the complement of fatty acids as well as the variation in the profiles is relatively minor. Although the UI is contrary to what would be expected, the contribution to total phospholipid fluidity of the PS fraction is probably relatively minor due to the low absolute values of polyunsaturates.

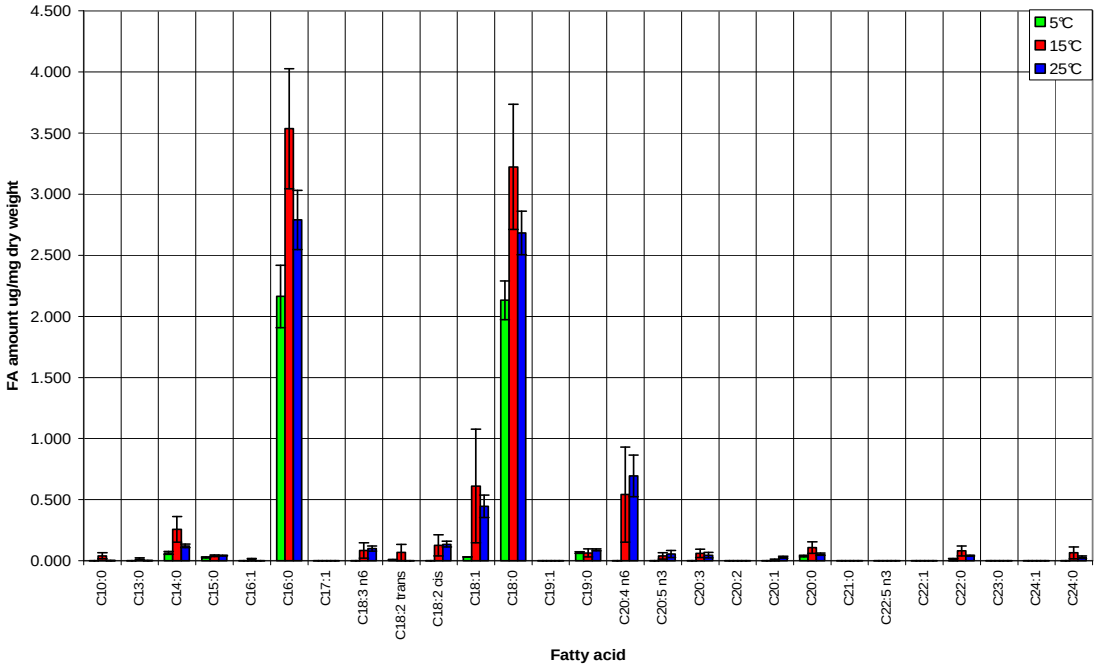


Figure 4-15. The PS fraction of *Mortierella alpina* grown under three temperatures. All peaks gave p values > 0.05, n = 3 and error bars are standard error of the mean.

4.2.2.4 Phosphatidylethanolamine fraction

The phosphatidylethanolamine (PE) fraction demonstrates the greatest diversity in fatty acid levels (figure 4-16). However it is suspected that the PE fraction also contains a large proportion of the neutral lipid fraction. This is evident from the increase in lipid observed with the increase in temperature, which is seen with the total lipid extract in *Mortierella*. The increase in lipid was not as extensive within any of the other phospholipid fractions. A subsequent TLC to separate the neutral and the PE fraction was run, although the results were inconclusive. The lack of separation between the neutral lipid and PE fraction is due to the large quantities of neutral lipid found within *Mortierella* species. Therefore any subsequent separations would require a silicic column separation of phospholipids from neutral lipids, followed by TLC of the eluted polar lipid fraction. In the combined fraction, 5°C growth results in much lower fatty acid abundances than at 15 and 25°C. The complement of fatty acids is similar to that observed within the TFA extract with VLCPUFAs such as $C_{20:5}$ n3 and $C_{20:4}$ n6 found within this combined fraction. 5°C growth results in the lowest fatty acid levels and is most likely the most representative of the PE fraction, due to the decreased total lipid at this temperature as shown with the total lipid extraction, indicating low neutral lipid levels. $C_{20:5}$ n3 is not produced to the greatest extent at 5°C as is seen within the PC fraction or in the TFA extraction, indicating that some $C_{20:5}$ n3 may be found within the neutral lipids due to the large increase observed at 15°C, when PE and neutral lipid fractions appear to merge.

Long chain saturates such as $C_{20:0}$, $C_{22:0}$ and $C_{24:0}$ appear to be mainly absent at 5°C growth, but experience rapid increases at both 15 and 25°C growth again indicating the amalgamation of the two fractions. The rapid rise in $C_{20:4}$ n6 levels at 15 and 25°C growth resembles that seen with the TFA fraction, indicating that both PE and neutral lipid fractions contain this highly unsaturated fatty acid, although the neutral lipid appears to contain the majority of this fatty acid due to the highly elevated levels. The third most unsaturated fatty acid, $C_{18:3}$ n6 demonstrates a similar trend to $C_{20:4}$ n6, with increasing temperature resulting in greater production. Other unsaturated C_{20}'s such as $C_{20:1/2/3}$ were absent or in low abundance in 5°C culture, although warmer temperature growth resulted in low levels of $C_{20:1/2}$ but higher abundances of $C_{20:3}$ n6 for both 15 and 25°C growth. The increased quantity of $C_{20:3}$ n6 comes as no surprise as this fatty acid is the precursor to $C_{20:4}$ n6. The localisation of $C_{20:3}$ n6 appears to be in both the neutral

and PE fraction, as it is present albeit at low quantities at 5°C growth with the substantial increase at 15 and 25°C indicating that a fraction may reside in the neutral lipids. The saturates $C_{16:0}$ and $C_{18:0}$ do not appear to be as prominent as in the TFA extract indicating that the neutral lipids may contain a large proportion of unsaturated fatty acids. Using a solvent system of hexane, acetic acid and water, it was found that polyunsaturated fatty acids had lower Rf than saturates, which would explain the lack of the saturates $C_{16:0}$ and $C_{18:0}$ detected in the PE fraction. The fatty acid $C_{18:1}$ also demonstrates lower levels, as the TFA show abundances similar to $C_{20:4}$ n6 at 25°C growth.

The unsaturation index of the PE fraction therefore is skewed due to the fact that it is thought to contain a large proportion of neutral lipid. 25°C growth produced the most unsaturated fatty acid profile, whereas 5°C growth produced the least unsaturated FA profile. The large error associated with the 5°C growth index was caused by several highly unsaturated fatty acids not being detected in one of the biological replicates. This resulted in a lower unsaturation index for the PE fraction at 5°C. This demonstrates that the TLC separation of phospholipid components can be prone to error. This error is carried forward to the total phospholipid unsaturation index, which shows 5°C growth producing the most saturated fatty acid profile whereas 25°C growth produces the most unsaturated fatty acid profile. If the PE fraction is removed from the calculation then 5°C and 25°C growth produce similar unsaturation indices around 0.8, although 25°C growth still produces the higher unsaturation index predominantly because of the $C_{20:4}$ n6 found in the PS fraction. It appears that the PE and PC fractions are responsible for the majority of fatty acid regulation within *Mortierella alpina* as these fractions contain the majority of the VLCPUFA, with the PC fraction demonstrating greater unsaturation with the decrease in temperature. However, it also appears that neutral lipid within the species comprises a large proportion of C_{20} VLCPUFA such as $C_{20:4}$ n6, especially at 25°C growth.

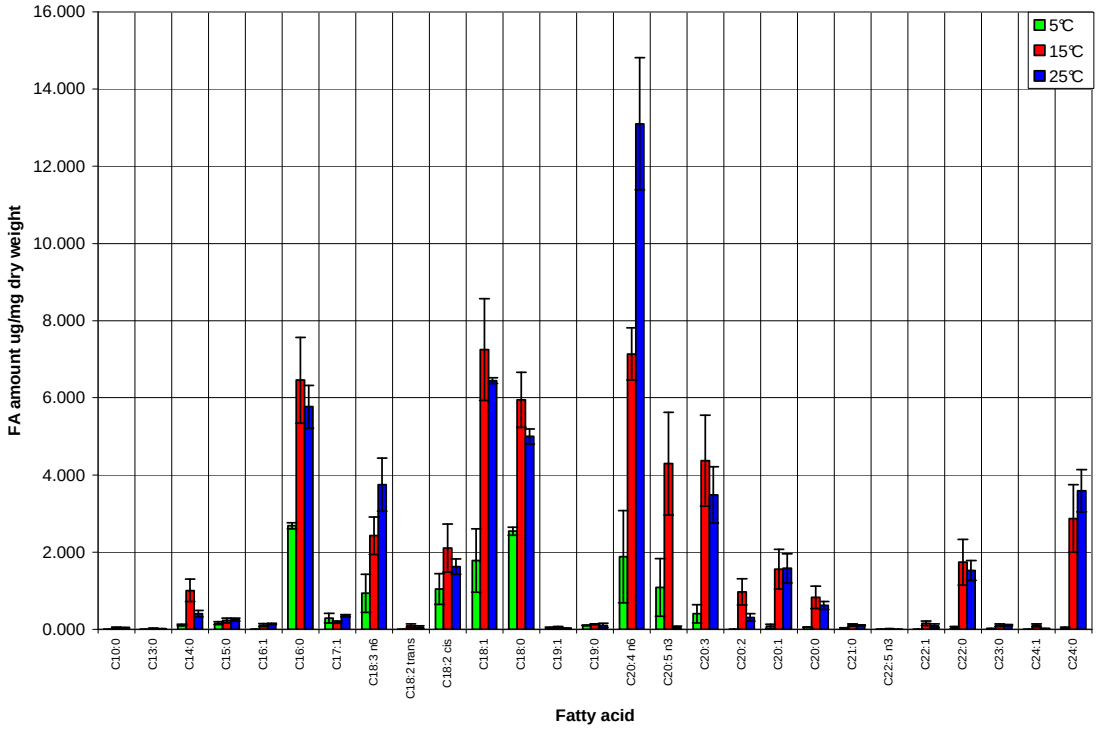


Figure 4-16. The PE fraction of *Mortierella alpina* grown under three temperatures. The PE fraction appears to be an amalgamation of the PE and neutral lipid fractions due to the high lipid production at 15 and 25°C. n = 3 and error bars are standard error of the mean. ANOVA was not performed on the data, due to the suspected mixing of the PE and neutral lipid fraction.

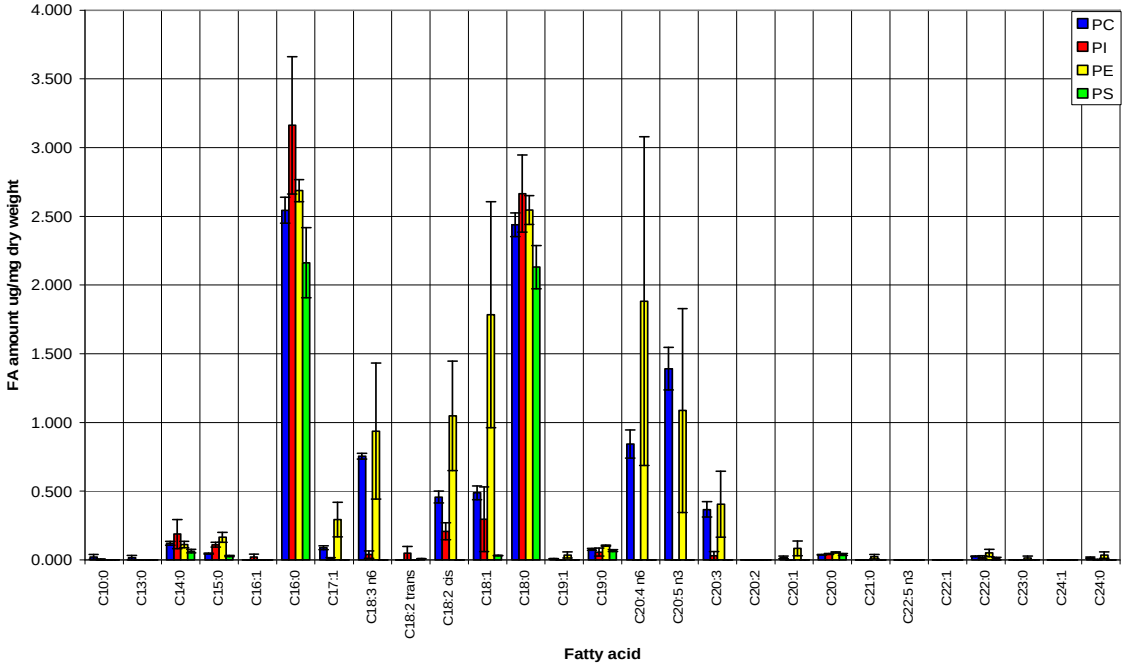


Figure 4-17. Comparison of phospholipid classes using a culture temperature of 5°C. The comparable lipid levels of the PE fraction indicates that 5°C growth results in minimal neutral lipid production and therefore the PE fatty acid profile suffers little contamination from the neutral lipid fraction. It can be clearly seen at the lowest growth temperature, the PE and PC fractions of *Mortierella alpina* appear to be responsible for membrane fluidity regulation. n = 3 and error bars are standard error of the mean.

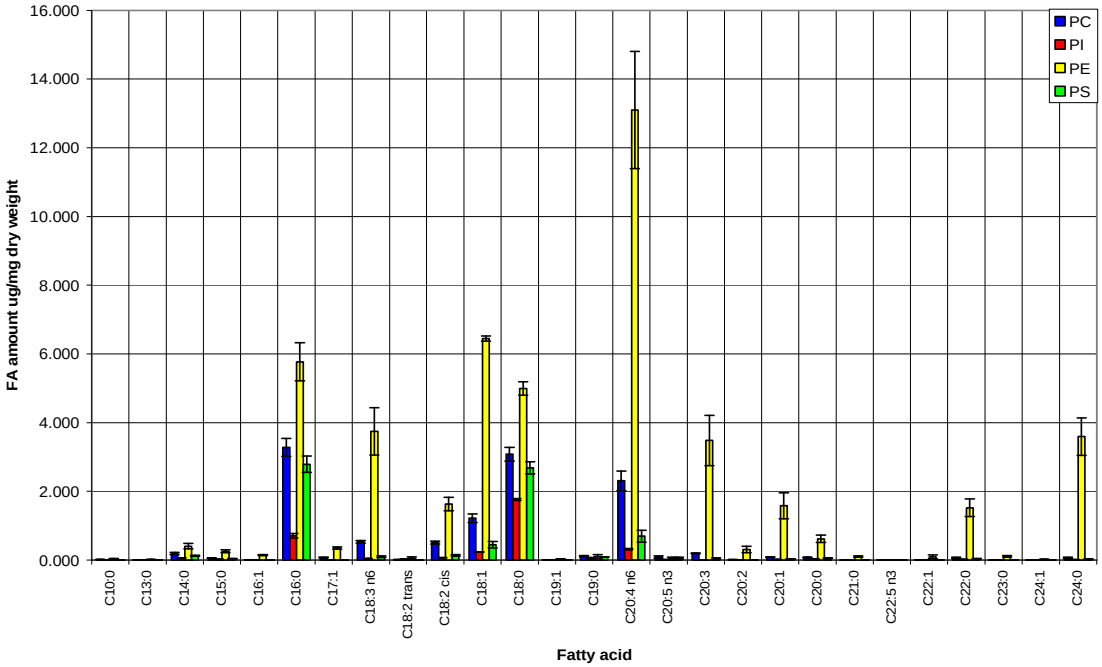


Figure 4-18. Comparison of phospholipid classes using a culture temperature of 25°C. The significantly increased levels of fatty acid associated with the PE fraction at 25°C growth indicate that neutral lipid has been incorporated into the TLC fraction. The large abundance of $C_{20:4}$ n6 indicates that this VLCPUFA is found as a major component of the neutral lipids. The greatly elevated levels of $C_{20:3}$ n6 and the saturates $C_{20:0}$, $C_{22:0}$ and $C_{24:0}$ compared with the other phospholipid fractions would indicate their predominant localisation within the neutral lipids as well. n = 3 and error bars are standard error of the mean.

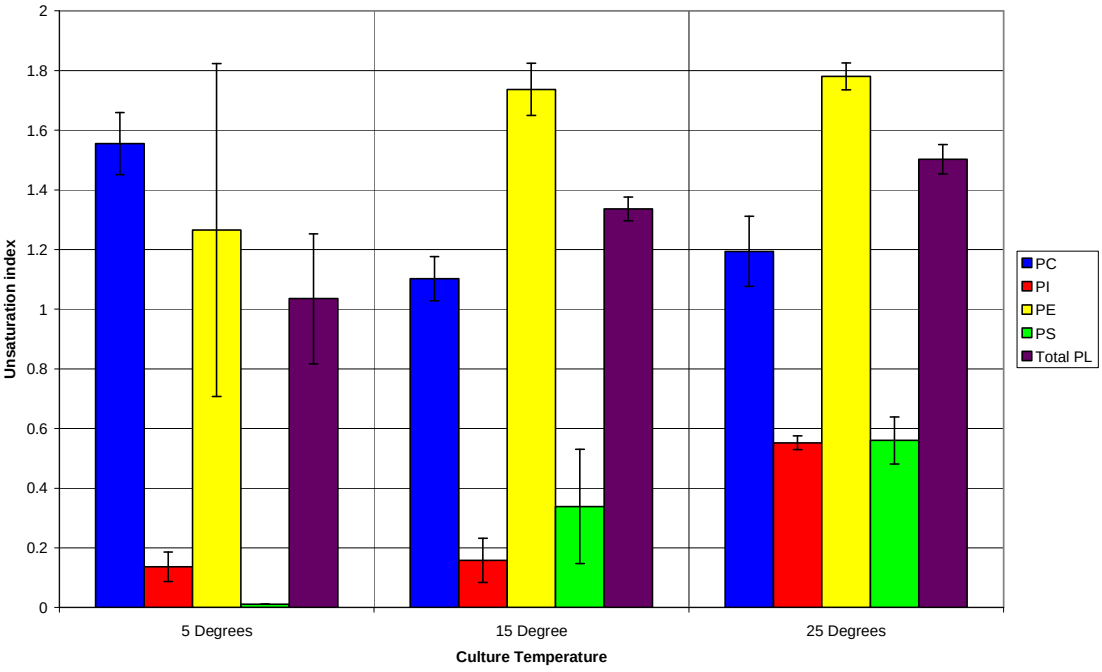


Figure 4-19. The unsaturation indices for the individual phospholipid components predominantly show an increase in desaturation with increasing temperature. The PC fraction however shows the greatest unsaturation at 5°C growth, and this phospholipid

fraction is thought to be one of the modulators of membrane fluidity within *Mortierella*. The increase seen in the PI fraction is due to a small quantity of $C_{20:4}$ n6 which is also responsible for the increase in desaturation found in the PS fraction. The small levels of precursors to $C_{20:4}$ n6 may indicate that this VLCPUFA may be a contaminant due to its high levels at higher temperatures. The PE fraction is proposed to contain neutral lipid, and as such demonstrates the highest unsaturation at 25°C due to the high proportion of $C_{20:4}$ n6. The total unsaturation index for all the fractions shows 25°C producing the greatest unsaturation. This is in contrast to the TFA extract, indicating that a large proportion of saturated lipid was not extracted. n = 3 and error bars are standard error of the mean. ANOVA was not performed, due to the suspected contamination with neutral lipid.

4.3 Discussion

In *Mucor racemosus* one of the primary responses to low temperature culture is to increase the quantity of fatty acids, with the majority of classes experiencing a substantial rise in abundance at 5°C. Culture at 15°C showed a decrease in a large number of fatty acids compared with 5°C and 25°C culture, specifically $C_{16:0,}$ $C_{18:0}$ and $C_{18:1}$, although $C_{14:0}$ and $C_{18:2}$ demonstrated this trend albeit with levels much closer to 25°C growth. The second response to low temperature culture was the high abundance of $C_{18:3}$ n6, with levels peaking at 5°C culture and then subsequently declining with the increase in temperature. For all the organisms studied except *M. alpina* strain 82072, the highest absolute amount of the most unsaturated fatty acids $C_{18:3}$ n3/6 or $C_{20:5}$ n3 was produced at the lowest culture temperature. However, although strain 82072 did not contain the greatest absolute concentration of $C_{20:5}$ n3, it did contain the greatest proportion of $C_{20:5}$ n3 as a percentage of the total fatty acids. This would appear to reinforce the theory that a mechanism exists that translates the rigidity of the membrane into the production of the most unsaturated fatty acid through the expression of several desaturases. In several of the cases examined, where $C_{18:3}$ n3/6 is the most unsaturated fatty acid, it could be inferred that 5°C culture appears to promote increased expression of either a Δ15 or a Δ6 desaturase. In the case of *Mucor racemosus* for example, increased $C_{18:3}$ n6 may partially be the result of increased levels of $C_{18:2}$ n6, the substrate for the Δ6 desaturase. In the case of $C_{20:5}$ n3, cold temperature growth appears to stimulate the expression of a Δ17 desaturase. The reason for incorporating the most highly unsaturated fatty acids is predominantly to maintain the fluidity of the membrane during cold conditions. The rise in the entire complement of fatty acids within *M. racemosus* during cold temperature growth indicates that FAs are being sent for storage. The logical conclusion would be the conversion of excess fatty acids into TAG lipids. When the unsaturation index values were studied for *M. racemosus*, it revealed that

culture at 15°C produced the most unsaturated fatty acids. What is not evident from the unsaturation index, as will be applicable to all samples, is the localisation of the FAs. If saturates were localised as storage lipids, then it may be found that the majority of unsaturates are localised within the membrane, which would increase the unsaturation index of these functional lipids. However, the initial extraction procedure used assessed total cellular lipids and therefore, no definite conclusion can be drawn as to where the unsaturation occurs or whether neutral lipids increase with the decrease in temperature. The most efficient growth of *Mucor racemosus* in terms of fatty acid and biomass production would be achieved by culture at 5°C, due to the minimal loss of biomass coupled with the substantial increase in total FA production as well as increases in $C_{18:3}$ n6, the most unsaturated FA produced by this organism.

Penicillium rugulosum demonstrates the highest unsaturation index of any of the organisms studied when grown at 5°C. It also produced the greatest biomass with over 2 g of per 100 ml of PD broth. This is quite remarkable as this organism contains the same fatty acid complement as the majority of the other fungi studied, but has fewer unsaturated fatty acids when compared with the *Mortierella alpina* strains. It appears that possessing the ability to produce fatty acids longer and more unsaturated than $C_{18:3}$ n3/6 is not a requisite for optimal low temperature growth. This highlights two points; firstly *P. rugulosum* contained the highest percentage of $C_{18:3}$ n3 at 5°C, accounting for 47.6% of the total fatty acids. When the other trienoic values are studied, values of 17.6% (*Mucor*), 20.1% and 19.1% (*Herpotrichia* strains 403016 and 403002), 13.9% and 21.4% (*Umbelopsis* strains Dis 260 and 195) are produced. Whilst all these organisms can produce the same fatty acids, the percentage composition of the most unsaturated seems to play an important role in the adaptation of the organism to low temperature environments. *Bjerkandera adusta* produced its most unsaturated fatty acid $C_{18:2}$ at 79.6% at 5°C. The unsaturation index of 1.65 is still lower than that observed for *Penicillium* which produced a value of 1.95. The predominant strategy for *B. adusta*, which only produced two major fatty acids, is to increase production of $C_{18:2}$ under low temperature conditions, again supporting the theory that cold temperatures favour unsaturated fatty acid production. Due to the low growth and low lipid levels it could be assumed that $C_{18:2}$ on its own is not sufficient to maintain optimal membrane fluidity.

The second point is that whilst *Penicillium* demonstrates the highest percentage of $C_{18:3}$ n3, is most likely adapted in other ways. The ability of proteins to function at such

low temperatures is also critical for the survival of the organism, with *Penicillium* most likely having evolved these adaptations, compared with the other organisms studied, which show relatively slow growth at low temperatures compared with *Penicillium* biomass production. Further, the low lipid level indicates low neutral lipid abundance. Another point in regards to cold adaptation is the fact that several organisms exhibited increases in lipid levels with decreasing temperature. It is thought that this may be a feature of restrictive protein synthesis whereby under low temperature conditions the inability to regulate fatty acid production leads to increased production (Suutari, 1995) (Bensch *et al.*, 1961). Protein malfunction at low temperatures may also explain the lower biomass levels for these organisms at low temperature. *P. rugulosum* compared with the other studied isolates appears to be a true psychrophile, indicating that a high proportional value of $C_{18:3}$ n3/6 is important for optimal growth at low temperatures. Whilst the majority of isolates studied were also capable of producing trienoic fatty acids they did so to a lesser degree. It also suggests that trienoic fatty acids can be equally or more effective than tetra- and pentaenoic acids at maintaining membrane fluidity at low temperatures. In regards to the *Bjerkandera* isolate, the importance of unsaturated fatty acids in relation to cold temperature survival is again highlighted, with the correlation between the most unsaturated fatty acid and the growth temperature. As to why only two fatty acids are predominantly made is unknown, although the fact that very low levels of precursors to $C_{18:2}$ are present would indicate either very efficient synthesis of $C_{18:2}$ or a novel synthesis route. The low biomass at 5°C coupled with the predominantly unsaturated fatty acid profile suggests that other factors may also be responsible for low growth at 5°C.

The unsaturation indices and fatty acid profiles suggest that the *Herpotrichia* strains employ different strategies to survive low temperatures. One of the common features between the two strains is that $C_{18:3}$ n3 abundance is directly linked with the growth temperature, with peak levels achieved at 5°C growth. Whilst strain 403016 shows elevated fatty acid levels at 5°C, strain 403002 has the most abundant lipid levels at 25°C. The elevated lipid levels at low temperatures could again be the result of restricted protein synthesis as discussed previously. $C_{18:2}$ levels in strain 403016 remain relatively constant throughout the temperature range, which is not found within any of the other samples. This fatty acid is probably utilised to maintain sufficient fluidity to counter the decrease in $C_{18:3}$ n3 levels at warmer temperatures, but it may also be stored as a non-membrane associated fatty acid due to its constantly high levels. In regards to

unsaturation index strain 403002 achieves a slightly higher unsaturation index than strain 403016, with strain 403002 showing the hypothesised decrease in UI with increase in temperature. Strain 403016 opposes this by demonstrating a marginally higher UI value at 25°C compared with 5°C. The reason for the high index is due to the lowering of all the fatty acid levels except $C_{18:2}$, which remains at a consistently high level. Why this fatty acid remains at such a constant level whilst all other fatty acids decline is unknown. The variation between the two isolates of *Herpotrichia* could be attributed to intraspecific variation, however this may lead to the classification of a new strain which is biochemically distinct. This can be ascertained through the use of multiple isolates and data analysis techniques such as PCA, as described in the previous chapter, whereby strains cluster together and intraspecific variation is observed by the scatter of the points making up the cluster. In this case, as no species identification was made it is impossible to deduce whether the variation is inter- of intra- species variation.

The genus *Mortierella* is worthy of note due to several factors. Firstly the capability of *Mortierella* spp. to accumulate large quantities of lipid, large proportions of which are thought to be stored as TAG in lipid droplets (Sorger & Daum, 2003) (Beopoulos *et al.*, 2010). The abundance of neutral lipids may interfere with accurate UI estimates due to the much larger ratio of neutral to polar lipid. As neutral lipid in storage is less likely to play a role in membrane fluidity the UI value may be lower than expected, as it is generally thought that neutral lipid is more saturated than in polar lipids (Mumma *et al.*, 1971) (Kendrick & Ratledge, 1992b) (Tonon *et al.*, 2002). High levels of saturates and monounsaturates would decrease the total fatty acid percentage of polyunsaturates such as $C_{18:3}$ n6, which in turn would lead to a lower unsaturation index. This lack of knowledge regarding localisation in high lipid producing organisms such as *Mortierella* may account for the 15°C culture in strain 82072 producing the most unsaturated profile. The unsaturation profile of strain 330997 correlated with other works (Jang *et al.*, 2005) and the proposed hypothesis. In regards to lipid accumulation, *Umbelopsis* strain Dis 206 produced the most lipid at 5°C with the greatest constituent being $C_{18:1}$. This negative correlation of increased temperature leading to decreased fatty acid production appears to differentiate this strain from both *Mortierella* isolates and *Umbelopsis* strain 195. As to why such large lipid quantities are produced at 5°C are unknown, although it is possible that protein regulation at low temperature growth may have a role as this strain is mesophilic. *M. alpina* strains are thought to be adapted to low temperature environments due to their production of VLCPUFAs such as $C_{20:5}$ n3,

however the mesophilic *Umbelopsis* isolates demonstrated that growth is possible without VLCPUFAs, although lesser unsaturation indices were achieved by both Dis 206 and 195 strains. This is in comparison to other mesophilic organisms, such as those studied by Suutari (Suutari, 1995), where mesophilic *Penicillium*, *Aspergillus* and *Trichoderma* demonstrated either bell shaped unsaturation index curves in relation to temperature, with an average temperature of 26°C producing the greatest unsaturation, or with sudden drops in unsaturation from 15°C to 10°C growth. This indicated that these mesophiles were unable to cope with low temperature growth, whereas in this study the unsaturation indices of the mesophilic *Umbelopsis* isolates seem to demonstrate the ability to cope with low temperature, with 5°C producing the greatest unsaturation indices.

Mortierella alpina is one of the few true fungi capable of producing VLCPUFAs. As has been demonstrated with the previously discussed samples, trienoic fatty acids seem to provide enough fluidity to allow survival in low temperature environments. In the case of *Penicillium rugulosum*, it affords more than adequate membrane fluidity at high proportional values. The VLCPUFA producing *M. alpina* at 5°C growth still showed reduced growth at low temperatures which subsequently increased with increasing temperature. It could be hypothesised that if such an organism possessed the ability to produce greatly unsaturated fatty acids, especially in light of the capabilities of fatty acid production within the majority of the fungi, it would thrive in low temperature environments. One can then only assume that other factors limit the growth of such an organism at low temperatures. Whilst *M. alpina* isolates 330997 and 82072 do not thrive as does *Penicillium*, when compared with the *Umbelopsis* isolates, biomass production at 5°C is enhanced. Compared with the majority of the other isolates biomass production at 5°C is also greater. At 15°C however any benefits that were attributed to VLCPUFAs in regards to membrane fluidity are lost as *Umbelopsis* isolates show a substantial increase in growth, with biomass production higher than in the *Mortierella*. The final point to consider is $C_{20:4}$ n6 and the high levels detected at the warmer growth conditions of 15 and 25°C. As to why such an unsaturated fatty acid is produced at warmer temperatures is unclear. If the fatty acid was localised to the membrane then one would assume that the membrane would be in a very fluid state, and if localised to neutral lipids what role does it play to warrant its accumulation, as so few true fungi have this capability. The fact that *Mortierella* are oleaginous organisms, would provide fatty acid biosynthesis with increased substrate, however depending on

the expression of specific elongases and desaturases, accumulation of specific fatty acids could occur anywhere within the pathway. This raises the question as to the function of VLCPUFAs within *Mortierella alpina*. It can be seen that the presence of VLCPUFAs correlate with an increase in biomass at 5°C, although when compared with *Penicillium* the apparent benefit they provide is small. However, low temperatures do induce $C_{20:5}$ n3 production indicating low temperature growth is a trigger for Δ17 gene expression. The roles of VLCPUFAs were discussed in the previous chapter but the relationship between the *Mortierella* and Chytridiomycota may indicate that VLCPUFA production may be a remnant, possibly vestigial phenotype from the Zygomycota ancestry. The fact that the majority of the Zygomycota have lost the ability to produce VLCPUFAs also indicates the redundant roles these VLCPUFAs may play in these true fungi. Counter to this is the fact that VLCPUFA production is still selected for and would indicate that some environmental pressure confers a benefit, leading to their perpetuation within the *Mortierella*.

Total fatty acid analysis provides a good indication that temperature affects the composition of the fatty acids within the cell. The unsaturation indices in some isolates demonstrated that the lowest temperature did not always induce the greatest unsaturation. This is possibly due to the increase in total fatty acid levels, with the unknown localisation of the unsaturated fatty acids. The segregation of lipids allows for a more definite conclusion on the role that fatty acids play within membrane lipids. The fungus *Mortierella alpina* was studied further as it is capable of producing fatty acids such as arachidonic acid and eicosapentaenoic acid. The TLC separation procedure alone failed to separate the neutral lipid from the PE fraction although in other cases the TLC method alone has been sufficient (Domergue *et al.*, 2003, Tan *et al.*, 2011). However the large increase in neutral lipid promoted by the increase in temperature led to insufficient separation and further analysis would require the prior separation of neutral lipids from polar lipids using a silicic column. On analysis of the phospholipid fractions, only the PC fraction produced the greatest unsaturation index value at the lowest culture temperature. When the total unsaturation index from all phospholipids fractions was analysed, a positive correlation with relation to temperature was observed. The low value of the 5°C unsaturation index was exacerbated primarily by the lack of detection of several PUFAs in the PE fraction replicates. Even with the removal of the merged PE fraction from the total phospholipid calculation, growth at 25°C still yielded the greatest unsaturation index. This is in opposition to the hypothesis that decreasing

culture temperature results in greater unsaturation within the phospholipids. Whilst the phospholipid fraction is thought to contain the majority of the polyunsaturated fatty acids, the *Mortierella* species studied contained a large proportion of the unsaturated fatty acids, specifically $C_{20:4}$ n6 within the neutral lipids, which was also shown by Aki *et al*., who showed that within *Mortierella alliacea* around 86% the total arachidonic acid was stored in neutral lipids. They also showed that VLCPUFAs were localised to the PC and PE fractions (Aki *et al.*, 2001) which was also found to be the case with the studied *Mortierella* isolate. The localisation to the PE and PC fractions was also described by Tan *et al*. (Tan *et al.*, 2011) on their study of the Zygomycete *Conidiobolus obscurus* which contains $C_{20:4}$ n6 and $C_{20:5}$ n3 and like the majority of fungi, the phospholipids were the most unsaturated fraction. PC and PE localisation of VLCPUFAs has also been documented in *Achlya* species (Aki *et al.*, 1998) and the Zygomycetes *Conidiobolus* and *Entomophthora* spp. (Kendrick & Ratledge, 1992c) although they also contained VLCPUFA within the PI fraction. The PC fraction is also thought to be the substrate for fungal Δ5, Δ6 (Domergue *et al.*, 2003) and Δ12 desaturases (Jackson & Fraser, 1998) and the observed increase in desaturation with decreased temperature seems to correlate with this finding. The low unsaturation at 5°C observed within both the PI and PS fractions would indicate these phospholipids play little role in membrane fluidity regulation, although the PS fraction shows a linear increase in unsaturation with increasing temperature whilst the PI fraction only experiences a large increase in unsaturation at 25°C. The lack of substrate for $C_{20:4}$ n6, as well as the small quantities found of this FA within these two fractions, alludes to the fact that the presence of this fatty acid is possibly due to the large proportion of neutral lipid contaminating other fractions, or that it was transferred from PC via an acyl-transferase. As such both the PS and PI fractions are unlikely to be heavily involved in membrane fluidity regulation and likely contain large proportions of $C_{16:0}$ and $C_{18:0}$.

If PC is indeed the substrate for the Δ5 desaturase, then the high levels of $C_{20:4}$ n6 found within the neutral lipids must be the result of efficient transfer between the two lipid fractions. It would also imply that the PE fraction is enriched with VLCPUFA from the PC fraction. Another point raised by Domergue *et al*. was the presence of bottlenecks which appeared in recombinant organisms transformed to produce $C_{20:4}$ n6. It was thought the differing substrate requirements for fungal and algal desaturases and elongases, acyl-PC and acyl-CoA respectively, resulted in inefficient conversion past $C_{18:3}$ n6 due to the requisite of elongases requiring acyl-CoA substrate. *Mortierella*

alpina appears to have an efficient method by which to produce sufficient $C_{20:3}$ n6 through elongation, followed by transfer to the PC fraction, which then drives the substantial $C_{20:4}$ n6 production due to desaturation, followed by subsequent transfers to the PE/neutral lipid fraction. Again, why $C_{20:4}$ n6 is produced in such large quantities when grown at temperatures above 15°C and subsequently stored in neutral lipid is unknown. What is noticeable within the PE and PC fraction is the lack of production of $C_{20:5}$ n3 at 25°C, mimicking the TFA profile. This would indicate the down regulation of the Δ17 desaturase at temperatures at or below 25°C. The PE fraction also demonstrated a lack of $C_{20:0}$, $C_{22:0}$ and $C_{24:0}$ at 5°C growth, which was also shown in the TFA profile at 5°C growth. This would indicate the down regulation or complete cessation of MALCE1 and MAELO, the elongases involved in these saturate's production. A small increase in total fatty acid associated with the phospholipid fractions, excluding the merged PE fraction, was seen between growth at 5 and 15°C, which then showed a small decline at 25°C growth compared with 15°C. This is possibly due to the increase in neutral lipid production which is usually stored in vesicles surrounded by phospholipid mono-layers. Hence, by increasing the number and size of vesicles, the more phospholipid is required to encapsulate these storage vesicles.

The reason as to why *Mortierella alpina* produces $C_{20:4}$ n6 to such a large degree is unknown, however the attachment of VLCPUFAs to the PC and PE phospholipids is possibly due to the physical properties of the head groups. Both PC and PE exhibit significantly higher melting temperatures than PI and PS with values on average of 230°C and 196°C compared with 160°C and 136°C (Chapman, 1975). However it is not just the melting points that determine the fluidity of the phospholipids, but rather the endothermic transition state, a mesomorphic state whereby the acyl chains "melt" and display greater molecular motion as described by Chapman. The transition to this state within the PE and PC lipids is directly correlated to the attached acyl chains, with shorter and more unsaturated chains resulting in lower transition temperatures, in essence increasing fluidity. PC demonstrates a much wider degree of transition temperatures with respect to attached acyl chains, with $C_{12\text{-}18}$ acyl chains affording transition temperatures from 0-50°C. This in effect means that fluidity can be maintained over a wide temperature range by altering only the chain length. This gives the organism much finer control over membrane fluidity. The PE fraction however demonstrates for acyl chains $C_{14\text{-}18}$ a transition temperature range of around 70-80°C. This range is much smaller and higher than that from PC, therefore highly unsaturated

fatty acids such as $C_{20:4}$ n6 and $C_{20:5}$ n3 would allow lower transition temperatures. Therefore, the PC fraction provides tight control over the membrane fluidity due to its large transition range achieved through acyl chain length and unsaturation. This would explain the specificity of Δ5 and Δ6 desaturases for the PC fraction as this fraction has the greatest impact on fluidity. The PE fraction has a small transition range which sits above ambient temperature and therefore necessitates the need for polyunsaturated fatty acids to maintain its fluidity. Once the acyl chains have passed through the transition temperature, the phospholipids are then in a liquid crystalline state. The significance of maintaining this liquid crystalline state is that acyl chains passing back through the transition temperature return to the phospholipid gel state. It is thought that during the transition between liquid crystal and gel states, temporary defects occur within the membrane resulting in membrane leakage (Crowe *et al.*, 1987), potentially causing cellular damage. To prevent leakage therefore, phospholipids must be prevented from transitioning to the gel state and is achieved through acyl chain modification, which maintains the phospholipids above their transition temperature. In the case of the PE fraction, which has a narrow but high transition temperature range, a high degree of unsaturation is required to maintain the liquid crystalline state.

4.4 Conclusion

It was found that low temperature growth resulted in the greatest formation of the most unsaturated fatty acid, in most cases the trienoic acid $C_{18:3}$ or $C_{20:5}$ n3. *Mortierella alpina* strain 82071 however displayed equal proportions of $C_{20:5}$ n3 when grown at 5 and 15°C. Six of the nine isolates studied demonstrated the greatest unsaturation index at 5°C, with two out of the nine isolates displaying the greatest unsaturation at 15°C growth. *Herpotrichia* sp. strain 403016 produced the greatest unsaturation at 25°C, which was marginally greater than that found at 5°C growth. *Penicillium rugulosum* demonstrated that trienoic acids are sufficient for survival in low temperature environments with the isolate demonstrating prolific growth at 5°C. The large degree of growth shown by the *Penicillium* isolate would indicate that other non-fatty acid factors also play a role in optimal growth under low temperature conditions, as isolates displaying the same fatty acid complement did not match the growth of *Penicillium*. Two of the isolates, *Mucor racemosus* and *Herpotrichia sp*. strain 403002, which produce trienoic fatty acids as their most unsaturated component, showed comparable biomass to *Mortierella alpina* isolates at 5°C growth. Other trienoic containing isolates

such as the *Umbelopsis* species showed noticeable lack of growth when compared to VLCPUFA producing *Mortierella alpina* isolates at 5°C growth, indicating VLCPUFAs confer some benefit at very low temperature growth. It was suggested that due to the lack of $C_{20:5}$ n3 production at 25°C in both *Mortierella alpina* strains that Δ17 desaturase transcription or translation was down regulated. The lack of long chain saturate production at 5°C growth in both *Mortierella alpina* strains suggested that either MAELO or MALCE1 production was down regulated, as both elongases are responsible for long chain saturate formation.

Whilst the PE and neutral lipid fractions from *Mortierella alpina* strain 330997 were not resolved the resulting data supports the role of PC as a major modulator of the membrane fluidity within *Mortierella alpina* due to the increased unsaturation observed with decreased temperature. This highlights PC's role as a key modulator of membrane fluidity due to its wide transition temperature range which makes it a suitable substrate for Δ5 and Δ6 desaturases. The 5°C profile of the PE fraction is most likely representative of the fraction and corresponds to reports of both PC and PE containing elevated levels of PUFAs, with those PUFAs located within the PE fraction lowering the transition temperature. As to how the PE fraction acquires PUFAs, as PC is the proposed major substrate for desaturation is unknown, although it is likely that PUFA from the PC fraction is transferred to the PE lipid. *Conidiobolus obscurus* is known to contain the majority of PUFA within the phospholipid fraction, although *Mortierella* displays a different strategy by placing large quantities of $C_{20:4}$ n6 and $C_{20:3}$ n6 into the storage lipids. Again, it is proposed that PUFA from the PC fraction is transferred to the neutral lipid fraction either through a phospholipase route or acyl-transferase route. It also appears that *Mortierella alpina* is highly efficient in producing VLCPUFA, due to the proposed differential specificities of fungal elongases and desaturases. *Mortierella alpina* appears to have developed, by current elongation and desaturation mechanics, an efficient transfer system between the acyl-CoA pool and PC bound lipid to sustain the high production of $C_{20:4}$ n6.

5 Development of a novel genetic screen for the detection of long chain polyunsaturated fatty acids within the fungi

5.1 Introduction

The favoured analysis of PUFAs from fungi, as well other organisms such as algae, is to extract and derivatise the fatty acids, followed by their separation on a suitable analytical platform such as GC-FID or GC-MS. This method is relatively simple and sensitive and enables a fatty acid profile of the organism to be acquired, which can be utilised in taxonomical classification. However, screening for a particular set of high value fatty acids such as the omega 3s, the relatively long growth and processing time, as well as the expense incurred from solvent use can be significant, especially when hundreds of samples are to be analysed. Typically, for an organism isolated from a hostile environment such as the Antarctic, growth on plates and in broth is slow and growth conditions are usually un-optimised. In addition, under sub-optimal growth conditions certain fatty acids may not be produced, as shown with *Mortierella alpina* and the lack of $C_{20:5}$ n3 production during 25°C culture. To complement metabolic analysis, DNA based approaches can be used for targeted screening, which can then be complemented by analytical technologies to validate production of the compound. This requires a specific DNA based target, which can differentiate the presence or absence, of a target gene. For poorly studied organisms or metabolic pathways this is not always possible, due to the lack of information available regarding the genome of that organism. Another problem, when analysing a wide spectrum of organisms is the lack of homology at the nucleotide level which may prevent efficient PCR based analysis. With the careful selection of gene and primers, it is possible to reduce the incidence of missing a novel producer of a desirable metabolite. Primers must be specific enough to bind only to the gene of interest but be lenient enough to allow polymorphisms within the sequence. Benefits of PCR based methods include shorter sample analysis times, due in part because broth culture is not required, and solvent use is also greatly reduced. The method also allows for the detection of the metabolites regardless of the culture conditions, due to the fact that genomic DNA is utilised for the screen, and not mRNA.

The aim of this section of work was to develop a PCR based screen to detect the presence of VLCPUFAs within fungal populations. The target gene utilised for the screen was GLELO, as it is responsible for the elongation of $C_{18:3}$ n6 to $C_{20:3}$ n6 as well as having been shown to have activity on the n3 substrates. The Δ6 elongase GLELO was selected due to its essential role in the formation of C_{20} VLCPUFAs such as $C_{20:3}$ n6 which are then subsequently desaturated by Δ5 and Δ17 desaturases to form $C_{20:4}$ n6 and $C_{20:5}$ n3 respectively. The elongase can also be utilised for both the n3 and n6 pathways, although GLELO has only been documented to be involved with the n6 pathway. Using a Δ5 or Δ17 desaturase as found in *Mortierella* may result in producers of VLCPUFAs not being detected due to the nature of their biosynthesis. A Δ17 desaturase as a genomic marker for VLCPUFAs is not viable due to the lack of organisms capable of producing $C_{20:5}$ n3. Organisms that produce $C_{20:4}$ n6 would be missed, as a Δ17 desaturase is not required for its formation. This argument cannot be applied to the Δ5 desaturase, as many organisms are capable of producing $C_{20:4}$ n6 limiting the incidence of missing VLCPUFA producers. However the Δ5 desaturase gene cannot be used because analysis of the genomic DNA revealed that there was no conserved region to develop primers for. There was also a lack of fungal Δ5 desaturase DNA sequences indicating that this enzyme is present in few fungi or in those that do contain it, has not been characterised. The Δ6 elongase, GLELO, whilst only being documented within *Mortierella* contains a conserved genomic region when compared to similar Δ6 elongases. This meant that primers could be designed around the conserved region of GLELO, which would allow for the detection of VLCPUFAs at the genomic level. The conserved genomic region and primer development are discussed in detail in section 5.2.1.

5.1.1 Aims

The aim of this study is to determine whether a suitable gene marker is present by which VLCPUFA production can be inferred. Once identified, suitable primers will be developed to allow amplification across a wide array of organisms within the fungi. The primers will then be tested to determine the correlation of the gene product and the fatty acids $C_{20:3}$ n6, $C_{20:4}$ n6 and $C_{20:5}$ n3.

5.2 Results

5.2.1 The identification of conserved regions of homology within Δ6 elongases from diverse species

The interrogation of known and putative Δ6 elongase sequences present in the public databases revealed conserved sequences unique to Δ6 elongases. The GLELO PCR primer pairs were based on the conserved region found within multiple Δ6 elongase sequences. For example GLELO cDNA from *Mortierella alpina* was BLAST searched, shown in figure 5-1. These sequences when aligned revealed an apparent 123-125 bp conserved region with other Δ6 type elongases from other organisms. Therefore it was hypothesised that this conserved region could be used to develop a PCR based screen to detect elongases from unstudied fungi, with *Mortierella alpina* the only true Fungus to have a Δ6 elongase characterised.

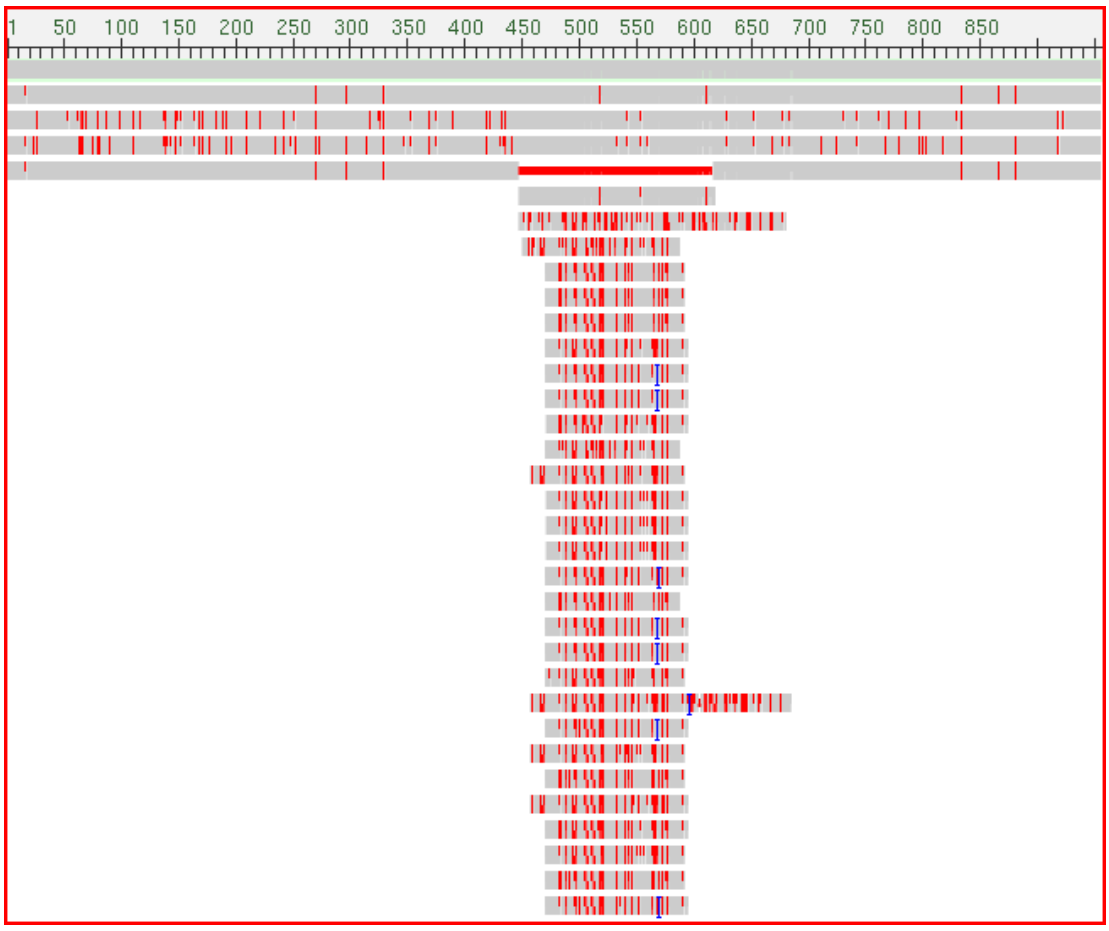


Figure 5-1. The Δ6 elongase (GLELO, top sequence) cDNA sequence from *Mortierella alpina* strain 330997 was BLAST searched. Within the sequence lies a 123-125 bp conserved region shared by Δ6 and ELOVL5 class elongases from other organisms which indicated that this region may correlate with VLCPUFA production. Red areas denote base pair differences from GLELO.

The design of these primers took into account other closely related Δ6 elongase sequences. The 123-125 bp conserved fragment contains within it nucleotide variations between organisms, although the first and last 18 bp were sufficiently conserved throughout different species to enable the generation of efficient degenerate primers. There were several base pairs however that varied between *Mortierella* and other species consistently, shown in figure 5-2. These variants were taken into consideration when designing several sets of robust primers. For example set two differed from set one due to the presence of 4 degenerate base pairs whilst the first set contained only one. The first set of primers, designated Uni 1 and Uni 2 were more specific for *Mortierella*, whilst the second pair, designated Uni 3 and Uni 4 were less specific but adapted for a wider range of organisms. The primer sequences are shown in table 5-1.

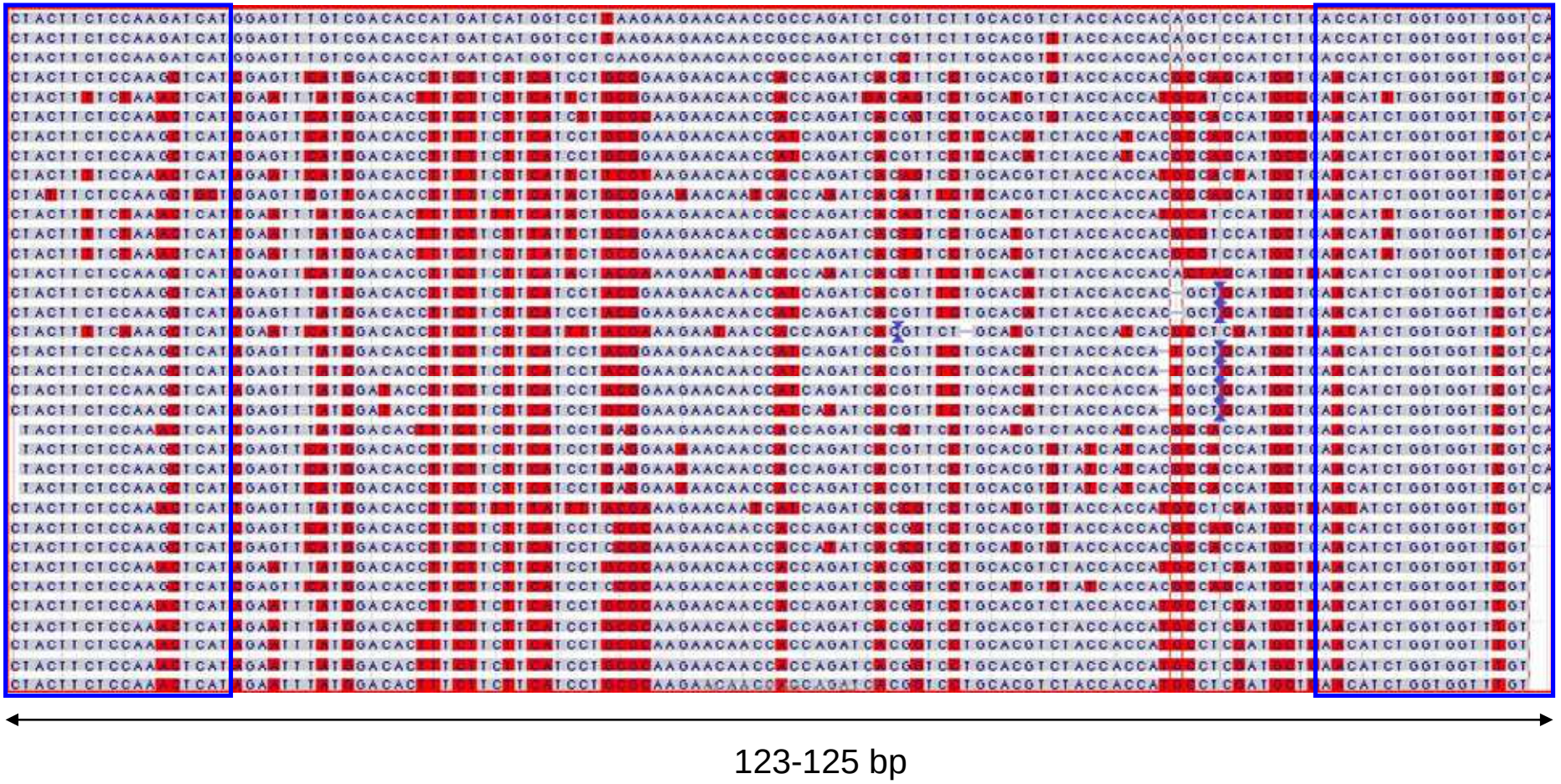


Figure 5-2. Two sets of primers were designed for the relatively conserved end regions of the 125 bp conserved region, highlighted by the two blue boxes. When the conserved region was compared between *Mortierella* and other organisms, several consistent base pair changes were evident in the primer design regions

Table 5-1. Primer sequences for pairs Uni 1 and 2, Uni 3 and 4. The primer pairs are designed to amplify the 125 bp region. Primer set 1+2 (Uni 1 and Uni 2) were designed to be more specific for the *Mortierella* sequence and contained only one degenerate base, whereas primers 3+4 (Uni 3 and Uni 4) contained four degenerate bases to account for the consistent base pair variations.

Primer name	Primer sequence
Uni 1	CTACTTCTCCAAGVTCAT
Uni 2	TGACCAACCACCAGATGGT
Uni 3	CTACTTCTCCAARVTCAT
Uni 4	TGACVAACCACCAGATGKT

The conditions for the PCR were established using the isolated GLELO gene inserted into TOPO 2.1, as described in Materials and Methods section 2.2.7.7, with this construct used as a positive control. Further to that, *Mortierella* cDNA was used as well as mouse genomic DNA, isolated from mouse liver as shown in figure 5-4. Mouse DNA was utilised as a Δ6 elongase exists in mice and no other true fungi have been documented to contain a Δ6 elongase. The PCR reaction and conditions were optimised as shown in figure 5-3, utilising several reaction conditions. It was found that the optimal reaction mix was:

- 1 μl DNA
- 2+2 μl primers at 10pmol/μl
- 20 μl DNA free water
- 1 taq bead (GE healthcare)

It was also found that a 3% agarose gel was required to resolve the 125 bp band sufficiently. The optimal PCR conditions were found by running the PCR reaction stated above, with an annealing temperature gradient. The optimal PCR thermocycler conditions are described in table 5-2.

Table 5-2. Thermocycler conditions for amplifying the 125 bp region using primers Uni 1 + 2 and Uni 3 + 4.

Temperature	Length	Role
95°C	2 minutes	Initial melting
94°C	30 seconds	Melting step
52°C	30 seconds	Annealing step
72°C	1 minute	Elongation step
35 cycles		
72°C	5 minutes	Final elongation

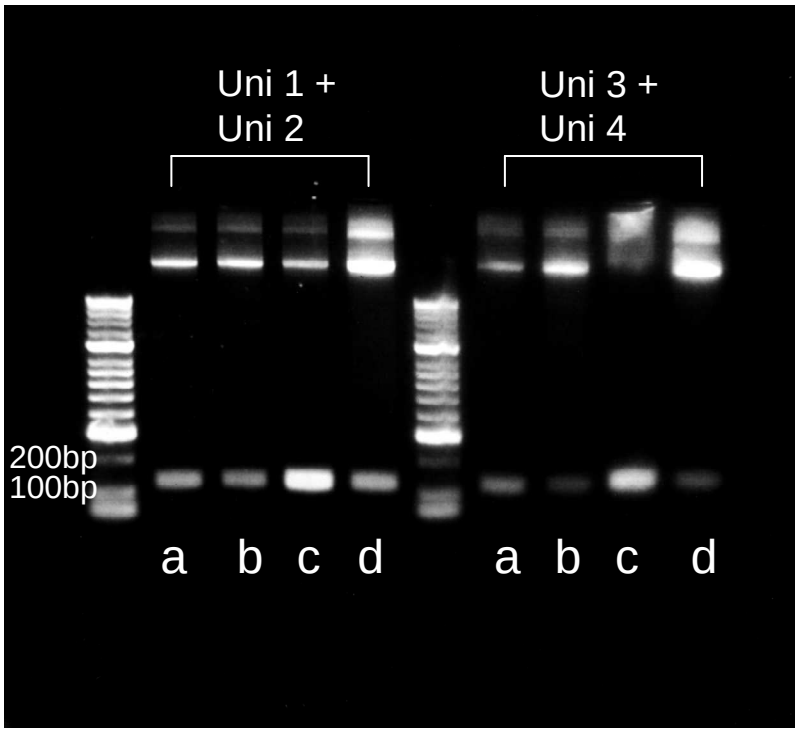


Figure 5-3. Optimisation of the Δ6 elongase PCR screen reaction mix for Uni primers. a = 1 µl TOPO positive control DNA, 1+1 µl primers, 22µl dH_20, 1 Taq bead. b = Addition of 1 µl 4% DMSO. c = Increase to 2+2 µl primers. d = Increase to 2 µl of TOPO positive control DNA. The initial trial conditions indicated 2 µl of primer produced the best amplification. The thermocycler conditions for Uni 1 and 2 primers were; Melting step - 94°C for 30 seconds, Annealing step – 50°C for 30 seconds, Elongation step – 72°C for 1 minute, 30 cycles. For primers Uni 3 and 4; Annealing – 48.5°C for 30 seconds. All other parameters kept the same. Thermocycler parameters were subsequently changed to an annealing temperature of 52°C and increased the number of cycles to 35.

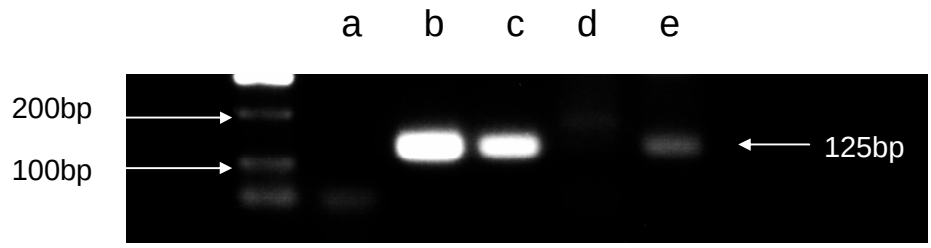


Figure 5-4. Amplification of Δ6 like elongase from *Mus musculus* using both sets of Uni primers. a = Blank primer Uni 1 and Uni 2, b = TOPO positive control Uni 1 and Uni 2, c = *Mortierella alpina* 330997 GLELO cDNA Uni 3 and Uni 4, d = *Mus musculus* DNA Uni 1 and Uni 2, e = *Mus musculus* Uni 3 and Uni 4. *Mus musculus* DNA at 296.9 ng/µl. The annealing temperature was set to 52°C with the number of cycles set to 35. The mouse genomic DNA was only amplified using the non-specific primers Uni 3 and 4. This indicated that the strategy to include four degenerate bases into the primers was effective at detecting the conserved region in an organism not related to the fungi.

5.2.2 *Mortierella* GLELO screen

In total, 9 *Mortierella* species were screened and compared with two mesophilic *Umbelopsis* isolates. The *Umbelopsis* isolates were used to represent typical zygomycetes with most species incapable of elongating past $C_{18:3}$ n6 unlike *Mortierella* species. It was therefore thought that *Umbelopsis* spp. would not display the 125 bp band, whereas those from the *Mortierella* would. Another two isolates were studied which were initially thought to be *Mortierella* species, but were subsequently identified as a *Penicillium* sp. and an isolate from the Clavicipitaceae, both from the Ascomycota. The fatty acid profiles were confirmed using GC-MS, as described in materials and methods, section 2.2.7. The first PCR utilised three *Mortierella alpina* species, IMI 330997, 82072 and 196057, two *Umbelopsis* species, Dis 195 and Dis 206 and an isolate from the Clavicipitaceae, Dis 169. Figures 5-5 and 5-6 show the absence of the 125 bp band from both *Umbelopsis* species and the isolate from the Clavicipitaceae, which correlates with the lack of fatty acids longer than $C_{18:3}$ found with GC-MS. All three *M. alpina* species demonstrated fatty acids up to $C_{20:5}$ n3 which correlated with the presence of the 125 bp fragment. *M. polycephala* (140468) and two uncharacterised Antarctic *Mortierella* strains were also studied. Strain 403341 was thought to be a *Mortierella* strain but was subsequently found to be contaminated with *Penicillium*. The *Penicillium* species demonstrated fatty acids up to $C_{18:3}$ n3, correlating with the lack of the 125 bp band. This was in contrast to *M. polycephala*, which clearly demonstrated the 125 bp band as well as producing C_{20} VLCPUFAs as did the uncharacterised Antarctic *Mortierella* strain 398111. The 125 bp fragments from 330997 and 196057 were sequenced to confirm the identity of the fragment. Three other fragments from

82072 (approx. 200 bp), 140468 (approx. 200 bp) and 398111 (approx. 300 bp) were sequenced which were larger than the GLELO fragment, and in the case of sample 82072, had a much greater concentration than the target 125 bp fragment. The sequencing results did not determine the identities of these larger fragments. It would therefore appear that in some cases the primers have a greater affinity for unknown genomic sequences rather than the conserved region. Both sets of primers were capable of amplifying the conserved region from *Mortierella* although primer set Uni 1 and 2 produce stronger bands indicating greater amplification. This is in line with the greater sequence similar between primer and target in this case.

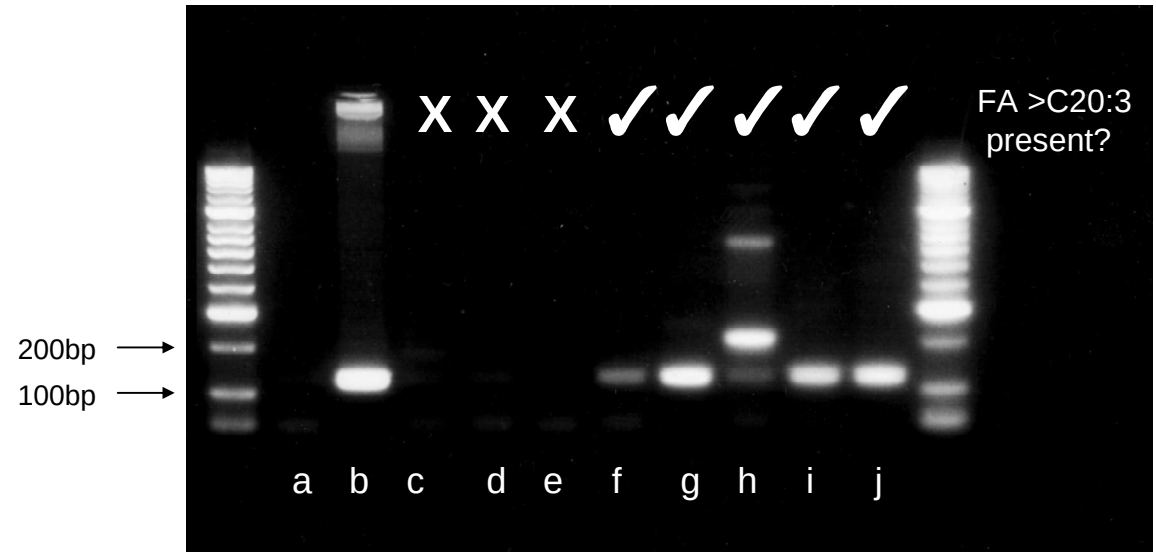


Figure 5-5. Δ6 elongase PCR screen of fungal isolates using primer set Uni 1 and 2. a = Blank, b = TOPO positive control, c = Dis 206, d = Dis 195, e = Dis 169, f = 330997, g = 196057, h = 82072, i = 140468, j = 398111. DNA concentration = 11 ng/µl excluding TOPO control. The amplification was performed using primers Uni 1+2.

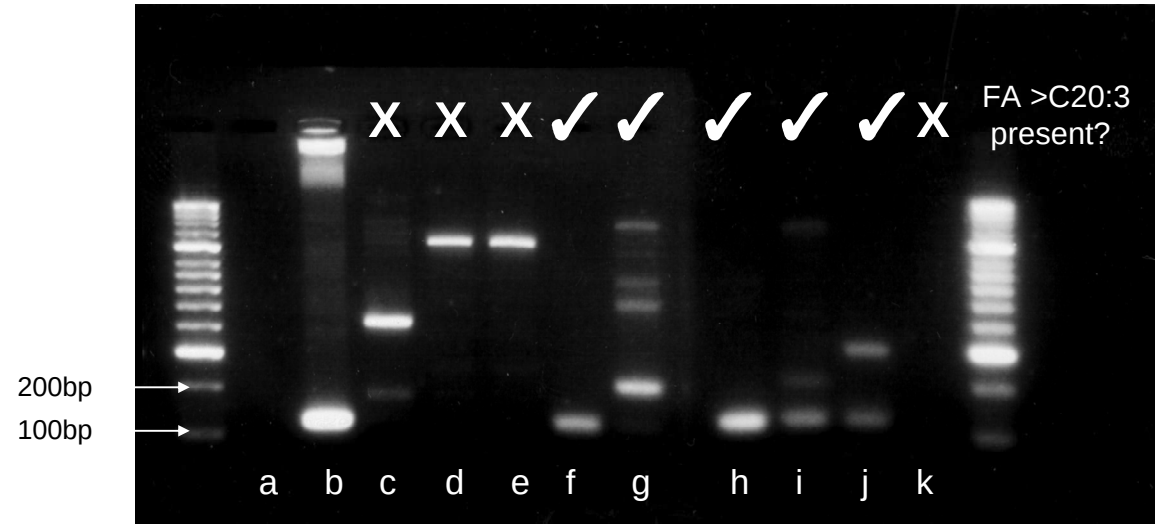


Figure 5-6. Δ6 elongase PCR screen of fungal isolates using primer set Uni 3 and 4. a = Blank, b = TOPO positive control, c = Dis 206, d = Dis 195, e = Dis 169, f = 330997, g = 82072, h = 196057, i = 140468, j = 398111, k = 403341. DNA concentration = 11 ng/µl excluding TOPO control. The amplification was performed using primers Uni 3+4.

The remaining four uncharacterised Antarctic *Mortierella* isolates, and an additional *Allomyces macrogynus* (332398) and *Geomyces sp*. (140037) were also screened as shown in figure 5-7. *A. macrogynus* is reported as producing $C_{20:4}$ n6 ($C_{20:4}$ n6) and the *Geomyces* isolate was isolated from the Antarctic. The four Antarctic *Mortierella* were found to produce C_{20} VLCPUFAs and were also found to produce the 125 bp band, again indicating the role GLELO plays in VLCPUFA formation. *Allomyces macrogynus* was also found to produce fatty acids up to $C_{20:4}$ n6 and clearly demonstrates the presence of the 125 bp band, however only with primers Uni 1 and 2. Subsequent PCR allowed amplification utilising Uni primers 3 and 4. *Geomyces* did not possess any fatty acids beyond $C_{18:3}$ n3, and the lack of the 125 bp fragment correlates with this. *Mortierella* isolate 398217 was found with both sets of primers to display a much brighter 125 bp band compared with the other isolates even though DNA concentrations between isolates were kept constant. This brighter band did not correlate with any significant increase in C_{20} VLCPUFA production.

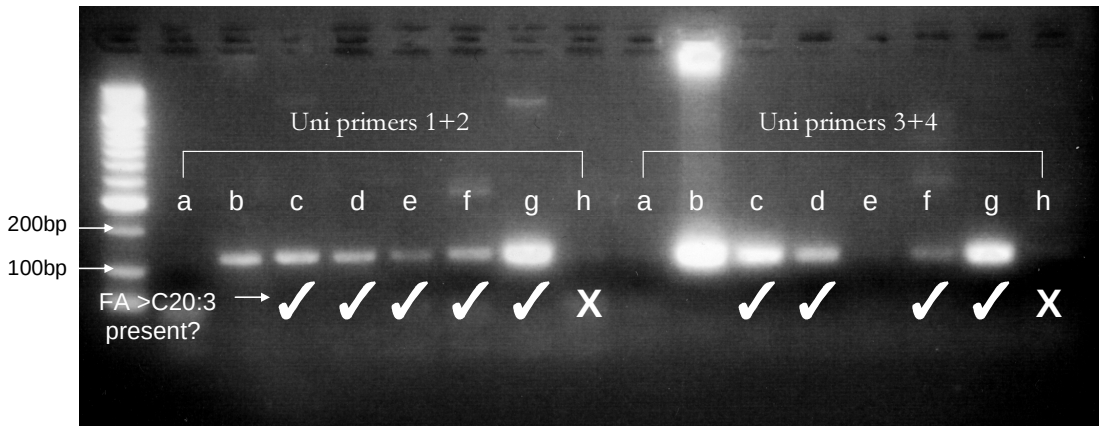


Figure 5-7. Δ6 elongase PCR screen of Antarctic *Mortierella* isolates. a = Blank, b = TOPO positive control, c = 398216, d = 398220, e = 332398, f = 398213, g = 398217, h = 140037. DNA concentration = 8.4 ng/µl excluding TOPO control. Both sets of primers amplified the 125 bp conserved region in all the uncharacterised *Mortierella* species

5.2.3 Identification and elucidation of Δ6 elongase in *Allomyces macrogynus* and *Saprolegnia parasitica*

The GLELO like enzyme detected in *Allomyces* was initially localised in the genome by searching the translated GLELO sequence from *Mortierella* with the whole genome shotgun sequence (WGS) of *Allomyces*. The search identified two possible contig sequences. The highest match sequence (accession no. ACDU01000340) was studied and was deemed to be the most likely candidate due to an intact open reading frame.

The 125 bp conserved region also aligned within this open reading frame, corresponding to the PCR product observed in the genomic screen. However the entire conserved region was not matched, with 64 nucleotides matched out of 125. The matching nucleotides were located at the end of the conserved sequence. The explanation for this will be explored subsequently. Four primers were then developed to allow for the sequencing of the gene; primers F1, F2, F3 and R1. Primer F1 when coupled to the primer R1 was designed to amplify the entire gene, F2 when coupled with the primer R1 was designed to amplify roughly 2/3rds of the gene, whilst the F3 and R1 primers where designed to amplify a third of the gene. The primer sequences have been described in the Materials and Methods section 2.1.4.2. The PCR reaction was modified to;

- 1 taq bead
- 3 µl DNA at 8.4 ng/µl
- 1 µl primers F1, F2, F3 at 10 pmol/µl
- 1 µl primer R1 at 10 pmol/µl
- 20 µl dH_20

The temperature program was modified with a 54°C annealing step for 30 seconds and still included 35 cycles. The F1xR1 fragment failed to amplify, the F2xR1 fragment produced a weak band and the F3xR1 fragment produced a strong 400 bp band (figure 5-8).

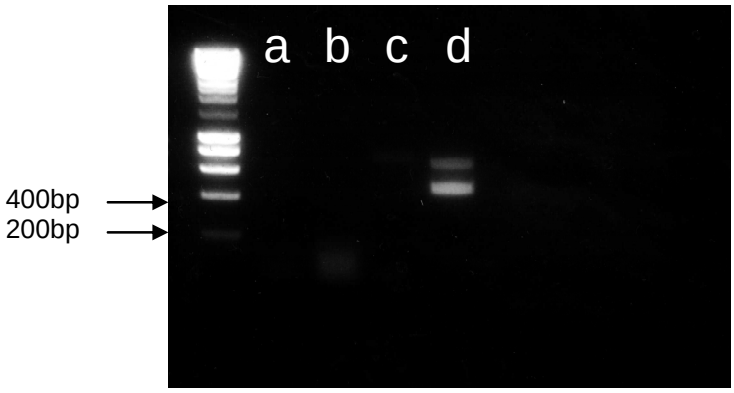


Figure 5-8. Amplification of the *Allomyces* Δ6 elongase. a = blank, b = F1xR1 primers, c = F2xR1 primers, d = F3xR1 primers. Amplification using primers F1 and R1 resulted in no band however, primers F2 and R1 produced a very weak band approximately 700 bp in size with primers F3 and R1 producing two bands, with the approximately 400 bp band the strongest. The amplified F2xR1 and 400 bp F3xR1 bands were excised from the gel and purified for sequencing.

The F2xR1 fragment failed to insert into the vector however the F3xR1 fragment was sequenced successfully. The 467 bp F3xR1 fragment yielded a high percentage match with the contig sequence (figure 5-9), indicating that this position on the contig sequence was indeed the location for the *Allomyces* Δ6 elongase.

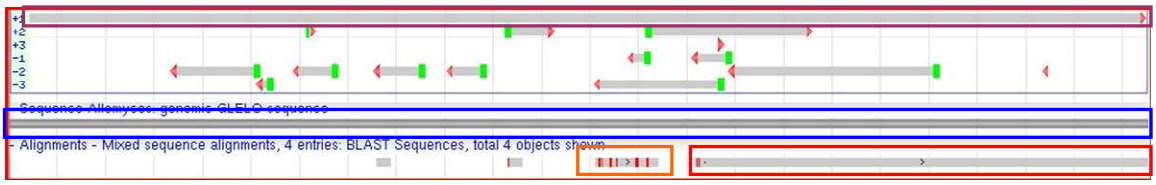


Figure 5-9. Alignment of the putative *Allomyces* Δ6 elongase with the F3xR1 fragment. The suspected *Allomyces* Δ6 elongase within the contig 3-340 (highlighted in blue) matched with; in orange, the 125 bp conserved region, in red, the sequenced F3xR1 fragment which is 467 bp. The open reading frame within the contig sequence is shown highlighted in

purple. Only part of the conserved region matched the genomic sequence when BLAST searched. This was found to be because of an inserted intron as shown in figure 5-12.

The translated *Mortierella* GLELO sequence when matched with the contig sequence, demonstrated a non-continuous alignment, indicating that introns were present within the contig sequence. Merging the exons yielded the putative cDNA for the *Allomyces* Δ6 elongase, whereas the entire ORF on the contig sequence is thought to be the putative genomic sequence. The putative introns and exons are shown in Figure 5-12. It was noted that the conserved genomic region contained an intron, which accounted for the incomplete BLAST search. The PCR amplification of the conserved region should produce a 220 bp band. This however, was found not to be the case with the genomic DNA producing a 125 bp band upon amplification. The cDNA sequence was translated, as shown in Figure 5-10, followed by protein BLAST searching to establish the closest match for the putative protein. The closest match was from a *Mortierella* GLELO (BAF97073.1, score 266), and long chain polyunsaturate elongases from other organisms. This leads to the conclusion that the function of this protein is indeed a Δ6 elongase.

```
                    20                  40                  60
MEATTDLIISAFAALEGRIAPITAPYEAHAVAFLADKFPEATTATKTFLASIRSPYADQLPLMN
          80                  100                 120
PAHVLFLVTAYLALIGGGRLIMSQVPFKLQVKTYALLNNVVLTSLSLFMAVEVLRQAFLGRYSL
      140                 160                 180
FGNPLVTPEQGGTGMAKIVAVFYLSKILEFNDTIIMVLKSTRLSENFHQISFLHVYHHASIFMI
    200                 220                 240
WWLVTLWAPTGESYFSAFLNSGIHVIMYGYYFLVRLSALGFKQVTFIKKYITSAQMTQFMLMMV
  260                 280                 300                 320
QATYNLIDGYVLRPEKAKAPNAYPLELSVLLWVYMVTMLALFGNFYRQSYKNKARAGKGAAVPV

AKKSS*
```

Figure 5-10. The putative translated Δ6 elongase from *Allomyces macrogynus* strain 332398.

It was found that the cDNA of the *Allomyces* Δ6 elongase had the highest match to a *Mortierella* GLELO. When BLAST compared, a region comprising 24% of the total gene had a similarity of 76% to the closest *Mortierella* GLELO (Accession AF206662.1). When the amino acid sequences against the best match *Mortierella* GLELO (BAF97073.10) were BLAST searched, it was found that 198 residues matched out of 319 (62%), although the first two residues failed to match when NCBI BLAST searched, shown in figure 5-11.

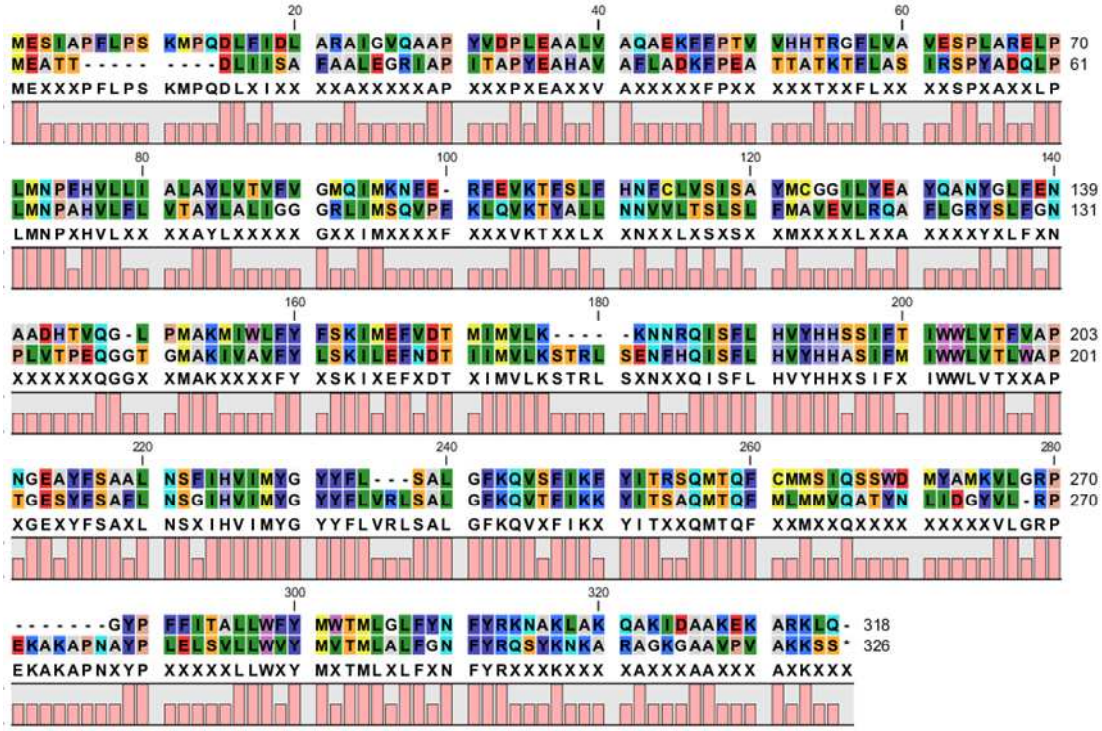


Figure 5-11. Comparison of the *Allomyces* and *Mortierella* Δ6 elongase sequences. The top sequence is the translated *M. alpina* GLELO and the bottom sequence is the translated *Allomyces macrogynus* Δ6 elongase. When the sequences were aligned it was found that 198 out of 319 residues matched giving a similarity of 62%.

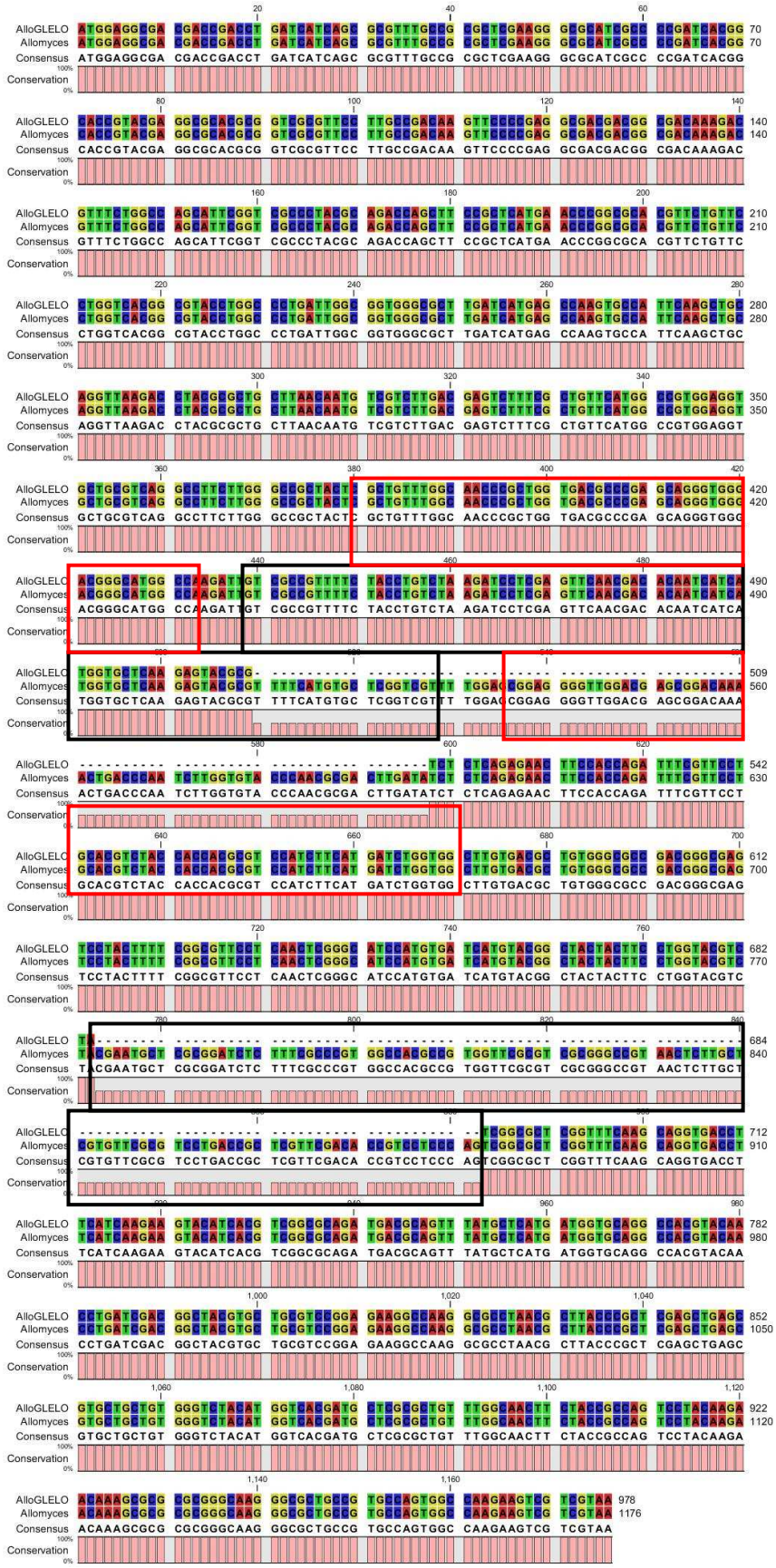


Figure 5-12. Comparison of the putative genomic *Allomyces* Δ6 elongase with the cDNA. Black boxes indicate the putative location of introns whilst red boxes indicate the location of the conserved region. It can be seen that in the case of *Allomyces* an intron sequence is found to separate the genomic conserved region.

Other organisms were also studied to identify whether they contained a Δ6 elongase. One of these organisms was *Saprolegnia*, which is a well known VLCPUFA producer. The translated GLELO sequence was found to match a region on a WGS contig (accession no. ADCG01000825) and the open reading frame was located. The conserved region was found to partially bind within this ORF, with 69 bp matching. However, it was found that PCR would not amplify this region due to the lack of binding of the reverse primer. Introns were not found by comparison to the *Mortierella* GLELO and so the sequence was converted to the amino acid sequence. When the amino acid sequence was BLAST searched, the most similar protein was a putative long chain fatty acid elongase from *Phytophthora infestans*. A Δ6 elongase from *Phaeodactylum tricornutum* was also highly similar suggesting that this protein is a Δ6 elongase.

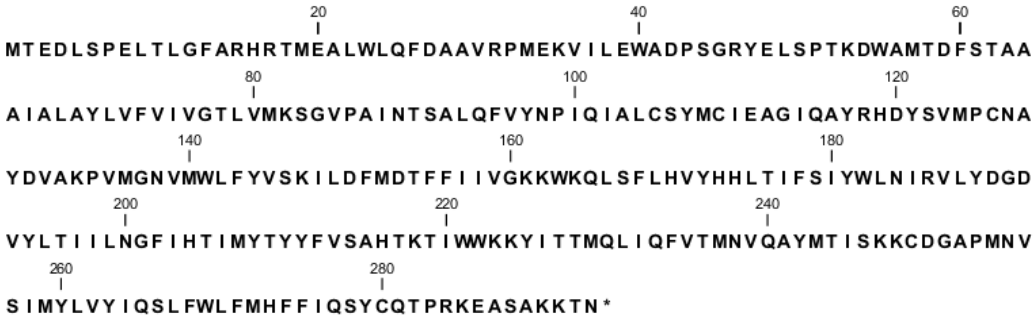


Figure 5-13. The translated putative sequence of *Saprolegnia parasitica* Δ6 elongase. No introns were detected whilst matching the *Mortierella alpina* GLELO sequence with the *Saprolegnia parasitica* WGS sequence.

5.2.4 Location of the conserved region

The conserved region amino acid sequence was elucidated in the correct frame from *Mortierella alpina* strain 330997, which is shown in Figure 5-14. The structure of several Δ6 elongases was studied further to see if a role for the conserved region could be found. The Δ6 elongases from *Mortierella alpina, Allomyces macrogynus* and *Saprolegnia parasitica* were found to share structural similarities further confirming their role as VLCPUFA elongases. The amino acid sequences were analysed using SMART from EMBL to elucidate functional domains, and TMHMM from CBS was used to generate probability graphs for trans-membrane regions. All three elongases were found to contain the ELO domain, which in turn contained 6-7 trans-membrane regions, as shown in Figures 5-15 to 5-17. It is possible that the *Saprolegnia* Δ6 elongase contains seven trans-membrane regions due to the elevated probability around

residues 220-245. The conserved region for all the elongases contained the third and fourth trans-membrane regions and a small extracellular region 6-21 residues long.

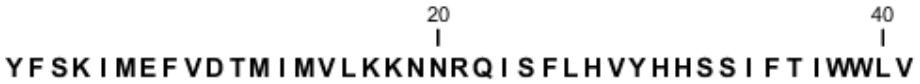


Figure 5-14. The amino acid sequence of the conserved region found within *Mortierella alpina* strain 330997. The conserved DNA was translated in the correct frame resulting in the removal of the first and last base pairs.

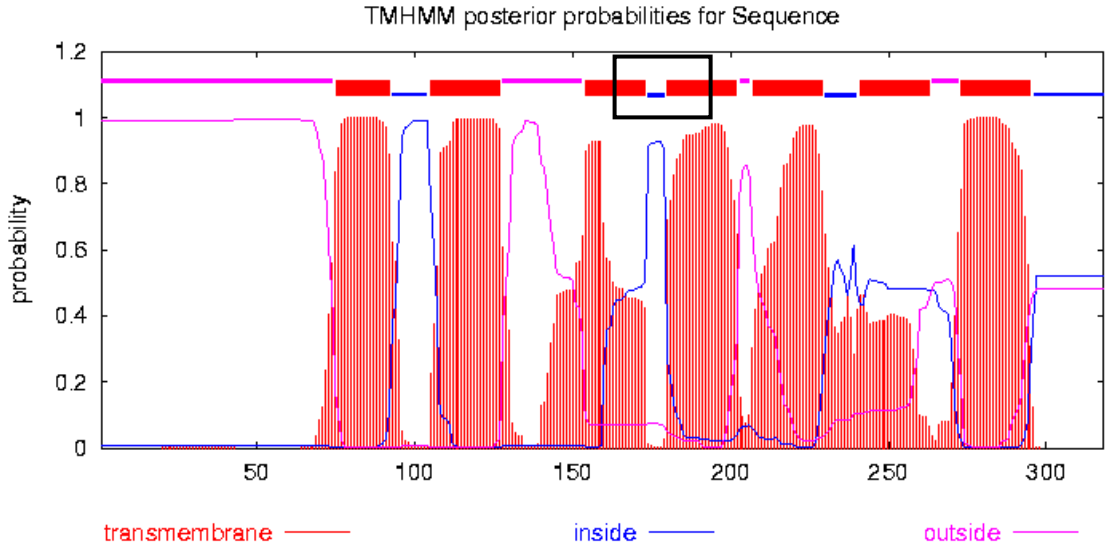


Figure 5-15. Probability plot of the trans-membrane domains found within the *Mortierella* GLELO. Trans-membrane domains are found between residues; 75-92, 105-127, 154-173, 180-202, 207-229, 241-263, 273-295. The ELO domain is between residues 69-307 and the conserved region between residues 158-198. The conserved region contains within it two trans-membrane domains and a small 6 amino acid hydrophilic region.

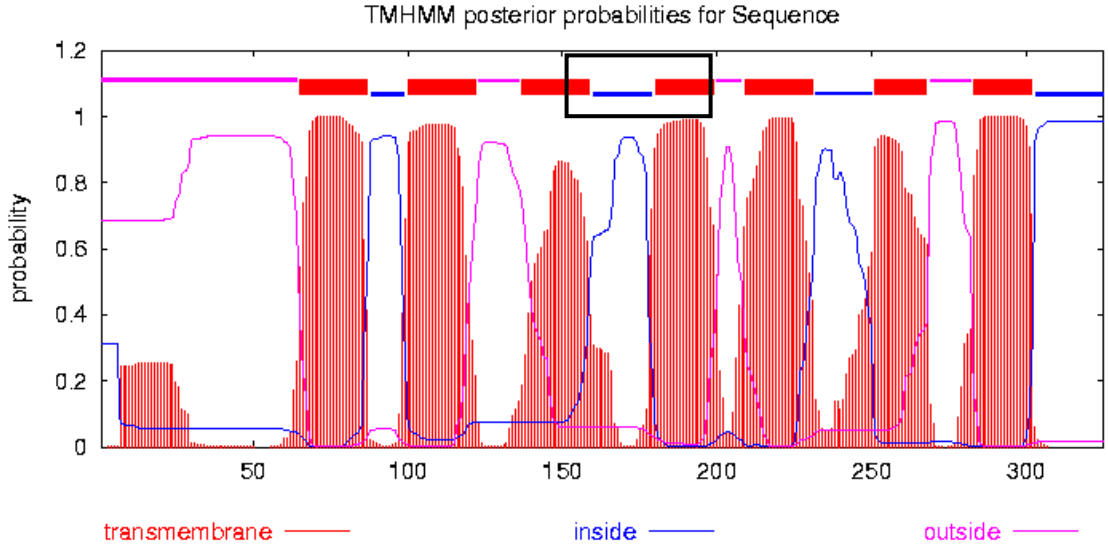


Figure 5-16. Probability plot of the trans-membrane domains in the *Allomyces* Δ6 elongase. Domains are found between residues; 65-87, 100-122, 137-159, 180-199, 209-231, 251-268, 283-302. The ELO domain is located between residues 60-314 and the conserved region between residues 151-196. The conserved region is found within two trans-membrane domains and an external hydrophilic domain.

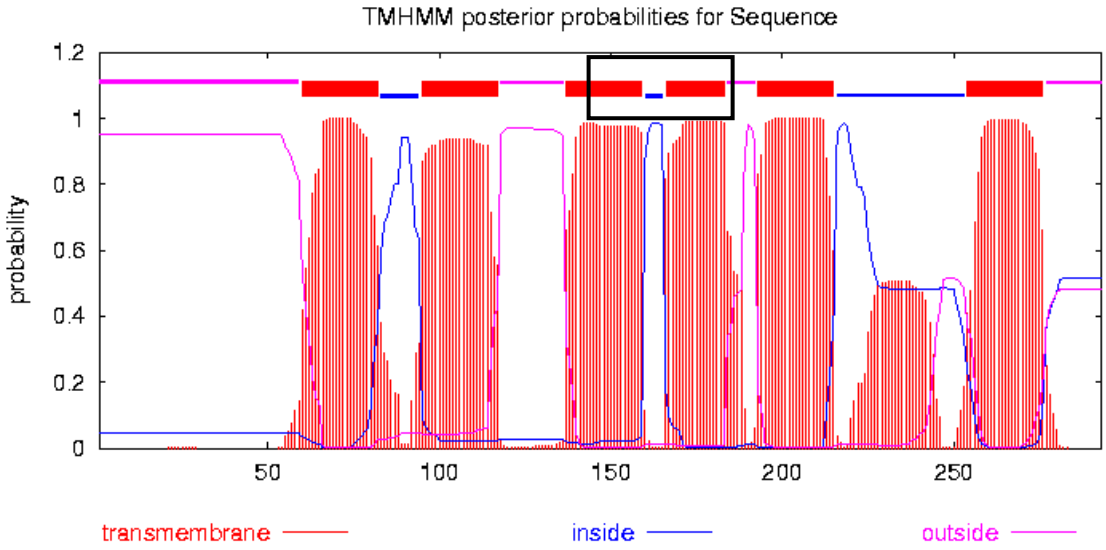


Figure 5-17. Probability plot of the trans-membrane domains in the *Saprolegnia parasitica* Δ6 elongase. Domains are found between residues; 60-82, 95-117, 137-159, 166-183, 193-215 and 254-276. A seventh trans-membrane domain might be found around residues 220-245 due to the elevated probability calculated by the algorithm. The ELO domain is located between residues 55-287 and the conserved domain between residues 144-183. The conserved region again is found to contain two trans-membrane domains and a small 6 residue hydrophilic region.

The amino acid sequence similarity was studied between Δ6 elongases and other elongases, such as those responsible for saturated and mono-unsaturated fatty acid elongation using the UPGMA method (Figure 5-18). Sequences from NCBI were used for the analysis as well as the putative Δ6 elongase sequences discussed previously. Interestingly, elongases responsible for saturated and mono-unsaturated fatty acid elongation grouped together, with the exception of ELOVL1 from *Mus musculus* and MALCE1. The MALCE1 elongase from *Mortierella alpina* shares greater similarity to the polyunsaturate accepting elongases, though it has been shown to elongate $C_{18:3}$ n6 its main function is the elongation of $C_{16:0}$ to $C_{18:0}$. The elongases from yeast, ELO1-3 as well as MAELO from *Mortierella alpina* form a clade distinguished by the fact that they are all saturate and mono-unsaturate accepting elongases, as well as being isolated from fungi. The basal nature of MAELO to the ELO elongases may indicate its ancestral nature. The next closest set of elongases are ELOVL3 and 6, again saturate and mono-unsaturate elongases from *Mus musculus*, alluding to the role of amino acid sequence and function. Several of these elongases have shared functionality in that they catalyse similar reactions. ELO1 catalyses a similar set of reactions to ELOVL6, ELO2 shares functional similarities to ELOVL3 and MALCE1, and ELO3 shares functional similarities to ELOVL1 and MAELO. It might be expected that elongases with similar

functions would cluster together, however it appears that the organism from which they are isolated plays an equally large role in their clustering. ELOVL1 demonstrates this to a large degree by grouping with LCPUFA accepting elongases and not saturate and mono-unsaturate accepting elongases. It does however, show greater similarity to elongases from vertebrates. Elongases that utilise PUFAs as their substrate cluster together, but several internal clades are observed. Primarily there are those isolated from vertebrates such the ELOVL2, ELOVL4 and ELOVL5 elongases which cluster together, with those from the same class showing greater similarity. The Plantae also demonstrate a defined PUFA elongating elongase clade, with members from terrestrial plants and algae. The putative *Allomyces macrogynus* Δ6 elongase falls into the fungal Δ6 elongase grouping indicating that the elongase identified is indeed a PUFA elongating enzyme. Finally, the putative *Saprolegnia parasitica* Δ6 elongase is found to group with the algal *Phaeodactylum tricornutum* Δ6 elongase, adding support for its function as well as illustrating the separation of the Chromista from the fungi. These two Δ6 elongases are the most distinct, as they separate out from the main grouping of PUFA accepting elongases.

Analysis of sequences with ClustalW2 (Figure 5-19) from EMBL-EBI, then displayed as a phylogram, showed similar trends. Predominantly, three clades are visible; one comprising saturate and mono-unsaturate fatty acid elongases from both fungi and vertebrates, one comprising PUFA accepting elongases from fungi and the Plantae and the final clade included PUFA accepting elongases from vertebrates. The *Allomyces macrogynus* Δ6 groups with GLELO and the *Saprolegnia parasitica* putative Δ6 falls into the fungal and plant PUFA accepting elongase clade. Also of interest is the early branching of the MALCE1 and MAELO elongases from the other fungal saturated and mono-unsaturated elongases. ELOVL1 from *Mus musculus* groups closer to the PUFA accepting ELOVL class of elongases.

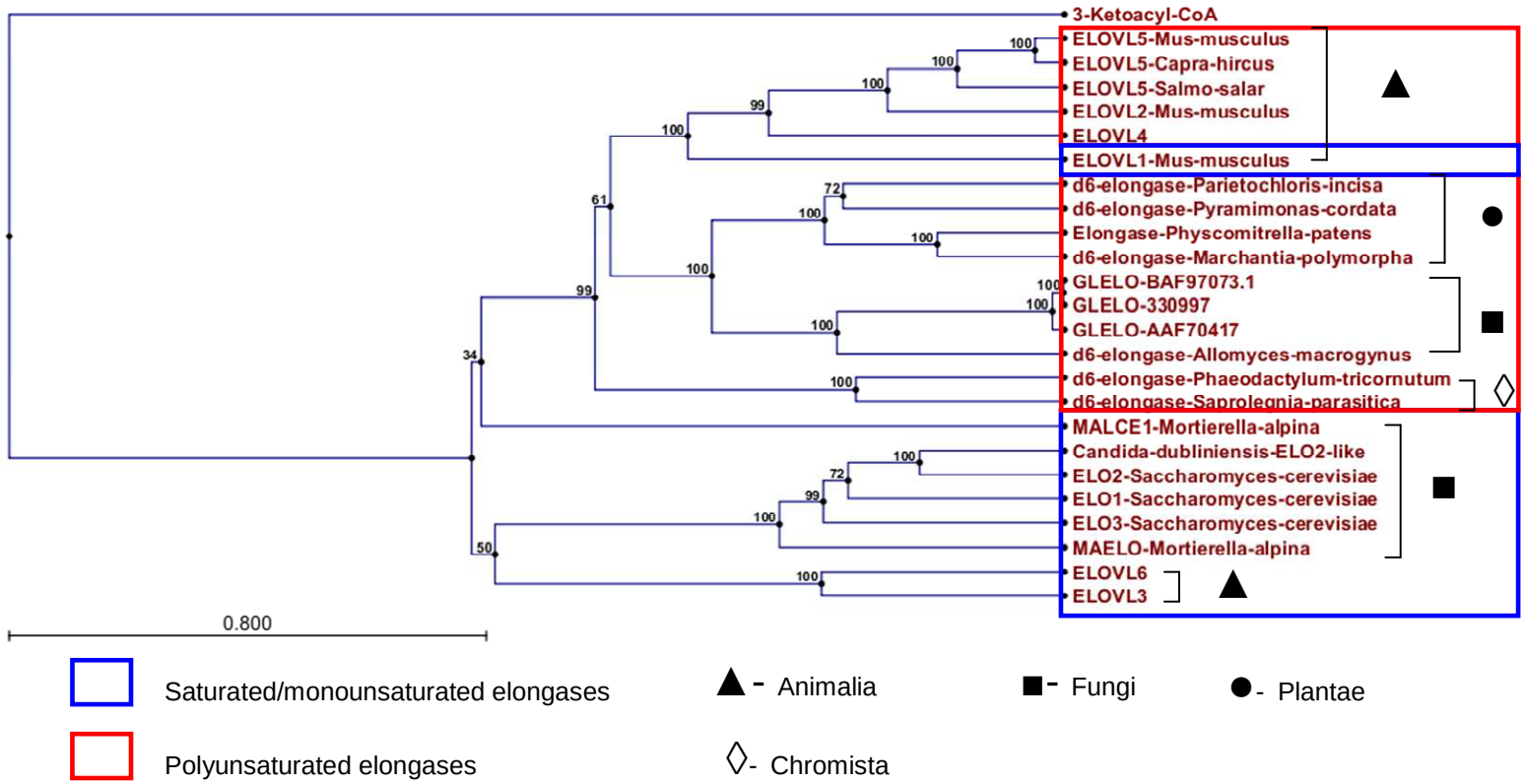


Figure 5-18. UPGMA sequence similarity analysis of PUFA and non-PUFA accepting elongases. The UPGMA method was run in CLC sequence viewer. There appear to be two major clades, with the polyunsaturate accepting elongases grouping together and the saturated/mono-unsaturated accepting elongases grouping together. Within the polyunsaturate clade, the ELOVL class form a grouping with ELOVL1 clustering on family rather than function. The *Mortierella* and *Allomyces* Δ6 elongases cluster together indicating their fungal lineage, however they are more similar to plant Δ6 elongases than animal elongases. The putative *Saprolegnia* Δ6 elongase is most similar to the algal elongase from *Phaeodactylum tricornutum*. The 3-Ketoacyl-CoA synthase from *Arabidopsis* was added to the analysis due to its similar function and in this analytical method, roots the tree.

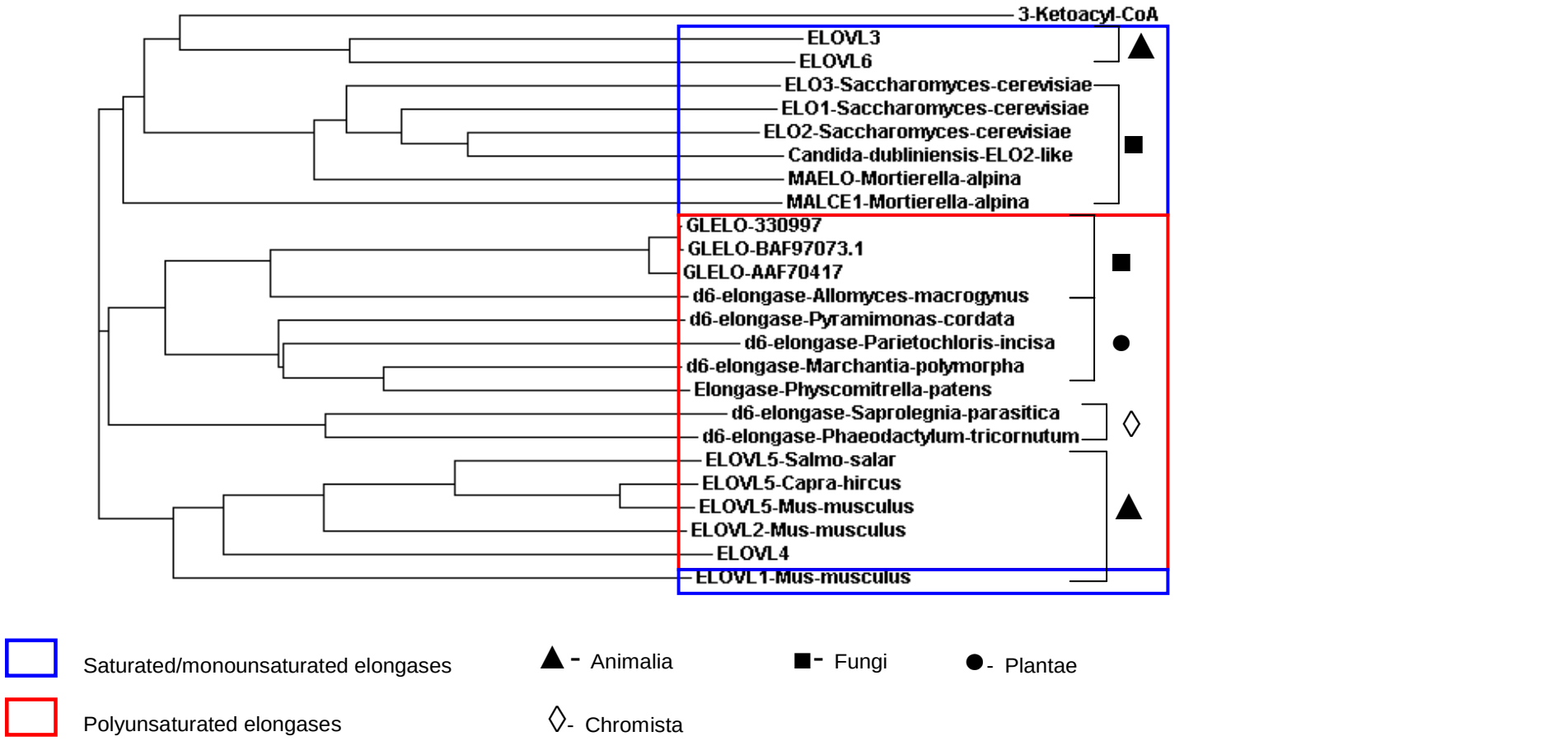


Figure 5-19. ClustalW2 sequence similarity analysis of PUFA and non-PUFA accepting elongases. The phylogram was created using ClustalW2 from EMBL-EBI. Three major clades are visible; Saturate and mono-unsaturate accepting elongases from fungi and vertebrates, PUFA accepting elongases from fungi and Plantae and PUFA accepting elongases from vertebrae. Both the putative *Allomyces macrogynus* and *Saprolegnia parasitica* Δ6 elongases cluster within the PUFA accepting elongase clade.

In order to identify the key parameters that differentiate polyunsaturate and saturate/monounsaturate accepting elongases, a closer look at the amino acid sequence is necessary. When the sequences are aligned, it can be seen that several residues appear conserved within the polyunsaturate accepting elongases. Within the conserved region, which contains highly conserved residues common to both classes of elongases, position 229 in the aligned sequence shows a conserved glutamine residue whilst at position 235 a conserved valine residue is found. At position 245 and 246, two conserved tryptophan residues are found. PUFA accepting elongases also display in general two extra residues located in positions 225-226, whilst the *Saprolegnia parasitica* and *Phaeodactylum tricornutum* display an addition residue at position 224. *Allomyces macrogynus* displays an additional five residues when compared to the other PUFA excepting elongases. It is found that *Saprolegnia parasitica* and *Phaeodactylum tricornutum* do not always display the conserved amino acids found within the PUFA elongating enzymes, which is one reason why these elongases find themselves grouped away from the main body of this class of elongase. Similarly, ELOVL1 displays several conserved amino acids found within the PUFA elongase class, which partially led to its grouping with the other ELOVL PUFA elongating enzymes. Other residue changes outside of the conserved region can be found at positions 161 and 162, where a conserved tyrosine and methionine residue are located. Position 255 shows a conserved glycine residue, whilst at position 273 a conserved serine is found. Finally, three conserved residues are found at positions 412, 413 and 422 with the amino acids being tyrosine, methionine and asparagine, respectively. These conserved amino acids are shown in Figures 5-20 to 5-23.

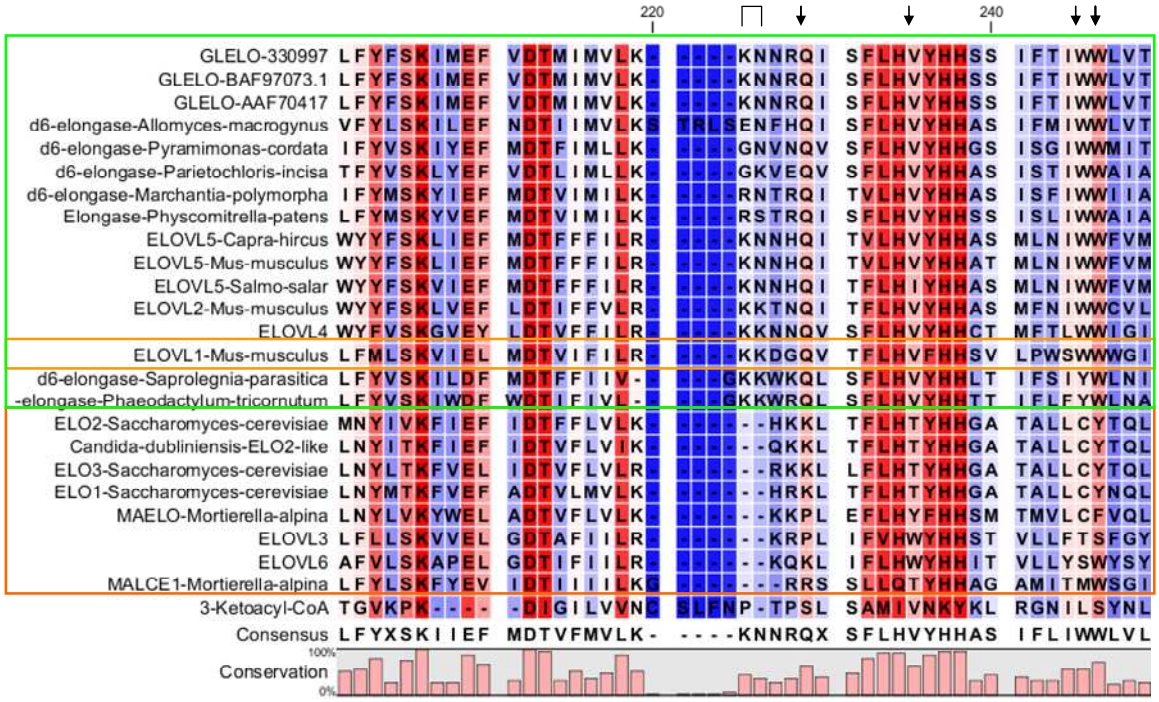


Figure 5-20. Sequence alignment of several ELO class elongases within the conserved region with the green boxes highlighting PUFA accepting elongases and the orange boxes highlighting saturated/mono-unsaturated accepting elongases. Arrows indicates residues which differ between PUFA accepting and saturate/monounsaturated accepting elongases. Notable distinguishing residue changes are located in positions 229, 235, 245 and 246 where the conserved amino acid residue is different between saturated and polyunsaturated substrate elongases. At residue number 225-226 there are extra amino acids present consistently within PUFA accepting elongases, which are absent from saturated/mono-unsaturated elongases. The conserved region contains two highly conserved amino acid regions indicated in red from 203-218 and 232-238, with the first region permeated with non-conserved amino acids.

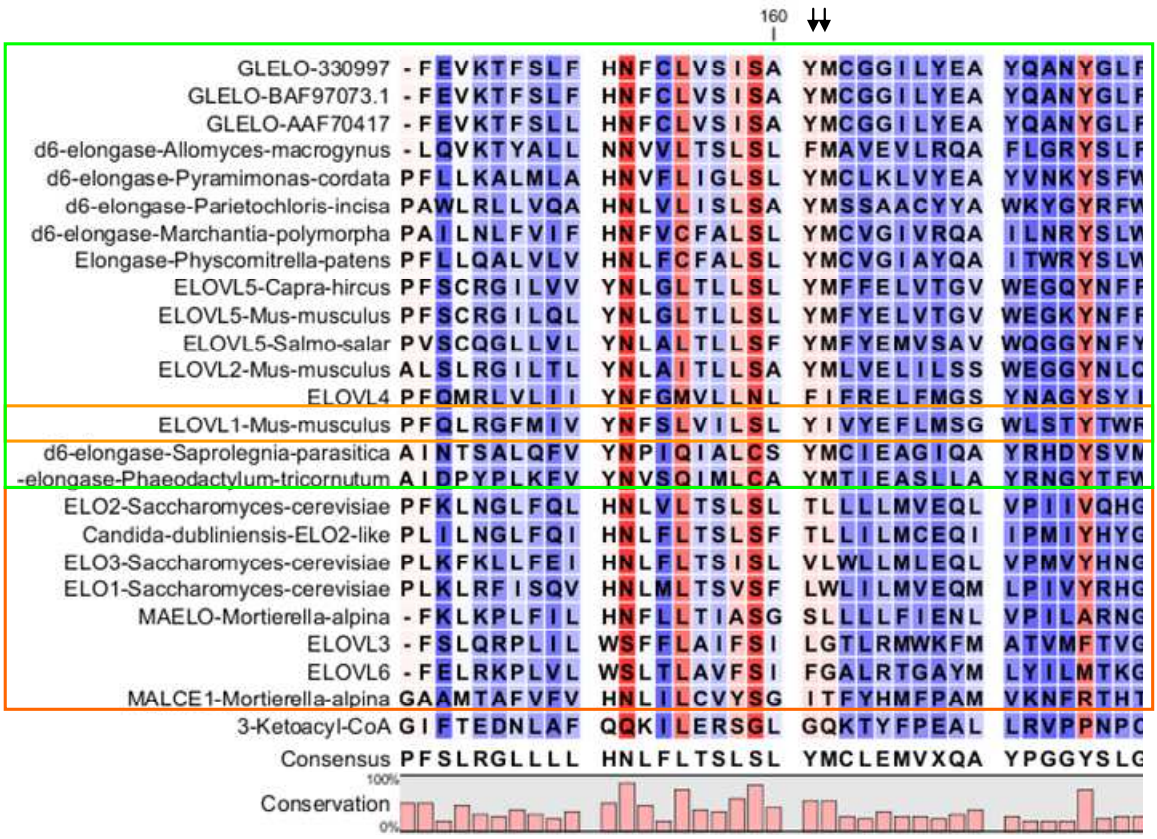


Figure 5-21. Sequence alignment of several ELO class elongases. Several changes to conserved amino acid residues occur at residue numbers 161 and 162, with the changes differentiating PUFA substrate from saturated/mono-unsaturate substrate elongases. ELOVL4 does not however have the amino acid residues common to PUFA elongating elongases. The green boxes highlight PUFA accepting elongases and the orange boxes highlight saturated/mono-unsaturated accepting elongases. Arrows indicates residues which differ between PUFA accepting and saturate/monounsaturated accepting elongases.

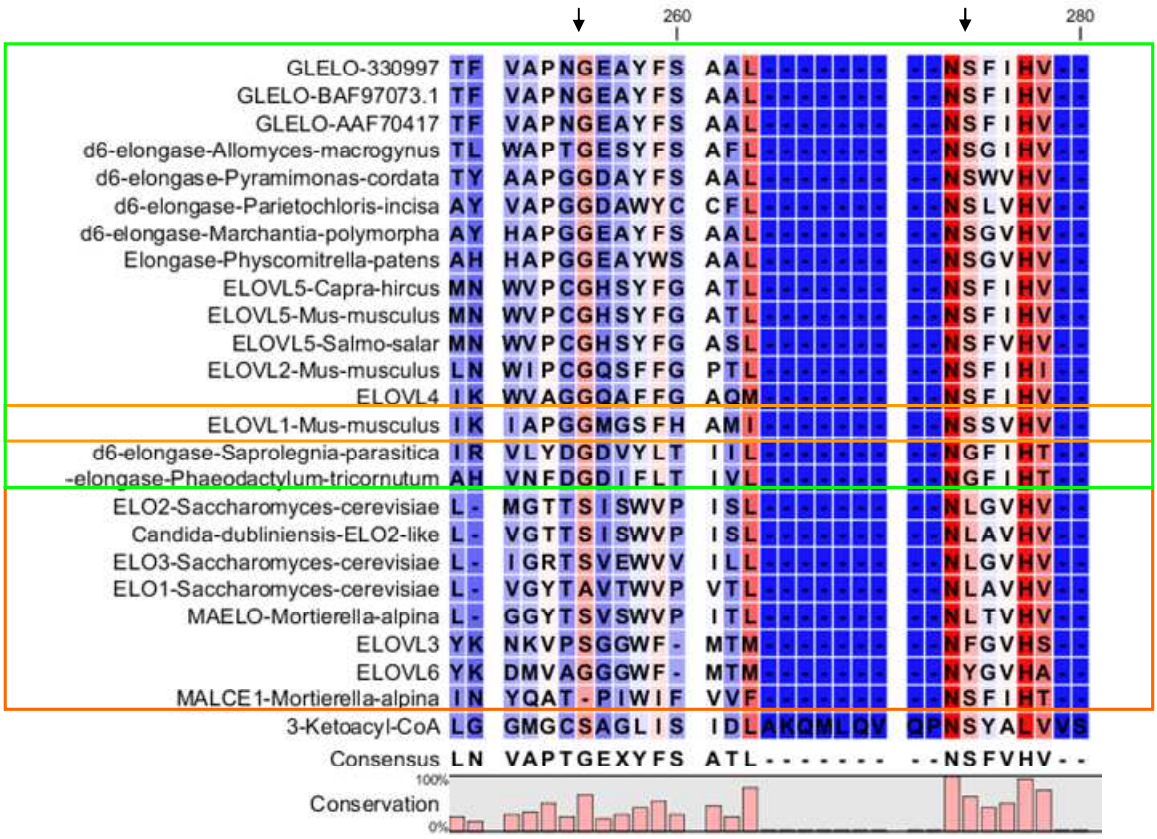


Figure 5-22. Two more distinguishing residue changes are detected at residue numbers 255 and 273 which separate PUFA and saturate/mono-unsaturate elongating elongases. ELOVL1 contains the same residue however as PUFA accepting elongases at position 255 whilst *Saprolegnia* and *Phaeodactylum* do not display the conserved amino acid residue at position 273 common to PUFA accepting elongases. Two highly conserved amino acid residues are present which are found within all the elongase, except residue number 277 which is not present within the FAE class 3-Ketoacyl-CoA. The green boxes highlight PUFA accepting elongases and the orange boxes highlight saturated/mono-unsaturated accepting elongases. Arrows indicates residues which differ between PUFA accepting and saturate/monounsaturated accepting elongases.

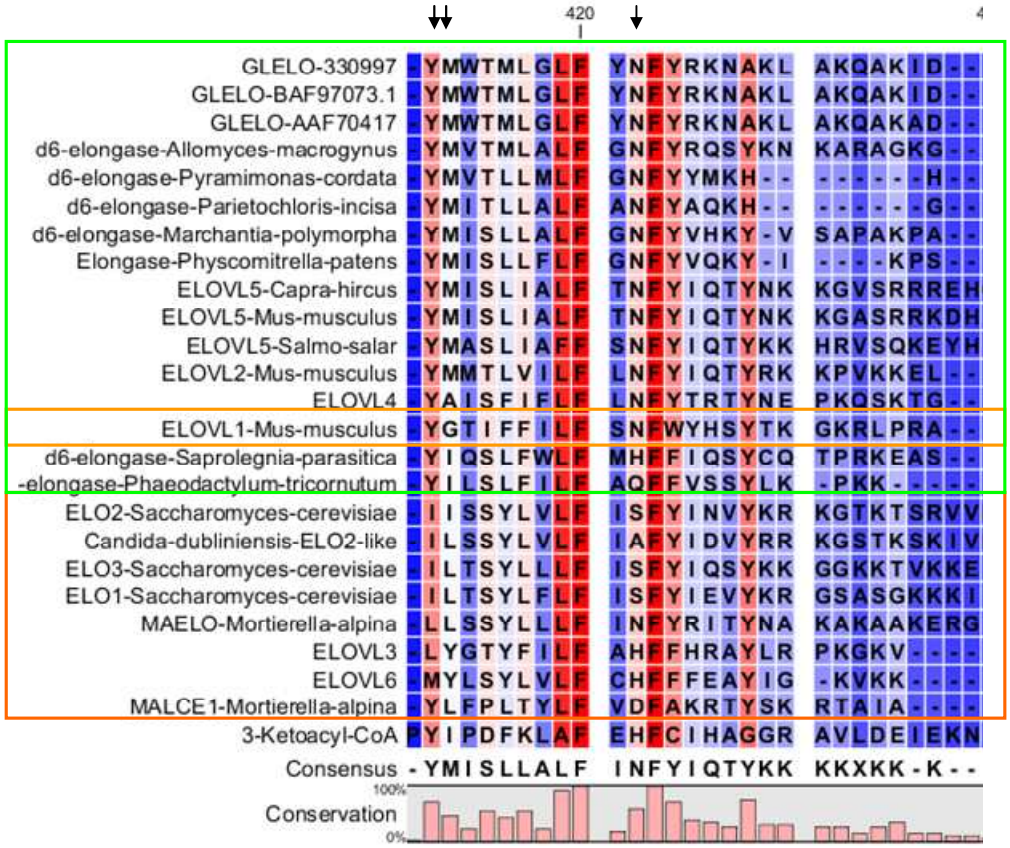


Figure 5-23. The final three distinguishing amino acid residues are detected at positions 412, 413 and 422. The first residue, 412, is also shared by ELOVL1 with residue 413 not shared by *Saprolegnia*, *Phaeodactylum* or ELOVL4. The final residue 422 is shared by ELOVL1 again but not *Saprolegnia* and *Phaeodactylum*. The region also contains three highly conserved amino acid residues found within all the elongases bar the FAE class 3-ketoacyl-CoA elongase. The green boxes highlights PUFA accepting elongases and the orange boxes highlights saturated/mono-unsaturated accepting elongases. Arrows indicates residue changes.

It is interesting to note that a highly conserved amino acid sequence is found within the conserved nucleotide region for both substrate classes of elongase, with two regions of conserved amino acids present within the conserved region with the sequences Y-X-S-K-X-X-E-F-X-D-T and F-L-H-V-Y-H-H. The conserved nucleotide sequence however, only appears to apply to PUFA accepting elongases and not saturate/mono-unsaturate accepting elongases. There are subtle differences in the conserved region amino acid sequence which distinguish the two classes, but it is possible that this region has some functional role within the elongase even though it is speculated to be within a trans-membrane domain. The conserved nucleotide sequence was sequence aligned using ELOVL1-6 class elongases from *Mus musculus*, ELO1-3 from *Saccharomyces cerevisiae*, MAELO and MALCE1 from *Mortierella alpina* to determine whether the conserved nucleotide sequence was present within these mainly saturate/mono-unsaturate accepting elongases (figure 5-24). The conserved sequence was also aligned

with the four Δ6 elongases from the Plantae. It was found that only ELOVL2, ELOVL5 and the Δ6 elongases from *Pyramimonas cordata* and *Parietochloris incisa* displayed any significant sequence alignment with the conserved sequence from *Mortierella alpina*. MAELO showed limited sequence alignment and ELOVL3 and ELOVL4 showed similarity in a small fragment. ELOVL1, ELO1-3 and MALCE1 showed no sequence similarity. This would indicate that the conserved region primers are unlikely to amplify genes coding for saturated and mono-unsaturated accepting elongases. It also indicates that the conserved nucleotide sequence is prevalent within vertebrates as well as the Kingdom fungi. The Oomycete *Saprolegnia parasitica* demonstrated a fragment of the conserved region however sequence similarity was lost at the primer binding region leading to the conclusion that the sequence is unlikely to be amplifiable using the current primer set. In regards to amplification of the conserved region, it is possible that ELOVL2 class elongases may not be amplified due to the loss of similarity toward the 3' end. The ELOVL4 class, which is thought of as an elongase capable of producing $C_{22:6}$ n3, does not show a significant proportion of the nucleotide conserved region. When the elongases from the Plantae were analysed, both organisms that demonstrated the conserved region were algae, whereas *Marchantia* and *Physcomitrella* are both terrestrial plants. Whilst all four elongases have the same function it appears that some amino acid residue and genomic changes have taken place between the algal and terrestrial Plantae. The terrestrial Δ6 elongases are more similar to one another than to the algal elongases and this is true genomically, due to their lack of the genomic conserved region. The algal elongases are more varied in regards to amino acid residues however both contain the genomic conserved region.

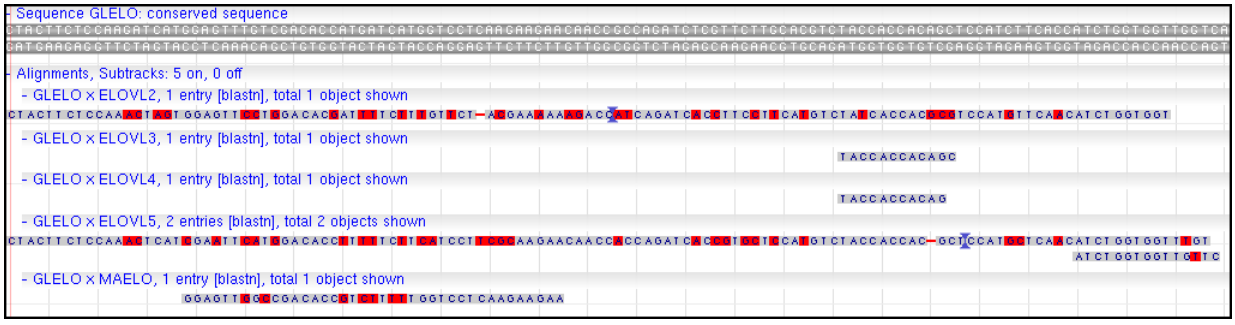


Figure 5-24. The genomic GLELO conserved region from *Mortierella alpina* was aligned with all ELOVL and ELO class elongase sequences as well as MAELO and MALCE. It was found that the sequence would only align with ELOVL2 and ELOVL5 sequences and partially aligned with the MAELO sequence. ELOVL3 and ELOVL4 showed a small fragment which aligned. This indicates that the primers will only bind with ELOVL2 and ELOVL5 class sequences which are PUFA elongating elongases.

5.3 Discussion

The ability to screen fungal samples for VLCPUFAs utilising a PCR based screen as oppose to a fatty acid GC-FID/MS screen at least as an initial exploratory probe seems feasible. The method has been shown to work for the genus *Mortierella* as well as for *Allomyces*, which is a chytrid. The fact that the PCR method correlated with the fatty acid profiles of all the isolates studied indicates that new, untested organisms will not be falsely flagged as containing VLCPUFAs. In general then it would be expected that this PCR approach would be successful in identifying VLCPUFA producing organisms from the Zygomycota as well as those from within the Chytridiomycota. The method has also been shown to work on mouse genomic material with the possibility of two PUFA accepting elongases being amplified, ELOVL2 and ELOVL5. This method in theory could be applied to invertebrates, however the actual application of such a probe for ELOVL2 and ELOVL5 is unclear. The primers did not amplify any genomic material from organisms within the Ascomycota, which correlated with the fatty acid profiles. Whilst only two organisms from this phylum were studied it is more than likely that the majority of ascomycetes and basidiomycetes will not contain Δ6 elongases due to their proposed evolutionary loss of such genes. Whilst not all organisms contain a PUFA elongating enzyme, all contain a complement of elongases capable of producing saturated and mono-unsaturated fatty acids.

The amino acid sequences show how similar the elongases are, especially within the conserved region, and this similarity could lead one to think that the nucleotide sequence would follow suit for all elongases. However, on aligning the genomic conserved region with elongases capable of elongating saturated and mono-unsaturated fatty acids it was found that no significant similarity existed, leading to the conclusion that the conserved primers will not amplify non-PUFA elongating elongase genes and that the region is specific for Δ6, ELOVL2 and ELOVL5 class elongases. What is interesting is that even though the conserved nucleotide sequence similarity is lost between classes, the amino acid sequences still share a high proportion of sequence similarity, which may have arisen because of preferential codon usage. Because of this, the conserved nucleotide region can be used as a conformational probe to deduce the function of the elongase, especially of putative identity. This was demonstrated when attempting to identify the Δ6 elongase from *Allomyces macrogynus* and *Saprolegnia parasitica*, that whilst arachidonic acid and eicosapentaenoic acid production in *Allomyces* and *Saprolegnia* has been documented respectively, the genes responsible for

this biosynthetic pathway have yet to be characterised to our knowledge. The elucidation of the sequence of the putative Δ6 elongases was primarily possible due to whole genome shotgun reads. This allowed for amino acid sequence matching, followed by confirmation by the identification of the nucleotide conserved region. Whilst this does not provide certain functional identification it does however provide further evidence that functional characterisation will likely yield a PUFA elongating elongase. It has to be noted however that there is a cut off point by which the conserved nucleotide region is deemed non-confirmatory. This is demonstrated with MAELO which does not display PUFA elongating capabilities but still contains a fragment of the genomic conserved fragment.

Regarding the conserved nucleotide sequence, *Allomyces* demonstrated a 125 bp fragment however, the whole genome shotgun read indicated that an intron was present between the two halves of the sequence. This would have lead to a noticeably larger fragment, but this was not the case. Explanations for this finding could be inaccuracies within the WGS sequence data or that the gene within the *Allomyces macrogynus* strain studied was different. It is also noticed that the *Allomyces* conserved region contained several extra amino acid residues, which most likely contributed to the larger internal region predicted by TMHMM. These extra residues could be spliced out with the conserved region intron and the fact that they were included may have been due to the algorithm used when the translated *Mortierella* GLELO and *Allomyces* WGS were aligned. The most definitive way to determine the sequence of the *Allomyces macrogynus* Δ6 elongase is through sequencing. The initial attempt to sequence the whole gene failed. This would indicate either that the PCR conditions need optimising or that the WGS sequence is not entirely accurate for the *Allomyces* strain in question and therefore revised primers may be needed. Both sets of primers were capable of amplifying the conserved region from all the *Mortierella* strains, however primers Uni 1 and 2 failed to amplify the conserved region from mouse DNA whilst primer set Uni 3 and 4 initially failed to amplify the conserved region from *Allomyces macrogynus*. When screening fungal isolates for VLCPUFAs, primer set Uni 1 and 2 appear to be the best choice as they detected all fungal conserved regions. Whilst Uni primers 3 and 4 detected the conserved region in mouse DNA, it is likely that fungi will contain Δ6 class elongases as oppose to ELOVL2 and 5 class elongases as found within mouse and other animals. In conclusion, the discovery of new elongases would allow greater diversification in biotechnological applications, as currently the *Mortierella* GLELO has been used to transform crop plants such as soybean. In conclusion, it can be seen

that this screening method is effective at identifying producers of VLCPUFAs, with it's detection of a putative Δ6 elongase in *Allomyces* and *Saprolegnia*. This detection across phyla makes the screen useful for a wide range of fungal species, not just *Mortierella*.

5.3.1 The Δ6 elongase GLELO distinguishes *Mortierella* from the rest of the Zygomycota

One of the characteristic features of the *Mortierella* genus since the reassignment of *Mortierella isabellina* and related species to *Umbelopsis* is the unique capability to elongate fatty acids beyond $C_{18:3}$ n6. *Umbelopsis* isolates were used as a comparison against *Mortierella* isolates as they represent the typical fatty acid profile exhibited by the majority of the zygomycetes; a preference for $C_{18:3}$ n6 over $C_{18:3}$ n3 and the inability to elongate past $C_{18:3}$ n6. Whilst testing the functionality of the conserved region primers the phenotypic trait which separates *Mortierella* spp. from the majority of the zygomycetes was confirmed genetically. The *Umbelopsis* strains Dis 195 and 206 were found not to contain the 125 bp fragment, which indicated the lack of GLELO. The ascomycetes *Penicillium* sp., *Geomyces* sp. and isolate from the Clavicipitaceae also did not contain the 125 bp band which correlated with the lack of fatty acids longer and more unsaturated than $C_{18:3}$ n3. On amplification of the genomic material from *Mortierella alpina* isolates 330997, 82072 and 196057 it was found that all three isolates demonstrated the 125 bp band, which correlated with the production of fatty acids longer and more unsaturated than $C_{18:3}$ n6. This was also found to be the case for *Mortierella polymorpha*, as well as the five Antarctic isolated *Mortierella* spp. studied. It can be assumed that the presence of the conserved fragment is indicative of a Δ6 elongase. The conclusion therefore is that the genus *Mortierella* is distinct from the rest of the zygomycetes due to the presence of the Δ6 elongase, GLELO. This allows for the production of fatty acids such as $C_{20:4}$ n6 and $C_{20:5}$ n3 not common in the zygomycetes. Exceptions to this however are *Entomophthora* sp. and *Conidiobolus* sp. (Kendrick & Ratledge, 1992b) which are capable of VLCPUFA production and reside within the Entomophthorales. The Entomophthorales have recently been classified within the sub phylum Entomophthoromycotina (Hibbett *et al.*, 2007), which groups these organisms away from the *Mortierella* sp. which can be seen in figure 5-25. Also of interest is the formation of the new phylum, the Blastocladiomycota formally part of the Chytridiomycota, which contains within it *Allomyces* spp.

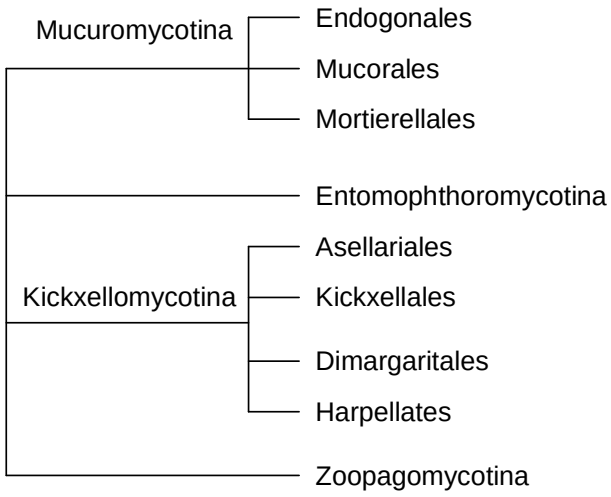


Figure 5-25. The formation of the new zygomycete sub-phyla Entomophthoromycotina as set out by Hibbet *et al.* (Hibbett *et al.*, 2007)**. Diagram adapted from the Tree of Life Web Program** (James & Kerry)**.**

It is also postulated that in fact the Entomophthorales are more related to the Blastocladiales (Tanabe *et al.*, 2004) with both *Allomyces* sp. and *Conidiobolus coronatus* clustering together based on the DNA dependent RNA polymerase II largest subunit sequence homology (RPB1) (Figure 5-26). It has been recognised that both the Zygomycota and Chytridiomycota are polyphylectic (James *et al.*, 2006) and the creation of new phyla and sub-phyla may be the first step to resolving this. The separation of the Blastocladiales and creation of the new sub-phylum Entomophthoromycotina, coupled with species from both groupings clustering together when analysed using RPB1 sequence homology could indicate that the Blastocladiales led to the evolution of the Entomophthorales. The loss of flagella is most likely the result of exploitation of a terrestrial niche by the newly evolved Entomophthorales. Therefore based on similarity, it could be postulated that the Blastocladiales diverged separating them from the Chytridiomycota. The move to a terrestrial environment resulted in flagellum loss and the evolution of the first zygomycetes, as evidenced by the apparent similarity between *Allomyces* sp. and *Conidiobolus coronatus*. Of note is the grouping of the two organisms away from the chytrids and the bulk of the zygomycetes, possibly indicating the transitional stage from chytrid to zygomycete. Both *Allomyces* and *Conidiobolus (Tan et al., 2011)* are capable of VLCPUFA production, and both contain a Δ6 elongase not present in higher fungi suggesting gene retention between phyla. *Mortierella* spp. are also found to have a Δ6 elongase, unlike the majority of the zygomycetes, which are capable of only $C_{18:3}$ n6 production, indicating the basal nature of the *Mortierella* species. Based on these data, it is possible that the Entomophthorales are progenitors to some of the

zygomycetes. The fact that *Mortierella elongata* appears basal to the rest of the Mucorales and has a Δ6 elongase may lead to the conclusion that a *Mortierella* like organism was a progenitor of the Mucorales. The same can be speculated about *Basidiobolus ranarum* from the Entomophthorales, that such an organism may be the progenitor to the rest of the zygomycete sub-phyla. It is probable that there are several ancestral organisms to the zygomycetes, as it is thought that the loss of the flagellum occurred independently several times from the transition from the Chytridiomycota to the Zygomycota (James *et al.*, 2006).

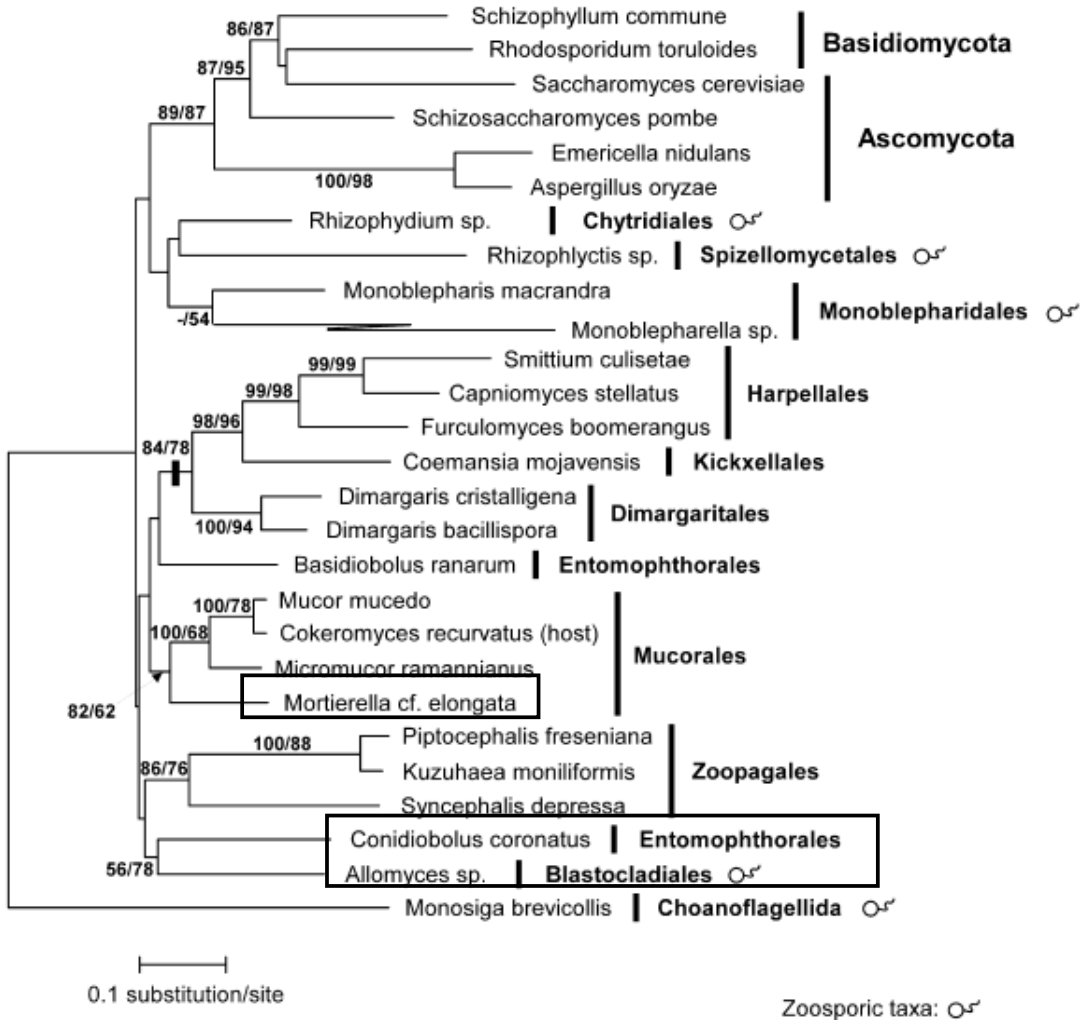


Figure 5-26. RPB1 sequence homology, diagram reproduced from Tanabe *et al.* (Tanabe *et al.*, 2004)**. As both the Chytridiomycota and Zygomycota are polyphylectic there are possibly multiple evolutionary lineages. The proposed chytrid to zygomycete lineage can be explained by the Blastocladiales being segregated from the rest of the Chytridiomycota, whilst the Entomophthorales due to their unique morphology, compared with other zygomycetes, have been designated a sub-phyla. When organisms from both the Blastocladiales (*Allomyces* sp.) and the Entomophthorales (*Conidiobolus coronatus*) are compared using RPB1 sequence homology it can be seen that both organisms group together. The rest of the flagellated chytrids group together, away from the zygomycetes and the Blastocladiales/Entomophthorales grouping.**

The production of fatty acids longer and more unsaturated than $C_{18:3}$ n6 with the presence of the 125 bp fragment confirms the reported role of GLELO as a Δ6 elongase. In regards to the evolution of the fungi, as to whether the fatty acid biosynthesis was immediately halted at the Δ6 elongase stage or whether the process was gradual due to the loss of later stage Δ5 or Δ17 desaturases first is unknown. The underlying reasons for the evolutionary loss of VLCPUFAs in fungi is unknown, but it is suspected they were an adaption to the aqueous environment in which the ancestors of the fungi inhabited, but with the migration to land permitted their loss. As to why the *Mortierella* maintain this phenotype is unknown, although the observation that *M. alpina* strains when compared with *Umbelopsis* species, as shown in chapter 4, display improved growth at 5°C and cease $C_{20:5}$ n3 production at warmer temperatures, indicates that low temperature is one of the key factors responsible for Δ6 elongase continuation within the genus. Also of interest is the grouping of the fungal saturated and mono-unsaturated substrate elongases when compared using ClustalW2 (figure 5-19). It can be seen that MALCE1, an elongase responsible for predominantly $C_{16:0}$ to $C_{18:0}$ elongation diverges earlier than the other similar function elongases. The fact that the zygomycete *Mortierella* predates *Saccharomyces cerevisiae* would lead to the conclusion that the ELO class elongases, especially ELO2 are derived from the zygomycete class of elongases. This appears to be the case using this type of analysis whereby the ELO class are more similar to themselves, with MALCE1 sitting outside the grouping. MAELO is also found to sit outside the grouping, which shows greater similarity to the ELO class. It again could be thought that MAELO is an ancestral protein to ELO3 as both share similar functionality. Therefore in regards to *Allomyces* and *Mortierella* Δ6 elongases, it would be thought that the chytrid elongases would be ancestral to zygomycete elongases due to the evolutionary theory regarding the two phyla. The putative *Saprolegnia* Δ6 elongase sits outside the main PUFA accepting elongase clade and groups with *Phaeodactylum tricornutum* another chromistan organism.

5.3.2 Elucidation of the Δ6 elongase from Allomyces macrogynus and Saprolegnia parasitica

A putative Δ6 elongase was identified from both *Allomyces macrogynus* and *Saprolegnia parasitica*. *Allomyces* was the first Δ6 elongase to be studied due to direct observation of the VLCPUFA complement. The detection of the 125 bp conserved region confirmed the presence of a GLELO like elongase and alignment of WGS translation sequences yielded a putative gene and protein. The same method was used

for *Saprolegnia parasitica* although this organism's fatty acid complement was not directly observed, however the related species *Saprolegnia diclina* was analysed. Owing to the lack of WGS sequence data for *S. diclina*, the sequence data of *S. parasitica* was utilised. The structure of the putative ELO sequences were analysed using TMHMM and SMART to locate functional domains within the sequences. It was found that both amino acid sequences displayed the ELO family domain, and all were characterised by 6-7 trans-membrane domains characteristic of this class of elongase. The translated conserved region was found to fall within trans-membrane regions 3 and 4 and also contained a small extra-membrane region which was larger within *Allomyces macrogynus*. It is this region that is thought to be in fact one large trans-membrane region due to the small size of the extra-cellular amino acid sequence.

Table 5-3. Calculated number of trans-membrane domains using TMHMM and the location of the conserved region amino acid sequence. TM = Trans-membrane region OM = outside membrane region No. = Number of trans-membrane domains.

ID	No.	Trans-membrane domains	Conserved region	Lies within
3-Ketoacyl-CoA	3	42-64, 84-106, 235-257		
d6 elongase *Allomyces macrogynus*	7	65-87, 100-122, 137-159, 180-199, 209-231, 251-268, 283-302	151-196	TM 3-4
d6 elongase *Saprolegnia parasitica*	6	60-82, 95-117, 137-159, 166-183, 193-215, 254-276	144-183	TM 3-4
d6 elongase *Phaeodactylum tricornutum*	7	48-70, 77-99, 119-141, 148-165, 175-197, 210-232, 247-269	126-165	TM 3-4
d6 elongase *Pyramimonas cordata*	7	54-73, 93-115, 135-157, 164-183, 193-215, 228-250, 260-282	142-182	TM 3-4
d6 elongase *Parietochloris incisa*	7	51-68, 92-114, 129-151, 158-180, 185-207, 220-242, 257-274	136-176	TM 3-4
d6 elongase *Marchantia polymorpha*	7	42-64, 77-99, 125-147, 154-173, 183-205, 217-239, 249-271	132-172	TM 3-4
Elongase *Physcomitrella patens*	7	44-66, 85-107, 127-149, 156-175, 185-207, 219-241, 251-273	134-174	TM 3-4
GLELO *M. alpina* AAF70417	7	75-92, 105-127, 154-173, 180-202, 207-229, 241-263, 273-295	158-198	TM 3-4
GLELO *M. alpina* BAF97073.1	7	75-92, 105-127, 154-173, 180-202, 207-229, 241-263, 273-295	158-198	TM 3-4
GLELO *M. alpina* 330997	7	75-92, 105-127, 154-173, 180-202, 207-229, 241-263, 273-295	158-198	TM 3-4
MALCE1 *Mortierella alpina*	6	18-40, 71-93, 120-142, 172-194, 209-231, 238-255	127-163	TM 3, OM 4
MAELO *Mortierella alpina*	5	55-72, 85-104, 180-202, 222-244, 259-281	138-167	OM 3
ELOVL1 *Mus musculus*	7	20-42, 63-85, 108-130, 137-154, 174-196, 203-221, 231-253	116-153	TM 3-4
ELOVL2 *Mus musculus*	7	28-50, 62-84, 116-135, 142-164, 179-201, 208-225, 235-254	120-160	TM 3-4
ELOVL3 *Mus musculus*	6	45-67, 79-98, 126-148, 176-198, 213-235, 248-270	135-164	TM 3, OM4
ELOVL4 *Mus musculus*	7	58-75, 88-110, 136-158, 165-184, 194-216, 228-250, 260-277	143-182	TM 3-4
ELOVL5 *Mus musculus*	7	26-48, 68-90, 110-132, 139-158, 168-187, 207-224, 229-251	117-157	TM 3-4
ELOVL6 *Mus musculus*	6	45-62, 77-99, 148-165, 170-192, 205-227, 242-264	130-164	OM 3, TM 3
ELOVL5 *Salmo salar*	7	32-50, 63-85, 110-132, 139-161, 176-198, 205-227, 232-251	117-157	TM 3-4
ELOVL5 *Capra hircus*	7	28-50, 63-85, 110-132, 139-158, 168-187, 207-224, 229-251	117-157	TM 3-4
ELO1 *Saccharomyces cerevisiae*	5	64-83, 96-118, 191-213, 233-255, 270-292	148-178	OM 3
ELO2 *Saccharomyces cerevisiae*	7	68-90, 97-119, 123-142, 147-169, 197-219, 232-254, 274-296	154-184	TM 4, OM 5

ELO3 *Saccharomyces cerevisiae*	6	73-95, 110-127, 181-203, 208-227, 240-262, 282-304	161-191	OM 3, TM 3
Candida dubliniensis ELO2 like	7	45-67, 87-109, 131-153, 160-179, 183-205, 222-244, 259-281	138-168	TM 3-4

When compared with the other classes, the PUFA elongating class contain seven trans-membrane domains with the exception of the *Saprolegnia* elongase. The conserved region also consistently falls within the third and fourth trans-membrane domains. Elongases which accept saturated and mono-unsaturated fatty acids display a varied number of trans-membrane domains from 5-7. It is possible that some cytoplasmic domains may in fact be trans-membrane regions as in some instances equal probabilities for both trans-membrane and extra-membrane domains were calculated for the same region. The number of trans-membrane domains and the localisation of the conserved region seem to follow the groupings seen by UPGMA and ClustalW2 with ELOVL1/2/4/5 showing seven trans-membrane domains, with the conserved region localised within the 3rd and 4th domain. ELOVL3 and 6 however only display six trans-membrane domains with the conserved region lying between a trans-membrane region and an extra-membrane region. The ELO2 elongase in *Saccharomyces* and *Candida* show greater similarity with each other and with the PUFA elongating class, as both have seven trans-membrane regions and *Candida* has a conserved region localised to the 3rd and 4th trans-membrane region.

The ELO and ELOVL class show differences in terms of localisation of conserved region and number of trans-membrane domains when shared functionality between elongases is observed. ELOVL1 contains one more trans-membrane domain compared with ELO3, with MAELO containing a further reduction in trans-membrane domains at five compared with the ELO counterpart, as well as the conserved region being wholly located within an extra-membrane region. ELOVL3 and MALCE1 share six trans-membrane regions as opposed to ELO2's seven trans-membrane domains. Both MALCE1 and ELOVL3 also share the same conserved region localisation, whereas in ELO2 it is located around trans-membrane domain four and not domain three. Finally ELOVL6 contains six trans-membrane regions compared with ELO1's five, with the conserved region localised completely to an extra-membrane region within ELO1. The consistency however between ELOVL5 and GLELO for example, seems to indicate the importance of seven trans-membrane domains in the function of PUFA accepting elongases. The consistent localisation of the conserved region to the third and fourth trans-membrane regions also appears to be a dominant feature of PUFA accepting elongases. As to why the conserved region localises to extra-membrane regions in

saturated and mono-unsaturated elongases is unknown although as mentioned earlier it is possible that the regions calculated as lying outside the membrane are in fact trans-membrane regions.

Within this region, there exists a large proportion of conserved amino acid residues indicating its importance, with the amino acid sequences Y-X-S-K-X-X-E-F-X-D-T and F-L-H-V-Y-H-H being highly conserved between the different classes. There are four residue changes that appear to be class specific within the conserved region, as well as the presence of up to seven additional amino acids which occur only within the PUFA elongation elongase class. The MALCE1 elongase does however contain an additional residue compared to the other saturated and mono-unsaturated elongases. The reasons for these residue changes and the protein elongation may be to allow the binding of the preferred substrate. It is assumed that the mechanism of action between all elongases is the same, the addition of two carbon units at the carboxyl terminus from malonyl-CoA via a condensation reaction. Therefore one would assume that any structural changes would be to accommodate the differing substrates. The introduction of *cis* double bonds subsequently causes the acyl chain to bend, with saturated fatty acids being relatively linear molecules. The addition of residues to PUFA accepting elongases may allow a greater degree of flexibility to bind, in the case of $C_{18:3}$ n6 and stearidonic acid, these curved carbon chains. To determine whether these additional amino acids as well as the conserved residues are instrumental for the substrate specificity of PUFA accepting elongases, it could be proposed that further work would aim to remove these additional residues or change conserved residues to those found within saturated/mono-unsaturate accepting elongases. This would give us a greater understanding of how substrate and elongase interact.

In regards to substrate specificity and whether the substrates shape as a whole or the shape adjacent to the carboxyl end only is important for acceptance by the elongase, it appears that the former is the case. The reason is highlighted by two examples. Firstly ELO1 is capable of elongating up to $C_{18:0}$ whereas ELO2 is capable of further elongation up to $C_{22:0}$. If only the shape near the carboxyl group where the modification occurs were the regulatory factor, then it would seem plausible that one elongase could elongate all the way up to $C_{26:0}$. Secondly ELO2 is capable of elongating both saturated and mono-unsaturated fatty acids however if the carboxyl terminus again was the regulating factor then why is $C_{20:2}$ not found as a major constituent, if at all, elongated from $C_{18:2}$? The elongation from $C_{18:1}$ to $C_{20:1}$ should be similar to the elongation of $C_{18:2}$

if the carboxyl terminus shape were the regulating factor as both $C_{18:1}$ and $C_{18:2}$'s closest double bond is at the Δ9 position. It can be assumed then that the shape of the acyl chain as a whole is the key factor as to whether it can be acted upon by an elongase.

Other amino acid residue changes are found throughout the sequences which distinguish PUFA and non-PUFA accepting elongases, although in several cases ELOVL1 is shown to contain residues which are conserved within PUFA accepting elongases whilst the *Saprolegnia* and *Phaeodactylum* elongases show a lack of several residues which appear consistent throughout the PUFA accepting class. This is visualised with the UPGMA, as well as with the ClustalW2 analysis, with ELOVL1 clustering together with the PUFA accepting ELOVL class, as oppose to the saturated and mono-unsaturated accepting ELOVL class. As to why the ELOVL1 enzyme groups with the PUFA elongating ELOVL class is unknown, but it is possible that this elongase, whose role it is to elongate up to $C_{26:0}$ may be derived from the PUFA elongating ELOVL class, and whose amino acid sequence was modified enough to accept long chain saturated fatty acids. The *Saprolegnia* elongase sits outside the main grouping of PUFA accepting elongases using the UPGMA analysis indicating its lowered similarity when compared to the rest of the class. In general though, the majority of elongases group according to their function, forming the PUFA accepting and the non-PUFA accepting elongase groupings as was also shown by Leonard *et al*. (Leonard *et al.*, 2004). However a division between the ELOVL and Δ6 class elongases is seen, as is a division between the ELOVL and ELO class. This division in protein similarity appears linked to the Kingdom from which the elongases are isolated, with fungi forming one distinct grouping with the ELO class and Δ6 class, and vertebrae forming another distinct grouping with the ELOVL class.

Interestingly the division seen in the PUFA accepting elongase appears to correlate with Cavalier-Smith's 6 Kingdom theory (Cavalier-Smith, 1998). Firstly, *Saprolegnia* groups with *Phaeodactylum tricornutum* both of which are chromists. Whilst both organisms are relatively distant from one another, the clustering of their Δ6 elongases confirms their grouping within the Chromista, indicating a possible shared lineage between the photosynthetic *Phaeodactylum* and the non phototrophic *Saprolegnia*. As stated by Cavalier-Smith, it is thought that animals and fungi derived from a common protozoan ancestor and this can be seen with the UPGMA analysis. It can be seen that the animal and fungal PUFA accepting elongases are more similar to themselves than to the chromist Δ6 elongases. Therefore it appears that the protozoan ancestor to the fungi

and Animalia diverged from the protozoan ancestor to the Chromista. The fact that Animalia and fungi shared a common ancestor means that the Δ6 elongase sequence from their ancestor experienced independent residue changes from the Δ6 elongase of the chromist ancestor, which is shown by the greater similarity experienced by animals and fungi. The point at which the Animalia and fungi diverged is the point at which independent differences in the Δ6 elongase sequences between the two Kingdoms developed, differentiating the two classes of elongase. However the reason as to why plant Δ6 elongases cluster with related fungal elongases is unknown. If the Chromista and Plantae had separate ancestors comparable with the relation exhibited by Chromista and fungi/Animalia then it would be expected the Δ6 elongase from plants would form a distinct cluster away from Animalia/fungi and the Chromista. Another theory proposed by Cavalier-Smith would have the Plantae and Chromista grouping closer together. This is because it is thought the protozoan, which merged with a cyanobacterium may have formed either an intermediary organism or an early plant, which may have been the host organism for the amalgamation with a red alga. This is thought to have been the ancestor to the Chromista. However the UPGMA method clearly shows plant Δ6 elongases clustering with fungal Δ6 elongases. Only a few terrestrial plants from the mosses and related liverworts appear to be capable of producing these VLCPUFAs. Also of interest is the division within the Plantae itself. It was shown that the genomic conserved region was only present in algae and not terrestrial plants. The fact that the terrestrial plant Δ6 elongases share similar amino acid sequences and lack the genomic conserved region confirms their shared lineage. Like the Animalia and fungi, the terrestrial plant ancestor, from the Charophyta, most likely lost the genomic conserved region at a point after the division of the Clorophyta from the Charophyta, with this loss passed on to the terrestrial plants, as observed with *Marchantia* and *Physcomitrella*. The greater sequence similarity between the terrestrial plants is because both are from the Charophyte lineage, with their elongase sequence experiencing independent change from the algae, which are more similar to themselves and further removed from the terrestrial plants as they are from the Chlorophyta lineage. It therefore seems the ancestral history of the elongases plays a role in their amino acid sequence and subsequent clustering.

5.4 Conclusion

The search for a PCR based method by which VLCPUFA production may be detected within the fungal kingdom appears to be a success. The screen is capable of identifying producers from the Zygomycota as well as from the Chytridiomycota. The method was also shown to work on mammalian ELOVL class elongases. It is unlikely that fungi from the Ascomycota or the Basidiomycota will contain the gene and to our knowledge no VLCPUFA producing isolate from these phyla has been identified. The method was extended to the Oomycete *Saprolegnia* which resides within the Chromista. However the conserved region was not amplifiable due to the loss of conservation at the primer binding sites and whether the primers would work on other Chromista is unknown. *Mortierella* and other fungal species containing the Δ6 elongase are thought to be basal organisms due to the loss of this gene from the higher fungi. Due to the similarity of *Allomyces* sp. with *Conidiobolus coronatus* using RPB1 sequence homology, it is proposed that the Blastocladiales are ancestral to the Entomophthorales as both species also contain the Δ6 elongase. The similarity in RPB1 sequence, which sets the Blastocladiales/Entomophthorales grouping apart from the other chytrid and zygomycete groupings, could indicate the transitional period between the two phyla. As *Mortierella* species are more removed from the rest of the Mucorales and contain the Δ6 elongase, it is proposed that they are one of the progenitors of this Δ6 elongase deficient phylum. The PCR screen offers a quick method for detecting the gene and therefore aiding in taxonomic classification. In regards to the conserved region, it's presence in the nucleotide sequence appears to be indicative of a PUFA accepting elongase, however at the protein level the conserved region is still found within all classes of elongase excluding 3-Ketoacyl-CoA elongases. Therefore it appears it is not the presence or absence of the amino acid conserved region which determines the elongase's function but rather the several residue changes found throughout the protein, as well as the additional amino acids present within trans-membrane domains three and four. As to why the nucleotide conserved region is indicative of PUFA accepting elongases is unknown. This is most evident between elongases within the same class, with the differentiation between ELOVL2/5 and ELOVL3/6. As these elongases are found together within the same organism it is possible that this nucleotide differentiation is an indication of the elongase's ancestry.

6 Heterologous transformation of *Phaffia rhodozyma* with Δ5, Δ6 desaturases and Δ6 elongase from *Mortierella alpina*

6.1 Introduction

The zygomycete fungus *Mortierella alpina* is a well studied organism, due in part because of its commercial use as an arachidonic acid producer. Because of this, many of the elongases and desaturases responsible for the formation of $C_{20:4}$ n6 and $C_{20:5}$ n3 are well characterised, with several desaturases being used in recombinant systems (Chen *et al.*, 2006). Plants such as soybean (*Glycine max)* (Damude & Kinney, 2008) and *Arabidopsis* (Qi *et al.*, 2004) have been modified with Δ5 and Δ6 desaturases, as well as the GLELO elongase to produce modest amounts of polyunsaturated fatty acids. *M. alpina* is used commercially because of its oleaginous nature and can produce large quantities of the desired fatty acid $C_{20:4}$ n6. *M. alpina* has also been trialled directly as a fish feed (Harel *et al.*, 2002) to supply the VLCPUFA $C_{20:4}$ n6. The aquaculture industry seeks to complement and replace existing feeds with those that are high in polyunsaturates. *Mortierella* has also been modified using *Agrobacterium tumefaciens* (Ando *et al.*, 2009), an organism traditionally associated with plant modification. Amenability to genetic manipulation should enable the tailoring of longer chain fatty acid production within this organism, as well as increase yields of already existing fatty acids. To date, the method has been utilised to improve levels of $C_{20:5}$ n3, although through the addition of an elongase and desaturase, the production of $C_{22:6}$ n3 in *Mortierella* is not difficult to envisage. Because of these factors, *Mortierella alpina* is a valuable organism and as such fully understanding the regulation of fatty acid synthesis would be beneficial industrially, as strain improvement and recombination within this organism seem likely. With respect to the elongases and desaturases of *Mortierella*, the fatty acid $C_{22:5}$ n3 as well as other C_{22} VLCPUFAs, were identified within *Mortierella alpina* strain 330997. Therefore one goal was the identification, expression and functional characterisation of the Δ6 elongase, GLELO.

As mentioned previously, the aquaculture sector is a major consideration for the development of VLCPUFA containing microorganisms. One factor in creating

nutritionally comparable fish is the addition of a polyunsaturated fatty acid source, traditionally from fish sources, and in the case of salmon the inclusion of an astaxanthin source to allow the production of the distinct colouration of the flesh of the fish, as well as the potential health benefits associated with this anti-oxidant xanthophyll. Health benefits include maintenance of retinal health and possible prevention of age related macular degeneration, reduction in inflammation, heart health through prevention of oxidation of LDL cholesterol and protection from certain tumour types such as prostate cancer (Guerin *et al.*, 2003). Astaxanthin is a C_{40} hydrocarbon, classified as a carotenoid. The general structure is a 20 carbon aliphatic chain, with methyl branches with cyclization of the terminal 6 carbons to form two ring structures. The aliphatic chain and ring structures are linked by a series of conjugated π bonds, which allow for delocalisation of the electrons. This in turn acts like a standing wave system, which has set integer values of energy which can be absorbed. When a photon carrying the correct energy arrives (energy being related to wavelength) at the system, the delocalised electrons absorb the energy removing that wavelength from the spectrum. As the delocalised system grows through the addition of carbon-carbon double bonds and keto groups for example, the wavelength absorbed increases. This results initially in UV range wavelengths being absorbed, resulting in a white colour being observed, due to UV not being observable to the human eye. Following UV absorption, wavelengths within the blue visible range are absorbed, leading to the colours observed within the carotenoids such as orange, pink and red. Astaxanthin displays a pinkish red colour, with the oxygenated rings which contain hydroxyl and keto groups classifying this molecule as a xanthophyll. The majority of carotenoids within nature are found to be in the trans- configuration and astaxanthin, due to the presence of the hydroxyl groups on both the 3 and 3' positions contains two chiral centres. This allows 3 enantiomers to exist, with the 3S, 3S' predominating. It is also possible, due again to the hydroxyl group, that fatty acids may form an ether bond with astaxanthin (Higuera-ciapara *et al.*, 2006) (Higuera-ciapara *et al.*, 2006). Astaxanthin has been found to be a potent antioxidant (Edge *et al.*, 1997), and it is this property that has been linked to many of its health promoting benefits.

The supplementation of salmon with astaxanthin has been linked to optimal growth of the juvenile fish, increased lipid content and increased astaxanthin content (Christiansen & Torrissen, 1996). Polyunsaturated fatty acids are also liable to oxidation due to their high level of unsaturation, forming reactive free radicals which can damage membranes through the propagation reaction of the radicals. Most feeds containing high

levels of PUFAs are in danger of becoming oxidised, which in turn when ingested by fish lead to oxidative stress (Nakano *et al.*, 1999). By combining astaxanthin, a potent anti-oxidant which is believed to be found within the phospholipid membrane with polyunsaturated fatty acids, several benefits may be observed. Firstly, astaxanthin is required by salmon to reduce the levels of reactive oxygen species and free radicals which in turn can lead to poor health. Secondly the polyunsaturates within the yeast *Phaffia rhodozyma*, which will be discussed later, will be protected from lipid peroxidation allowing more non-oxidised PUFAs to be taken up by salmon. Thirdly, by extracting the oil of *Phaffia*, one would hope that the pigment would decrease the rate of rancidification within the oil. As mentioned previously, most carotenoids are thought to be localised within the membrane, due to their predominantly hydrophobic nature. Astaxanthin, which contains two polar end groups due to the hydroxyl moieties, is thought to span the membrane (Britton, 1995) with the polar groups interacting with the phosphate region of the phospholipids. Due to this spanning of the membrane, free radicals and reactive oxygen species may be dealt with within both the aqueous and interior hydrophobic phases, unlike carotenoids such as β-carotene, which are thought to remain fully within the interior non-polar region, allowing them to only access free radicals and harmful species within this region. By having a molecule span the membrane, such as astaxanthin, it is also thought that membrane rigidity increases. This may however, lead to increased desaturation of FAs within the membrane. Therefore one would expect a higher proportion of PUFAs within the membrane to compensate. As seen in chapter 3, VLCPUFAs such as $C_{20:5}$ n3 are rarely produced by fungi, so it is likely that in *Phaffia* more $C_{18:3}$ n3 will be introduced into the membrane to maintain fluidity.

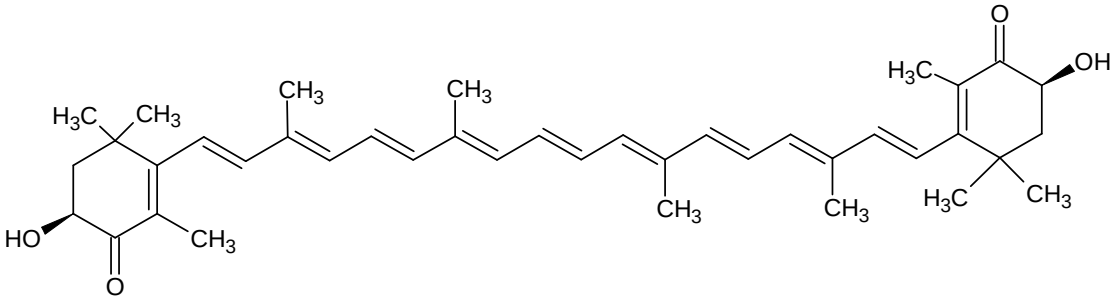


Figure 6-1. The structure of astaxanthin (3, 3'-dihydroxy-β-carotene-4, 4'-dione). A C_{40} hydrocarbon with polar cyclic groups, enabling it to reside between membrane layers. The pink colouration is due to the delocalised electron system from the conjugated double bonds (π bonds).

A combination of PUFAs and astaxanthin are required for the breeding of salmon as well as other fish, and a feed organism which could provide both compounds would be

beneficial due to its cost effectiveness. As mentioned previously, the organism chosen to incorporate the elongase and desaturase system is *Phaffia rhodozyma*, which is used as an astaxanthin feed for salmon (Whyte & Sherry, 2001). The organism itself could be used as a human dietary supplement, or following lipid extraction the compounds may be encapsulated. *Phaffia* is a budding pigmented yeast from the Basidiomycota, which produces carotenoid, primarily astaxanthin, compared with other pigmented yeast such as *Rhodotorula* and *Sporobolomyces* which produce torulene and torularhodin (Miller *et al.*, 1976). Utilising the elongase and desaturases of *M. alpina* it is hoped that a dual compound producing organism can be created. By utilising the organism *Phaffia rhodozyma*, which naturally produces astaxanthin in large quantities it is hoped the fatty acid synthesis pathway can be expanded to produce $C_{20:5}$ n3. The proposed alteration to the biosynthetic pathway in Phaffia is shown in Figure 6-2. In *Mortierella alpina* the fatty acid biosynthetic pathway follows the n6 route progressing from linoleic to $C_{18:3}$ n6 via a Δ6 desaturase. Following this, the Δ6 elongase elongates $C_{18:3}$ n6 to $C_{20:3}$ n6, which is then desaturated to $C_{20:4}$ n6 by a Δ5 desaturase. A Δ17 desaturase then crosses the n6-n3 bridge by forming $C_{20:5}$ n3. The three enzymes, GLELO and the Δ5/6 desaturases were isolated from *M. alpina*.

Phaffia rhodozyma produces $C_{18:3}$ n3 as opposed to $C_{18:3}$ n6 as found in *M. alpina*, which raises questions about substrate specificity. The GLELO has been shown to have specificity for stearidonic acid (Parker-Barnes *et al.*, 2000), which was converted to $C_{20:4}$ n3, as was demonstrated by supplying the yeast, which heterologously expressed the elongase, with stearidonic acid. Parker-Barnes *et al.* also showed that the Δ5 desaturase had specificity for $C_{20:4\ n3}$, converting it to $C_{20:5}$ n3, as well as demonstrating the extension of the fatty acid biosynthetic pathway. The Δ6 desaturase has also been shown to convert the n3 substrate $C_{18:3}$ n3 into stearidonic acid (Domergue *et al.*, 2002). A similar method was tried by Chen *et al.* (Chen *et al.*, 2006), with all three enzymes described previously used to transform *Glycine max*. In this case however, the n3 route was blocked by RNAi to prevent the formation of $C_{18:3}$ n3, therefore mimicking the path taken in *M. alpina*. The result showed that $C_{20:4}$ n6 could be formed in a recombinant system, which utilised 3 genes from the fungus *M. alpina*. However, the yields for the majority of FAs after $C_{18:2}$ were low. This was probably due to the low percentage of $C_{18:3}$ n6 within the cells. Without an efficient conversion from $C_{18:2}$ to $C_{18:3}$ n6, all subsequent fatty acid yields would be expected to be low. In this case, a more efficient Δ15 desaturase inhibition could have resulted in an increased $C_{18:3}$ n6 substrate pool, available for elongation to $C_{20:4}$ n6.

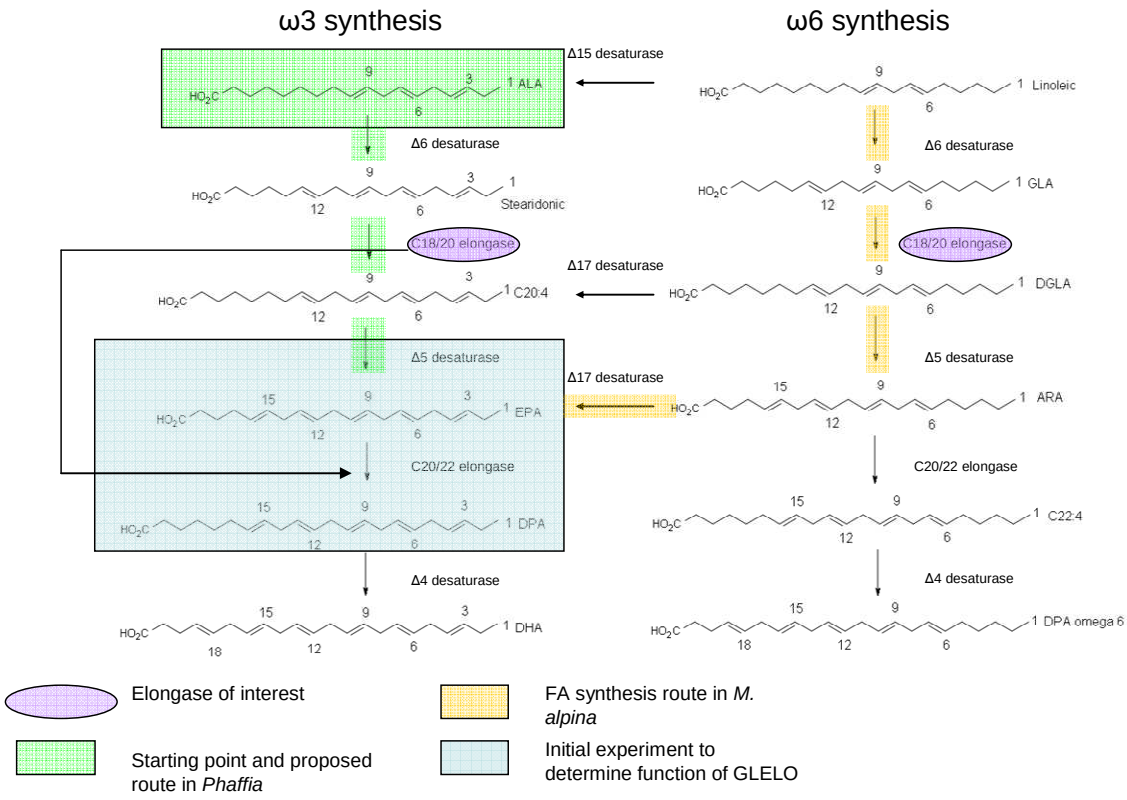


Figure 6-2. The fatty acid biosynthesis pathway in *Mortierella alpina* and the proposed recombinant fatty acid biosynthesis pathway in *Phaffia rhodozyma*. *Mortierella alpina* follows the n6 biosynthetic route, by elongating $C_{18:3}$ n6 to $C_{20:3}$ n6, followed by the subsequent Δ5 desaturation resulting in $C_{20:4}$ n6 and crossover desaturation by a Δ17 desaturase to produce $C_{20:5}$ n3. *Phaffia rhodozyma* produces $C_{18:3}$ n3 and so the recombinant strain will contain a Δ6 desaturase to produce $C_{18:4}$ n3, which will be acted upon by the Δ6 elongase (GLELO) to produce $C_{20:4}$ n3. The final Δ5 desaturase will result in the formation of $C_{20:5}$ n3. The $C_{22:5}$ n3 found within *Mortierella alpina* is proposed to be formed by the Δ6 elongase acting upon $C_{20:5}$ n3.

This highlights the issue of selecting a suitable organism for modification, one trait of which should be the pool of starting substrate available to the organism. With the *Phaffia* transformation only one substrate is available for enzyme action, which in turn should increase the efficiency of conversion. Recently the fatty acid biosynthetic pathway was extended in *Saccharomyces cerevisiae* using a novel Δ6 elongase and Δ6 desaturase from the zygomycete *Conidiobolus obscurus* to produce the fatty acid $C_{20:4}$ n3 (Tan *et al.*, 2011). It was also confirmed that the Δ6 desaturase and elongase could act upon the n3 substrates and that low temperature growth induced greater transcription of both recombinant genes.

In conclusion, two recombinant *Phaffia rhodozyma* strains will be produced; one expressing only the Δ6 elongase, whereas the other strain will express all three genes to extend the fatty acid profile in *Phaffia rhodozyma*. *E. coli* BL21(DE3)pLysis* will also

be transformed with the Δ6 elongase to ascertain whether the bacteria is capable of expressing the elongase, which can then be functionally characterised. In regards to Δ6 elongase expressing strains of *Phaffia* and *E. coli* the substrate specificity of the elongase can be determined either through growth of the organisms inoculated with precursor fatty acid followed by cellular fatty acid extraction (Domergue *et al.*, 2003, Tan *et al.*, 2011), or through microsomal/membrane fraction separation, purification and testing *in vitro* with acyl-CoA substrates to determine elongase substrate specificity (Jump, 2009, Moon *et al.*, 2001, Ohno *et al.*, 2010). It is hoped that by incubating with various fatty acid substrates such as $C_{18:3}$ n6 and $C_{20:5}$ n3 the specificity of the Δ6 elongase can be determined.

6.1.1 Aims

The aim of this study is to establish whether the *Mortierella alpina* fatty acid elongation pathway can be heterologously reconstituted in the astaxanthin producing yeast *Phaffia rhodozyma*. The elucidation of the substrate specificity of the Δ6 elongase expressed in *Phaffia* and *E. coli* was also attempted, to ascertain whether the elongase can accept $C_{20:5}$ n3, in turn producing $C_{22:5}$ n3.

6.2 Results

All three genes were first identified using the NCBI database and primers developed based on these sequences. cDNA from *Mortierella alpina* strain 330997 was obtained from reverse transcription of *Mortierella alpina* RNA. Primers 1+2 were used for Δ6 desaturase amplification, primers 3+4 were used for Δ5 desaturase amplification and primers 5+6 were used for Δ6 elongase amplification. Primer sequences are described in the materials and methods section 2.1.4.2 and contained no intentional restriction sites. Taq polymerase was used for amplification as the TOPO 2.1 vector required a single adenine residue overhang at each 3' end. All three genes were amplified successfully, confirmed using agarose gel electrophoresis, ligated with the sequencing vector TOPO 2.1 and amplified in TOP10 chemically competent *E. coli* cells as shown in figure 6-3. Aliquots of the purified DNA were sent for sequencing which confirmed the identities of the amplified products. The sequences of the three genes are shown in figures 6-4, 6-5 and 6-6. The three genes were sent to Prof. Gerhard Sandermann at Goethe University, Frankfurt for transformation into *Phaffia rhodozyma*.

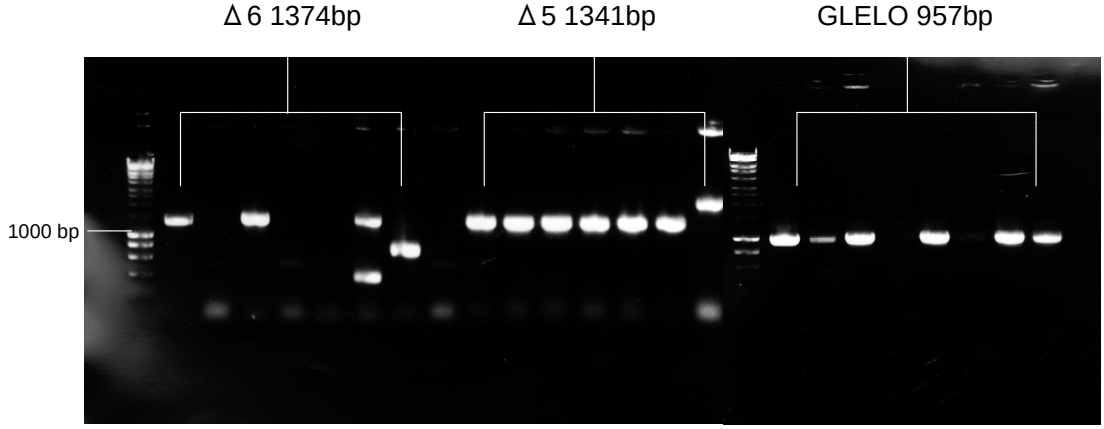


Figure 6-3. Colony PCR to detect the presence of the three amplified genes within TOP10 *E. coli* cells. Those colonies displaying the correct size amplification product were then harvested from broth and the DNA extracted. Following DNA extraction, aliquots were sent for sequencing to confirm the TOPO 2.1 gene inserts.

```
ATGGAGTCGATTGCGCCATTCCTCCCCTCAAAGATGCCGCAAGATCTGTTTATTGACCTTGCAAGGGCCATCGGTGTCCAGGCCGCACCCTATGTCG
ACCCTCTCGAGGCAGCGCTTGTGGCCCAGGCCGAGAAGTTCTTCCCCACGGTCGTTCATCACACGCGCGGCTTTTTGGTCGCGGTCGAGTCACCCTT
GGCCCGTGAGCTGCCCTTGATGAACCCCTTCCACGTGCTGTTGATCGCGCTCGCTTACTTGGTCACGGTCTTTGTCGGCATGCAGATCATGAAGAAC
TTTGAGCGGTTCGAGGTCAAGACGTTCTCGCTCTTCCATAACTTTTGTCTGGTCTCGATCAGTGCCTACATGTGCGGCGGGATCTTGTACGAGGCTT
ACCAGGCCAACTATGGACTGTTTGAGAACGCGGCCGATCATACCGTCCAGGGTCTTCCTATGGCCAAGATGATCTGGCTCTTCTACTTCTCCAAGAT
CATGGAGTTTGTCGACACCATGATCATGGTCCTCAAGAAGAACAACCGCCAGATCTCGTTCTTGCACGTCTACCACCACAGCTCCATCTTCACCATC
TGGTGGTTGGTCACCTTTGTTGCACCCAACGGTGAAGCCTACTTCTCGGCTGCGTTGAACTCGTTCATCCACGTGATCATGTACGGCTACTACTTCC
TGTCCGCCTTGGGCTTCAAGCAGGTGTCGTTCATCAAGTTCTACATCACGCGTTCGCAGATGACGCAGTTCTGCATGATGTCGATCCAGTCCTCCTG
GGACATGTATGCCATGAAGGTGCTTGGCCGCCCCGGATACCCCTTCTTCATCACCGCCTTGCTTTGGTTCTACATGTGGACCATGCTCGGTCTCTTC
TACAACTTTTACAGAAAGAACGCCAAGTTGGCCAAGCAGGCCAAGATCGATGCTGCCAAGGAGAAGGCAAGGAAGTTGCAGTAA
```

Figure 6-4. *Mortierella alpina* isolate 330997 Δ6 elongase (GLELO) cDNA sequence.

```
ATGGGTGCGGACACAGGAAAAACCTTCACCTGGCAAGAACTCGCGGCGCATAACACCGAGGACAGCCTCCTTTTGGCTATCCGTGGCAATGTATACGA
TGTCACAAAGTTCTTGAGCCGTCATCCTGGTGGAACGGATACTCTTCTGCTCGGAGCTGGCCGAGATGTCACTCCGGTCTTTGAGATGTACCACGAGT
TTGGAGCTGCAGAGGCTATCATGAAGAAGTACTATGTTGGCACACTGGTCTCAAATGAGTTGCCCATCTTCCCGGAGCCAACGGTGTTCCATAAGACC
ATCAAGGGCAGAGTTGAGGCATACTTTAAGGACCGGAACATGGATTCCAAGAACAGACCAGAGATCTGGGGACGATATGCTCTCATCTTCGGATCCTT
GATCGCCTCTTACTACGCGCAGCTCTTTGTACCGTTCGTTGTCGAACGTACATGGCTCCAGGTGGTGTTTGCTATCATCATGGGATTTGCGTGCGCGC
AAGTCGGACTGAACCCTCTTCACGATGCCTCCCACTTTTCAGTGACCCACAACCCCACCGTTTGGAAGATTCTCGGAGCCACGCACGACTTTTTCAAC
GGAGCATCGTATCTCGTGTGGATGTACCAACATATGCTCGGCCATCATCCCTATACCAACATTGCTGGAGCCGATCCCGATGTGTCGACCTCTGAGCC
CGATGTTCGTCGTATCAAGCCCAACCAAAAGTGGTTCGTCAACCACATCAACCAGCACATGTTTGTTCCTTTCCTGTACGGACTGCTGGCGTTCAAGG
TGCGAATCCAGGACATCAACATCTTGTACTTTGTCAAGACCAATGACGCCATTCGTGTCAACCCCATCTCGACTTGGCACACCATCATGTTCTGGGGC
GGAAAGGCCTTCTTTGTCTGGTACCGCTTGATCGTTCCTATGCAGTATCTGCCCCTGAGCAAGGTGTTGCTCTTGTTCACCGTCGCGGACATGGTCTC
TTCTTACTGGCTGGCCCTGACCTTCCAGGCGAACCACGTTGTTGAGGAGGTTCAGTGGCCATTGCCTGATGAGAATGGAATCATCCAAAAGGATTGGG
CAGCCATGCAGGTCGAGACCACTCAGGATTACGCCCACGATTCGCACCTCTGGACCAGCATCACGGGCAGCTTGAACTACCAGGCCGTTCACCATCTG
TTCCCAAATGTGTCCCAGCACCACTACCCTGATATCCTGGCCATCATCAAGGACACCTGCAGCGAGTACAAGGTGCCATACCTCGTCAAGGATACCTT
TTGGCAAGCGTTTGCTTCACATTTGGAGCACTTGCGTGTACTTGGTCTTCGTCCCAAGGAAGAGTAA
```

Figure 6-5. *Mortierella alpina* isolate 330997 Δ5 desaturase cDNA sequence.

Figure 6-6. *Mortierella alpina* isolate 330997 Δ6 desaturase cDNA sequence.

The Δ6 elongase was also expressed in BL21(DE3)pLysis* *E. coli* cells. The Δ6 elongase was amplified using primers P1+P2 which contained NdeI and HindIII restriction sites for insertion into the pET-23b vector, as well as removing the stop codon to allow the translation of the attached His tag protein. The Δ6 elongase was successfully amplified using the primer set and ligated with the pET-23b vector. The vector was successfully inserted and amplified in BL21(DE3)pLysis* *E. coli* cells. After induction with IPTG, the total cell lysate underwent SDS-PAGE and silver staining which revealed no apparent expression of the Δ6 elongase as shown in figure 6-7. Western blotting followed by anti-His antibody and BCIP/NBT staining showed a band with a molecular mass of around 38 KDa for BL21(DE3)pLysis* cells expressing the Δ6 elongase within pET-23b shown in figure 6-7, with the molecular weight of the His tagged Δ6 elongase being 38.3 KDa. Negative control cells demonstrated a much fainter band which was not uniform throughout the lane indicating possible contamination from the adjacent sample. This would indicate the successful expression of the Δ6 elongase within *E. coli* cells.

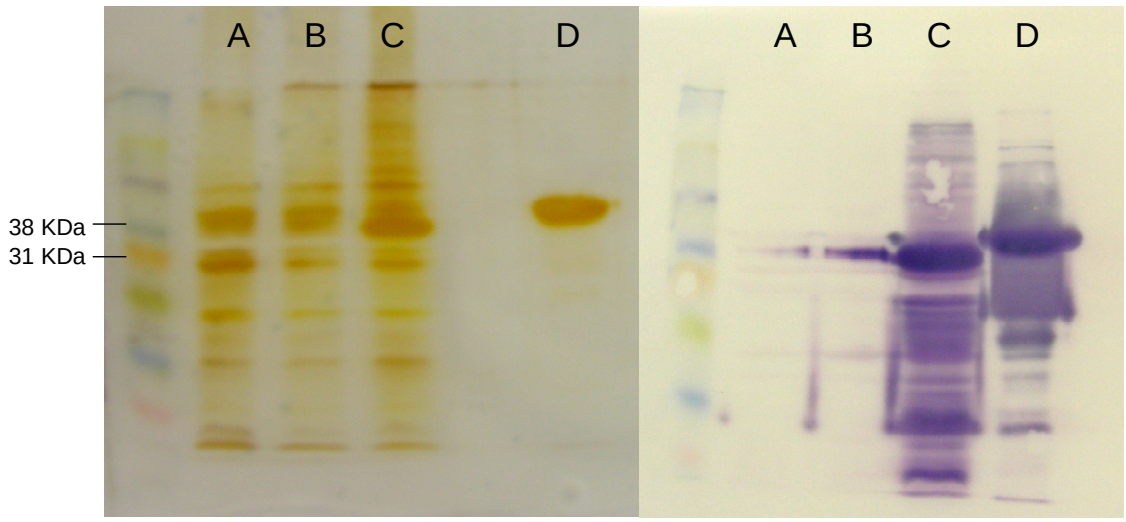


Figure 6-7. Sonicated BL21(DE3)pLysis* *E. coli* cells run on SDS-PAGE with silver staining (left) and Western blot using anti-His antibodies, stained with BCIP/NBT. A = pET-23b-blank B = pET-23b-Δ6 elongase C = *E. coli* lysate expressing 36KDa His-tagged protein D = Purified 41KDa His-tagged protein. The silver stained gel failed to highlight the production of the 38.3KDa His tagged Δ6 elongase protein, however following antibody binding a band corresponding to the size of the elongase is detected in the *E. coli* cells containing pET-23b-Δ6 elongase. The negative control cells however do display a faint, non-uniform band possibly indicating contamination from the adjacent lane. The relative production of the putative Δ6 elongase is low compared to the *E. coli* cells in lane C.

Protein purification through His-tagged affinity column chromatography failed to isolate the location of the Δ6 elongase when used with silver staining. This is probably due to the low expression of the elongase, as seen with the antibody stain and the comparison with the other two His-tagged proteins. It is likely however that the elongase is located within a membrane due to the seven trans-membrane domains, as the elongase usually localises to the ER, which is absent within Bacteria. This may account for the slower growth of the Δ6 expressing strain, as expressed elongase may interfere with membrane processes or other cellular functions.

Of the three genes sent for transformation into *Phaffia rhodozyma*, only the Δ5 desaturase and the Δ6 elongase were successfully transformed into individual isolates. The Δ6 desaturase and isolates containing different combinations of the three genes were not successful. Freeze dried material of two control strains, two Δ5 desaturase recombinant strains and ten Δ6 elongase recombinant strains were received. These were subjected to fatty acid analysis by GC-FID and GC-MS as stated in the materials and method sections 2.2.5 and 2.2.6. The fatty acid profiles are shown in table 6-1. It was initially noted that both control strains contained much lower abundances of total lipid compared with the recombinant strains, in most cases containing values half or less that of the recombinant isolates. Whilst the complete pathway was not successfully

incorporated into *Phaffia* to allow end point production of $C_{20:5}$ n3, the inserted enzymes did display minor activity. The Δ5 desaturase demonstrated an additional three peaks compared against the control, with the Δ6 elongase also producing an additional three peaks, one of which was shared with the Δ5 desaturase. The addition of the Δ5 desaturase was thought to produce $C_{18:3}$ n6 and two $C_{18:2}$ isomers, with $C_{18:2}$ isomer (a) putatively identified as $C_{18:2}$ Δ14, 17 based on the NIST GC-MS library as the highest hit compound. The retention time of isomer (a) fell around the elution time of $C_{18:1}$ on both GC-FID and GC-MS (figure 6-9). The mass spectrum of isomer (a) indicated two double bonds, although the isolate is characteristically more non-polar as it elutes earlier on the more polar GC-FID column, and elutes later on the more non-polar GC-MS column compared with the standard $C_{18:2}$ Δ9, 12 isomer. Further structural detail will be discussed in the subsequent section. The second $C_{18:2}$ isomer detected by GC-FID (figure 6-8), found to be produced by the Δ5 desaturase could not be identified by GC-MS as it was presumed to elute at the same time as the high abundance $C_{18:1}$ or $C_{18:2}$ Δ9, 12 fatty acids. Therefore the putative $C_{18:2}$ isomer identity was given based on the GC-FID retention time, eluting after $C_{18:2}$ Δ9, 12 but before the $C_{18:3}$ fatty acids. This isomer was given the designation (b) in the following table.

Table 6-1. Total lipid fatty acid composition of each recombinant *Phaffia* isolate. Isolates 6938 and 6939 are controls with non-recombinant plasmids. Δ5 isolates contain the addition of the Δ5 desaturase, whilst elo isolates contain the addition of the Δ6 elongase (GLELO). Distinguishing peaks were a putative $C_{18:2}$ isomer (a), found in all elo isolates and Δ5 n7 isolates, another putative $C_{18:2}$ isomer (b), found only in Δ5 n7 isolates. $C_{18:3}$ n6 was found only in isolate Δ5 n7. $C_{20:2}$ n6 and $C_{20:3}$ n3 were found only in elo isolates. Unknown compounds were given their relative retention times as identifications. Fatty acid values are stated as the % (w/w) of the total lipid fraction

Organism ID	% FA	C14:0	C15:0	C16:0	C16:1	C17:1	C18:0	C18:2 isomer (a)	C18:1 cis	1.17	1.198	1.206	C18:2 cis	C20:0	C18:2 isomer (b)
6938	Avg	0.34	0.00	12.54	0.80	0.00	3.55	0.00	24.17	0.00	0.24	0.00	49.03	0.72	0.00
	St Err	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
6939	Avg	0.31	0.00	12.73	0.80	0.00	3.57	0.00	24.50	0.00	0.11	0.00	49.41	0.72	0.00
	St Err	0.00	0.00	0.02	0.01	0.00	0.00	0.00	0.03	0.00	0.06	0.00	0.12	0.01	0.00
Δ5 n2	Avg	0.49	0.15	14.28	0.42	0.26	4.41	0.00	29.57	0.33	0.18	0.00	45.15	1.03	0.00
	St Err	0.00	0.00	0.07	0.00	0.00	0.05	0.00	0.01	0.00	0.00	0.00	0.24	0.01	0.00
Δ5 n7	Avg	0.44	0.13	13.75	0.30	0.17	3.74	0.54	32.45	0.13	0.59	0.39	40.81	1.57	0.67
	St Err	0.00	0.00	0.04	0.00	0.00	0.05	0.08	0.04	0.00	0.06	0.05	0.11	0.00	0.01
elo n2	Avg	0.57	0.11	14.52	0.62	0.11	3.03	0.20	27.80	0.00	0.31	0.11	45.66	0.91	0.00
	St Err	0.00	0.00	0.09	0.01	0.00	0.05	0.04	0.15	0.00	0.06	0.05	0.27	0.00	0.00
elo n3	Avg	0.63	0.12	14.18	0.75	0.12	2.35	0.24	25.93	0.10	0.42	0.19	47.80	0.83	0.00
	St Err	0.00	0.00	0.02	0.00	0.01	0.02	0.01	0.16	0.00	0.01	0.01	0.02	0.00	0.00
elo n4	Avg	0.57	0.19	15.78	0.45	0.53	3.63	0.34	36.58	0.58	0.40	0.00	35.49	1.34	0.00
	St Err	0.00	0.00	0.03	0.00	0.00	0.02	0.14	0.06	0.00	0.12	0.00	0.16	0.00	0.00
elo n5	Avg	0.78	0.22	17.77	0.90	0.34	2.31	0.32	27.71	0.15	0.37	0.12	43.79	0.54	0.00
	St Err	0.00	0.00	0.10	0.01	0.00	0.01	0.00	0.11	0.00	0.09	0.00	0.49	0.00	0.00
elo n6	Avg	0.52	0.18	13.92	0.39	0.37	3.26	0.87	28.30	0.41	0.53	0.31	44.29	1.18	0.00
	St Err	0.01	0.00	0.08	0.02	0.01	0.04	0.27	0.24	0.01	0.04	0.05	1.17	0.02	0.00
elo n7	Avg	0.39	0.19	12.14	0.30	0.47	4.10	0.13	28.90	0.34	0.19	0.00	43.43	1.23	0.00
	St Err	0.01	0.00	0.21	0.01	0.00	0.03	0.00	0.56	0.00	0.00	0.00	0.94	0.02	0.00
elo n7a	Avg	0.51	0.24	14.43	0.40	0.66	3.64	0.03	33.27	0.68	0.18	0.00	39.99	1.32	0.00
	St Err	0.00	0.00	0.05	0.00	0.00	0.03	0.00	0.21	0.01	0.00	0.00	0.40	0.02	0.00
elo n8	Avg	0.56	0.24	15.59	0.45	0.65	3.80	0.06	34.93	0.92	0.16	0.00	36.47	1.29	0.00
	St Err	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.21	0.07	0.01	0.00	0.28	0.01	0.00
elo n9	Avg	0.56	0.23	15.07	0.48	0.64	3.38	0.20	33.33	0.66	0.30	0.08	40.36	1.22	0.00
	St Err	0.00	0.00	0.16	0.00	0.00	0.03	0.07	0.43	0.00	0.06	0.04	0.76	0.03	0.00
elo n10	Avg	0.66	0.10	15.21	0.62	0.10	2.66	0.65	29.92	0.11	0.50	0.28	40.10	1.03	0.00
	St Err	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.07	0.02	0.06	0.06	0.23	0.01	0.00

Organism ID	$C_{18:3}$ n6	1.284	1.289	C20:1	$C_{18:3}$ n3	C21:0	C20:2 n6	C22:0	1.43	C20:3 n3	1.487	C24:0	total lipid mg/g
6938	0.00	0.00	0.00	0.00	1.37	0.00	0.00	0.35	0.00	0.00	0.00	0.35	14.55
	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
6939	0.00	0.00	0.00	0.00	1.36	0.00	0.00	0.36	0.00	0.00	0.00	0.35	14.44
	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.26
Δ5 n2	0.00	0.00	0.00	0.00	1.08	0.00	0.00	0.66	0.00	0.00	0.00	0.65	29.42
	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.59
Δ5 n7	0.09	0.00	0.00	0.18	1.27	0.00	0.00	1.16	0.10	0.00	0.02	0.95	42.39
	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.01	0.00	0.00	0.00	0.01	1.04
elo n2	0.00	0.00	0.00	0.17	1.00	0.00	0.90	0.81	0.00	0.18	0.03	0.96	30.68
	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.00	0.00
elo n3	0.00	0.00	0.10	0.39	1.04	0.00	2.31	0.78	0.00	0.37	0.06	0.95	36.82
	0.00	0.00	0.01	0.00	0.00	0.00	0.02	0.01	0.00	0.00	0.00	0.01	0.13
elo n4	0.00	0.01	0.09	0.19	0.41	0.05	0.67	0.95	0.10	0.07	0.04	0.81	50.38
	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.01	6.32
elo n5	0.00	0.03	0.07	0.15	1.14	0.00	0.82	0.61	0.07	0.18	0.05	0.68	49.98
	0.00	0.00	0.01	0.00	0.03	0.00	0.10	0.05	0.00	0.00	0.00	0.00	0.99
elo n6	0.00	0.13	0.11	0.17	0.57	0.00	1.25	0.98	0.11	0.23	0.00	0.64	36.53
	0.00	0.00	0.01	0.00	0.03	0.00	0.29	0.11	0.00	0.07	0.00	0.01	1.20
elo n7	0.00	0.12	0.05	0.00	0.84	0.00	0.20	0.90	0.00	0.00	0.00	0.79	42.39
	0.00	0.00	0.00	0.00	0.02	0.00	0.01	0.02	0.00	0.00	0.00	0.01	1.04
elo n7a	0.00	0.12	0.09	0.21	0.47	0.06	0.77	0.94	0.00	0.04	0.04	0.75	34.97
	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.03	0.00	0.00	0.00	0.01	0.32
elo n8	0.00	0.06	0.07	0.34	0.44	0.06	1.33	0.90	0.06	0.15	0.04	0.78	50.71
	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.00	4.96
elo n9	0.00	0.12	0.09	0.10	0.51	0.06	0.35	0.86	0.00	0.00	0.04	0.74	50.35
	0.00	0.01	0.00	0.00	0.01	0.00	0.01	0.02	0.00	0.00	0.00	0.02	0.45
elo n10	0.00	0.00	0.00	0.88	0.44	0.00	3.73	0.91	0.16	0.31	0.04	0.90	53.72
	0.00	0.00	0.00	0.01	0.00	0.00	0.02	0.00	0.01	0.00	0.00	0.01	2.82

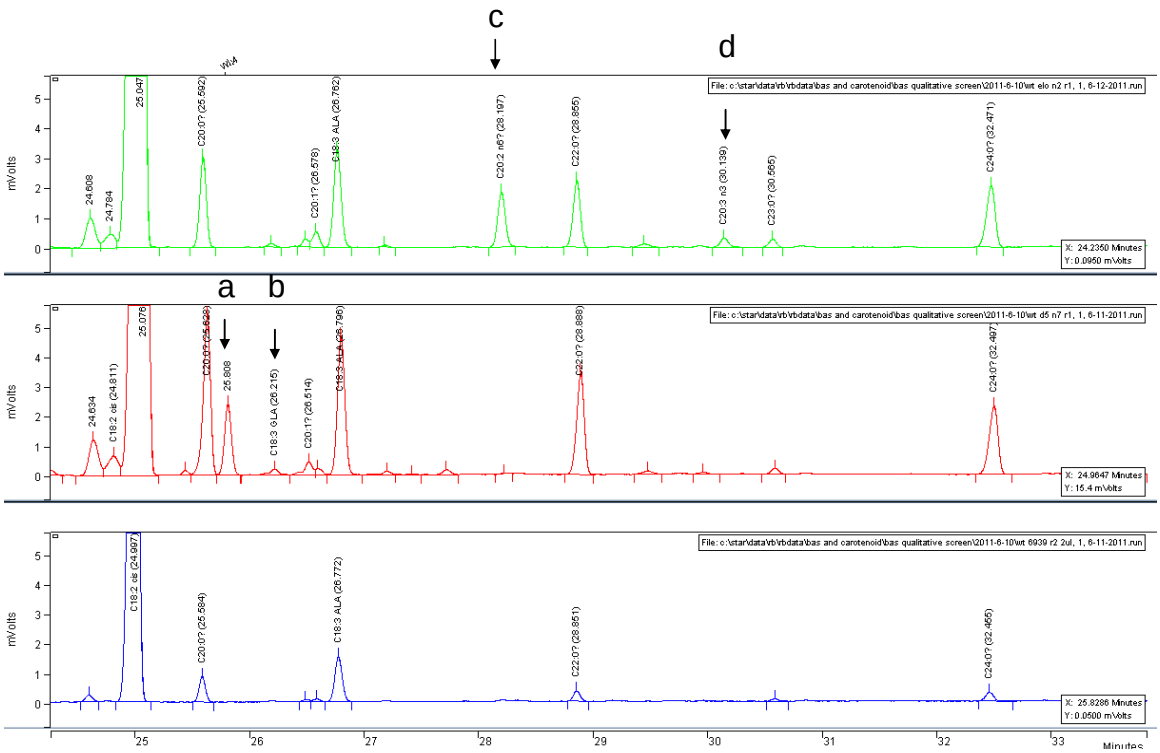


Figure 6-8. GC-FID trace of elo n2 (top), Δ5 n7 (middle) and control 6939 (bottom). Highlighted peaks thought to be the result of the recombination are; a = $C_{18:2}$ isomer (b), b = $C_{18:3}$ n6, c = $C_{20:2}$ n6, d = $C_{20:3}$ n3. The putative $C_{18:2}$ isomer (a) is not shown. The identity of (b) as an isomer of $C_{18:2}$ was made on the basis of elution time, as the peak elutes after $C_{18:2}$ Δ 9, 12 but before $C_{18:3}$ n6. No GC-MS data was obtained for this peak. Also of note are the much lower lipid levels of the control.

The two Δ5 isolates however are different as the Δ5 no. 7 isolate contains all three novel fatty acids whilst isolate no. 2 contains none of the novel fatty acids. The fatty acid complement of isolate no. 2 is therefore very similar to the control isolates although strain Δ5 no. 2 does contain $C_{15:0}$, $C_{17:1}$ and a peak with the RRT of 1.17, as well as higher lipid levels, similarities shared with the other recombinant isolates. The absence of the three peaks from the control samples may be due to the general low abundance of lipid found in these isolates. The Δ6 elongase recombinant isolates share the production of $C_{18:2}$ isomer (a) with isolate Δ5 no. 7, as well as producing two unique peaks, identified as $C_{20:2}$ n6 and $C_{20:3}$ n3. $C_{20:2}$ n6 was identified by retention index on both GC-FID and GC-MS, as well as through the custom built fatty acid mass spectrometry library. $C_{20:3}$ n3 could not be detected using GC-MS due to the low abundance of the compound but was again identified by retention index.

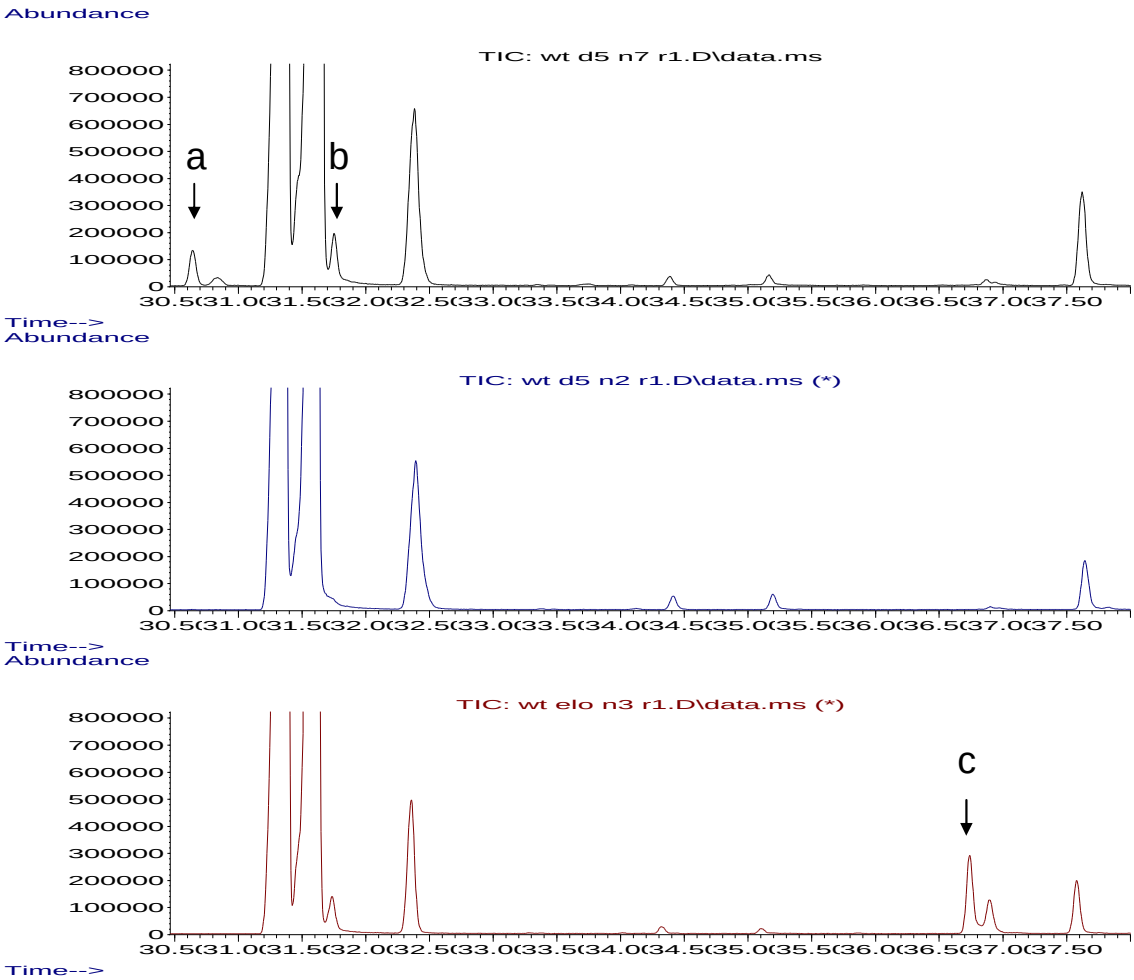


Figure 6-9. GC-MS trace of Δ5 n7 (top), Δ5 n2 (middle) and elo n3 (bottom). Highlighted peaks thought to be a result of the recombination are; a = $C_{18:3}$ n6, b = $C_{18:2}$ isomer (a), c = $C_{20:2}$ n6. $C_{18:3}$ n6 was found present in isolate Δ5 n7 only, although a peak of similar elution time was found in elo isolates using GC-FID. $C_{18:2}$ (a) was searched using the NIST library and putatively identified as $C_{18:2}$ Δ14, 17. This peak was only found in elo and Δ5 n7 isolates. $C_{20:2}$ n6 was found only in elo isolates though $C_{20:3}$ n3 was not identified using GC-MS, due to low abundance.

6.3 Discussion

The initial aim of transforming *Phaffia rhodozyma* with the three genes responsible for $C_{20:5}$ n3 production was unsuccessful. However the individually inserted genes exhibited some minor activity by producing novel fatty acids within the yeast. The identities of the fatty acids were made either through retention indices or mass spectrum, although the new complement of fatty acids could mostly be explained through the action of the inserted enzymes. Starting with the recombinant elo isolates, $C_{20:2}$ n6 and $C_{20:3}$ n3 could be explained by the action of the Δ6 elongase on $C_{18:2}$ n6 and $C_{18:3}$ n3 respectively. The Δ6 elongase is reported to act predominantly on $C_{18:3}$ n6 and $C_{18:4}$ n3, presumably because both share a double bond in the Δ6 position. The catalysis

by the Δ6 elongase of $C_{18:2}$ n6 and $C_{18:3}$ n3 however, is relatively minimal although the higher abundance of $C_{20:2}$ n6 over $C_{20:3}$ n3 is likely explained by the much higher abundance of the precursor $C_{18:2}$ n6 to $C_{18:3}$ n3 respectively. The average conversion rate was estimated to be 2.8% and 17.2% for $C_{18:2}$ and $C_{18:3}$ n3 respectively. The most effective method for calculating substrate conversion is the isolation of the Δ6 elongase into microsomes, followed by the supplementation of single fatty acids in CoA form. The data presented here would indicate that $C_{18:3}$ n3 is more effectively used by the Δ6 elongase than $C_{18:2}$, implying that the number of double bonds is important for the function of the elongase even though neither fatty acid contains a double bond in the Δ6 position. These results also demonstrate that the Δ6 elongase from *Mortierella alpina* can work with the other endogenous components of the fatty acid elongation system found within *Phaffia rhodozyma*, such as the two reductases and dehydrase, which are separate from the condensing elongase enzyme.

The $C_{18:2}$ isomer (a) was found in all Δ6 elongase isolates as well as Δ5 desaturase isolate no. 7. This was putatively identified as $C_{18:2}$ Δ14, 17 due to NIST mass spectrometry library searching. The localisation of double bonds in mono- and dienes using electron impact mass spectrometry is considered not particularly feasible due to the relocation of double bonds during the ionisation process. The only definitive method for double bond localisation is to derivatize the double bond into more stable structures such as dimethyl disulphide abducts (Christie, 1998). The mass spectrum data do indicate that the $C_{18:2}$ (a) isomer shares features of both a monoene and a diene. The molecular ion for $C_{18:2}$ (a) is predominantly at 294 m/z, indicating a diene. The subsequent loss of the CH_3O ($M\text{-}31^+$) group indicates that the isomer is a monoene, as a mass peak of 264 m/z is predominant in both $C_{18:1}$ and in $C_{18:2}$ (a) with ($M\text{-}32^+$) common for this class, indicating a loss of an addition proton. The isomer also produces a clear ion at 222 m/z, shared with $C_{18:1}$. This is possibly the product of a dual fragmentation as shown in figure 6-11. An ion at 180 m/z is also shared with the monoene $C_{18:1}$. Evidence for the isomer being a diene lies in the homologous series of smaller fragments, with ions such as 67, 81 and 95 m/z distinguishing the isomer from the monoene $C_{18:1}$ and placing it with the diene $C_{18:2}$ Δ9, 12. The consistent mass difference of 2 m/z units between the isomer and $C_{18:1}$ in the mid-range m/z homologous series also indicates the presence of another double bond. Finally, the retention index of the compound places it with the monoenes, however it is likely that it is a diene which shares monoene characteristics. Regarding the Δ5 desaturase's activity it is possible that

$C_{18:1}$ Δ9 has been desaturated further to $C_{18:2}$ Δ5, 9, although how this would be produced via the Δ6 elongase is unknown.

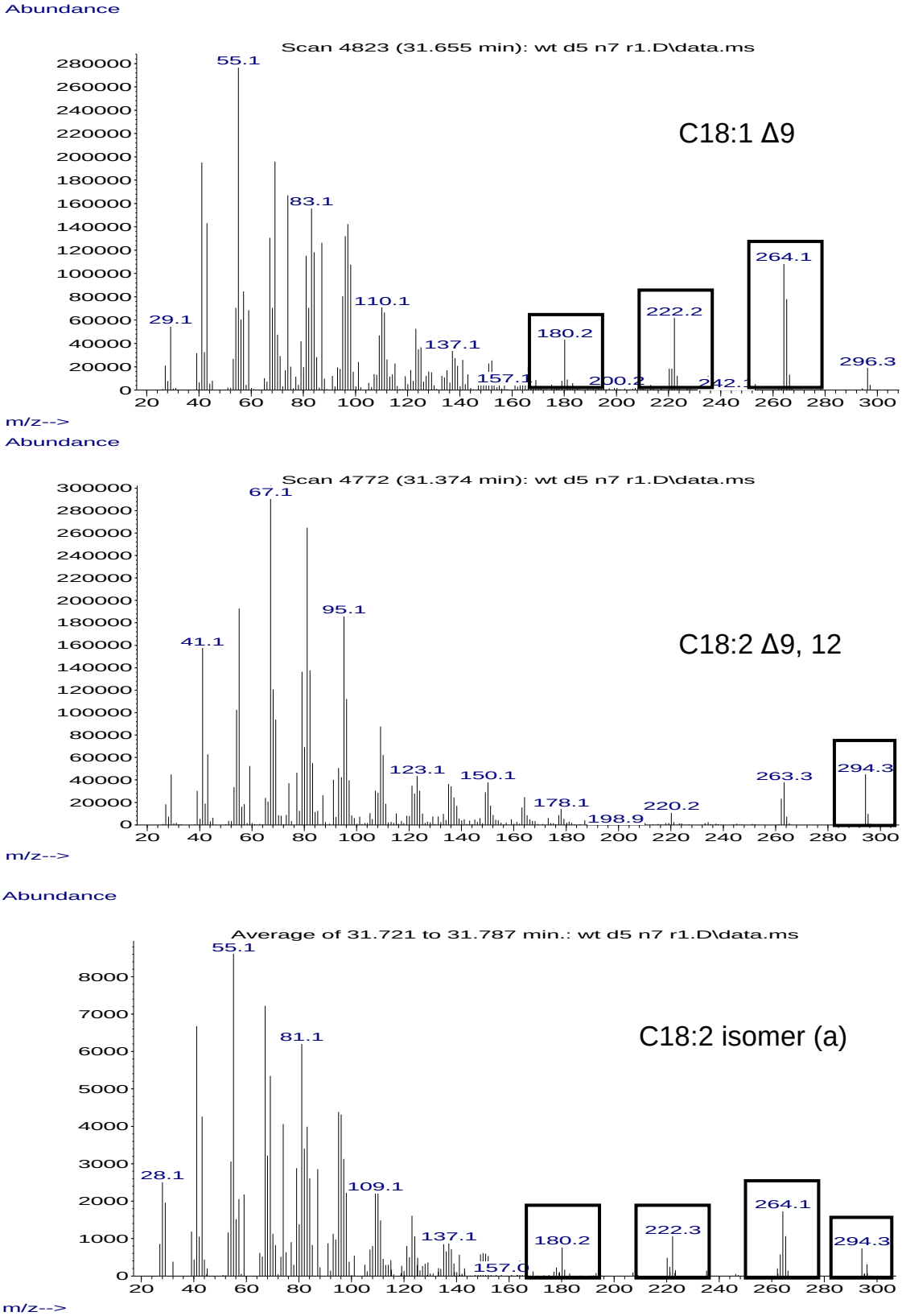


Figure 6-10. Mass spectrums of $C_{18:1}$ Δ9, $C_{18:2}$ Δ9, 12 and the proposed $C_{18:2}$ isomer (a). Isomer (a) shares features with both mono and dienes. The molecular mass indicates that the isomer is a diene (294 m/z), however the three subsequent ions (264, 222, and 180 m/z)

are indicative of a monoene. The homologous series of 55, 81, 95 and 109 m/z fragments indicate that the isomer contains two double bonds.

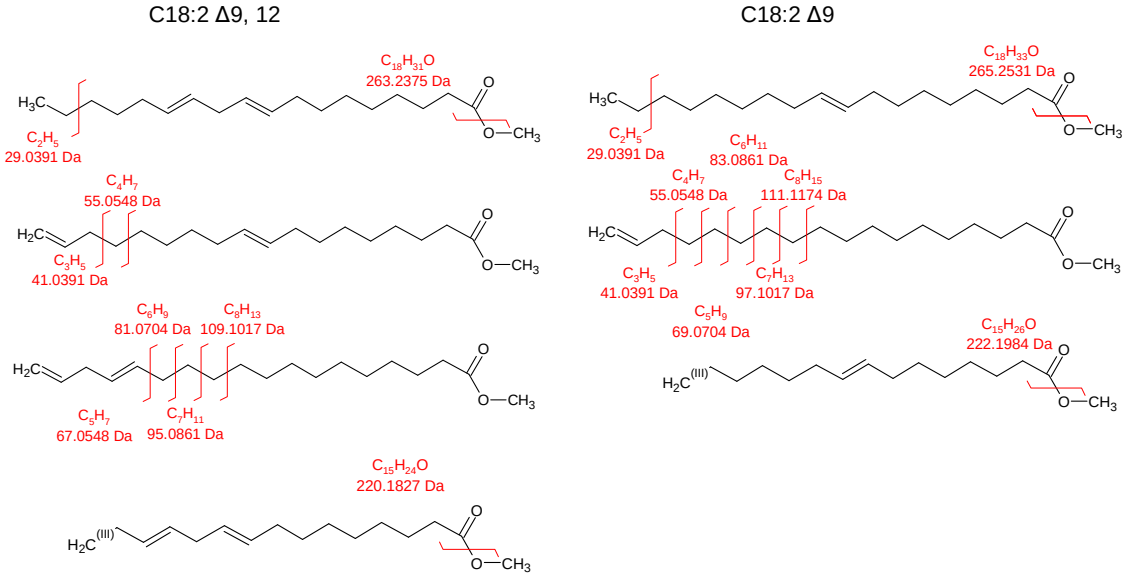


Figure 6-11. The proposed fragmentation pattern displayed by $C_{18:1}$ Δ9 and $C_{18:2}$ Δ9, 12. Electron impact mass spectrometry of mono and dienes is less effective at localising double bond positions, due to the migration of double bonds to the omega end of the chain. This migration of bonds explains the fragmentation pattern differences between monoenes and dienes. The cleavage of the CH_3O group results in a mass loss of ($M-31^+$) for dienes, though monoenes experience ($M-32^+$) resulting in a fragment of 264 m/z and not 265 m/z as shown here, indicating an additional proton has been lost. The fragment at 222 m/z can be explained through a double fragmentation, whereby the terminal propyl group is lost, as well as the CH_3O group.

The $C_{18:2}$ isomer (b) was only detected using GC-FID, possibly because the peak was obscured by the much larger $C_{18:1}$ Δ9 and $C_{18:2}$ Δ9, 12 peaks on the GC-MS. Therefore the only information regarding this peak is the retention time, which places the fatty acid as a late eluting $C_{18:2}$ isomer or as an early eluting $C_{18:3}$ isomer. This could mean, taking into account the activity of the Δ5 desaturase, the fatty acid in question may be $C_{18:3}$ Δ5, 9, 12, with $C_{18:2}$ Δ9, 12 acting as the substrate. The presence of $C_{18:3}$ n6 in the Δ5 desaturase isolate no. 7 is difficult to explain, as a double bond is inserted into the Δ6 position, which may be an unspecific reaction of the Δ5 desaturase. In general the conversion efficiency for both enzymes was relatively poor, due to the fact that the intended substrate was not present for the inserted enzymes to act upon. Also of note was that the $C_{18:3}$ n3 pool was relatively low within this strain of *Phaffia rhodozyma* and that if all three genes had been successfully inserted into the yeast, the yield of end product $C_{20:5}$ n3 would likely be low in abundance. Therefore additional strains of *Phaffia rhodozyma* require examination in search of elevated levels of $C_{18:3}$ n3. The specificities of the two inserted enzymes have been studied with the Δ5 desaturase

having been found by one group to desaturate only fatty acids containing a Δ8 double bond (Hornung *et al.*, 2005), although the recombinant yeast did not produce any $C_{18:2}$. This was confirmed by Domergue *et al*. who also showed that the Δ5 desaturase had activity on $C_{20:2}$ Δ11, 14 (Domergue *et al.*, 2003). The Δ5 desaturase has been found to minimally desaturate $C_{18:1}$ Δ9 to $C_{18:2}$ Δ5, 9 as well as $C_{18:1}$ Δ11 to $C_{18:2}$ Δ5, 11 (Saito *et al.*, 2000). The Δ5 desaturase studied was from the slime mould *Dictyostelium discoideum*, although the production of $C_{18:2}$ Δ5, 9 in Δ5 expressed recombinant yeast supports the putative production in *Phaffia*. The production of $C_{18:2}$ Δ5, 11 is less likely in *Phaffia* as the Δ5 desaturase recombinant yeast was supplied with exogenous $C_{18:1}$ Δ11. The yeast however was incapable of producing any trienoic fatty acids and therefore it is increasingly likely that isomer (b) is in fact $C_{18:3}$ Δ5, 9, 12. The Δ6 elongase was found to have additional activity on $C_{18:2}$ Δ9, 12 by converting it to $C_{20:2}$ Δ11, 14 (n6) as was documented in a patent by Mukerji *et al.* (Mukerji *et al.*, 2003), although supplementation with $C_{18:3}$ n3 did not yield $C_{20:3}$ n3. The elongation of $C_{18:2}$ Δ9, 12 was also reported in a recombinant *Aspergillus* expressing the Δ6 elongase (Takeno *et al.*, 2005).

The expression of the Δ6 elongase within *E. coli* appeared to be successful due to the presence of a 38.3 KDa band on the Western blot, detected using anti-His antibodies and stained using BCIP/NBT. The possible presence of the same band in the control may be due to contamination from the adjacent lane, inferred due to the non-uniformity of the band. The initial aim of inserting the Δ6 elongase into *E. coli* was as an alternative route to determining the substrate specificity of the elongase. With the successful Δ6 elongase transformation of *Phaffia rhodozyma*, functional characterisation of the elongase could be carried out within the yeast using previously mentioned exogenous fatty acid supplementation studies or endosome production methods. The bacterial expression of the elongase and the establishment of whether ELO class elongases can function within bacteria and utilise the other components required for successful elongation may in itself be novel. There are several points that need to be considered regarding recombinant systems and their use for VLCPUFA production in both yeast and plants. Firstly, the low production of fatty acids longer and more unsaturated than $C_{20:3}$ stems from the poor conversion between substrate classes. As discussed in the introductory chapter, elongases in fungi require acyl-CoA substrates whereas fungal desaturases require phospholipid linked acyl substrate. Specifically, it is thought that one problem arises after the Δ6 desaturation step, which occurs in phosphatidylcholine, position sn-2. Endogenously produced $C_{18:3}$ n6 formed in PC is

thought to be transferred to neutral lipid via several acyl-CoA independent pathways (Domergue *et al.*, 2003). This segregation of $C_{18:3}$ n6 from the acyl-CoA pool reduces the substrate available for the Δ6 elongase. As explored by Domergue *et al.* supplementation of exogenous $C_{18:3}$ n6 results in an increase in the $C_{18:3}$ n6 acyl-CoA pool which is subsequently elongated to $C_{20:3}$ n6. The acyl-CoA pool in yeast it appears can be indiscriminately transferred by a variety of acyl-CoA transferases into most lipid classes. Therefore, it is the transfer between substrate carriers that is thought to be a major bottleneck for the efficient production of VLCPUFA. For efficient production of fatty acids beyond $C_{20:3}$, alternating pools of phospholipid linked substrate are required for desaturation, specifically PC in position sn-2, although the Δ12 desaturase has been found not to be head group or position specific (Domergue *et al.*, 2003). The acyl chain then needs to be converted to acyl-CoA by an acyl-CoA:lyso-phosphatidylcholine to increase the available substrate for elongation. Following elongation, the acyl chain needs to be attached again to PC by the same enzyme. It is this enzyme which in plants is thought to also be bottleneck, with some plant transferases incapable of transferring acyl chains with a Δ6 double bond (Domergue *et al.*, 2005). Therefore an efficient acyl-CoA:lysophospholipid acyltransferase (LPLAT) enzyme capable of accepting acyl chains with a Δ6 double bond is essential for efficient VLCPUFA production. Currently the LPLAT from *C. elegans* has been studied as a potential transferase to improve VLCPUFA yields (Renz *et al.*, 2009). It is also possible that *Mortierella alpina* contains an efficient transferase system which allows for high accumulation of $C_{20:4}$ n6. Other issues which could effect VLCPUFA production may arise if acyl chains are transferred to the wrong phospholipid class or to the wrong position. This would prevent efficient desaturation by specific desaturases. One proposed solution is the use of mammalian like desaturases which use acyl-CoA substrates instead, such as the Δ6 desaturase from *Marchantia polymorpha* (Kajikawa *et al.*, 2004). This would mean only one pool of substrate is required for elongation and desaturation with both sets of reactions being able to occur sequentially.

6.4 Conclusion

The initial reconstitution of the *Mortierella alpina* fatty acid elongation pathway within *Phaffia rhodozyma* was unsuccessful. However some enzymatic activity was detected which correlated with the inserted gene. The insertion of the Δ6 elongase resulted in the elongation of $C_{18:2}$ n6 and $C_{18:3}$ n3 to $C_{20:2}$ n6 and $C_{20:3}$ n3 respectively.

The recombinant *Phaffia* strain expressing the Δ5 desaturase was found to produce two novel fatty acids, one thought to be $C_{18:2}$ Δ5, 9 and the other putatively identified as $C_{18:3}$ Δ5, 9, 12. The expression of all three genes however would likely lead to a poor conversion to the end product $C_{20:5}$ n3, due to a low $C_{18:3}$ n3 substrate pool within the *Phaffia* strain used. The fact that elongation and desaturation require different substrate pools, that endogenous Δ6 desaturation may result in desaturated product being sequestered within neutral lipid in an acyl-CoA independent manner, and that LPLAT in certain organisms may select against fatty acids to transfer between the acyl-CoA pool and phospholipid carriers, may also contribute to low conversion efficiency. The identification however of these bottlenecks may allow for their circumvention by selection of substrate specific and efficient LPLAT enzymes, the use of acyl-CoA accepting desaturases and selection of high $C_{18:3}$ n3 producing strains of *Phaffia*. The first goal however is the successful VLCPUFA biosynthesis pathway within *Phaffia rhodozyma*. The expression of the Δ6 elongase within *E. coli* appears to be successful with the visualisation of a protein using His tag Western blotting matching the expected size of the protein. The function of the expressed bacterial protein was not tested, however the expression within *Phaffia* may provide a more reliable method for establishing substrate specificity.

7 Conclusions

In the search for novel producers of VLCPUFAs it was found that the location of isolation was not the only consideration when identifying new sources of VLCPUFA producing fungi. A key consideration is the taxonomy of the organism. Organisms from the Zygomycota, Chytridiomycota and Oomycota have been demonstrated to be the few phyla capable of producing these desirable fatty acids and as such should be the primary targets in further screening efforts (Table 3-5). Studying species within these phyla isolated from low temperature environments may increase the chance of finding VLCPUFA producing fungi. Whilst the majority of zygomycetes produce only up to $C_{18:3}$ n6 the fact that several known species such as *Mortierella, Conidiobolus* and *Entomophthora* are capable of VLCPUFA production would indicate that more species from within this phyla are capable of VLCPUFA production. The current suggestion that Blastocladiales and Entomophthorales are more related than to other organisms from within their traditional phyla may suggest an ancestral link from the Chytridiomycota to the Zygomycota (section 5.3.1). The presence of the Δ6 elongase, which appears only in basal organisms, and its position within the Mucorales cluster (Figure 5-26) suggests that *Mortierella* like species may have led to the evolution of the Mucorales. Additionally chytrids and Oomycetes, both of which are capable of inhabiting aqueous environments are very likely to contain VLCPUFAs due to the proposed role these highly unsaturated fatty acids play in the motility of the flagellated zoospore stage, as well as possible protection from osmotic stress (section 3.3.2). The move to terrestrial ecosystems has led to the loss of VLCPUFA production from the Zygomycetes, the direct descendants of the Chytridiomycetes, having lost the flagellated zoospore stage as well as VLCPUFA production in most cases. What remains is the precursor, $C_{18:3}$ n6 which once led to the production of $C_{20:4}$ n6 and $C_{20:5}$ n3.

It was found that the loss of VLCPUFA production was in part due to the loss of a Δ6 elongase known as GLELO which catalyses the reaction $C_{18:3}$ n6 to $C_{20:3}$ n6 within the genus *Mortierella*. The loss of this elongase differentiates *Mortierella* from the majority of the zygomycetes as well as the ascomycetes and basidiomycetes. The correlation between fatty acids longer and more unsaturated than $C_{18:3}$ n6 and the presence of the GLELO gene, confirms the gene's proposed function, as without it polyunsaturated fatty acid production halts at $C_{18:3}$ n6 (Figures 5-5, 5-6). The Ascomycota and

Basidiomycota have evolved further and lost the similarities shared between the Zygomycota and the Chytridiomycota with the total loss of the Δ6 elongase and the shift in fatty acid pathway to the n3 route as oppose to the n6 route (Table 3-4). Low temperature environments have not appeared to prevent the loss of the Δ6 elongase, nor has it stimulated a change in function of an elongase to elongate past the end point fatty acid $C_{18:3}$ n3. However it does appear that this complement of fatty acids is sufficient for organism growth at low temperatures as was demonstrated with *Penicillium rugulosum*, which produced the greatest growth at 5°C, greater than any other organism studied (Figure 4-12). This would appear to indicate that other factors limit growth at low temperatures such as protein adaptation, and that either trienoic fatty acids provide enough membrane fluidity at low temperatures or other unstudied compounds within the membrane assist in maintaining the required fluidity. The role of unsaturated fatty acids was highlighted by 5°C growth promoting the greatest production of the most unsaturated fatty acid in all isolates studied except *Mortierella alpina* isolate 82072, where almost equal amounts of $C_{20:5}$ n3 at both 5 and 15°C growth were produced. This highlights the importance highly unsaturated fatty acids play in low temperature survival. In regards to unsaturation index, total fatty acid unsaturation indices showed six out of nine organisms displaying the greatest unsaturation at 5°C growth, whilst two of the nine displayed the greatest unsaturation at 15°C growth. *Herpotrichia* sp. isolate 403016 showed the greatest unsaturation index at 25°C although 5°C gave a very similar average value (Figure 4-10). This confirms the idea that low temperatures generally induce the greatest unsaturation of fatty acids as well as promoting the greatest formation of the most unsaturated fatty acid.

The localisation of the fatty acid to individual lipid components failed to conclusively segregate the lipids due to the large proportion of neutral lipid within the *Mortierella* isolate. However it does appear that PUFAs reside within the PE and PC fractions as well as within the neutral lipids (Figures 4-13, 4-16). Whilst the total phospholipid unsaturation index displayed a positive correlation against temperature the PC fraction illustrated the expected greatest unsaturation value under the lowest culture temperature (Figure 4-19). The localisation of VLCPUFAs to the PC fraction is confirmed by the finding that acyl linked PC is the substrate for the Δ5 and Δ6 desaturases. As such, it is possible that the PC fraction is the primary modulator of membrane fluidity, as the acyl chains attached to this membrane lipid are directly desaturated. Other supporting evidence for the localisation of VLCPUFAs to the PC and PE fractions is due to the physical properties of the head groups. PC has a wide ranging transition temperature

range allowing for precise alteration in fluidity in response to temperature by acyl chain modification. PE on the other hand has a high and relatively narrow transition temperature range, requiring highly unsaturated fatty acids to maintain the fluidity of the acyl chains when attached to this phospholipid (Section 4-3). Finally it was thought that *Mortierella alpina* possesses an efficient mechanism for transferring acyl chains between the acyl-CoA pool and the phospholipid head group due to the high levels of $C_{20:4}$ n6, allowing for elongation in the CoA form, and desaturation in the phospholipid form. It is this transfer of acyl substrate from phospholipid to acyl-CoA pools and back which poses a significant bottleneck to engineering high yield VLCPUFA producers (section 6-3). Utilising and directly modifying organisms capable of transcending this apparent bottleneck, such as *Mortierella alpina*, is one solution whilst another is the introduction of efficient acyl-CoA:lysophospholipid acyltransferase like enzymes alongside the required desaturases and elongases. Such limitations are likely to reduce initial yields of VLCPUFAs from new recombinant organisms engineered for high value lipid production. Initial fatty acid modification studies in the yeast *Phaffia rhodozyma* have shown that the Δ5 desaturase and the Δ6 elongase are functionally active when expressed individually, even when the preferred substrate is not present (Table 6-1). The production of $C_{20:2}$ n6 and $C_{20:3}$ n3, elongated from $C_{18:2}$ n6 and $C_{18:3}$ n3 respectively, demonstrate the functional activity of the Δ6 elongase. The putative identification of $C_{18:2}$ Δ5, 9 and $C_{18:3}$ Δ5, 9, 12 indicates that the Δ5 desaturase expressed in *Phaffia rhodozyma* is also functional. It is hoped that the co-expression of both aforementioned genes and a Δ6 desaturase within *Phaffia rhodozyma* will result in the production of $C_{20:5}$ n3. The potential value of a high astaxanthin, $C_{20:5}$ n3 producing *Phaffia* strain is of potential interest to the aquaculture industry, where both compounds are essential for the growth of farmed fish such as salmon.

Regarding the future screening of novel low temperature isolated fungi, ascomycetes and basidiomycetes do not appear to be prime targets for VLCPUFA production due to their evolutionary loss of the Δ6 elongase as well as the ability of trienoic fatty acids to regulate membrane fluidity sufficiently under low temperature conditions. The close ties to the chytrid ancestors make the zygomycetes a good choice when screening for VLCPUFAs, which under low temperature environments may promote the continued selection of the Δ6 elongase within a species, as well as inducing more efficient production of these desirable fatty acids. Chytrid and Oomycete isolates are also prime candidates for further screening due to their physiology and habitat, as well as being documented producers of VLCPUFAs. The screening of several hundred isolates

however is costly and time consuming. The development of a novel genomic screen was undertaken, which eliminates the need for preliminary fatty acid screening. A screen based on one gene, which allows for the prediction of specific metabolites across phyla is not always a simple task, and sometimes not possible at all. Specific issues that were demonstrated in this work for example were lack of conserved regions between genes and lack of characterised fungal gene sequences. This was shown by the Δ5 desaturase which demonstrated no highly homologous regions for which robust primers could be developed, as well as a lack of characterised fungal Δ5 desaturases. In other situations the presence of the gene does not specifically correlate with the production of the metabolite. In certain cases transcriptional regulation may prevent the metabolite from being produced, for example high temperature culture of *Mortierella alpina* strains resulted in the cessation of production of $C_{20:5}$ n3 (Figures 4-6, 4-7), presumably through down regulation of the Δ17 desaturase. Therefore for poorly studied metabolic pathways or novel metabolites, the first stage of screening will usually require biochemical analysis using platforms such GC-MS or HPLC. Only with greater understanding, is it possible to develop more efficient and less time consuming and resource intensive screening procedures. In this instance however, the screen was effective at identifying all *Mortierella* isolates that contained VLCPUFAs (Figures 5-5 to 5-7) and did not amplify elongases with different functionalities. The screen was also shown to work with the chytrid *Allomyces macrogynus* indicating that the screen may be utilised for both zygomycete and chytrid preliminary screening.

The Δ6 like elongase gene from *Allomyces macrogynus* was described utilising whole genome shotgun data and the amino acid sequence was hypothetically confirmed utilising SMART, which identified seven trans-membrane regions which constituted the ELO domain (Figure 5-16). When BLAST searched, the closest match for the protein was GLELO from *Mortierella alpina*. The putative *Allomyces* Δ6 elongase is sufficiently different to the currently used *Mortierella alpina* GLELO to warrant its use in a recombinant system (Figure 5-11). Currently, the Δ6 elongase from the zygomycete *Conidiobolus obscurus* (Tan *et al.*, 2011) is the most recent to be isolated and functionally characterised, with the final aim of fatty acid pathway elongation. The 125 bp conserved genomic region shared by all Δ6 like elongases from the fungi and Animalia was used for primer construction with the sequence found in only PUFA elongating elongases such as the Δ6, ELOVL2 and ELOVL5 classes (Figure 5-24). Conservation of the genomic sequence meant that ELOVL2 and ELOVL5 elongases can be detected within mammalian systems such as within *Mus musculus*, which was

used to test the specificity of the primer sets. The chromist *Saprolegnia parasitica* was found to contain a smaller fragment of the genomic conserved region preventing amplification. The genomic conservation was not found within terrestrial plants such as *Marchantia polymorpha* and *Physcomitrella patens*, however was found within the algae *Pyramimonas cordata* and *Parietochloris incisa* with all four organisms found within the Plantae. The close resemblance of the two terrestrial plant's Δ6 elongases when compared with the chlorophyte green algal elongases appear to confirm that Liverworts and mosses, which diverged from the Charophyta green algae, may have shared a common ancestor. The early branching of the Liverwort and Mosses from the terrestrial plant lineage bears resemblance to the evolution of the fungi. With the emergence of terrestrial species from aquatic environments, the presence of a flagellated cell during the life cycle and the capability of VLCPUFA production appear to be retained in basal plants and fungi. Several zygomycetes demonstrate an intermediary stage, producing VLCPUFA but no flagellated cell, which culminated in the abandonment of VLCPUFA production, as can be seen in the majority of zygomycetes and higher fungi. As with the higher fungi, no known vascular plant species is capable of VLCPUFA production, suggesting the loss of the Δ6 elongase. The elongase clustering also showed the relationship between the fungi and Animalia (Figures 5-18, 5-19), which are thought to have stemmed from a common ancestor, whereas the Chromista followed a different evolutionary path out from the Protozoa. This fungal/animal ancestor underwent change independently from the Chromista and incorporated changes that are both common to fungi and Animalia. The subsequent divergence of the two Kingdoms fungi and Animalia led again to independent changes between the two. This is seen in the elongase clustering, with the fungi grouped away from the Animalia, but both Kingdoms cluster together when compared with the Chromista, due to their common ancestor. As to why Plantae share similar sequence homology to the fungi is unknown. It is possible perhaps that residue changes occurred independently within both phyla which have coincidentally led to their greater similarity.

The amino acid translation of the conserved region was present in all elongases studied (Figure 5-20). The amino acid conserved region was consistently located between the third and fourth trans-membrane regions in PUFA accepting elongases (Table 5-3) indicating some possible functional role within this class of elongase although the location of the sequence within other elongase classes varied. The predicted number of trans-membrane domains for Animalia and fungal PUFA

elongating elongases was seven although the Oomycete *Saprolegnia parasitica* was calculated to contain only six. Non-PUFA elongating elongases contained five to seven trans-membrane domains, however these are predicted and may account for the varied numbers of domains as well as the differential localisation of the amino acid conserved region. Several amino acid residues were detected which distinguished PUFA accepting from non-PUFA accepting elongases (Figures 5-20 to 5-23) as were additional residue which characterised PUFA accepting elongases, and these were thought to play a role in substrate binding, possibly in allowing greater flexibility of the enzyme to accept the non-linear structure of PUFAs as functionally, the elongation procedure is identical. On comparing the elongases amino acid structure, the elongases clustered predominantly based on their function with the non-PUFA elongating ELO class grouping together and the PUFA elongating Δ6, ELOVL2 and ELOVL5 classes forming another large grouping (Figure 5-18, 5-19). In support of the evolutionary origins of the ascomycetes, protein analysis using ClustalW2 revealed the *Mortierella* elongases MALCE1 and MAELO branched away and were basal to the ELO class, indicating that these two elongases may be the progenitors of the ELO class found within *Saccharomyces cerevisiae*.

The development of this novel genomic screen based on the Δ6 elongase should provide an effective initial screen which will reduce the time and resources required for searching for novel VLCPUFA producers. The conserved nucleotide and amino acid sequences allow for a degree of elongase function determination, with whole genome sequences allowing for the subsequent isolation and functional characterisation of novel Δ6 elongases. Whilst the discovery of completely new fungal producers of VLCPUFAs is comparatively rare, the genetic recombination route provides an alternative pathway for the production of these desired fatty acids. Whilst novel fungal isolates may be capable of VLCPUFA production their amenability as an industrial organism may be questionable, with factors such as growth rate, biomass production, growth conditions and fatty acid composition playing a key role. This then makes the genes responsible, such as the Δ6 elongase valuable as they may be inserted into more industrially favourable organisms.

7.1 Future work

Further work would primarily focus on elongation of the fatty acid profile in *Phaffia rhodozyma*. Initial studies demonstrated that the individual expression of the Δ5 desaturase and Δ6 elongase within *Phaffia* resulted in minor modification of the fatty acid profile. It is hoped that all three genes may be successfully expressed within the yeast resulting in $C_{20:5}$ n3 production. With a successful transformation, the efficiency of VLCPUFA production can be studied, as can the effect fatty acid modification has on astaxanthin production. The efficiency of VLCPUFA production may be enhanced through the use of alternative LPLAT enzymes to enable efficient transfer of acyl substrate between phospholipid and CoA substrate pools. Such an enzyme may be found within *Mortierella alpina* due to the proficiency with which this organism produces $C_{20:4}$ n6, indicating efficient transfer between the two substrate pools. Identification, characterisation and expression of this gene/s may allow for greater yields of VLCPUFA within recombinant organisms. Further future work could continue the study of the remaining BAS isolates, however it should focus primarily on those species found from within the phyla Chytridiomycota and Zygomycota. The expression of the Δ6 elongase within *E. coli* may also be studied further to ascertain whether fungal elongases may function within Bacteria and to further characterise the Δ6 elongase through exogenous fatty acid feeding experiments. Finally, the separation of phospholipid components in *Mortierella* could be refined to allow more accurate localisation of fatty acids and in turn result in unsaturation indices which support the hypothesis that low temperature result in greater unsaturation within phospholipid fractions.

7.2 Bibliography

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7.3 Appendix

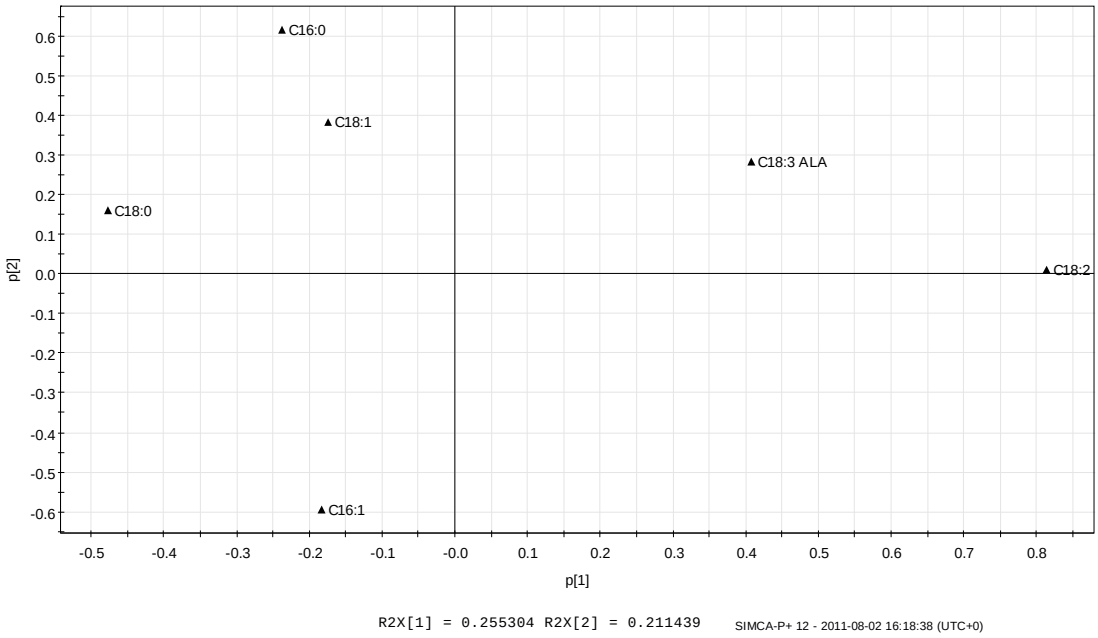

Figure 7-1. PLS-DA score plot for Figure 3-11.

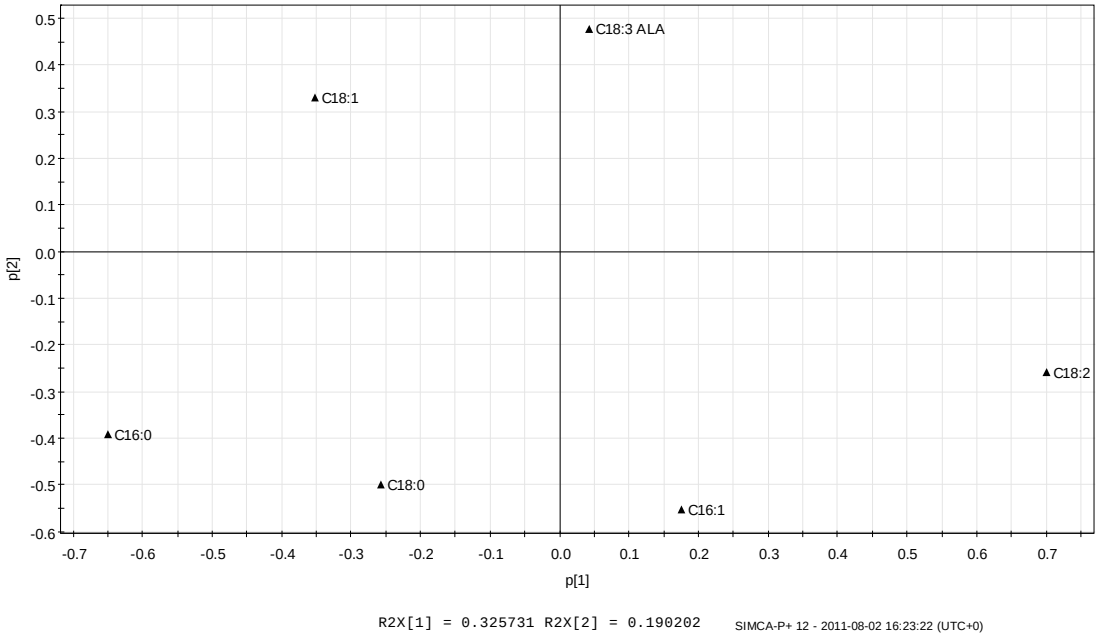

Figure 7-2. PLS-DA score plot for Figure 3-12.

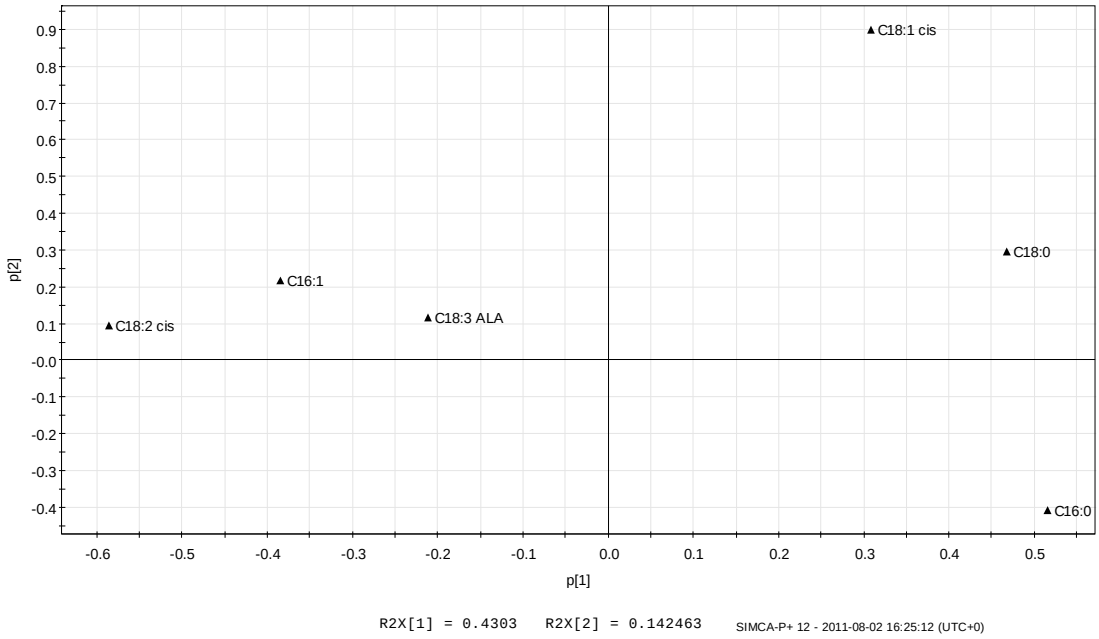

Figure 7-3. PLS-DA score plot for Figure 3-13.

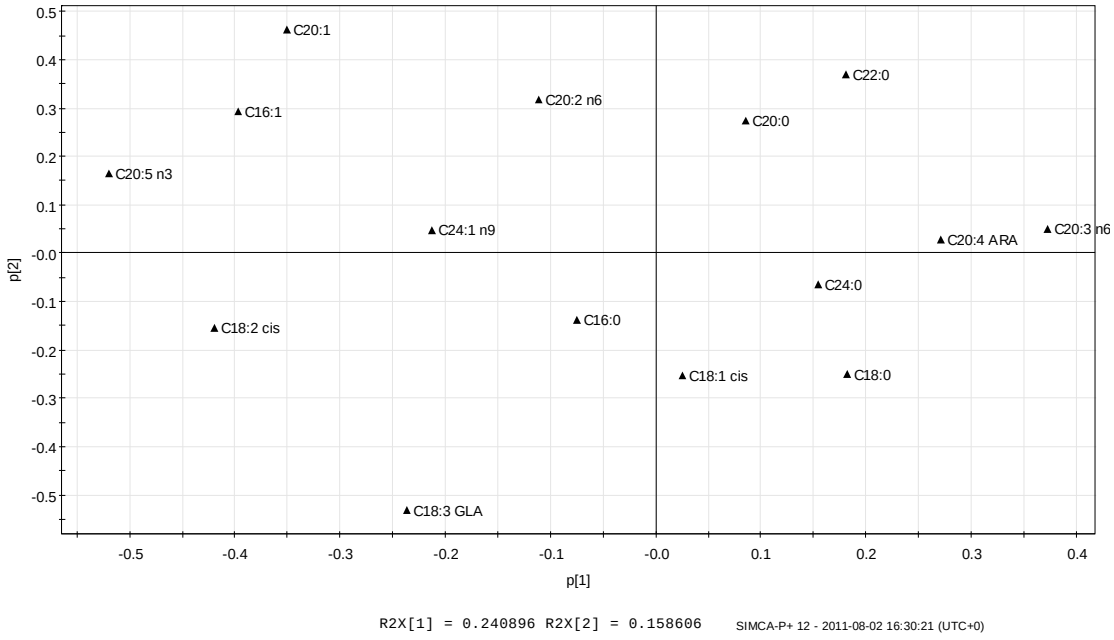

Figure 7-4. PLS-DA score plot for Figure 3-14.

Table 7-1. Fatty acid profiles of low temperature isolated fungi incapable of elongation beyond $C_{18:3}$ grown on MA and YES. Fatty acid values are stated as the % (w/w) of the total lipid fraction.

Organism	IMI/media	C14:0	C15:0	C16:0	C16:1	C17:1	C18:0	C18:1 cis	C18:2 cis	C20:0	C20:1	C18:3 ALA	C22:0	C22:1 n9	C24:0	C24:1 n9	Total lipid mg/g
	396505 yes	0.2	0.0	17.0	0.6	0.3	3.7	19.0	45.0	0.0	0.2	10.4	0.0	0.1	0.0	0.0	57.3
Herpotrichia sp.	403002 yes	0.0	0.0	15.4	1.7	0.0	6.0	18.6	41.1	0.0	0.0	7.7	0.0	0.0	0.0	0.0	9.6
Ascomycota	403004 yes	0.0	0.0	14.5	0.0	0.0	4.5	23.3	49.5	0.0	0.0	8.2	0.0	0.0	0.0	0.0	2.9
Leptodontidium sp.	403011 yes	0.0	0.5	13.5	1.2	0.5	1.9	42.5	26.8	0.0	0.0	8.6	0.0	0.0	0.0	0.0	18.5
Leptodontidium sp.	403012 yes	0.2	0.2	15.3	0.7	0.2	0.5	17.2	29.6	0.0	0.5	28.6	0.0	0.4	0.0	0.0	44.7
Herpotrichia sp.	403014 ma	0.0	0.0	23.2	1.5	0.0	5.4	33.2	33.5	0.0	0.0	2.3	0.4	0.0	0.0	0.0	61.3
Herpotrichia sp.	403015 ma	0.2	0.0	22.4	1.9	0.2	4.9	41.4	26.6	0.2	0.0	1.5	0.3	0.0	0.0	0.0	119.6
Herpotrichia sp.	403016 ma	0.0	0.0	16.1	1.3	0.0	2.6	20.8	53.1	0.0	0.0	5.6	0.6	0.0	0.0	0.0	25.8
Ascomycota	403017 ma	0.8	0.0	25.9	9.4	3.1	2.4	21.1	33.3	0.0	0.0	4.0	0.0	0.0	0.0	0.0	65.8
Ascomycota	403019 ma	0.2	0.0	21.6	6.5	0.6	4.0	34.7	28.6	0.0	0.0	3.7	0.0	0.0	0.0	0.0	52.5
Ascomycota	403020 ma	0.2	0.2	17.7	0.5	0.1	12.3	23.1	29.9	0.0	0.0	14.1	0.0	0.7	0.0	0.0	117.2
Ascomycota	403021 ma	0.0	0.0	21.4	1.6	0.2	5.5	49.6	19.1	0.3	0.0	1.6	0.2	0.0	0.0	0.0	174.4
Ascomycota	403023 ma	0.0	0.0	15.9	1.6	0.0	4.6	48.5	28.0	0.0	0.0	1.5	0.0	0.0	0.0	0.0	56.0
Ascomycota	403024 ma	0.0	0.0	18.8	1.6	0.0	4.1	43.5	26.2	0.0	0.0	2.4	0.0	0.0	0.0	0.0	56.9
Ascomycota	403025 ma	0.1	0.1	15.6	0.1	0.1	21.3	34.3	19.7	0.0	0.0	6.8	0.0	0.3	0.0	0.0	151.0
Pleosporales	403026 ma	0.2	0.0	21.6	5.3	0.4	4.0	43.7	22.6	0.2	0.0	0.0	0.0	0.0	0.0	0.0	114.3
Pleosporales	403027 yes	0.0	0.2	10.5	0.9	0.5	2.3	15.7	50.7	0.0	0.4	18.1	0.0	0.0	0.0	0.0	37.7
Pleosporales	403028 yes	0.0	0.0	12.2	1.4	1.8	5.0	17.0	38.3	0.0	0.0	22.1	0.0	0.0	0.0	0.0	9.0
Pleosporales	403029 ma	0.0	0.0	17.1	2.0	0.0	3.6	49.2	24.7	0.0	0.0	3.4	0.0	0.0	0.0	0.0	52.0
Pleosporales	403032 yes	0.0	0.0	11.7	0.9	0.5	2.6	22.5	49.9	0.0	0.0	8.8	0.0	0.0	0.0	0.0	18.3
Herpotrichia sp.	403037 yes	0.0	0.0	12.0	0.0	0.0	3.7	21.7	51.7	0.0	0.0	11.0	0.0	0.0	0.0	0.0	24.0
Pleosporales	403038 yes	0.0	0.0	11.0	0.0	0.0	3.4	19.5	42.3	0.0	0.0	5.0	0.0	0.0	0.0	0.0	21.9
Pleosporales	403041 yes	0.0	0.0	17.4	0.0	0.5	2.3	8.7	39.8	0.0	0.0	12.2	0.0	0.0	0.0	0.0	16.0
Pleosporales	403042 yes	0.0	0.0	11.3	1.0	0.0	2.2	24.1	42.8	0.0	0.0	4.0	0.0	0.0	0.0	0.0	29.8
Pleosporales	403043 yes	0.0	0.0	11.3	0.6	0.1	6.5	16.3	37.7	0.0	0.0	4.4	0.0	0.4	0.0	0.0	62.7
Pleosporales	403046 yes	0.0	0.0	13.3	2.9	0.7	3.3	24.2	47.7	0.0	0.0	5.2	0.0	0.0	0.0	0.0	17.2
Cadophora/polyscytalum	403059 yes	0.0	0.0	10.7	0.0	0.0	6.0	16.3	54.5	0.0	0.0	8.5	0.0	0.0	0.0	0.0	17.0
Leptodontidium sp.	403060 yes	0.0	0.0	14.1	0.0	6.2	3.5	18.5	40.4	0.0	0.0	9.9	0.0	0.0	0.0	0.0	31.9
Leptodontidium sp.	403061 yes	0.0	0.0	17.8	0.0	1.7	11.2	46.1	18.3	0.6	0.0	2.8	0.0	0.0	0.9	0.0	36.8
Leptodontidium sp.	403062 yes	0.4	0.0	19.2	0.6	0.0	6.6	44.8	20.3	0.0	0.0	3.7	0.0	0.0	0.0	0.0	57.5
Leptodontidium sp.	403063 yes	0.0	0.0	11.1	0.0	0.0	4.3	43.2	30.7	0.0	0.0	7.6	0.0	0.0	0.0	0.0	16.6
Leptodontidium sp.	403065 yes	0.0	0.0	13.0	0.0	0.0	4.1	40.3	33.6	0.0	0.0	6.7	0.0	0.0	0.0	0.0	12.8
Leptodontidium sp.	403067 yes	0.0	0.0	16.0	0.0	1.2	7.0	36.0	29.7	0.5	0.0	7.2	0.8	0.0	1.7	0.0	38.6
Leptodontidium sp.	403069 yes	0.0	0.0	15.6	0.0	0.0	5.6	46.6	27.2	0.0	0.0	4.3	0.0	0.0	0.0	0.0	13.9
Leptodontidium sp.	403070 yes	0.0	0.2	12.6	0.4	0.3	2.4	11.6	31.4	0.0	0.6	36.8	0.2	0.5	0.0	0.0	41.8
Leptodontidium sp.	403073 yes	0.0	0.0	19.1	0.4	3.6	7.4	27.8	17.4	0.4	0.0	0.9	0.4	0.0	0.0	0.0	41.3
Leptodontidium sp.	403074 yes	0.0	0.0	21.9	0.0	0.0	6.7	27.0	18.7	0.0	0.0	1.1	0.0	0.0	0.0	0.0	39.0
Leptodontidium sp.	403075 yes	0.0	0.0	15.7	0.0	10.0	4.5	21.6	28.4	0.0	0.0	19.8	0.0	0.0	0.0	0.0	13.4
Leptodontidium sp.	403077 yes	0.0	0.0	14.2	0.0	0.0	5.2	39.7	32.9	0.0	0.0	8.0	0.0	0.0	0.0	0.0	9.3

Organism	IMI/media	C14:0	C15:0	C16:0	C16:1	C17:1	C18:0	C18:1 cis	C18:2 cis	C20:0	C20:1	C18:3 ALA	C22:0	C22:1 n9	C24:0	C24:1 n9	Total lipid mg/g
Leptodontidium sp.	403078 yes	0.0	0.0	12.6	0.9	0.3	3.2	15.4	58.4	0.0	0.0	9.2	0.0	0.0	0.0	0.0	33.2
Leptodontidium sp.	403079 yes	0.0	0.0	21.4	0.0	4.4	8.6	35.8	25.1	0.0	0.0	4.7	0.0	0.0	0.0	0.0	27.8
Leptodontidium sp.	403080 yes	0.0	0.0	18.1	0.4	2.1	10.2	38.3	20.9	0.6	0.0	7.6	0.8	0.0	1.1	0.0	48.5
Leptodontidium sp.	403082 yes	0.0	0.0	13.2	0.4	1.7	5.2	34.8	30.2	0.4	0.0	11.5	0.6	0.0	0.7	0.0	32.6
Mollisia sp.	403087 ma	0.0	0.0	20.6	1.5	0.6	3.1	16.9	48.3	0.0	0.0	8.4	0.0	0.0	0.0	0.0	28.8
Mollisia sp.	403088 yes	0.0	0.0	21.2	0.0	15.0	5.8	23.5	22.4	0.0	0.0	4.8	0.0	0.0	0.0	0.0	26.6
Mollisia sp.	403090 yes	0.0	0.0	19.7	0.0	0.0	6.4	20.2	26.1	0.0	0.0	4.1	0.0	0.0	0.0	0.0	22.8
Mollisia sp.	403091 yes	0.4	0.0	20.3	0.0	0.0	6.3	30.1	15.2	0.0	0.0	3.2	0.0	0.0	0.0	0.0	47.3
Mollisia sp.	403092 yes	0.0	0.0	17.8	0.0	0.0	3.9	19.2	22.5	0.0	0.0	5.9	0.0	0.0	0.0	0.0	14.8
Mollisia sp.	403099 yes	0.4	0.0	22.7	0.0	16.8	4.9	24.0	23.8	0.0	0.0	5.8	0.0	0.0	0.0	0.0	40.6
Mollisia sp.	403100 yes	0.0	0.0	14.7	0.0	0.0	2.8	18.7	14.7	0.0	0.0	4.3	0.0	0.0	0.0	0.0	14.3
Helotiaceae	403104 yes	0.0	0.0	16.2	0.8	0.0	2.9	20.7	41.3	0.0	0.0	10.2	0.0	0.0	0.0	0.0	38.2
Gyoerffyella sp.	403109 yes	0.4	0.0	19.0	1.4	0.0	4.0	36.5	17.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	41.6
Penicillium rugulosum	403110 yes	0.0	0.8	11.2	1.2	0.7	6.6	21.4	29.9	0.0	0.9	26.9	0.0	0.0	0.0	0.0	27.0
Gyoerffyella sp.	403111 ma,	0.0	0.0	16.0	0.0	0.0	4.8	17.5	46.5	0.5	0.0	7.6	0.8	0.4	0.0	0.0	49.8
Gyoerffyella sp.	403112 yes	0.0	0.0	12.6	1.1	0.0	5.6	8.5	47.3	0.0	0.0	23.1	0.0	0.0	0.0	0.0	28.2
	403116 yes	0.0	0.0	17.4	2.5	0.5	9.6	22.8	41.9	0.0	0.0	2.6	0.4	0.0	0.0	0.8	58.2
	403119 yes	0.0	0.4	10.4	1.0	0.0	13.2	7.7	44.0	0.0	0.0	17.8	0.0	0.7	0.0	0.0	20.5
Ascomycota	403127 yes	0.0	0.0	11.6	0.0	0.0	2.9	9.8	20.2	0.0	0.0	9.1	0.0	0.0	0.0	0.0	6.5
	403131 yes	0.0	0.0	19.7	0.0	6.2	7.3	27.1	20.7	0.0	0.0	3.7	0.4	0.0	0.0	0.0	45.5
Mollisia sp.	403134 yes	0.0	0.0	14.0	0.0	13.9	4.2	16.0	19.2	0.0	0.0	11.6	0.0	0.0	0.0	0.0	29.7
	403142 yes	0.0	0.0	14.5	0.0	0.8	2.5	9.1	24.8	0.0	0.0	9.7	0.0	0.0	0.0	0.0	22.1
	403143 yes	0.0	0.0	18.3	0.0	0.0	4.2	20.7	36.9	0.0	0.0	5.9	0.0	0.0	0.0	0.0	10.1
	403147 yes	0.0	0.0	16.7	0.6	2.7	3.5	29.4	23.5	0.0	0.0	3.4	0.0	0.0	0.0	0.0	35.8
	403151 yes	0.0	0.0	13.3	0.0	1.8	6.9	9.3	45.5	0.0	0.0	20.3	0.0	0.0	1.6	0.0	21.4
	403158 yes	0.0	0.0	8.7	4.1	0.0	3.2	8.1	48.8	0.0	0.0	8.1	0.0	0.0	0.0	0.0	9.7
	403178 yes	0.0	0.0	13.3	0.0	0.8	5.0	20.2	28.3	0.0	0.0	17.4	0.0	0.0	0.8	0.0	42.7
Anarctomyces psychrotrophicus	403306 yes	0.0	0.0	3.7	0.4	0.3	0.9	33.8	21.3	0.0	0.7	30.1	0.0	0.3	0.0	0.0	73.4
Anarctomyces psychrotrophicus	403307 yes	0.0	0.0	6.8	0.5	0.0	3.7	29.8	42.6	0.0	0.0	10.1	0.0	0.0	0.0	0.0	37.1
	403308 yes	0.0	0.0	3.2	1.7	1.2	1.0	61.2	25.6	0.0	0.0	4.1	0.0	0.0	0.0	0.0	50.0
Thelebolaceae	403310 yes	0.0	0.0	5.3	0.0	0.0	2.6	8.2	20.0	0.0	0.0	3.6	0.0	0.0	0.0	0.0	27.4