6 APPENDIX

Appendix A - Vin*life*® product information

Certificate of Analysis

Vinlife

PRODUCT: **Vin*life*® Grape Seed Extract**
Batch No.: N03010

Retest Date: 01/03/2006

Appearance: Light brown powder with characteristic herbal/tannic odour and astringent mouth feel.

Results:	**Analysis**	**Specification**
Appearance	complies	
Solubility	complies	Freely soluble in H_2O
Phenolic Content (as % gallic acid equivalent)	46	45 to 55
Oligomeric proanthocyanidins (% as gallic acid equivalent)	38	37 to 46
pH (1% aqueous solution)	4.4	4.0 to 4.5
Moisture (%w/w)	2.1	< 5
Arsenic (mg/kg)	< 1	< 1
Lead (mg/kg)	< 1	< 2
Pesticide Residues	complies	< MRL's
Microbiology:		
Total Plate Count (cfu/mL)	< 10	< 1000
Yeast & Mould (cfu/mL)	< 10	< 100
Coagulase +ve Staphylococci (/g)	< 100	< 100
Coliform (MPN/g)	< 3	< 3
E.coli (MPN/g)	< 3	< 3
Pseudomonas sp (per g)	< 10	<10
Nutritional Information: (per 100 g)		
Energy (kJ)	1480	
Protein (g)	4.5	
Fat (g)	0.2	
Carbohydrates - total (g)	79.1	
- sugars (g)	19.7	
Sodium (mg)	300	
Potassium (mg)	2565	

Declarations

No Added preservatives.
No Added carriers.

Michael Oatey
Laboratory/QA Manager

Confidential

Printed 17/05/2005

Appendix B – Buffers/Reagents/Solutions/Media

All reagents listed below were of molecular grade and obtained from Sigma-Aldrich® (Australia) unless otherwise specified. Solutions requiring sterilization were autoclaved on a fluid cycle at 121°C for 15 min.

B.1 Luria Bertani (LB) medium

LB broth and agar:

1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl (Chem Supply, SA, Australia) and 1.5% (w/v) agar (Amresco, Ohio, USA) (if required). The pH was adjusted to 7 and autoclaved.

Selective LB broth and agar:

The LB broth or agar was allowed to cool to 50°C after autoclaving. This was followed by the addition of 100 μg mL^{-1} of ampicillin.

B.2 Yeast Extract Peptone Dextrose (YPD) broth and agar:

5% (w/v) YPD broth or 6.5% (w/v) YPD agar was prepared.

B.3 Synthetic minimal defined medium for yeast without uracil (SC-U):

0.67% (w/v) yeast nitrogen base without amino acids, 0.192% (w/v) yeast synthetic drop-out media supplement without uracil, 2% (w/v) carbon source (glucose, raffinose or galactose) and 2% (w/v) agar (Amresco) (if required). All reagents, except the carbon source, were autoclaved. The medium was allowed to cool to 50°C after autoclaving. The carbon source was added after 0.2 μm filter sterilization.

B.4 Agarose gel electrophoresis:

50X TAE buffer

24.2% (w/v) Tris-base (Amresco), 50 mM EDTA (Merck Pty. Ltd., Australia) pH 8 and 5.71% (v/v) glacial acetic acid (Chem Supply).

SDS-PAGE gel electrophoresis:

B.5 Resolving gel – 10%:

375 mM Tris-HCl pH 8.8, 0.1% (w/v) SDS (Amresco), 10:0.27% (w/v) acrylamide-bis (Amresco), 0.4% (v/v) TEMED (Amresco) and 0.05% (w/v) ammonium persulphate.

B.6 Stacking gel – 4%:

125 mM Tris-HCl pH 6.8, 0.1% (w/v) SDS (Amresco), 4:0.11% (w/v) acrylamide-bis (Amresco), 0.2% (v/v) TEMED (Amresco) and 0.05% (w/v) ammonium persulphate.

B.7 Running buffer:

1.44% (w/v) glycine (Chem Supply), 0.3% (w/v) Tris-base (Amresco) and 0.1% (w/v) SDS (Amresco).

B.8 SDS-PAGE sample buffer:

0.05 M Tris-HCl pH 6.8, 2% (w/v) SDS (Amresco), 10% (v/v) glycerol, 2 mM EDTA (Merck), 50 mM DTT, 12 M Urea and a few grains of bromophenol blue.

Western Blots:

B.9 Transfer buffer

0.58% (w/v) Tris-base (Amresco), 2.9% (w/v) glycine (Chem Supply), 0.1% (w/v) SDS (Amresco) and 20% (v/v) methanol.

B.10 TBS buffer:

10 mM Tris-HCl pH 7.5 and 150 mM NaCl (Chem Supply).

B.11 Blocking buffer:

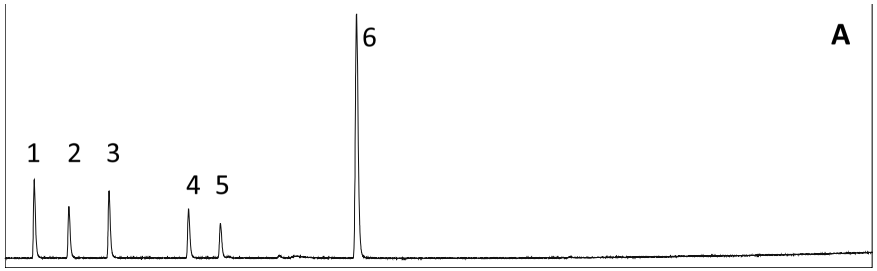
3% (w/v) BSA in TBS buffer

B.12 TBS-Tween/Triton buffer:

20 mM Tris-HCl pH 7.5, 500 mM NaCl (Chem Supply), 0.05% (v/v) Tween 20 and 0.2% (v/v) Triton X-100.

Appendix C – *S. cerevisiae* fatty acid profiles

C.1 Identification of the endogenous *S. cerevisiae* fatty acids. The *S. cerevisiae* were transformed with the empty pYES2 vector and grown under inducing conditions for 24 h. The FAME were extracted as outlined in section 3.2.31 from cultures supplemented with 18:3n-3 (**A**), 18:4n-3 (**B**), 20:5n-3 (**C**), 22:5n-3 (**D**), 18:2n-6 (**E**), 18:3n-6 (**F**), 20:4n-6 (**G**) or 22:4n-6 (**H**). The following fatty acids are labelled in the GC profiles: 16:0 (**1**), 16:1n-7 (**2**), 17:0_ITSD (**3**), 18:0 (**4**), 18:1n-9 (**5**), 18:3n-3 (**6**), 18:4n-3 (**7**), 20:5n-3 (**8**), 20:4n-6 (**9**), 22:5n-3 (**10**), 18:2n-6 (**11**), 18:3n-6 (**12**), 22:4n-6 (**13**). See following page.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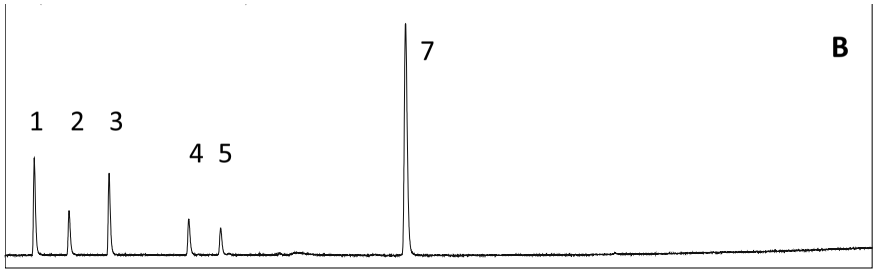
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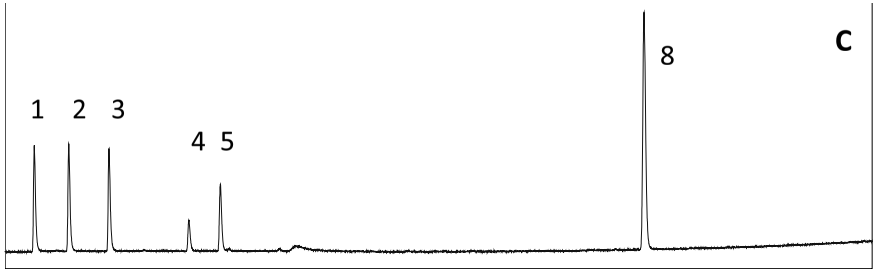
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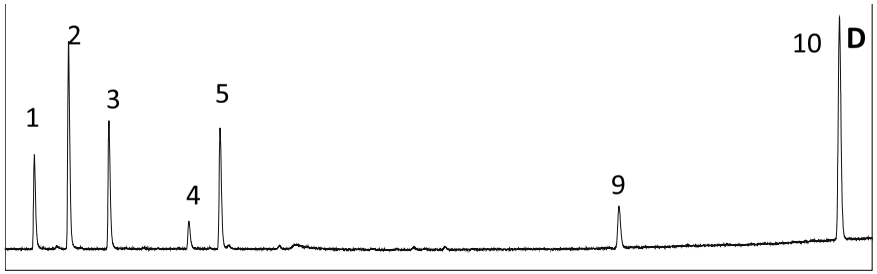
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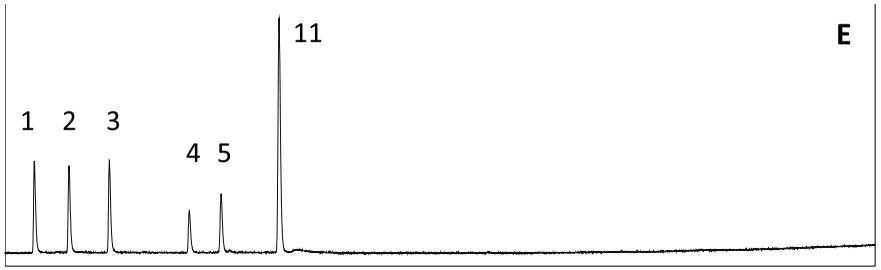
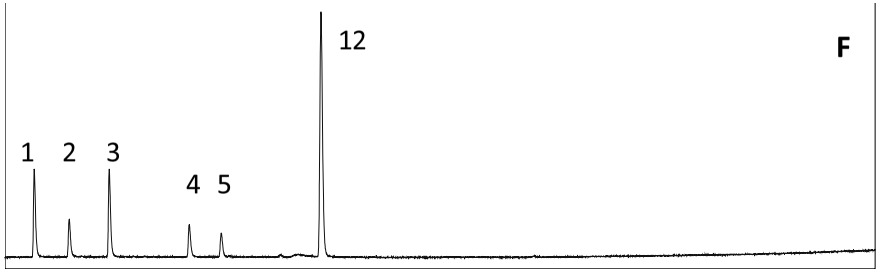
1 2 3 4 5 9 10


E
1 2 3 4 5 11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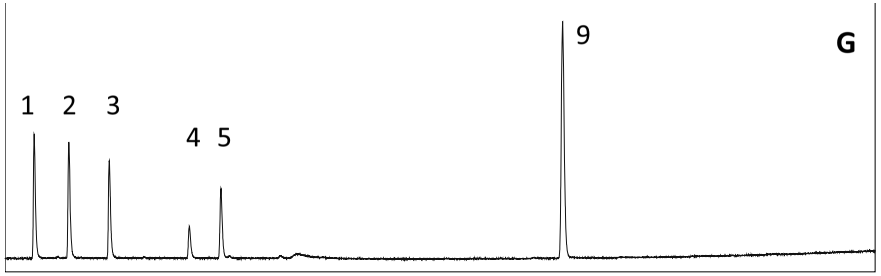
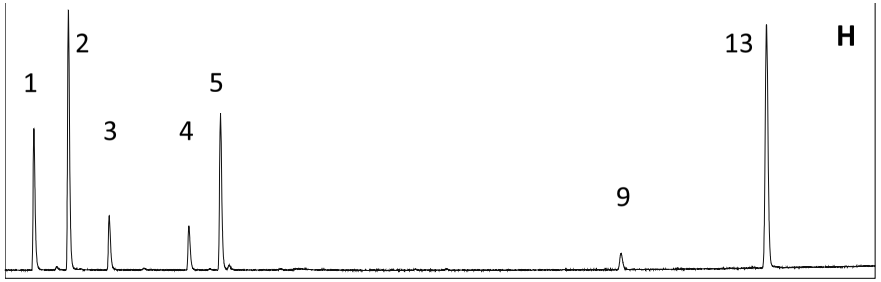
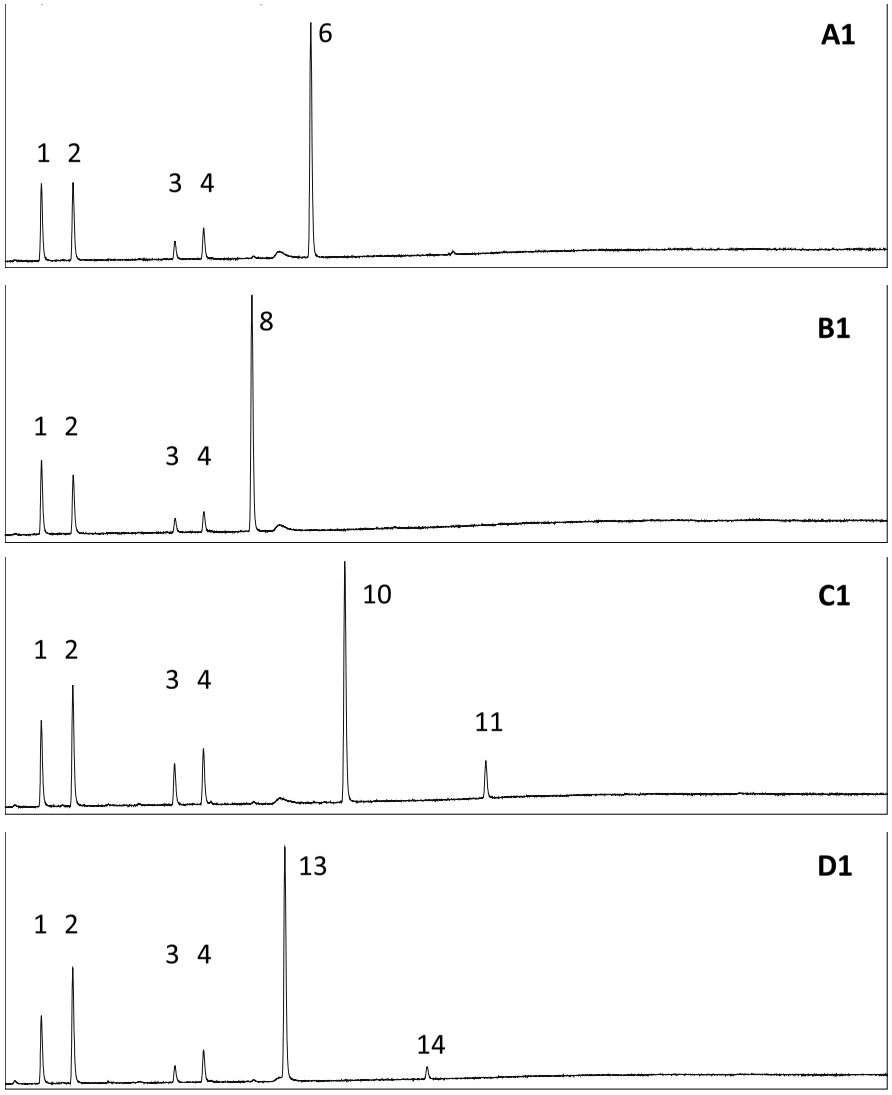

G
1 2 3 4 5 9


H
1 2 3 4 5 9 13

C.2 Identification of fatty acid elongation products in transgenic *S. cerevisiae*. The *S. cerevisiae* were transformed with the pYES2-sbt*Elovl5* vector and grown under non-inducing or inducing conditions for 24 h. The FAME were extracted as outlined in section 3.2.31 from cultures supplemented with 18:3n-3 – uninduced (**A1**) or induced (**A2**), 18:2n-6 – uninduced (**B1**) or induced (**B2**), 18:4n-3 – uninduced (**C1**) or induced (**C2**), 18:3n-6 – uninduced (**D1**) or induced (**D2**), 20:5n-3 uninduced (**E1**) or induced (**E2**), 20:4n-6 uninduced (**F1**) or induced (**F2**), 22:4n-6 uninduced (**G1**) or induced (**G2**) or 22:5n-3 uninduced (**H1**) or induced (**H2**). The following fatty acids are labelled in the GC profiles: 16:0 (**1**), 16:1n-7 (**2**), 18:0 (**3**), 18:1n-9 (**4**), 18:1n-7 (**5**), 18:3n-3 (**6**), 20:3n-3 (**7**), 18:2n-6 (**8**), 20:2n-6 (**9**), 18:4n-3 (**10**), 20:4n-3 (**11**), 22:4n-3 (**12**), 18:3n-6 (**13**), 20:3n-6 (**14**), 22:3n-6 (**15**), 20:5n-3 (**16**), 22:5n-3 (**17**), 24:5n-3 (**18**), 20:4n-6 (**19**), 22:4n-6 (**20**). See following page.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1 2
3 4
6
B1
1 2
3 4
8
C1
1 2
3 4
10
11
D1
1 2
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13
14
A2
1 2
3 4
5
6
7
B2
1 2
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D2
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3 4
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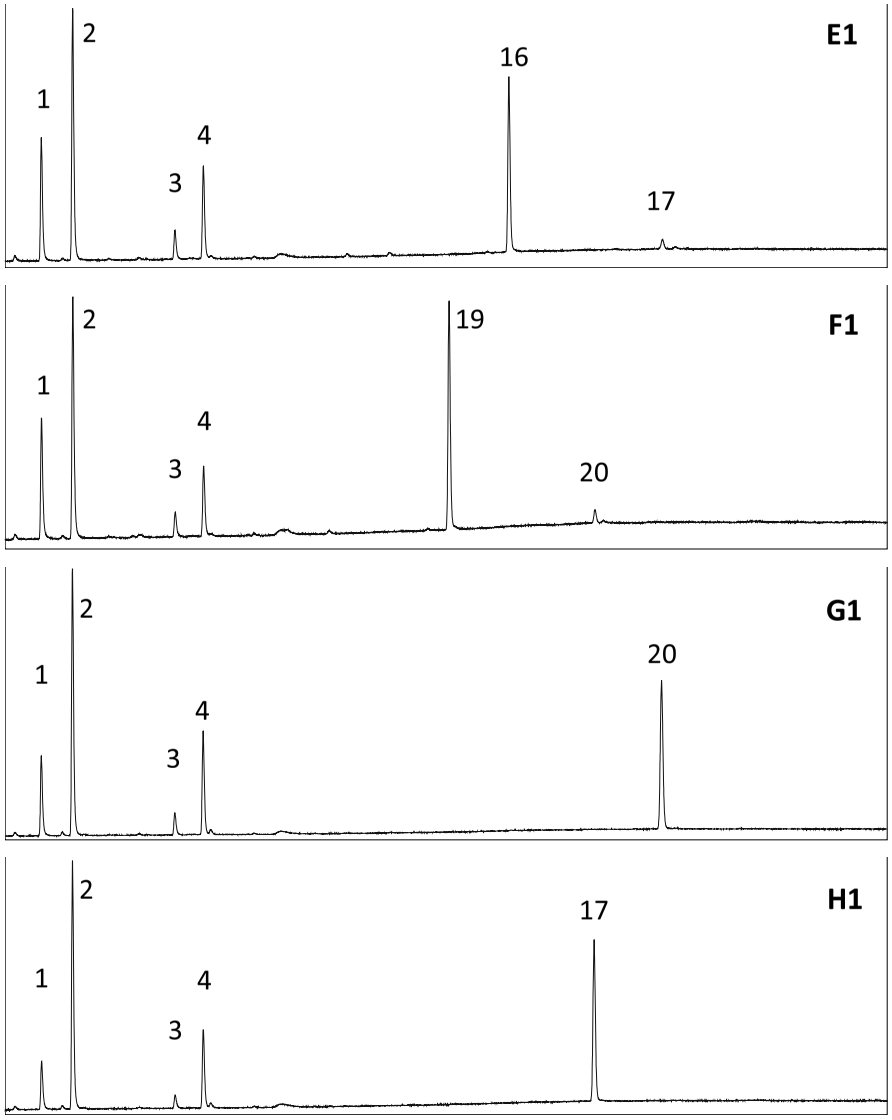
13
14
15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16
17
F1
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G1
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H1
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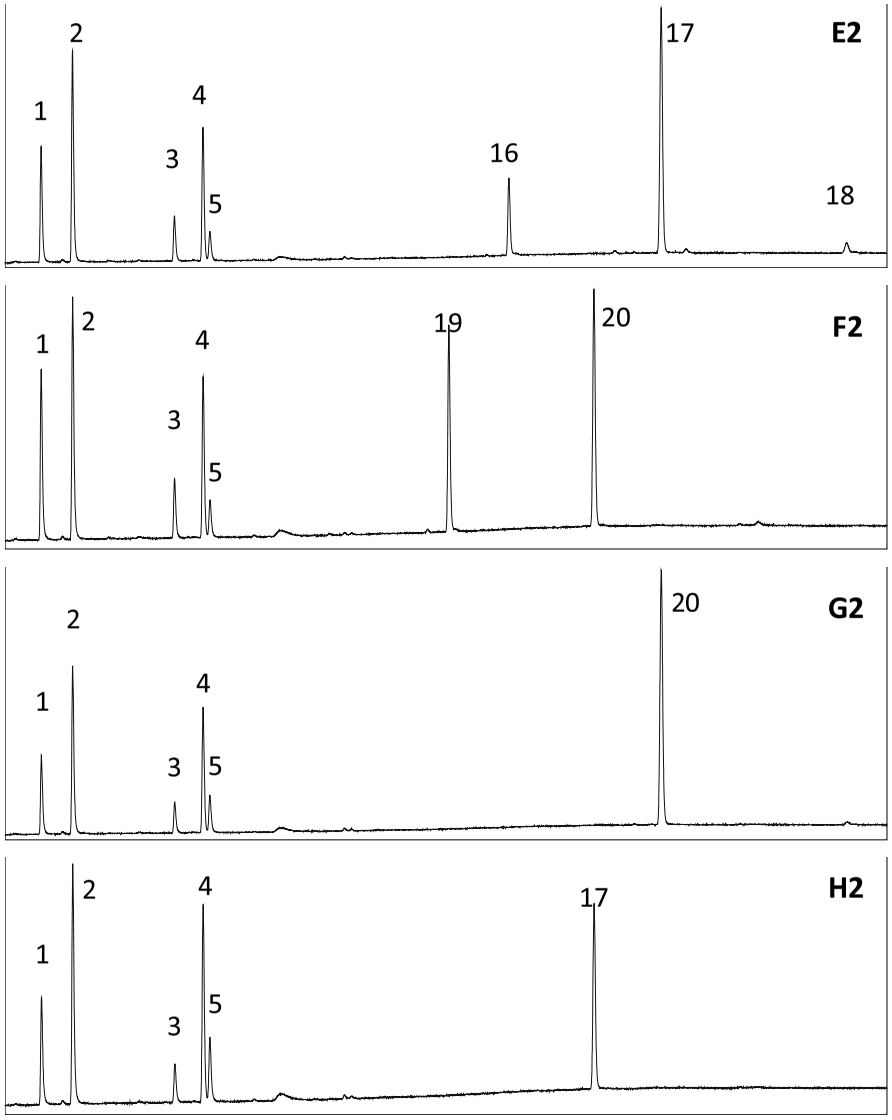
4
17


E2
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5
16
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18
F2
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4
5
19
20
G2
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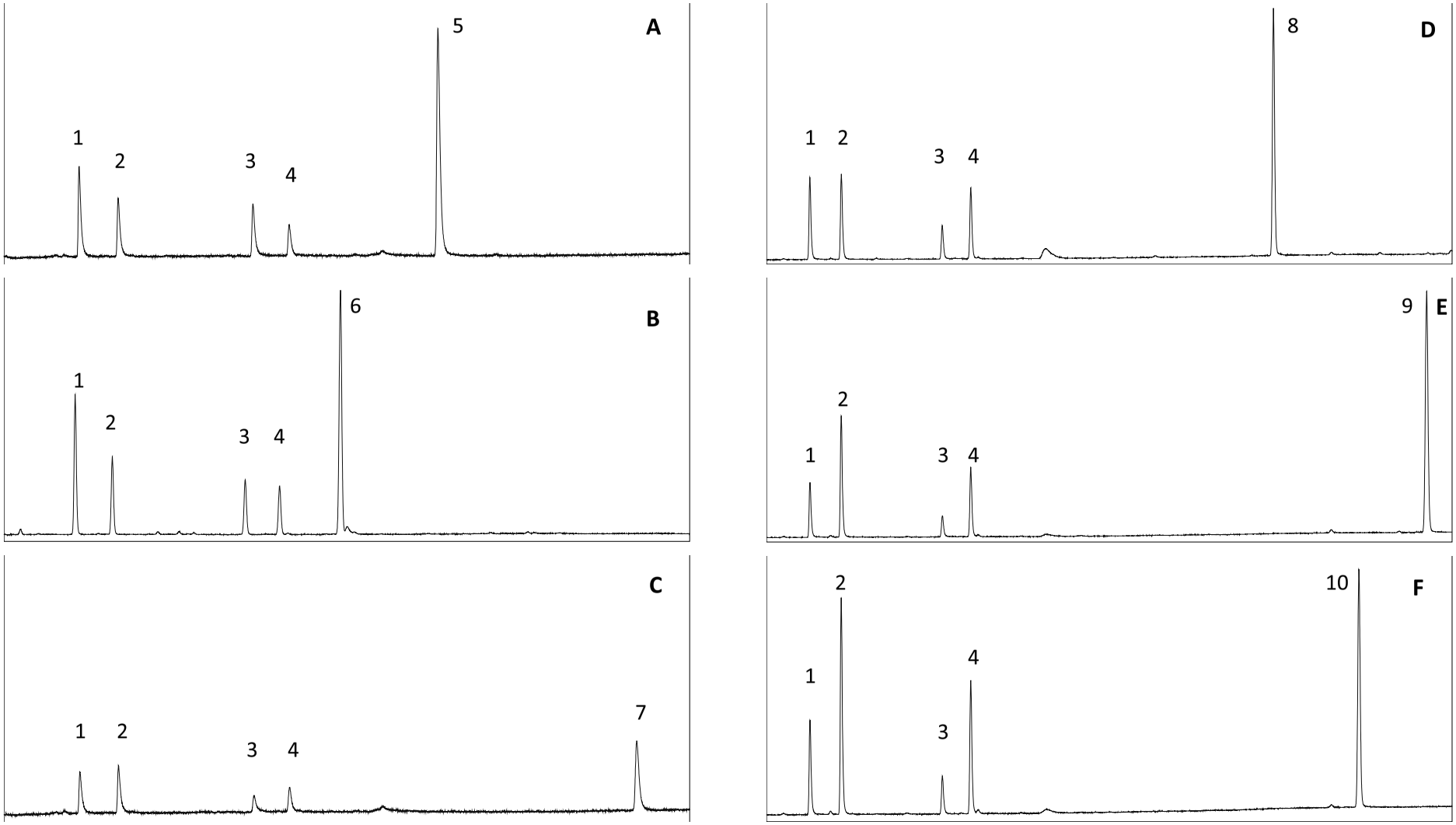
2
3
4
5
20
H2
1
2
3
4
5
17

C.3 Identification of fatty acid desaturation products in transgenic *S. cerevisiae*. The *S. cerevisiae* were transformed with the pYES2-sbt*Δ6des* vector and grown under inducing conditions for 24 h. The FAME were extracted as outlined in section 3.2.31 from cultures supplemented with 18:3n-3 (**A**), 18:2n-6 (**B**), 20:3n-6 (**C**), 20:5n-3 (**D**), 22:5n-3 (**E**) or 22:4n-6 (**F**). The following fatty acids are labelled in the GC profiles: 16:0 (**1**), 16:1n-7 (**2**), 18:0 (**3**), 18:1n-9 (**4**), 18:3n-3 (**5**), 18:2n-6 (**6**), 20:3n-6 (**7**), 20:5n-3 (**8**), 22:5n-3 (**9**), 22:4n-6 (**10**). See following page.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B
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4
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C
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2
3
4
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D
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E
1
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3
4
9
F
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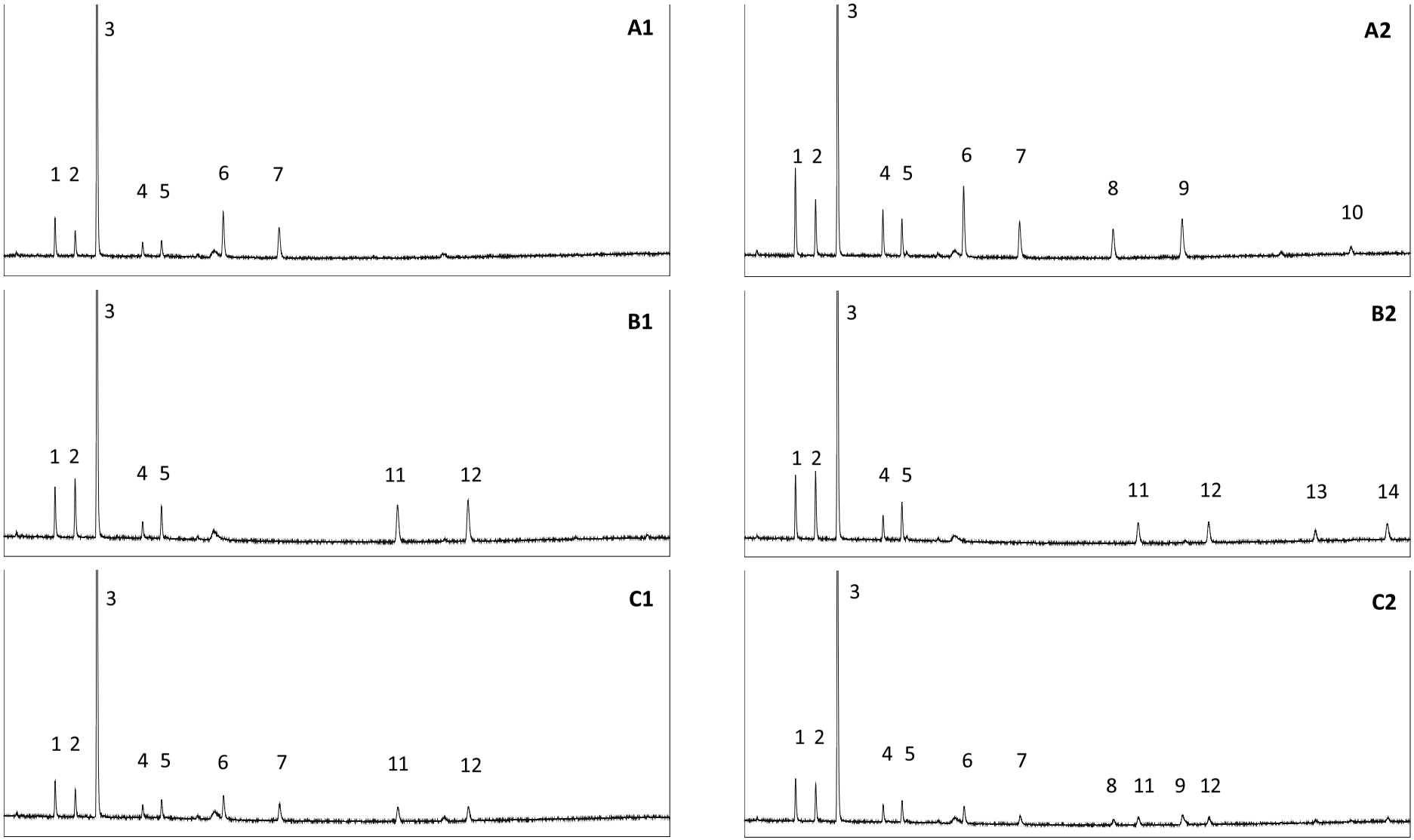
10

C.4 Identification of fatty acid elongation products in transgenic *S. cerevisiae*. The *S. cerevisiae* were transformed with the pYES2-sbt*Elovl5* vector and grown under non-inducing or inducing conditions for 24 h. The FAME were extracted as outlined in section 3.2.31 from cultures supplemented with 500 μM in total of 18:3n-6 and 18:4n-3 – uninduced (**A1**) or induced (**A2**), 20:4n-6 and 20:5n-3 – uninduced (**B1**) or induced (**B2**) or 18:3n-6, 18:4n-3, 20:4n-6 and 20:5n-3 – uninduced (**C1**) or induced (**C2**). The following fatty acids are labelled in the GC profiles: 16:0 (**1**), 16:1n-7 (**2**), 17:0_ITSD (**3**), 18:0 (**4**), 18:1n-9 (**5**), 18:3n-6 (**6**), 18:4n-3 (**7**), 20:3n-6 (**8**), 20:4n-3 (**9**), 22:4n-3 (**10**), 20:4n-6 (**11**), 20:5n-3 (**12**), 22:4n-6 (**13**), 22:5n-3 (**14**). See following page.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4 5
6
7
A2
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4 5
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8
9
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B1
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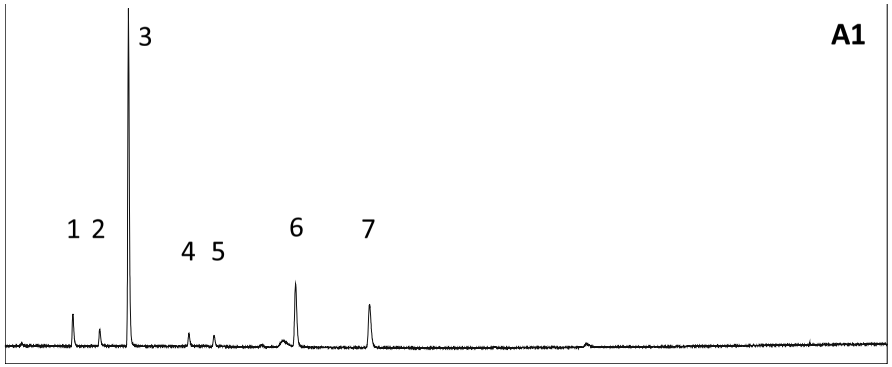
11
12
B2
3
1 2
4 5
11
12
13
14
C1
3
1 2
4 5
6
7
11
12
C2
3
1 2
4 5
6
7
8
11
9
12

C.5 Identification of fatty acid elongation products in transgenic *S. cerevisiae*. The *S. cerevisiae* were transformed with the pYES2-sbt*Elovl5* vector and grown under non-inducing or inducing conditions for 24 h. The FAME were extracted as outlined in section 3.2.31 from cultures supplemented with 500 μM each of 18:3n-6 and 18:4n-3 – uninduced (**A1**) or induced (**A2**), 20:4n-6 and 20:5n-3 – uninduced (**B1**) or induced (**B2**) or 18:3n-6, 18:4n-3, 20:4n-6 and 20:5n-3 – uninduced (**C1**) or induced (**C2**). The following fatty acids are labelled in the GC profiles: 16:0 (**1**), 16:1n-7 (**2**), 17:0_ITSD (**3**), 18:0 (**4**), 18:1n-9 (**5**), 18:3n-6 (**6**), 18:4n-3 (**7**), 20:3n-6 (**8**), 20:4n-3 (**9**), 22:4n-3 (**10**), 20:4n-6 (**11**), 20:5n-3 (**12**), 22:4n-6 (**13**), 22:5n-3 (**14**). See following page.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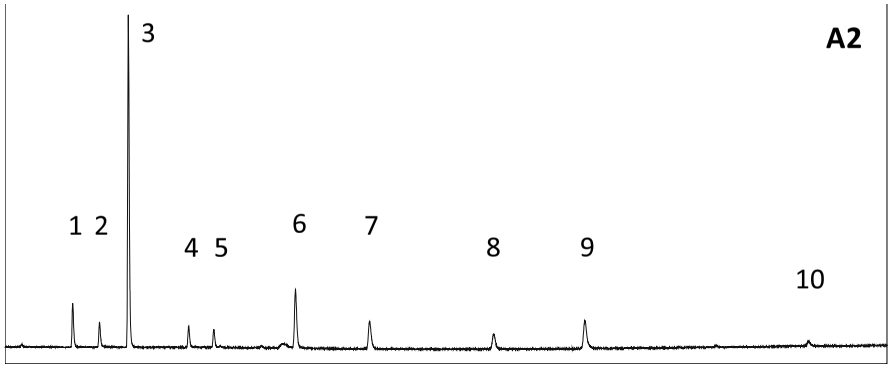
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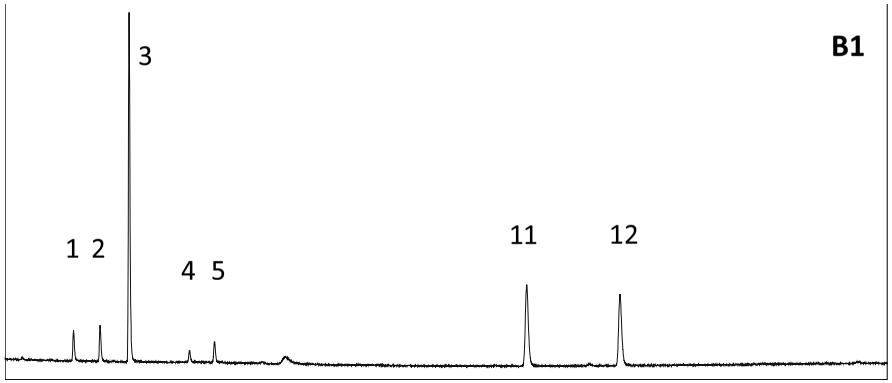
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4 5
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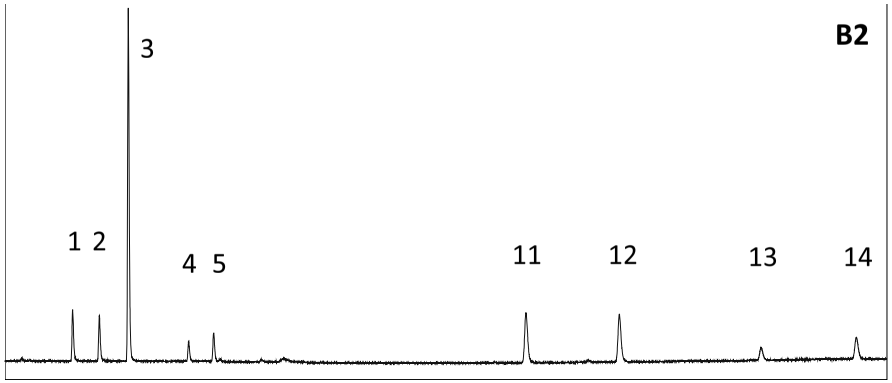
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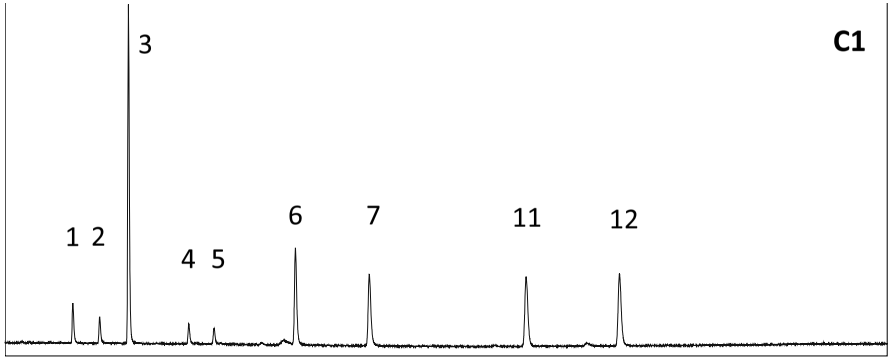
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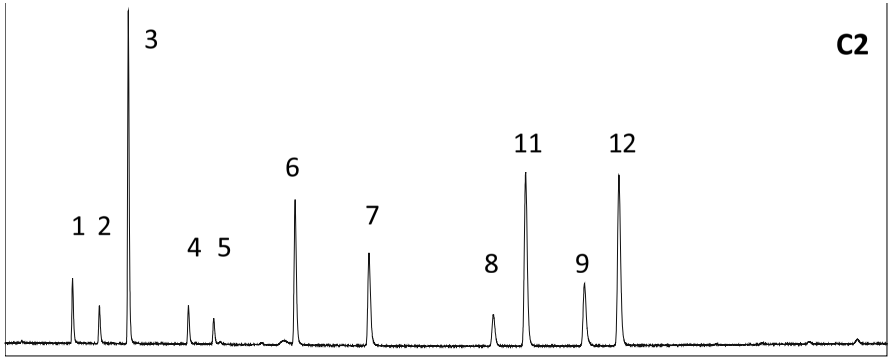
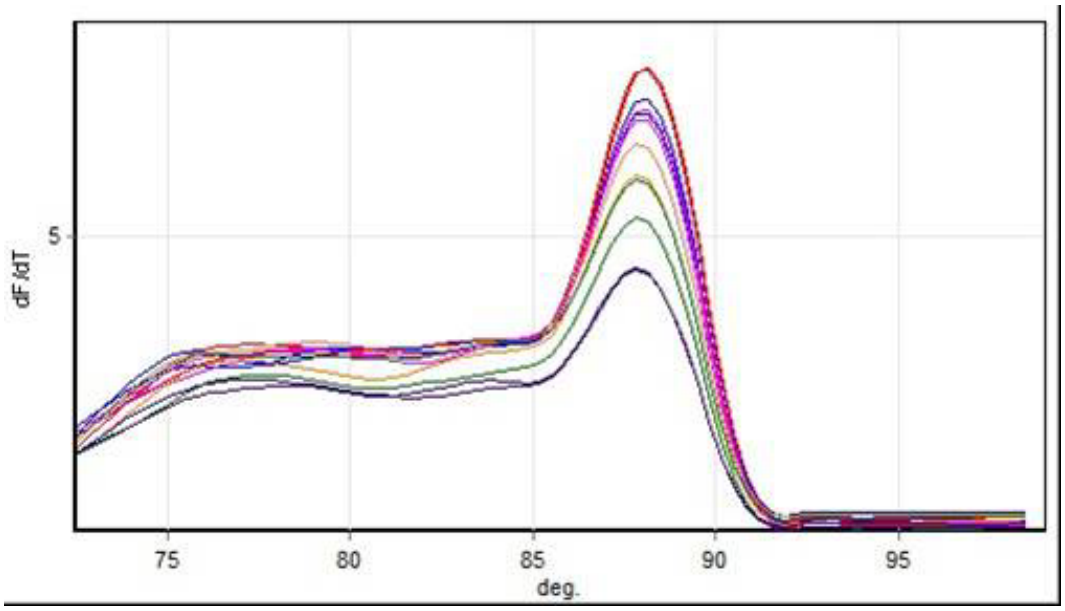

C2
3
1 2
4 5
6
7
8
9
11
12

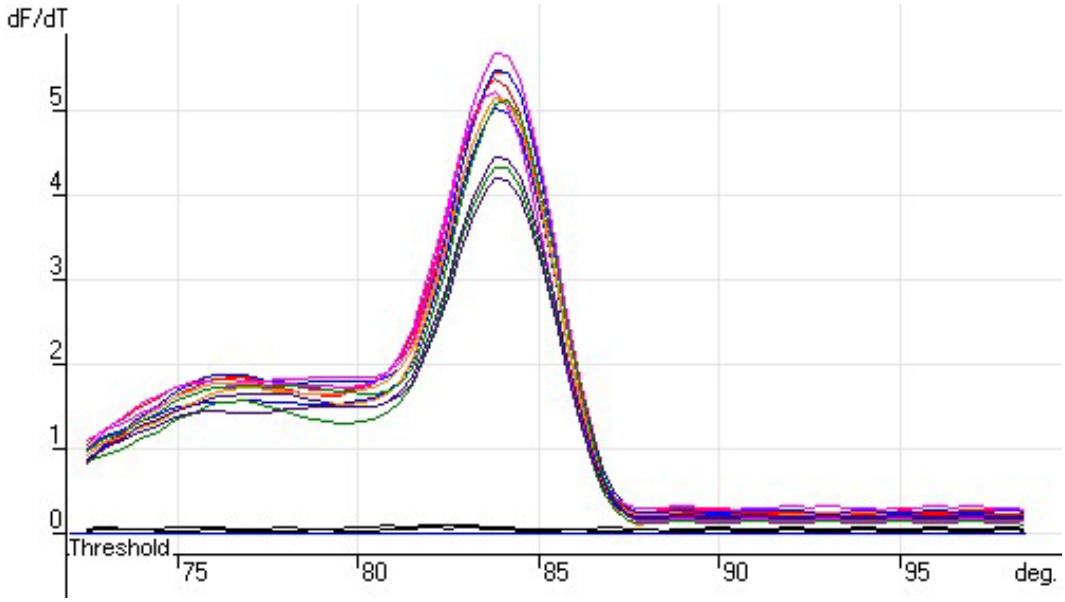
Appendix D – Quantitative real time PCR (qRT-PCR)

The following are examples of melt curves generated from qRT-PCR.

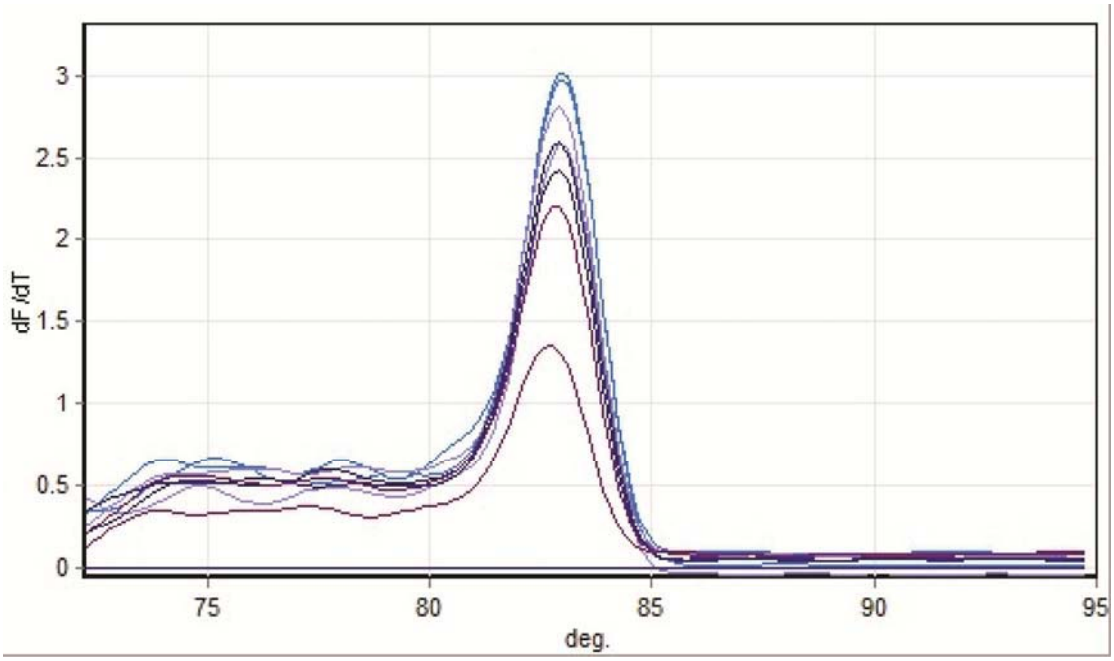
D.1 The Δ6desaturase plasmid standard melt curve obtained during qRT-PCR using SBT tissues



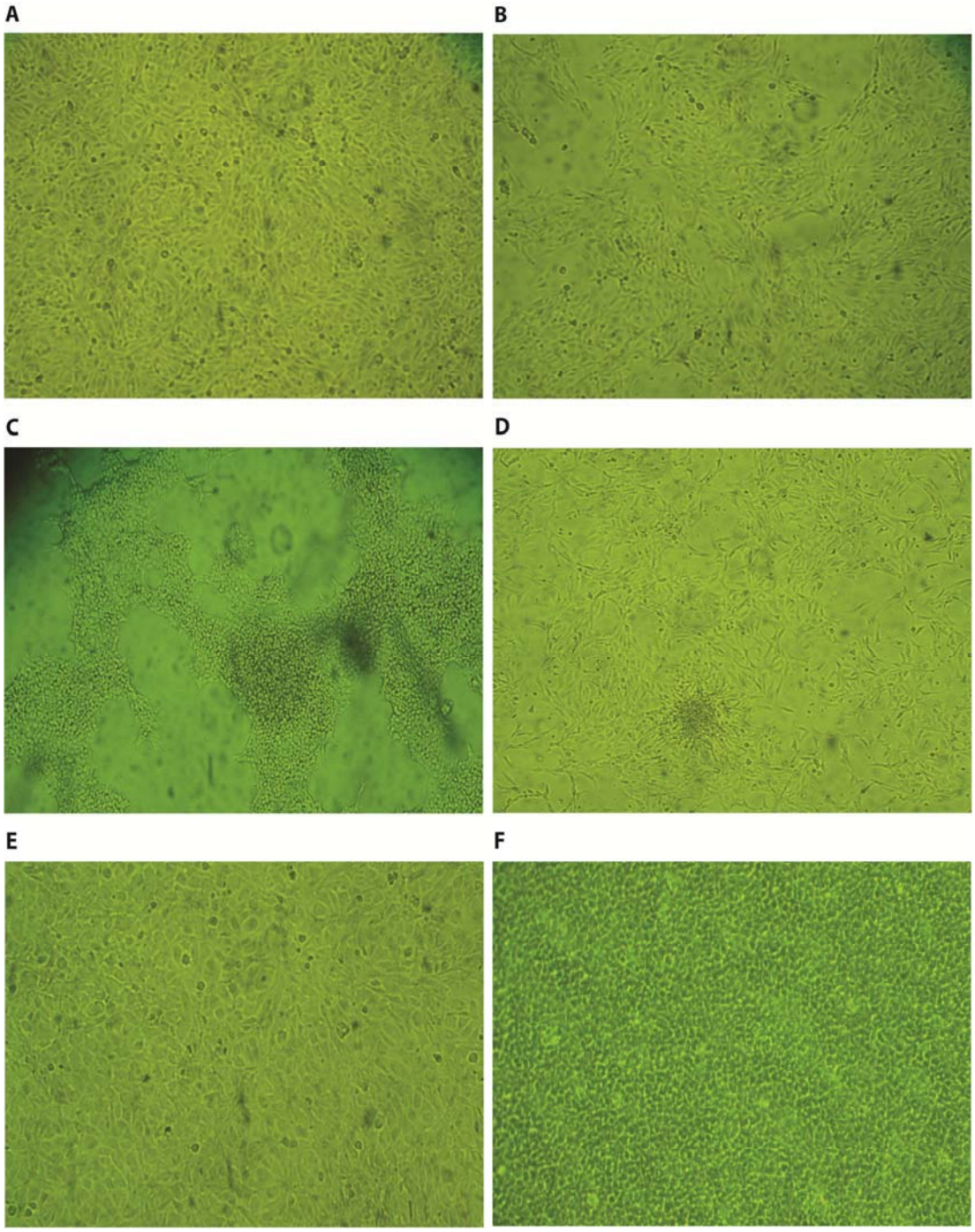
D.2 The Elovl5 plasmid standard melt curve obtained during qRT-PCR using SBT tissues



D.3 The Δ6desaturase melt curve obtained during qRT-PCR using FHM cells



Appendix E



The morphology of the fish cell lines used in this study. The CHSE-214 (**A**), BF-2 (**B**), KF-1 (**C**), LCK (**D**), RTG-2 (**E**) and FHM (**F**) cell lines were maintained as described in section 4.2.1.

Appendix F

Comparative Biochemistry and Physiology, Part B 155 (2010) 178–185

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

Cloning and functional characterisation of a fatty acyl elongase from southern bluefin tuna (*Thunnus maccoyii*)

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Fatty acyl elongase
Long chain polyunsaturated fatty acids
Southern bluefin tuna
Yeast expression

ABSTRACT

The synthesis of long chain polyunsaturated fatty acids (LCPUFA), such as eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), involves fatty acyl desaturase and elongase enzymes. The marine fish species southern bluefin tuna (SBT) can accumulate large quantities of omega-3 (n-3) LCPUFA in its flesh but their capacity to synthesize EPA and DHA is uncertain. A cDNA, sbt*Elovl5*, encoding a putative fatty acyl elongase was amplified from SBT liver tissue. The cDNA included an open reading frame (ORF) encoding 294 amino acids. Sequence comparisons and phylogenetic analyses revealed a high level of sequence conservation between sbt*Elovl5* and fatty acyl elongase sequences from other fish species. Heterologous expression of the sbt*Elovl5* ORF in *Saccharomyces cerevisiae* confirmed that it encoded a fatty acyl elongase capable of elongating $C_{18/20}$ polyunsaturated fatty acid (PUFA) substrates, but not C_{22} PUFA substrates. For the first time in an Elovl5, the substrate competition occurring in nature was investigated. Higher activity towards n-3 PUFA substrates than omega-6 (n-6) PUFA substrates was exhibited, regardless of substrate chain length. The sbtElovl5 preferentially elongated 18:4n-3 and 18:3n-6 rather than 20:5n-3 and 20:4n-6. The sbtElovl5 enzyme also elongated saturated and monounsaturated fatty acids.

1. Introduction

Dietary omega-3 (n-3) long chain polyunsaturated fatty acids (LCPUFA), in particular eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), have beneficial health effects in humans, particularly in relation to neurodevelopment (Smithers et al., 2008), the treatment of inflammatory and autoimmune diseases (Proudman et al., 2008) and reduced risk of sudden cardiac death (Metcalf et al., 2008). Fish and seafood in general are the major sources of n-3 LCPUFA in the human diet. Wild fish stocks, particularly small pelagic fish such as anchovy, mackerel, herring and sardine, are currently being depleted in an attempt to supply the n-3 fatty acids found in the farmed species (Naylor et al., 2000; Jenkins et al., 2009). Farming of carnivorous species, such as southern bluefin tuna (SBT, *Thunnus maccoyii*), requires large quantities of fish meal and fish oil to supply essential amino acids and LCPUFA which are not found in plant derived proteins and oils (Naylor et al., 2000). The partial substitution of valuable fish oils with more readily available vegetable oils in aquaculture feeds is accepted within the aquaculture industry (Naylor et al., 2000). Vegetable oils are rich in C_{18} polyunsaturated fatty acids (PUFA), such as α-linolenic acid (ALA; 18:3n-3) and linoleic acid (LA; 18:2n-6), but low in n-3 LCPUFA, such as EPA and DHA (Regost et al., 2003; Whelan and Rust, 2006). Marine fish are widely considered to be unable to synthesize LCPUFA from C_{18} PUFA precursors, possibly due to an enzyme deficiency or structural modification of one or more of the key enzymes in the synthesis pathway (Regost et al., 2003; Tocher, 2003; Agaba et al., 2004). The LCPUFA synthesis pathway requires three elongation and three desaturation steps (Sprecher, 2000). The same enzymes may be involved in both the n-3 and n-6 pathway. The Δ5desaturase and Δ6desaturase enzymes introduce a double bond at the Δ5 or Δ6 position of the carbon chain, respectively (Park et al., 2009). The fatty acyl elongase enzymes, Elovl2 and Elovl5, add two carbons to their respective C_{18}, C_{20} or C_{22} PUFA substrates. In mammals the Elovl5 enzyme prefers $C_{18/20}$ PUFA substrates and the Elovl2 enzyme prefers $C_{20/22}$ PUFA substrates (Leonard et al., 2000, 2002; Inagaki et al., 2002).

Fatty acyl elongase genes have been cloned and functionally characterised from the fungus *Mortierella alpina* (Parker-Barnes et al., 2000), the nematode *Caenorhabditis elegans* (Beaudoin et al., 2000), rat (Inagaki et al., 2002), mouse (Leonard et al., 2002), human (Leonard et al., 2000) and fish species, including Nile tilapia (*Oreochromis nilotica*), North African catfish (*Clarius gariepinus*), Atlantic cod (*Gadus morhua*), gilthead sea bream (*Sparus aurata*), turbot (*Psetta maxima*) (Agaba et al., 2005), zebrafish (*Danio rerio*) (Agaba et al., 2004), Atlantic salmon (*Salmo salar*) (Hastings et al., 2005; Morais et al., 2009), rainbow trout (*Oncorhynchus mykiss*) (Meyer et al., 2004), cherry salmon (*Onchorhynchus masou*)

Abbreviations: ALA, α-linolenic acid (18:3n-3); DHA, docosahexaenoic acid (22:6n-3); EPA, eicosapentaenoic acid (20:5n-3); LA, linoleic acid (18:2n-6); LCPUFA, long chain polyunsaturated fatty acids; n-3, omega-3; n-6, omega-6; ORF, open reading frame; PUFA, polyunsaturated fatty acids; SBT, southern bluefin tuna.

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(Alimuddin et al., 2008) and cobia (*Rachycentron canadum*) (Zheng et al., 2009). Until recently, all characterised fish fatty acyl elongases were considered to be Elovl5-like, as they prefer $C_{18/20}$ PUFA substrates. Morais et al. (2009) have recently characterised an Elovl2-like elongase in Atlantic salmon which prefers $C_{20/22}$ PUFA substrates.

Tuna species have unique fatty acid profiles which contain high levels of DHA and a very high DHA/EPA ratio compared to other marine fish species (Mourente and Tocher, 2003). Tuna are at the top of the marine food chain and have the ability to selectively accumulate and retain a large quantity of DHA originating from their diet (Saito et al., 1997; Mourente and Tocher, 2003). SBT flesh is particularly rich in DHA, containing over 44%, and has a high DHA/EPA ratio of 9 (Nichols et al., 1998). These values are elevated compared with other tuna species found in Australian waters. The yellowfin tuna (*Thunnus albacares*) and albacore (*Thunnus alalunga*) contain 38.9% and 34.3% DHA, respectively and have DHA/EPA ratios of 7 and 6, respectively (Nichols et al., 1998). The special nature of SBT positions them in a different ecological niche to the fish species used in previous characterisation studies of fatty acyl elongases. Conducting SBT feeding trials to provide a better understanding of the possible impacts formulated vegetable oil-based diets will have on the fatty acid profile are not feasible. Therefore, to elucidate the enzymatic regulation in the LCPUFA synthesis pathway this study aimed to characterise a fatty acyl elongase gene. Here we report on the cloning and functional characterisation of sbtElovl5, an Elovl5-like elongase from SBT.

2. Materials and methods

2.1. Cloning of an internal fragment of the sbtElovl5 cDNA

SBT liver tissue was obtained from a commercial farm located near Port Lincoln in SA. The tissue was stored in RNA*later*® (Ambion, TX, USA) at −80 °C until the RNA isolation was performed. Total RNA was extracted from 100 mg of the liver tissue using the RiboPure™ Kit (Ambion). An internal fragment of the sbt*Elovl5* cDNA was cloned using reverse transcriptase-polymerase chain reaction (RT-PCR). First strand cDNA was synthesized using 0.3 μg of total RNA with 4 U Omniscript™ RT (Qiagen, Australia). PCR primers to amplify an internal fragment of the sbt*Elovl5* cDNA were designed based on an alignment of Atlantic cod (AY660881), Atlantic salmon (AY170327), cherry salmon (DQ067616), rainbow trout (AY605100), turbot (AF465520) and zebrafish (AF532782) fatty acyl elongase sequences obtained from the GenBank database. PCR amplification using primer set A (Table 1) and Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen, Australia) was performed with an initial denaturation step at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, and extension at 68 °C for 1 min, followed by a final extension at 72 °C for 5 min. The gel purified PCR products were cloned into the pGEM®-T Easy Vector (Promega, WI, USA) and then sequenced at the Australian Genome Research Facility (AGRF) (Brisbane, Australia) using Big Dye Terminator sequencing technology and capillary separation on an AB 3730*xl* 96-capillary sequencer.

Table 1
Primers used for amplifying the sbt*Elovl5* cDNA and ORF.

Primer Set	Primer Name	Sequence (5′→3′)
A	sbt*Elovl5* F	GGT CTA CAA TCT GGG CCT C
	sbt*Elovl5* R	CCA CCA TAG ATA GGG CCG
B	sbt*Elovl5* 3′ RACE	CAC ATC TAC CAC CAC GCT AGC ATG C
	sbt*Elovl5* 5′ RACE	CTT CCA CCA AAG ATA CGG CCG CAT G
C	sbt*Elovl5* Full Length F	GGT TAC ACA GCC GCG TTC TCC G
	sbt*Elovl5* Full Length R	GGA TAT ATG GGG CTA TGG CTT ATT TCA
D	sbt*Elovl5 Eco*R1 F	GCG *GAA TTC* AGG TGA CAA ATG GAG ACT TTC AAT
	sbt*Elovl5 Not*1 R	GAC TGC GGC CGC TCT CAA ATG TCA ATC CAC CCG CAG

Restriction enzyme sites are indicated by italics and the start or stop codons are underlined.

2.2. Cloning of the 3′ and 5′ ends of the sbtElovl5 cDNA

The 3′ and 5′ ends of the sbt*Elovl5* cDNA were cloned using 3′ and 5′ rapid amplification of cDNA ends (RACE)-PCR with the SMART™ RACE cDNA Amplification Kit and the Advantage® 2 PCR Kit (Clontech, CA, USA). The primers for RACE-PCR (Table 1, primer set B) were designed based on the known sequence of the internal fragment of the sbt*Elovl5* cDNA. RACE-PCR cycling conditions were 5 cycles of 94 °C for 30 s and 72 °C for 2 min, followed by 5 cycles of 94 °C for 30 s, 70 °C for 30 s and 72 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 2 min. The RACE-PCR products were cloned into the pGEM®-T Easy Vector (Promega) and the constructs were sequenced as previously described. The sequence of the sbt*Elovl5* cDNA was assembled by aligning the sequences of the RT-PCR and 3′ and 5′ RACE-PCR products using Sequencher version 4.1.4 (Gene Codes, MI, USA).

2.3. Cloning of the full length sbtElovl5 cDNA

PCR amplification of the full length sbt*Elovl5* cDNA was performed using primer set C (Table 1) and Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen) with an initial denaturation step at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, and extension at 68 °C for 1.5 min, followed by a final extension at 68 °C for 5 min. The resulting sbt*Elovl5* cDNA was cloned into the pGEM®-T Easy Vector (Promega) and sequencing was performed as previously described.

2.4. Sequence analyses

The sbt*Elovl5* open reading frame (ORF) was translated and the deduced amino acid sequence was aligned with other fatty acyl elongase sequences using ClustalX. Maximum parsimony (MP) analysis was conducted with phylogenetic analysis using parsimony (*and other methods) (PAUP*) version 4.0b 10 (Swofford, 2003). MP analyses were performed with heuristic searches using 100 random sequence additions and tree bisection reconnection (TBR) branch swapping. Node support was assessed using 1000 bootstrap replicates. The choice of out-group was limited by the availability of fatty acyl elongase sequences relating to the basal animals in terms of evolutionary progression of animal phylogeny in the GenBank database. Therefore, *M. alpina* and *C. elegans* were chosen to represent basal phyla.

2.5. Heterologous expression of the sbtElovl5 ORF in Saccharomyces cerevisiae

Functional characterisation was done by expressing the sbt*Elovl5* ORF in *S. cerevisiae*. PCR was used to introduce an EcoRI restriction site upstream of the putative translation initiation codon and a NotI restriction site downstream of the putative translation termination codon in the sbt*Elovl5* cDNA. PCR amplification was performed using the pGEM®-T Easy Vector-sbt*Elovl5* construct with primer set D (Table 1) and Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen) with an initial denaturation of 94 °C for 2 min, followed by 25 cycles of 94 °C for 30 s, 57 °C for 30 s and 68 °C for 1.5 min, followed by a final extension of 68 °C for 5 min. The resulting sbt*Elovl5* PCR product and the pYES2 vector (Invitrogen) were restricted with EcoR1 and Not1 (Promega). The digested pYES2 vector was treated with shrimp alkaline phosphatase (SAP) (1 U/μg plasmid DNA) (Promega) to prevent re-circularization of the linearized plasmid. The restriction enzyme treated PCR product was ligated into the pYES2 vector using T4 DNA ligase (1.5

Weiss units) (Promega). The resulting construct, pYES2-sbt*Elovl5*, was used to heat-shock transform Subcloning Efficiency™ DH5α™ Competent *E. coli* cells (Invitrogen). Putative transformants were selected using 100 μg/mL ampicillin and PCR screening. Recombinant plasmids were purified and the presence of the sbt*Elovl5* insert in the correct orientation was confirmed via sequencing.

The pYES2-sbt*Elovl5* construct was then used to transform *S. cerevisiae* strain INVSc1 (Invitrogen) for the production of recombinant protein, using the *S. c.* EasyComp™ Transformation Kit (Invitrogen). Successfully transformed yeast cells were selected on uracil dropout medium. Recombinant yeast cells were maintained in synthetic minimal defined medium for yeast without uracil (SC-U) containing 2% raffinose. Upon fatty acid supplementation, raffinose was replaced with 2% galactose for induction of gene expression. Recombinant yeast were supplemented with 500 μM of one of the following PUFA substrates: 18:3n-3, 18:2n-6, 18:4n-3, 18:3n-6, 20:5n-3, 20:4n-6, 22:5n-3 or 22:4n-6; or 500 μM in total of 18:4n-3 and 18:3n-6, 20:5n-3 and 20:4n-6 or 18:4n-3, 18:3n-6, 20:5n-3 and 20:4n-6. Recombinant yeast grown in SC-U medium containing raffinose and supplemented with PUFA substrates were used as the uninduced controls. Cells were harvested for analysis after 24 h of fatty acid supplementation. Approximately the same number of yeast cells was harvested from each culture by determining the OD_{600}. The cells were pelleted by centrifugation at 1500 *g* for 5 min and the cell pellets were washed twice with water. The cell pellets were stored at −80 °C in 1 mL of water until fatty acid analysis was performed. The results were expressed as the means of biological replicates ± S.D. ($n = 3$).

2.6. Fatty acid analysis

Total lipid was extracted from yeast cells with chloroform/isopropanol (2:1, v/v) or from SBT liver tissue with chloroform/methanol (2:1, v/v) and methylated in 1% sulphuric acid in methanol (v/v) at 70 °C for 3 h to prepare fatty acid methyl esters (FAME). FAME were extracted in heptane and analysed using a Hewlett-Packard 6890 gas chromatograph (Hewlett Packard, Palo Alto, CA, USA) fitted with a flame ionisation detector with a BPX-70 50 m capillary column coated with 70% cyanopropyl polysilphenylene-siloxane (0.25 mm film thickness and 0.32 mm internal diameter, SGE, Australia). The carrier gas was helium at a flow rate of 2.0 mL min^{-1} and the split-ratio was 20:1. The injection port temperature was 250 °C and the detector temperature was 300 °C. The initial column temperature was 140 °C and it was increased to 220 °C at a rate of 4 °C min^{-1} and held at 220 °C for up to 8 min. The identity of each fatty acid peak in the chromatogram was ascertained by comparing its retention time to authentic lipid standards (NuChek Prep, MN, USA and Larodan Fine Chemicals, Sweden). The identities of 20:4n-3 and 22:4n-3 were confirmed by gas chromatography–mass spectrometry (GC–MS). FAME were quantified using GC Chemstation software (Agilent Technologies, Australia). All solvents contained 0.005% (w/v) butylated hydroxyanisole (BHA) as antioxidant.

The amount of each fatty acid was expressed as a percentage of the total amount of all fatty acids. This was done by expressing the peak area for an individual fatty acid as a percentage of the total peak area for all fatty acids. The proportion of substrate fatty acid converted to longer chain fatty acid product was calculated as [% product/(% product + % substrate)] × 100.

3. Results

3.1. SBT liver fatty acid profile

The fatty acid profile of the SBT liver tissue, from which the sbt*Elovl5* was cloned, was determined (Table 2). The major fatty acids were 16:0, 16:1n-7, 18:0, 18:1n-7, 18:1n-9, 20:5n-3, 22:5n-3 and 22:6n-3. These fatty acid components accounted for more than 85% of the total fatty acids in the SBT liver. The profile was comprised of 36.5% n-3 PUFA, 33.6% saturated fatty acids, 25.6% monounsaturated fatty acids and 4% n-6 PUFA. The largest distribution of an individual fatty acid was DHA, with 18.3%. Notably, the liver tissue contained 0.6% ALA, 10.9% EPA and 18.3% DHA and a DHA/EPA ratio of 1.7.

Table 2
SBT liver fatty acid profile.

Fatty acid	% distribution
14:0	1.60
15:0	0.37
16:0	17.20
18:0	13.10
Total saturated	33.63
16:1n-7	2.13
18:1n-9	14.65
18:1n-7	4.66
20:1n-9	1.50
22:1n-11	1.14
24:1n-9	0.58
Total monounsaturated	25.55
18:2n-6	1.37
20:2n-6	0.32
20:4n-6	1.61
22:5n-6	0
Total n-6	4.03
18:3n-3	0.57
18:4n-3	0.81
20:5n-3	10.93
22:5n-3	5.33
22:6n-3	18.28
Total n-3	36.48
n-3/n-6	9.05
DHA/EPA	1.67

3.2. Sequence analyses

Overlapping fragments of a putative fatty acyl elongase cDNA were amplified from SBT liver RNA using a combination of RT-PCR with 3′- and 5′-RACE-PCR. The fragments were assembled to give a sequence of 1120 base pairs (bp) which was deposited in the GenBank database as accession number FJ156735. The 1120 bp sequence included an ORF of 885 bp plus a 42 bp 5′-untranslated region (UTR) and a 193 bp 3′-UTR. Subsequently, the ORF was amplified as one fragment and deposited in GenBank (GQ204105). The ORF encoded a predicted protein of 294 amino acids (Fig. 1). The predicted protein sequence included all of the characteristic features of a microsomal fatty acyl elongase, including the conserved motifs KxxExxDT (Box 1), QxxFLHxYHH containing the single histidine box redox centre motif HxxHH (Box 2), NxxxHxxMYxYY (Box 3) and TxxQxxQ (Box 4) and lysine or arginine residues close to the C terminus which may function as an endoplasmic reticulum (ER) retention signal (Fig. 1) (Agaba et al., 2005; Jakobsson et al., 2006). Fatty acyl elongases contain multiple transmembrane regions and the sbtElovl5 is predicted to contain four transmembrane regions (SOSUI software, http://bp.nuap.nagoya-u.ac.jp/sosui/).

Pairwise comparisons of the deduced amino acid sequences of fish and human fatty acyl elongases revealed that the sbtElovl5 sequence was most similar to the sequences from gilthead sea bream (AAT81404), cobia (ACJ65150), turbot (AAL69984) and Nile tilapia (AAO13174), with percentage identities of 92%, 92%, 87% and 84%, respectively. There are seven different human fatty acyl elongases (ELOVL1–ELOVL7) in the GenBank database, of which only ELOVL2 and ELOVL5 elongate PUFA substrates. The sbtElovl5 was most similar to ELOVL5 with a percentage identity of 71%, compared to ELOVL2 with a percentage identity of 55%. Phylogenetic analysis revealed that the sbtElovl5 formed a clade with the gilthead sea bream, cobia, turbot and Nile tilapia sequences, which was reasonably well supported by a

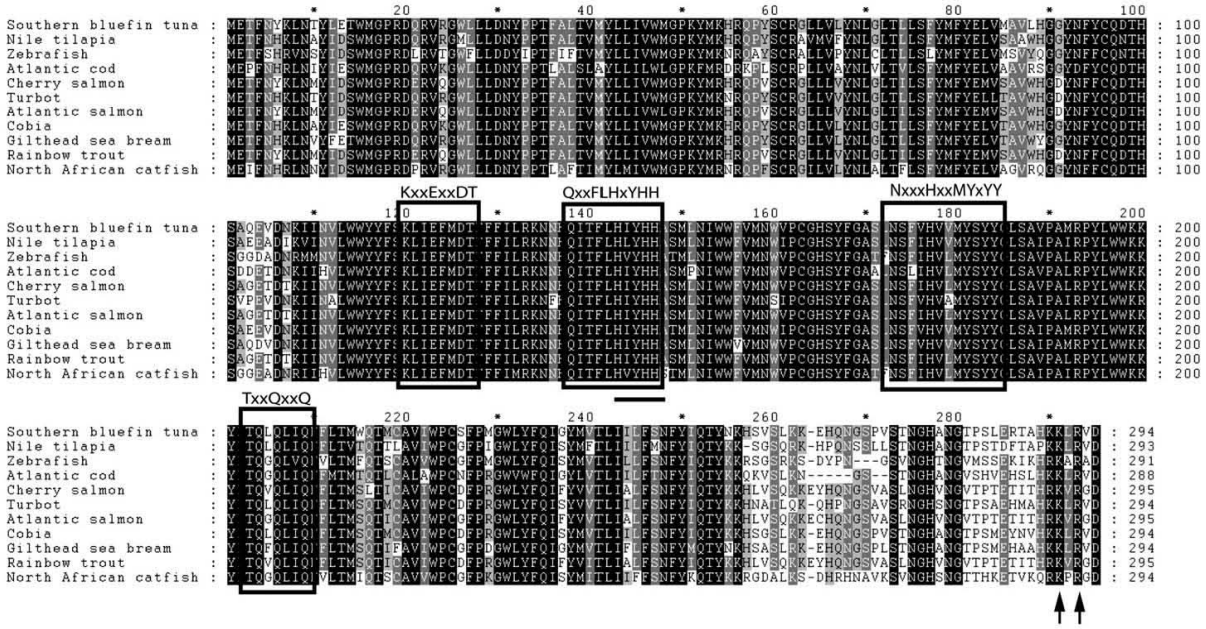

Fig. 1. A deduced amino acid sequence comparison of the sbtElovl5 from southern bluefin tuna with fatty acyl elongases from Nile tilapia, zebrafish, Atlantic cod, cherry salmon, turbot, Atlantic salmon, cobia, gilthead sea bream, rainbow trout and North African catfish. Identity/similarity shading was based on the Gonnet series matrix produced by ClustalX where primary black shading indicates identical residues and secondary and tertiary grey shading indicates similar residues with an 80% and 60% cut off, respectively. Highly conserved motifs are boxed, the histidine box is underlined and the ER retention signal is indicated by arrows.

bootstrap value of 89 (Fig. 2). The andronomous salmonids (cherry salmon, rainbow trout and Atlantic salmon) formed another clade which was strongly supported by a bootstrap value of 100.

3.3. Functional characterization of the sbtElovl5 ORF

The sbt*Elovl5* ORF was expressed in *S. cerevisiae* to investigate its functionality. In a preliminary experiment, induced cells were supplemented with 18:4n-3 to establish the best length of time for substrate incorporation and product conversion, for all subsequent experiments with all other PUFA substrates. The time-course experiment showed that 18:4n-3 was incorporated into the cells within 12 h and that it was progressively elongated to 20:4n-3 followed by 22:4n-3 over a period of at least 60 h (data not shown). Since the most dramatic changes occurred within the first 24 h, this time-point was chosen for harvesting the recombinant *S. cerevisiae* cells for all subsequent experiments.

Recombinant *S. cerevisiae* cells were cultured in the presence of various C_{18}, C_{20} and C_{22} PUFA substrates to determine the sbtElovl5 enzyme's substrate specificity (Fig. 3). The results showed that all of the substrates were incorporated into the cells. The uninduced cells showed low levels of elongation products from the C_{18} and C_{20} PUFA substrates, suggesting there was some 'leaky' sbtElovl5 expression, in the absence of the inducer, galactose. When the cells were induced in the presence of one PUFA substrate, sbtElovl5 showed high C_{18-20} elongase activity, converting 83.5% of 18:4n-3 to 20:4n-3 and 60.9% of 18:3n-6 to 20:3n-6 (Fig. 3A and B). The sbtElovl5 also showed high C_{20-22} elongase activity, converting 76.8% of 20:5n-3 to 22:5n-3 and 59.7% of 20:4n-6 to 22:4n-6 (Fig. 3C and D). In contrast, the C_{22-24} elongase activity of sbtElovl5 was much lower with only 1.7% of 22:5n-3 converted to 24:5n-3 and no detectable level of conversion of 22:4n-6 to 24:4n-6 (Fig. 3E and F). The cells supplied with the C_{18} substrates also showed detectable levels of the expected C_{22} products. This provided evidence that further elongation of the C_{20} intermediates was occurring. The sbtElovl5 could also elongate 18:3n-3 to 20:3n-3 and 18:2n-6 to 20:2n-6 with 15.7% and 10.8% conversion, respectively (Table 3). The percentage conversion data are summarised in Table 3.

Substrate supplementations with 18:4n-3 and 18:3n-6 or 20:5n-3 and 20:4n-6 confirmed that the sbtElovl5 had high C_{18-20} and C_{20-22} elongase activity. When two or four substrates were supplied approximately the same amount of each of the substrates was incorporated into the cells (data not shown). The sbtElovl5 converted 58.9% of 18:4n-3 to 20:4n-3 and 35.8% of 18:3n-6 to 20:3n-6 (Fig. 4A). Similarly to the individual supplementation with 18:4n-3 or 18:3n-6 a detectable level of the 22:4n-3 was seen, with 16.7% of 20:4n-3 being further elongated to 22:4n-3, but there was no detectable 22:3n-6. The sbtElovl5 converted 50.4% of 20:5n-3 to 22:5n-3 and 40.8% of 20:4n-6 to 22:4n-6 (Fig. 4B). The C_{18} and C_{20} substrates were not further elongated to C_{24} elongation products. Substrate supplementation with 18:4n-3, 18:3n-6, 20:5n-3 and 20:4n-6 showed that 18:4n-3 and 18:3n-6 as substrates were preferentially elongated to C_{20} products compared to 20:5n-3 and 20:4n-6 substrate elongation to C_{22} products (Fig. 4C). Under these conditions the sbtElovl5 converted 60.4% of 18:4n-3 to 20:4n-3 and 31.4% of 18:3n-6 to 20:3n-6, whilst the conversion of 20:5n-3 to 22:5n-3 was not detectable and 9.8% of 20:4n-6 was converted to 22:4n-6. It is clear that the sbtElovl5 exhibited a preference for n-3 over n-6 PUFA substrates. However, appreciable activity was still exhibited with the n-6 PUFA substrates.

The main endogenous fatty acids in the yeast cells were 16:0, 16:1n-7, 18:0 and 18:1n-9. The sbtElovl5 converted the endogenous 16:0, 16:1n-7 and 18:1n-9 fatty acids to 18:0, 18:1n-7 and 20:1n-9, respectively (data not shown). Thus, the sbtElovl5 was capable of elongating saturated and monounsaturated fatty acids as well as PUFA.

4. Discussion

Here we report on the cloning and functional characterisation of a cDNA encoding a fatty acyl elongase from the fish species SBT. The cDNA encoded a predicted protein which had the main structural characteristics of microsomal fatty acyl elongases from mammals and

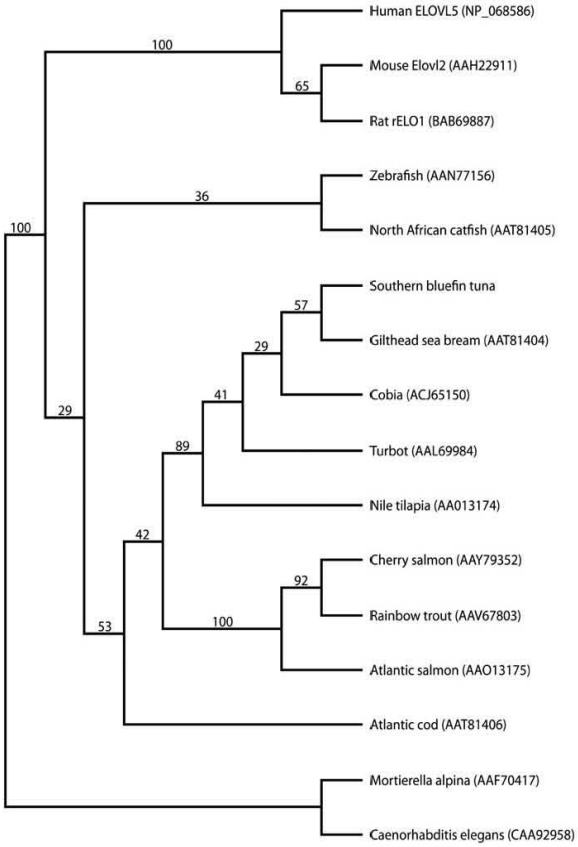

Fig. 2. A phylogenetic tree comparing deduced amino acid sequences of fatty acyl elongases from southern bluefin tuna, other fish species (zebrafish, North African catfish, gilthead sea bream, cobia, turbot, Nile tilapia, cherry salmon, rainbow trout, Atlantic salmon and Atlantic cod) and mammals (human, mouse and rat). The accession numbers are from the GenBank database. The tree was constructed using maximum parsimony analysis with ClustalX and PAUP* (Swofford, 2003). The numbers on the branches represent the frequencies with which the tree topology presented was replicated after 1000 bootstrap iterations.

other fish (Agaba et al., 2005; Jakobsson et al., 2006). The structural features included the four conserved motifs, a histidine box (HxxHH), C-terminal ER retention signal and multiple transmembrane regions. Sequence comparisons and phylogenetic analyses revealed that the sbtElovl5 predicted protein was most closely related to previously described fatty acyl elongases from the marine fish gilthead sea bream, cobia and turbot. In a comparison with the human fatty acyl elongases, the sbtElovl5 more closely resembled the human ELOVL5 than the ELOVL2.

Functional characterization of Elovl5-like fatty acyl elongases from fish species has been reported for zebrafish (Agaba et al., 2004), rainbow trout (Meyer et al., 2004), Nile tilapia, North African catfish, Atlantic cod, gilthead sea bream, turbot (Agaba et al., 2005), Atlantic salmon (Hastings et al., 2005), cherry salmon (Alimuddin et al., 2008) and cobia (Zheng et al., 2009). All of these fatty acyl elongases preferred C_{18} and C_{20} PUFA substrates over C_{22} PUFA substrates. The catfish, sea bream, zebrafish and Atlantic salmon elongases showed more C_{18-20} than C_{20-22} PUFA conversion (Agaba et al., 2004, 2005; Hastings et al., 2005) whereas the tilapia, turbot and cobia fatty acyl elongases showed approximately equal C_{18-20} and C_{20-22} PUFA conversion (Agaba et al., 2005; Zheng et al., 2009). The sbtElovl5 was more like the second group showing approximately equal 18:4n-3 and 20:5n-3 conversion, with percentage conversion values of 83.5% and 76.8%, respectively. The sbtElovl5 was able to elongate both the C_{20} and C_{22} n-3 PUFA substrates through to C_{24} PUFA products. The sbtElovl5 exhibited higher activity towards the n-3 substrates than the n-6 substrates, regardless of the chain length of the PUFA substrate (Table 3). This finding has also been reported for other fish species such as Atlantic salmon, zebrafish, catfish and tilapia (Agaba et al., 2005).

The LCPUFA synthesis pathway may use the same rate-limiting enzymes in both the n-3 and n-6 pathway, so it is important to understand the sbtElovl5 activity when more than one PUFA substrate is present. In the LCPUFA synthesis pathway 18:4n-3 and 18:3n-6 are in direct competition for elongation by the sbtElovl5 (Fig. 5). Similarly, after Δ5desaturation, 20:5n-3 and 20:4n-6 are competing for elongation by the sbtElovl5 (Fig. 5). This direct competition showed that the sbtElovl5 strongly preferred n-3 over n-6 PUFA substrates. Our multiple substrate supplementation experiments have uniquely addressed the issue of substrate competition which is found in nature. The four immediate substrates of sbtElovl5, 18:4n-3, 18:3n-6, 20:5n-3 and 20:4n-6 all compete for elongation at the same step in the LCPUFA synthesis pathway (Fig. 5). When equal amounts of all of the potential sbtElovl5 substrates were available, the sbtElovl5 preferentially utilized 18:4n-3 and 18:3n-6 over 20:5n-3 and 20:4n-6. In nature the availability of 18:4n-3, 18:3n-6, 20:5n-3 and 20:4n-6 may not be in equal proportions and so the competition at the sbtElovl5 position of the LCPUFA synthesis pathway would be intensified. For example, a high intake of dietary vegetable oil would make more 18:4n-3 and 18:3n-6 available and the efficiency of the sbtElovl5 to elongate 20:5n-3 and 20:4n-6 may be compromised.

Broad substrate specificity is a notable feature of vertebrate fatty acyl elongases compared to the lower eukaryote fatty acyl elongases from *M. alpina* (Parker-Barnes et al., 2000) and *C. elegans* (Beaudoin et al., 2000). The lower eukaryote enzymes are highly specific for C_{18} PUFA substrates. The human elongase ELOVL5 and the rat elongase rELO1, were both found to elongate C_{18} and C_{20} PUFA substrates, but not C_{22} PUFA substrates (Leonard et al., 2000; Inagaki et al., 2002). The mouse Elovl2 was shown to be active in elongating C_{20} and C_{22} PUFA substrates, whilst only a small amount of conversion of C_{18} PUFA substrates occurred and no saturated or monounsaturated fatty acids were elongated (Leonard et al., 2002). The human ELOVL2 was only active in elongating C_{20} and C_{22} PUFA substrates, with no conversion of C_{18} PUFA or saturated and monounsaturated fatty acids (Leonard et al., 2002). The sbtElovl5 substrate preference suggests that it is most similar to the mammalian ELOVL5 and rELO1 enzymes (Leonard et al., 2000; Inagaki et al., 2002).

The recombinant sbtElovl5 enzyme was very efficient at elongating the immediate precursors 18:4n-3 and 18:3n-6 but it was also capable of elongating 18:3n-3 and 18:2n-6, albeit with much lower efficiencies. The sbtElovl5 converted 15.7% of 18:3n-3 to 20:3n-3 and 10.8% of 18:2n-6 to 20:2n-6, respectively. Interestingly, ELOVL5 (Leonard et al., 2000), rELO1 (Inagaki et al., 2002) and the *C. elegans* fatty acyl elongase (Beaudoin et al., 2000) have all been shown to elongate 18:3n-3 and 18:2n-6 at a lower efficiency than 18:4n-3 and 18:3n-6. 20:3n-3 and 20:2n-6 are not considered intermediates of the accepted metabolic pathway for the synthesis of LCPUFA from C_{18} PUFA (Fig. 5). An alternative pathway involving Elovl5 followed by Δ8desaturase (18:3n-3/18:2n-6 → 20:3n-3/20:2n-6 → 20:4n-3/20:3n-6) rather than the traditional Δ6desaturase followed by Elovl5 (18:3n-3/18:2n-6 → 18:4n-3/18:3n-6 → 20:4n-3/20:3n-6) would synthesize 20:4n-3 and 20:3n-6 for further Δ5desaturation and continuation through the pathway. Park et al. (2009) have shown that the baboon FADS2 has both Δ6desaturation and Δ8desaturation activity when the substrates 18:3n-3 and 20:3n-3 or 18:2n-6 and 20:2n-6 were available. The introduction of vegetable oils into the SBT diet will increase the abundance of 18:3n-3 and 18:2n-6. The high enzyme activity of sbtElovl5 towards C_{18} PUFA substrates may enable an alternative Elovl5-Δ8desaturase pathway to be used.

The sbtElovl5 was active towards the endogenous yeast saturated and monounsaturated fatty acids. Elongation of the endogenous saturated fatty acids was made apparent by an increased 18:0/16:0

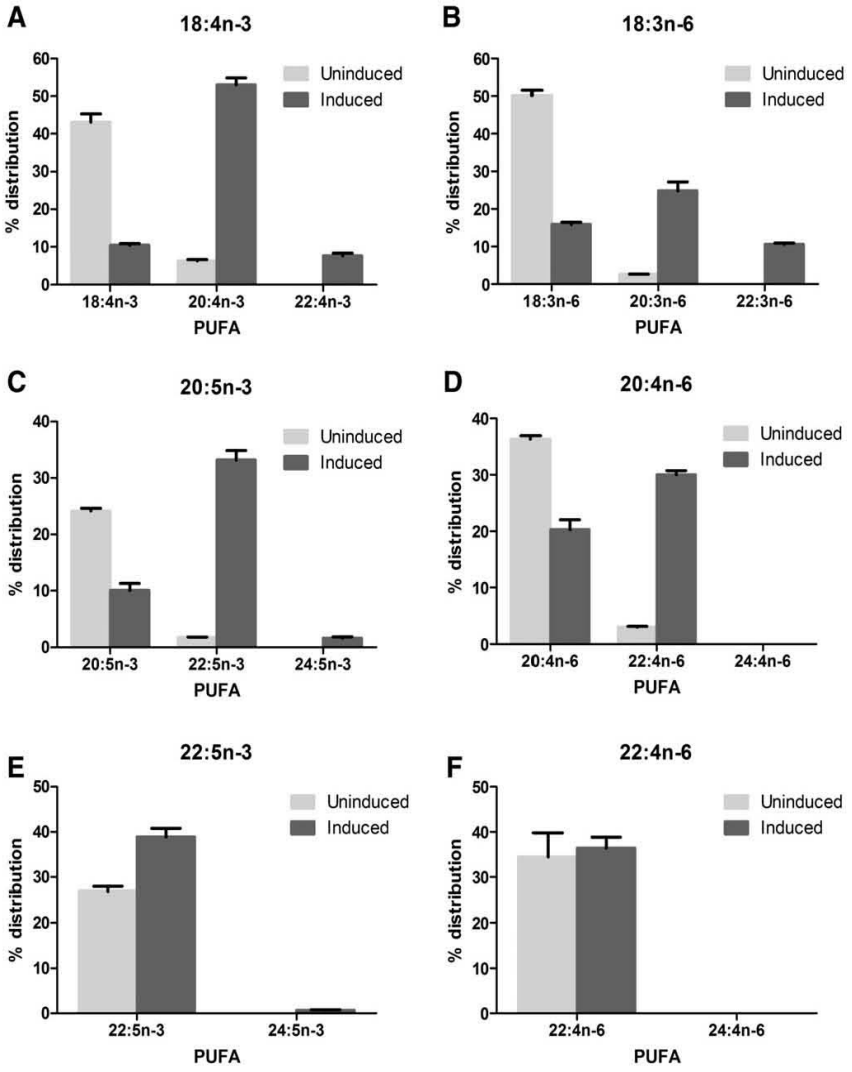

Fig. 3. Functional characterisation of the sbt*Elovl5* ORF in recombinant *S. cerevisiae* grown in the presence of 18:4n-3 (A), 18:3n-6 (B), 20:5n-3 (C), 20:4n-6 (D), 22:5n-3 (E) or 22:4n-6 (F) fatty acids. The yeast were cultured in either non-inducing media containing raffinose or inducing media containing galactose. Fatty acids were extracted from yeast transformed with the pYES2 vector containing the sbt*Elovl5* ORF. The amount of each fatty acid was expressed as a percentage of the total amount of all fatty acids. This was done by expressing the peak area for an individual fatty acid as a percentage of the total peak area for all fatty acids. The results are the means ± S.D. ($n=3$).

Table 3
Functional characterization of the sbtElovl5.

Substrate	Product	Conversion (%)	Substrate	Product	Conversion (%)
18:3n-3	20:3n-3	15.7 ± 1.0	18:2n-6	20:2n-6	10.8 ± 5.2
	22:3n-3	13.6 ± 3.1		22:2n-6	N.D.
	Total	29.3		Total	10.8
18:4n-3	20:4n-3	83.5 ± 0.8	18:3n-6	20:3n-6	60.9 ± 1.5
	22:4n-3	12.7 ± 1.2		22:3n-6	30.1 ± 2.7
	Total	96.2		Total	91
20:5n-3	22:5n-3	76.8 ± 1.9	20:4n-6	22:4n-6	59.7 ± 1.5
	24:5n-3	4.8 ± 0.8		24:4n-6	N.D.
	Total	81.6		Total	59.7
22:5n-3	24:5n-3	1.7 ± 0.4	22:4n-6	24:4n-6	N.D.

The results are expressed as the percentage of fatty acid substrate converted to elongated fatty acid product. The calculation used was [% product/(% product + % substrate)] × 100. Percentage conversion into intermediary products along the elongation pathway is also shown. The results are the means ± S.D. ($n=3$). N.D., not detected.

ratio for all of the n-3 and n-6 PUFA substrates. These data are consistent with the zebrafish fatty acyl elongase activity towards saturated fatty acids (Agaba et al., 2004). In contrast no such activity was observed with the Atlantic salmon (Hastings et al., 2005), North African catfish, turbot, Nile tilapia or gilthead sea bream fatty acyl elongases (Agaba et al., 2005). The elongation products of the endogenous monounsaturated fatty acids 16:1n-7 and 18:1n-9 were 18:1n-7 and 20:1n-9, respectively. Zebrafish, Atlantic salmon and gilthead sea bream fatty acyl elongases also elongated 16:1n-7 and 18:1n-9, whilst North African catfish, Nile tilapia and turbot only elongated 16:1n-7 (Agaba et al., 2004, 2005; Hastings et al., 2005). Thus, it appears that the sbtElovl5 is not only active with PUFA substrates, but also with saturated and monounsaturated fatty acid substrates.

Production of C_{24} PUFA, in particular 24:5n-3, is paramount because it is the precursor to DHA, which is widely accepted to be vital for neural tissue development and function and various human health benefits (Tocher, 2003; Agaba et al., 2004). In this study we saw very low production of C_{24} PUFA from shorter chain precursors. The liver is

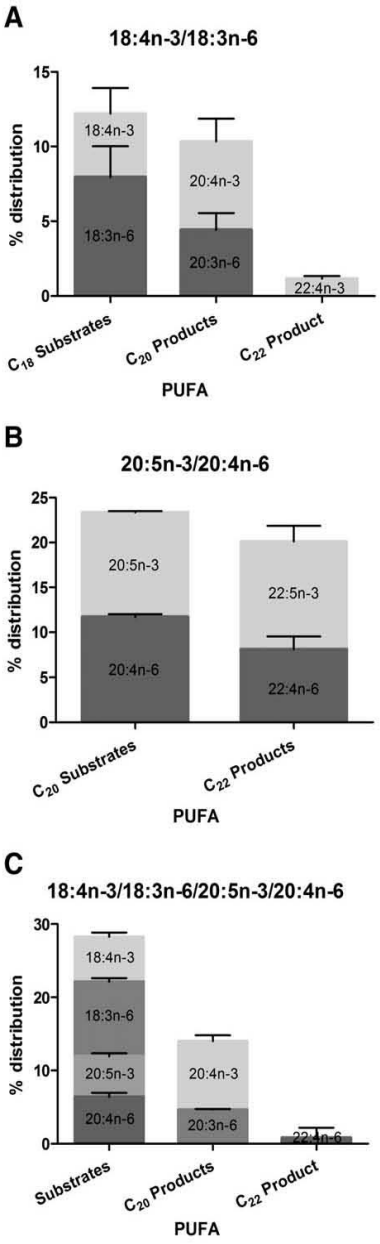

Fig. 4. Functional characterisation of the sbt*Elovl5* ORF in recombinant *S. cerevisiae* grown in the presence of 18:4n-3 and 18:3n-6 (A), 20:5n-3 and 20:4n-6 (B) or 18:4n-3, 18:3n-6, 20:5n-3 and 20:4n-6 (C) fatty acids. The yeast were cultured in inducing media containing galactose. Fatty acids were extracted from yeast transformed with the pYES2 vector containing the sbt*Elovl5* ORF. The amount of each fatty acid was expressed as a percentage of the total amount of all fatty acids. This was done by expressing the peak area for an individual fatty acid as a percentage of the total peak area for all fatty acids. The results are the means ± S.D. ($n = 3$).

the site of lipid synthesis in tuna and is not considered to store large quantities of lipid like other fish species (Mourente et al., 2002). This was evident by the lower DHA content and DHA/EPA ratio in the SBT liver than the flesh (Nichols et al., 1998). However, the fatty acid profile of the SBT liver tissue was particularly rich in n-3 PUFA, with an n-3/n-6 ratio of 9. As seen with ALA, EPA and DHA, the n-3 PUFA content increased as the chain length of the n-3 PUFA increased. Due to the structure of the marine food chain, marine fish species such as

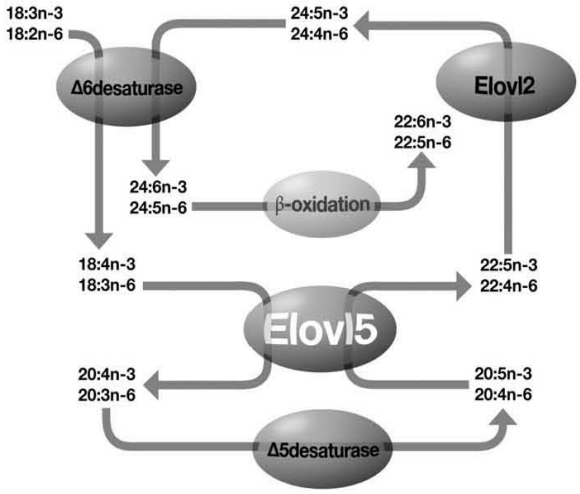

Fig. 5. Long chain polyunsaturated fatty acid (LCPUFA) synthesis in vertebrates highlighting the role of the fatty acyl elongase enzymes, Elovl2 and Elovl5.

SBT are expected to have a lesser capability to synthesize C_{24} PUFA. SBT may display a reduced capacity to elongate C_{22} PUFA because they have adapted to diets containing sufficient levels of C_{24} PUFA. Therefore, the accumulation and retention of DHA from the diet and possibly the selective use of saturated and monounsaturated fatty acids for use as energy (Saito et al., 1997), highlights the uniqueness of SBT as a marine fish species.

As discussed above, the sbtElovl5 substrate preference was most similar to the vertebrate elongases from human (ELOVL5), rat (rELO1) and Nile tilapia. This finding is significant because the freshwater fish species Nile tilapia can survive on diets containing vegetable oils which are rich in C_{18} PUFA but low in n-3 LCPUFA (Agaba et al., 2005). A shift in the SBT aquaculture industry to a sustainable vegetable oil-based pellet diet will require the SBT to utilize the abundance of C_{18} PUFA to produce LCPUFA. Previous work has shown that Atlantic salmon can survive and grow on a vegetable oil-based diet but the concentration of EPA and DHA in their flesh is lowered, thus lowering the human health value (Tocher et al., 2003). To enable freshwater fish and salmonids to synthesize DHA from ALA all of the enzymes in the LCPUFA synthesis pathway must be active (Tocher, 2003). Atlantic salmon is the only non-mammalian vertebrate with a functionally characterised Elovl2 (Morais et al., 2009). On the contrary, the marine species SBT may not have another fatty acyl elongase which elongates C_{22} PUFA substrates. Searches of the ENSEMBL genomes suggest that other carnivorous fish species *Tetraodon nigroviridis* and *Gasterosteus aculeatus* and even omnivorous fish species *Takifugu rubripes* and *Oryzais latipes* do not contain *Elovl2* homologs (Morais et al., 2009).

Future research could include genetically screening for fish with superior fatty acyl elongase enzyme activities or introducing a foreign fatty acyl elongase into an aquaculture species. For example, Alimuddin et al. (2008) have over-expressed a cherry salmon fatty acyl elongase in zebrafish to improve the synthesis of EPA and DHA by 1.3-fold each. If SBT are not capable of elongating C_{22} PUFA to C_{24} PUFA, introducing a foreign fatty acyl elongase, perhaps from a freshwater fish species which is capable of performing this conversion, would enable SBT to chain elongate C_{18} PUFA to C_{24} LCPUFA.

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