Author/s (year)	[redacted]
Title (version Nº)	**Characterisation of *Pavvvlova salina* Δ5-Desaturase**
Owner	**Nuseed Pty Ltd**
Date	**December 15, 2016**
Report Nº	**2016-009**
Testing Facility	**CSIRO** **Black Mountain Laboratories** **Canberra, ACT** **Australia**
Test method	**Not Applicable**
Good Laboratory Practice	**No GLP**
Confidentiality	**None**

TITLE:
CHARACTERIZATION OF *PAVLOVA SALINA* Δ5-DESATURASE

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ABBREVIATIONS

ALA	α-Linolenic acid, $18:3^{\Delta 9,12,15}$ (ω3)
BMGY	Buffered glycerol-complex medium
BMMY	Buffered complex medium containing methanol
CoA	Coenzyme A
DHA	Docosahexaenoic acid $22:6^{\Delta 4,7,10,13,16,19}$ (ω3)
DHA canola	Genetically modified canola, event NS-B50027-4
DPA	Docosapentaenoic acid $22:5^{\Delta 7,10,13,16,19}$ (ω3)
EPA	Eicosapentaenoic acid $20:5^{\Delta 5,8,11,14,17}$ (ω3)
ETA	Eicosatetraenoic acid $20:4^{\Delta 8,11,14,17}$ (ω3)
FAME	Fatty acid methyl ester
GC	Gas chromatography
GLA	γ-linolenic acid, $C18:3^{\Delta 6,9,12}$ (ω6)
kDa	Kilo dalton
LA	Linoleic acid, $18:2^{\Delta 9,12}$ (ω6)
Lackl-Δ12D	*Lachancea kluyveri* Δ12-desaturase
Micpu-Δ6D	*Micromonas pusilla* Δ6-desaturase
MMT	Million metric ton
MQ	MilliQ water
OA	Oleic acid, $18:1^{\Delta 9}$
ω3 LC-PUFA	Omega-3 long-chain (≥C20) polyunsaturated fatty acids
ORF	Open reading frame
Pavsa-Δ4D	*Pavlova salina* Δ4-desaturase
Pavsa-Δ5D	*Pavlova salina* Δ5-desaturase
pI	Theoretical isoelectric point
Picpa-ω3D	*Pichia pastoris* Δ15-/ω3-desaturase
Pyrco-Δ5E	*Pyramimonas cordata* Δ5-elongase
Pyrco-Δ6E	*Pyramimonas cordata* Δ6-elongase
SDA	Stearidonic acid, $18:4^{\Delta 6,9,12,15}$ (ω3)
SP	Secretion peptide
X:Y	A fatty acid containing X carbons with Y double bonds
YPD	Yeast extract-Peptone-Dextrose

EXECUTIVE SUMMARY

The purpose of this report was to characterise the yeast *Pavlova salina* Δ5-desaturase (Pavsa-Δ5D) protein, its amino acid sequence and homology to other proteins, and its enzymatic activity in different expression systems.

The results of the study demonstrated that Pavsa-Δ5D was a functional enzyme that desaturated eicosatetraenoic acid acid (ETA) to eicosapentaenoic acid (EPA) in different cells for accumulating more precursor of omega-3 long-chain (≥C20) polyunsaturated fatty acids (ω3 LC-PUFA). Pavsa-Δ5D protein contains 425 amino acid residues and shares high homology to other Δ5-desaturases that have been consumed as food, used in food production or in animal feeds. The molecular weight of Pavsa-Δ5D is predicted to be 48.2 kDa, with an estimated isoelectric point (pI) of 8.18.

I. INTRODUCTION

The omega-3 long-chain (≥C20) polyunsaturated fatty acids (ω3 LC-PUFA) eicosapentaenoic acid (EPA, 20:5ω3), docosapentaenoic acid (DPA, 22:5ω3) and docosahexaenoic acid (DHA, 22:6ω3) are widely recognised for their beneficial roles in human health, particularly those related to cardiovascular and inflammatory health. EPA, DPA and DHA are primarily sourced from wild-caught fish oils and algal oils, with algae being the primary producer in the marine food web. These sources are under pressure due to increasing demand for ω3 LC-PUFA by aquaculture, nutraceutical and pharmaceutical applications. Additional sources of these fatty acids can be produced by engineering land-based oilseed crops to convert native fatty acids to marine-type ω3 LC-PUFA which are then accumulated in seed oil. Canola is a commonly grown oilseed with 67 million metric tons (MMT) of rapeseed produced globally in 2015/16[1].

In collaboration with the Commonwealth Scientific and Industrial Research Organization (CSIRO), Nuseed Pty Ltd has developed genetically modified canola event NS-B50027-4 (DHA canola), which accumulates significant amounts of DHA in the seed oil.

[1] http://www.ers.usda.gov/data-products/oil-crops-yearbook/oil-crops-yearbook/#World Supply and Use of Oilseeds and Oilseed Products

In this DHA canola, seven fatty acid desaturases and elongases were introduced to convert oleic acid (OA) to DHA in a single pathway expression vector. The pathway (Figure 1) was consisted of *Lachancea kluyveri* Δ12-desaturase (Lackl-Δ12D, Watanabe et al. 2004), *Pichia pastoris* ω3-/Δ15-desaturase (Picpa-ω3D, Zhang et al. 2008), *Micromonas pusilla* Δ6-desaturase (Micpu-Δ6D, Petrie et al. 2010b), *Pyramimonas cordata* Δ6-elongase (Pyrco-Δ6E, Petrie et al. 2010a), *Pavlova salina* Δ5-desaturase (Pavsa-Δ5D, Zhou et al. 2007), *P. cordata* Δ5-elongase (Pyrco-Δ5E, Petrie et al. 2010a) and *P. salina* Δ4-desaturase (Pavsa-Δ4D, Zhou et al. 2007). The functionalities and activities of these enzymes have been demonstrated in different heterologous expression systems (see Report Nºs 2016-005, 2016-006, 2016-007, 2016-008, 2016-009, 2016-010, 2016-011) and in transgenic Arabidopsis or Camelina seeds (Petrie et al. 2012; Petrie et al. 2014). Based on the sequence similarity and functionality, these seven proteins can be classified into three groups, (1) yeast acyl-CoA type fatty acid desaturases including Lackl-Δ12D and Picpa-ω3D (Figure 1, blue) that introduce a double bond at the Δ12 and Δ15 positions, respectively; (2) algae fatty acid elongases including Pyrco-Δ6E and Pyrco-Δ5E (Figure 1, purple) that add two carbons to the carboxyl end of fatty acids; and (3) algae front-end fatty acid desaturases that introduce a double bond between an existing double bond and the carboxyl end of fatty acids (Zhou et al. 2007) including Micpu-Δ6D, Pavsa-Δ5D and Pavsa-Δ4D (Figure 1, green).

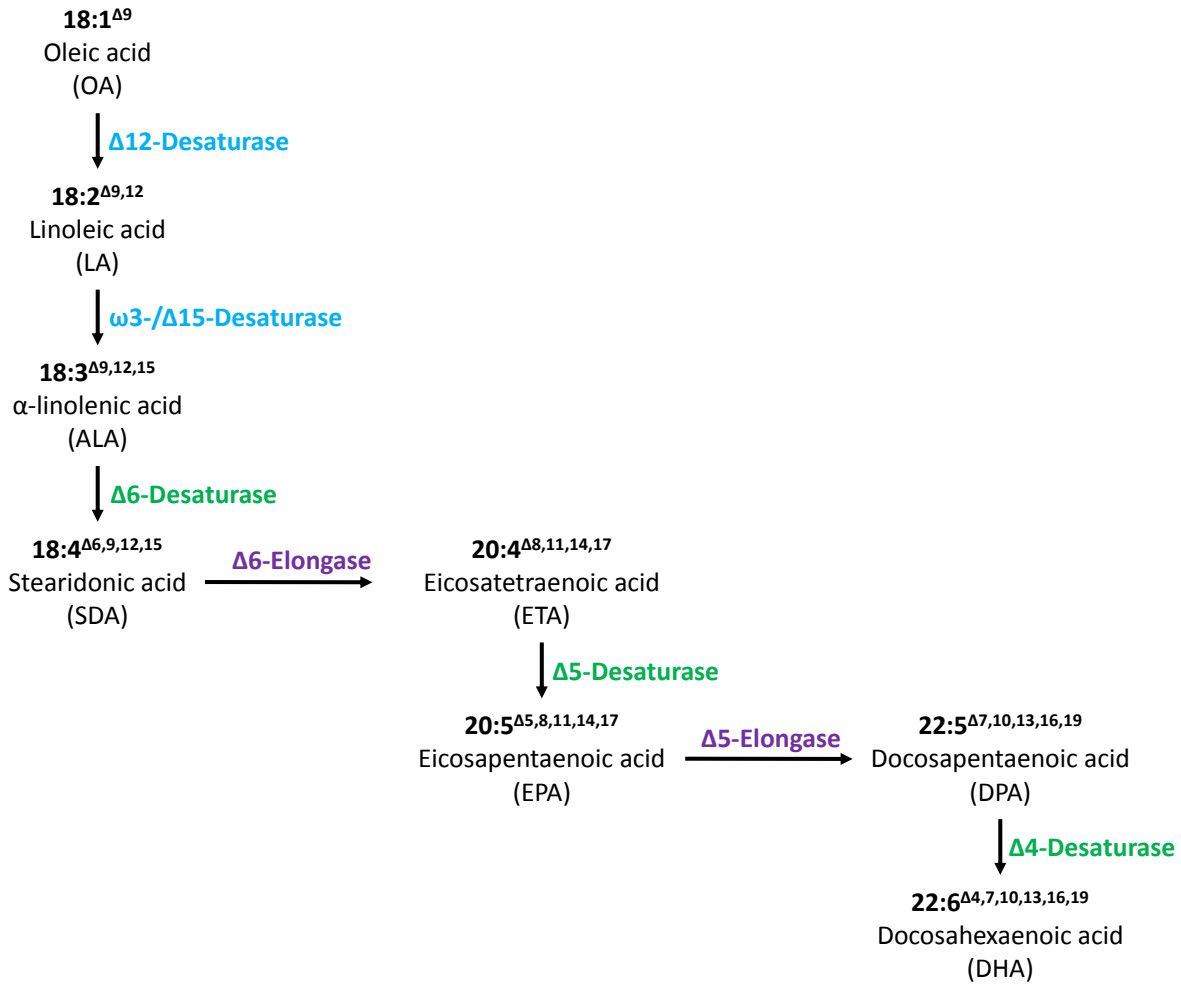

Figure 1. DHA biosynthesis pathway engineered into DHA canola event NS-B50027-4. Seven enzymes introduced in canola to convert oleic acid to final product docosahexaenoic acid were grouped into 3 classes, two fatty acid desaturases from yeast in blue, two elongases from microalgae in purple, and three front-end desaturases from microalgae in green (see text for detail).

II. PURPOSE

The purpose of this study was to characterise the fatty acid biosynthesis enzymes used in the engineering of DHA canola, including the amino acid sequences, homology to other proteins with similar function or presented in consumed food or used in food production, and their enzymatic activities in heterologous expression systems. This particular report is focusing on the *P. salina* Δ5-desaturase (Pavsa-Δ5D) protein to catalyse the desaturation of ETA at the Δ5 position to EPA ($20:4^{\Delta 8,11,14,17} \rightarrow 20:5^{\Delta 5,8,11,14,17}$).

III. MATERIALS

A. TARGET PROTEIN

The Δ6-desaturase gene used in DHA canola event was previously cloned from microalga *P. salina* (Zhou et al. 2007). The Pavsa-Δ5D protein was expressed as native sequence in yeast cells (Zhou et al. 2007), *Nicotiana benthamiana* leaf (Wood et al. 2009), Arabidopsis seed (Petrie et al. 2012) and Camelina seed (Petrie, 2014), as well as in yeast *P. pastoris* cell as His-tag fusions with or without *Saccharomyces cerevisiae α*-mating type signal peptide as secretion peptide (SP). The His-tag fusion vectors contained a coding sequence encoding a His-tag (His_{10}) and a PreScission protease cleavage site (SLEVLFQ$^{\downarrow}$GP) fused to the codon optimized *Pavsa-Δ5D* gene.

B. OTHER MATERIALS

The *Pavsa-Δ5D* gene was synthesized at GeneArt (Life Science Technologies, Germany), based on the sequence (DQ995517, Zhou et al. 2007) as a His-tag fusion with or without Pichia secretion peptide, and cloned into the Pichia expression vector pPinkα-HC (Invitrogen, Carlsbad, CA, USA).

IV. METHODS

A. SEQUENCE COMPARISON

The *Pavsa-Δ5D* gene was previously cloned from microalga *P. salina* (Zhou et al. 2007). The translated amino acid sequence was compared to other published Δ5-desaturases or related fatty acid desaturase presented in food or used in food production, with Vector NTI software (Version 11, Invitrogen).

B. TRANSFORMATION OF PICHIA CELL

Pichia transformation was essentially done according to published protocol (Chen et al. 2013). Pichia expression vector DNA containing *Pavsa-Δ5D* gene was first linearized by single restriction enzyme digestion in vector backbone, and precipitated with 1/10 volume of 3 M sodium acetate and 2 volume of 100% ethanol overnight at -20ºC. The precipitated DNA

was resuspended in 10 µL of MilliQ (MQ) water for yeast transformation. The yeast PichiaPink™ strain 4 (Invitrogen) was first activated from the stab culture on a fresh Yeast extract-Peptone-Dextrose (YPD) plate at 28ºC for 3 to 4 days. Single colony was inoculated in 10 mL of YPD medium and cultured at 28ºC with shaking (200 rpm) for 24 hours, followed by inoculating 100 mL of culture to OD_{600}=0.2 from the previous 10 mL culture and allowed to grow at 28ºC for 8 to 10 hrs until OD_{600}=1.0 to 1.5. The cells were spun down at 3000 rpm for 10 min and washed with 100 mL chilled MQ water, followed by washing with 50 mL of chilled MQ water then by 10 mL of cold 1 M sorbitol. The cells were resuspended in 300 µL of 1 M sorbitol and dispensed into 80 uL aliquots in Eppendorf tubes. The prepared Pichia competent cells were mixed with 10 µL of linearized DNA, incubated on ice for 5 min and electroporated. After electroporation, the cells were recovered from electroporation cuvettes with 1 mL of YPD with sorbitol (Invitrogen), incubated at 28ºC without shaking for 4 hrs, then plated out on Pichia Adenine Dropout Dextrose (Invitrogen) plate and incubate at 28ºC for 2-4 days. White yeast colonies were selected for further analysis.

C. Enzyme Activity Analysis in Pichia Cell

Individual white colonies of Pichia were inoculated into 10 mL Buffered Glycerol-complex Medium (BMGY, 1% yeast extract; 2% peptone; 100 mM potassium phosphate, pH 6.0; 1.34% Yeast Nitrogen Base; 0.0004% biotin; 1% glycerol) in 50 ml Falcon tubes and cultured for 48 hrs at 28ºC with shaking at 250 rpm. From each sample, 2.5 mL of the above culture were inoculated into 50 mL of BMGY medium in 250 mL flasks and grown at 28ºC for 24 hrs at 250 rpm. Yeast cells were collected by centrifugation at 3000 rpm for 10 min and resuspended in 5 mL induction medium (BMMY, 1% yeast extract; 2% peptone; 100 mM potassium phosphate, pH 6.0; 1.34% Yeast Nitrogen Base; 0.0004% biotin; 0.5% methanol) at 28ºC for 3 days, by adding 50 uL of methanol to the culture every 24 hrs. In the case of fatty acid substrate feeding was needed, the fatty acid was added to the final concentration of 0.5 mM with 1% of detergent NP-40 in BMMY medium. The cells were harvested by centrifuging at 3000 rpm for 5 min, and washed with 1% NP-40, 0.5% NP-40 then water as described (Zhou et al. 2006).

D. Fatty Acid Analysis

For fatty acid analysis, cells were dried overnight with freezing-vacuum dryer. Fatty acid methyl esters (FAME) were prepared with 0.75 mL of 1N methanolic-HCl at 80ºC for 3 hrs, and extracted with 0.5 mL 0.9% NaCl followed by 0.3 mL of hexane after cooling down to room temperature. The hexane phase containing FAMEs were recovered after centrifuging at

3000 rpm for 5 min, transferred to gas chromatography (GC) vials, dried down to 30 µL with nitrogen. GC analysis was done according to previously described (Zhou et al. 2011).

V. RESULTS AND DISCUSSION

A. GENE SOURCE AND DONOR ORGANISM

The *Pavsa-Δ5D* gene was previously cloned from microalga *P. salina* (Zhou et al. 2007). The open reading frame (ORF) of *Pavsa-Δ5D* gene consisted of 1278 bp, and is shown in Figure 2.

ATGCCGCCGCGCGATAGCTACTCGTACGCCGCCCCGCCGTCGGCCCAGCTGCACGAGGT
CGATACCCCGCAGGAGCATGATAAGAAGGAGCTCGTCATCGGTGACCGCGCGTACGACG
TGACCAACTTTGTGAAGCGCCACCCGGGTGGCAAGATCATCGCATACCAGGTTGGCACA
GATGCGACGGACGCGTACAAGCAGTTCCATGTGCGGTCTGCCAAGGCGGACAAGATGCT
CAAGTCGCTGCCTTCGCGCCCGGTGCACAAGGGCTACTCGCCCCGCCGCGCTGACCTCA
TTGCCGACTTCCAGGAGTTCACCAAGCAGCTGGAGGCGGAGGGCATGTTTGAGCCGTCG
CTGCCGCACGTGGCATACCGCCTGGCGGAGGTGATCGCGATGCACGTGGCCGGCGCCGC
GCTCATCTGGCACGGGTACACCTTCGCGGGCATTGCCATGCTCGGCGTTGTGCAGGGCC
GCTGCGGCTGGCTCATGCACGAGGGCGGCCACTACTCGCTCACGGGCAACATTGCTTTT
GACCGTGCCATCCAAGTCGCGTGCTACGGCCTTGGCTGCGGCATGTCGGGCGCGTGGTG
GCGCAACCAGCACAACAAGCACCACGCGACGCCGCAGAAGTTGCAGCACGACGTCGACC
TCGACACCCTCCCGCTCGTCGCCTTCCACGAGCGGATAGCCGCCAAGGTGAAGAGCCCC
GCGATGAAGGCGTGGCTTAGTATGCAGGCGAAGCTCTTCGCGCCAGTGACCACGCTGCT
GGTCGCGCTGGGCTGGCAGCTGTACCTGCACCCGCGCCATATGCTGCGCACCAAGCACT
ACGACGAGCTCGCGATGCTCGGCATTCGCTACGGCCTTGTCGGCTACCTCGCGGCGAAC
TACGGCGCGGGGTACGTGCTCGCGTGCTACCTGCTGTACGTGCAGCTCGGCGCCATGTA
CATCTTCTGCAACTTTGCCGTGTCGCACACACACCTGCCGGTTGTCGAGCCTAACGAGC
ACGCAACGTGGGTGGAGTACGCCGCGAACCACACGACCAACTGCTCGCCCTCGTGGTGG
TGCGACTGGTGGATGTCGTACCTCAACTACCAGATCGAGCACCACCTCTACCCGTCCAT
GCCGCAGTTCCGCCACCCGAAGATTGCGCCGCGGGTGAAGCAGCTCTTCGAGAAGCACG
GCCTGCACTACGACGTGCGTGGCTACTTCGAGGCCATGGCGGACACGTTTGCCAACCTT
GACAACGTCGCGCACGCGCCGGAGAAGAAGATGCAG**TGA**

Figure 2. ORF nucleotide sequence of native Pavsa-Δ5D gene.
Start codon (ATG) and stop codon (TGA) are in bold.

B. PROTEIN SEQUENCE

The translated *P. salina* Δ5-desaturase (Pavsa-Δ5D, ABL96295) contained 425 amino acid residues (Figure 3). The molecular weight of Pavsa-Δ5D is predicted as 48.2 kDa, with estimated pI of 8.18.

```
MPPRDSYSYAAPPSAQLHEVDTPQEHDKKELVIGDRAYDVTNFVKRHPGGKIIAYQVGT
DATDAYKQFHVRSAKADKMLKSLPSRPVHKGYSPRRADLIADFQEFTKQLEAEGMFEPS
LPHVAYRLAEVIAMHVAGAALIWHGYTFAGIAMLGVVQGRCGWLMHEGGHYSLTGNIAF
DRAIQVACYGLGCGMSGAWWRNQHNKHHATPQKLQHDVDLDTLPLVAFHERIAAKVKSP
AMKAWLSMQAKLFAPVTTLLVALGWQLYLHPRHMLRTKHYDELAMLGIRYGLVGYLAAN
YGAGYVLACYLLYVQLGAMYIFCNFAVSHTHLPVVEPNEHATWVEYAANHTTNCSPSWW
CDWWMSYLNYQIEHHLYPSMPQFRHPKIAPRVKQLFEKHGLHYDVRGYFEAMADTFANL
DNVAHAPEKKMQ
```

Figure 3. Amino acid sequence of Pavsa-Δ5D.

The fatty acid Δ5-desaturases have been cloned from protozoon (Venegas-Calerón et al. 2007), alga (Zhou et al. 2007), amoeba (Saito et al. 2000), moss (Kaewsuwan et al. 2006), liverwort (Kajikawa et al. 2004), fungus (Michaelson et al. 1998), octopus (Monroig et al. 2012), zebra fish (Hastings et al. 2001), scallop (Liu et al. 2014) and mammals (Leonard et al. 2000). The Pavsa-Δ5D shared high homology to other Δ5-desaturase proteins as shown in Figure 4.

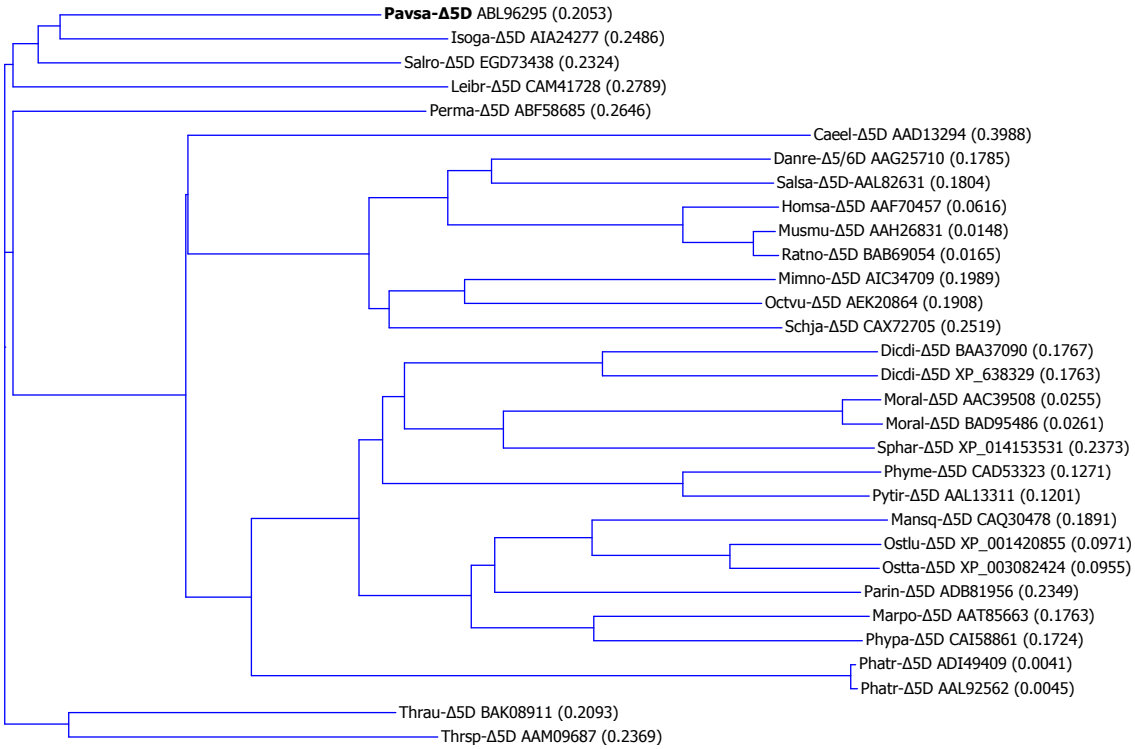

Figure 4. Phylogenetic tree for sequence comparison of Pavsa-Δ5D with representative Δ5-desaturases.

The phylogenetic tree was generated with Vector NTI software (Invitrogen, Carlsbad, USA). The protein sequences were named as 5-letter initial of species name, followed by NCBI accession numbers.

Caeel, *Caenorhabditis elegans* (nematode); Danre, *Danio rerio* (zebrafish); Dicdi, *Dictyostelium discoideum* (amoeba); Homsa, *Homo sapiens* (human); Isoga, *Isochrysis galbana* (alga); Leibr, *Leishmania braziliensis* (protozoa); Mansq, *Mantoniella squamata* (alga); Marpo, *Marchantia polymorpha* (liverwort); Mimno, *Mimachlamys nobilis* (scallop); Moral, *Mortierella alpina* (fungus); Musmu, *Mus musculus* (mouse); Octvu, *Octopus vulgaris* (octopus); Ostlu, *Ostreococcus lucimarinus* CCE9901 (alga); Ostta, *O. tauri* (alga); Parin, *Parietochloris incisa* (alga); Pavsa, *Pavlova salina* (alga); Perma, *Perkinsus marinus* (protozoan); Phatr, *Phaeodactylum tricornutum* (diatom); Phyme, *Phytophthora megasperma* (fungus); Phypa, *Physcomitrella patens* (moss); Pytir, *Pythium irregulare* (fungus); Ratno, *Rattus norvegicus* (rat); Salro, *Salpingoeca rosetta*; Salsa, *Salmo salar* (salmon); Schja, *Schistosoma japonicum* (parasite); Sphar, *Sphaeroforma arctica* (protist); Thrau, *Thraustochytrium aureum* (protist); Thasp, *T. sp.* ATCC21685 (protist). Δ5D, Δ5-desaturase; Δ5/6D, bifunctional Δ5- and Δ6-desaturase.

C. Similarity of Pavsa-Δ5D to Other Proteins in Consumed Foods, Used in Food Production or in Animal Feeds

The Pavsa-Δ5D protein shares similarity to desaturase proteins present in food that is directly consumed, used in food production or in animal feeds (Table 1). Pavsa-Δ5D was cloned from *P. Salina,* one of the microalgae used in mariculture (Brown 1991). *P. lutheri* is used for oyster larvae and clam larvae feeds (Brown et al. 1997). Pavsa-Δ5D shares 53% sequence identity to *P. lutheri* Δ5-desaturase (ALE15225, partial sequence) in overlapping region.

Pavsa-Δ5D shares amino acid sequence identities to many other fatty acid desaturases from a wide range of species. Pavsa-Δ5D shares 53% sequence identity to marine microalga *Isochrysis galbana* Δ5-desaturase (AIA24277). *I. galbana* is used to make functional sweet biscuits with enriched LC-PUFA (Gouveia et al. 2008). *I. galbana* and *Diacronema vlkianum* are of substantial interest in aquaculture, principally to feed mollusk larvae, as well as fish and crustaceans in the early stages of growth (Fradique et al. 2013).

Pavsa-Δ5D shares 47% sequence identity to Thraustochytrium Δ5-desaturase (BAK08911). Thraustochytrids are single-celled eukaryotic protists, classified as oleaginous microorganisms for microbial production of omega-3 fatty acids DHA and EPA (Gupta et al. 2012).

Pavsa-Δ5D shares 18% sequence identity to *Mortierella alpina* Δ5-desaturase (AAC72755). *M. alpine* is currently used for the commercial production of arachidonic acid for fortification of baby food. Several LC-PUFAs are also commercially produced by using *Mortierella* fungi species (Sakuradani and Shimizu 2009).

Pavsa-Δ5D shares 25%, 23% or 23% sequence identity to octopus, salmon or human Δ5-desaturases (AEK20864, AAL82631, AAF70457).

Pavsa-Δ5D shares 21% sequence identity to Δ6-desaturases from *Oenothera biennis* (evening primrose) (ACB47482), *Echium plantagineum* (echium, AAZ08559) and *Borago officinalis* (borage, O04353). These species have been used to produce oils that are relatively high in γ-linolenic acid ($C18:3^{\Delta 6,9,12}$, GLA) and/or SDA for human consumption. The oils produced by these species have been studied extensively for their anti-inflammatory effects on leukotriene and prostaglandin biosynthesis (Fan and Chapkin, 1998), and are sold as cold-pressed oils for use as dietary supplements. Evening primrose oil is commonly sold in Australian health food shops. Additionally, the flowers of *Echium* sp. have been consumed as medicinal plants in countries such as Iran (Heidari et al., 2006). Evening primrose plants have been used as ornamentals, food sources, and as medicinal herbs for more than 50 years.

Table 1. Amino acid sequence identity between Pavsa-Δ5D in DHA canola (event NS-B50027-4) and other desaturase proteins present in consumed foods, used in food production or in animal feeds

No.	Protein	Accession	Common Name	Sequence identity								
				1	2	3	4	5	6	7	8	9
1	NS-B50027-4 Pavsa-Δ5D			100	52.7	52.6	46.8	24.9	22.9	22.9	18.0	20.8
2	Pavlu-Δ5D*	ALE15225	Alga		100	86.6	43.3	26.8	24.7	26.6	18.7	20.7
3	Isoga-Δ5D	AIA24277	Alga			100	42.5	25.2	24.1	24.6	17.4	19.4
4	Thrau-Δ5D	BAK08911	Protist				100	23.8	24.2	23.0	16.1	21.4
5	Octvu-Δ5D	AEK20864	Octopus					100	49.7	51.0	19.9	24.7
6	Homsa-Δ5D	AAF70457	Human						100	57.2	21.8	23.7
7	Salsa-Δ5D	AAL82631	Salmon							100	20.0	23.0
8	Moral-Δ5D	AAC72755	Fungus								100	20.7
9	Onebi-Δ6D	ACB47482	Evening primrose									100

Δ5D, Δ5-desaturase; Δ6D, Δ6-desaturase. Homsa, *Homo sapiens* (human); Isoga, *Isochrysis galbana* (alga); Moral, *Mortierella alpina* (fungus); Octvu, *Octopus vulgaris* (octopus); Onebi, *Oenothera biennis* (evening primrose); Pavlu, *Pavlova lutheri* (alga); Pavsa, *P. salina* (alga); Salsa, *Salmo salar* (salmon); Thrau, *Thraustochytrium aureum* (protist). *Pavlu-Δ5D is partial sequence. The sequence identity was based on the overlap region only.

D. HETEROLOGOUS EXPRESSION

The enzyme functionality of Pavsa-Δ5D has been confirmed in different heterologous expression systems, including yeast cell (Zhou et al. 2007), *Nicotiana benthamiana* leaf (Wood et al. 2009), Arabidopsis seed (Petrie et al. 2012) and Camelina seed (Petrie et al. 2014). In this study, Pavsa-Δ5D was expressed in *P. pastoris,* as fusion proteins designated as SP::His_{10}::Pavsa-Δ5D or His_{10}::Pavsa-Δ5D. In SP::His_{10}::Pavsa-Δ5D, the Pavsa-Δ5D sequence was fused to *Saccharomyces cerevisiae α*-mating type signal peptide (SP), followed by His-tag (His_{10}) and PreScission protease cleavage site (SLEVLFQ$^{\downarrow}$GP) at its N-terminal (Figure 5). In His_{10}::Pavsa-Δ5D, the Pavsa-Δ5D sequence was fused to His-tag (His_{10}) and PreScission protease cleavage site (SLEVLFQ$^{\downarrow}$GP) at its N-terminal (Figure 6). No secretion peptide was used in His_{10}::Pavsa-Δ5D.

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNST
NNGLLFINTTIASIAAKEEGVSLEKRPHHHHHHHHHHSLEVLFQGPMPPRDSYSYAAPP
SAQLHEVDTPQEHDKKELVIGDRAYDVTNFVKRHPGGKIIAYQVGTDATDAYKQFHVRS
AKADKMLKSLPSRPVHKGYSPRRADLIADFQEFTKQLEAEGMFEPSLPHVAYRLAEVIA
MHVAGAALIWHGYTFAGIAMLGVVQGRCGWLMHEGGHYSLTGNIAFDRAIQVACYGLGC
GMSGAWWRNQHNKHHATPQKLQHDVDLDTLPLVAFHERIAAKVKSPAMKAWLSMQAKLF
APVTTLLVALGWQLYLHPRHMLRTKHYDELAMLGIRYGLVGYLAANYGAGYVLACYLLY
VQLGAMYIFCNFAVSHTHLPVVEPNEHATWVEYAANHTTNCSPSWWCDWWMSYLNYQIE
HHLYPSMPQFRHPKIAPRVKQLFEKHGLHYDVRGYFEAMADTFANLDNVAHAPEKKMQ

Figure 5. Amino acid sequence of SP::His10::Pavsa-Δ5D.
Pavsa-Δ5D was expressed in *P. pastoris*, fused to mating type alpha signal peptide as secretion peptide (SP, underlined), followed by His-tag (His_{10}, double underlined) and PreScission protease cleavage site (SLEVLFQ$^{\downarrow}$GP, dotted underlined) at its N-terminal.

MRPHHHHHHHHHHSLEVLFQGPMPPRDSYSYAAPPSAQLHEVDTPQEHDKKELVIGDRA
YDVTNFVKRHPGGKIIAYQVGTDATDAYKQFHVRSAKADKMLKSLPSRPVHKGYSPRRA
DLIADFQEFTKQLEAEGMFEPSLPHVAYRLAEVIAMHVAGAALIWHGYTFAGIAMLGVV
QGRCGWLMHEGGHYSLTGNIAFDRAIQVACYGLGCGMSGAWWRNQHNKHHATPQKLQHD
VDLDTLPLVAFHERIAAKVKSPAMKAWLSMQAKLFAPVTTLLVALGWQLYLHPRHMLRT
KHYDELAMLGIRYGLVGYLAANYGAGYVLACYLLYVQLGAMYIFCNFAVSHTHLPVVEP
NEHATWVEYAANHTTNCSPSWWCDWWMSYLNYQIEHHLYPSMPQFRHPKIAPRVKQLFE
KHGLHYDVRGYFEAMADTFANLDNVAHAPEKKMQ

Figure 6. Amino acid sequence of His10::Pavsa-Δ5D.
Pavsa-Δ5D was expressed in *P. pastoris*, fused to His-tag (His_{10}, double underlined), and PreScission protease cleavage site (SLEVLFQ$^{\downarrow}$GP, dotted underlined) at its N-terminal.

The enzyme activity of SP::His_{10}::Pavsa-Δ5D fusion protein was confirmed in *P. pastoris* yeast cells, as shown in Table 2. The result showed the desaturation activity on $20{:}4^{\Delta 8,11,14,17}$ (ω3) at Δ5-position producing $20{:}5^{\Delta 5,\ 8,11,14,17}$ (ω3), although the conversion efficiency was low, where the vector alone led to no 20:5 product.

Table 2. Activity of Pavsa-Δ5D fusion protein in P. pastoris cells.

Sample	Substrate (%)		Product (%)		Conversion (%)	
Vector	20:4	6.2 ± 1.1	20:5	0.0 ± 0.0	0.0 ± 0.0	n=3
SP::His_{10}::Pavsa-Δ5D		5.3 ± 0.7		0.05 ± 0.01	0.9 ± 0.3	n=8

Substrate and product are shown in percentage of total fatty acid in cells. Conversion rate is based on the product 20:4 $^{\Delta 8,11,14,17}$ (ω3) compared to the total of product 20:5$^{\Delta 5,8,11,14,17}$ (ω3) and remaining substrate 20:4 $^{\Delta 8,11,14,17}$ (ω3). SP, secretion peptide. n = repeats with individual colonies. The yeast cell culture was fed with 0.1 mM 20:4$^{\Delta 8,11,14,17}$ (ω3) substrate.

E. GLYCOSYLATION ANALYSIS

Several classes of glycans exist, which are widely distributed in nature, including *N*-linked glycans glycolipids, *O*-GlcNac, and glycosaminoglycans. *N*-linked glycosylation occurs when glycans are attached to asparagine residues on the protein. *O*-linked glycans are most commonly attached to serine or threonine residues through the *N*-Acetylgalactosamine residue. N-linked glycans are the most common in plants, and typically, can only be found as a linkage to an asparagine residue (N) where it is flanked on the C-terminal side by X-S or X-T. For the Pavsa-Δ5D protein, there are two potential glycosylation sites within this amino acid sequence derived from the nucleotide sequence of the inserted DNA (Figure 7, highlighted in green).

MPPRDSYSYAAPPSAQLHEVDTPQEHDKKELVIGDRAYDVTNFVKRHPGGKIIAYQVGT
DATDAYKQFHVRSAKADKMLKSLPSRPVHKGYSPRRADLIADFQEFTKQLEAEGMFEPS
LPHVAYRLAEVIAMHVAGAALIWHGYTFAGIAMLGVVQGRCGWLMHEGGHYSLTGNIAF
DRAIQVACYGLGCGMSGAWWRNQHNKHHATPQKLQHDVDLDTLPLVAFHERIAAKVKSP
AMKAWLSMQAKLFAPVTTLLVALGWQLYLHPRHMLRTKHYDELAMLGIRYGLVGYLAAN
YGAGYVLACYLLYVQLGAMYIFCNFAVSHTHLPVVEPNEHATWVEYAANHTTNCSPSWW
CDWWMSYLNYQIEHHLYPSMPQFRHPKIAPRVKQLFEKHGLHYDVRGYFEAMADTFANL
DNVAHAPEKKMQ

Figure 7. Theoretical glycosylation site (NXT/NXS) in Pavsa-Δ5D.

F. SEQUENCE CONFIRMATION IN TRANSGENIC CANOLA

The genome sequence including the T-DNA insertions in DHA canola was analysed. The translated amino acid sequence of Pavsa-Δ5D in the insert was confirmed to be identical to the original sequence (Figure 8).

```
                        1                                                  50
Pavsa-D5D_vec     (1)   MPPRDSYSYAAPPSAQLHEVDTPQEHDKKELVIGDRAYDVTNFVKRHPGG
NS-B50027-4       (1)   MPPRDSYSYAAPPSAQLHEVDTPQEHDKKELVIGDRAYDVTNFVKRHPGG
                        51                                                 100
Pavsa-D5D_vec    (51)   KIIAYQVGTDATDAYKQFHVRSAKADKMLKSLPSRPVHKGYSPRRADLIA
NS-B50027-4      (51)   KIIAYQVGTDATDAYKQFHVRSAKADKMLKSLPSRPVHKGYSPRRADLIA
                        101                                                150
Pavsa-D5D_vec   (101)   DFQEFTKQLEAEGMFEPSLPHVAYRLAEVIAMHVAGAALIWHGYTFAGIA
NS-B50027-4     (101)   DFQEFTKQLEAEGMFEPSLPHVAYRLAEVIAMHVAGAALIWHGYTFAGIA
                        151                                                200
Pavsa-D5D_vec   (151)   MLGVVQGRCGWLMHEGGHYSLTGNIAFDRAIQVACYGLGCGMSGAWWRNQ
NS-B50027-4     (151)   MLGVVQGRCGWLMHEGGHYSLTGNIAFDRAIQVACYGLGCGMSGAWWRNQ
                        201                                                250
Pavsa-D5D_vec   (201)   HNKHHATPQKLQHDVDLDTLPLVAFHERIAAKVKSPAMKAWLSMQAKLFA
NS-B50027-4     (201)   HNKHHATPQKLQHDVDLDTLPLVAFHERIAAKVKSPAMKAWLSMQAKLFA
                        251                                                300
Pavsa-D5D_vec   (251)   PVTTLLVALGWQLYLHPRHMLRTKHYDELAMLGIRYGLVGYLAANYGAGY
NS-B50027-4     (251)   PVTTLLVALGWQLYLHPRHMLRTKHYDELAMLGIRYGLVGYLAANYGAGY
                        301                                                350
Pavsa-D5D_vec   (301)   VLACYLLYVQLGAMYIFCNFAVSHTHLPVVEPNEHATWVEYAANHTTNCS
NS-B50027-4     (301)   VLACYLLYVQLGAMYIFCNFAVSHTHLPVVEPNEHATWVEYAANHTTNCS
                        351                                                400
Pavsa-D5D_vec   (351)   PSWWCDWWMSYLNYQIEHHLYPSMPQFRHPKIAPRVKQLFEKHGLHYDVR
NS-B50027-4     (351)   PSWWCDWWMSYLNYQIEHHLYPSMPQFRHPKIAPRVKQLFEKHGLHYDVR
                        401                425
Pavsa-D5D_vec   (401)   GYFEAMADTFANLDNVAHAPEKKMQ
NS-B50027-4     (401)   GYFEAMADTFANLDNVAHAPEKKMQ
```

Figure 8. Alignment of protein sequences of Pavsa-Δ5D.
Δ5D sequence translated from sequenced T-DNA insert in DHA canola NS-B50027-4 event was identical to the original Δ5D sequence from *P. salina* in binary vector (Pavsa-Δ5D_vec).

VI. CONCLUSIONS

The results of this study demonstrated that the cloned yeast Pavsa-Δ5D protein has activity in heterologous expression systems, including in DHA canola, event NS-B50027-4. The Pavsa-Δ5D protein shares similarity to desaturase proteins present in consumed food, used in food production or in animal feeds. The enzyme functionality of Pavsa-Δ5D has been confirmed in several different heterologous expression systems. Data for Pavsa-Δ5D expressed in Pichia as fusion proteins confirmed this functionality.

Pavsa-Δ5D protein contains 425 amino acid residues. The molecular weight of Pavsa-Δ5D is predicted to be 48.2 kDa, with an estimated pI of 8.18. For the Pavsa-Δ5D protein, there are two potential glycosylation sites within this amino acid sequence derived from the nucleotide sequence of the inserted DNA. The study also demonstrates that canola event NS-B50027-4 contains T-DNA insertions that are translationally identical to the original Pavsa-Δ5D protein sequence.

VII. REFERENCES

Brown, MR. 1991. The amino-acid and sugar composition of 16 species of microalgae used in mariculture. *J Exp Mar Biol Ecol* 145: 79-99.

Brown, MR, Jeffrey, SW, Volkman, JK, Dunstan, GA. 1997. Nutritional properties of microalgae for mariculture. *Aquaculture* 151: 315-331.

Chen, HQ, Gu, ZN, Zhang, H, Wang, MX, Chen, W, Lowther, WT, Chen, YQ. 2013. Expression and purification of integral membrane fatty acid desaturases. *PLoS ONE* 8:e58139.

Fan, Y-Y and Chapkin, RS. 1998. Importance of dietary gamma-linolenic acid in human health and nutrition? *J Nutr* 128:1411-1414.

Fradique, M, Batista, AP, Nunes, MC, Gouveia, L, Bandarra, NM, Raymundo, A. 2013. *Isochrysis galbana* and *Diacronema vlkianum* biomass incorporation in pasta products as PUFA's source. *LWT - Food Sci Technol* 50: 312-319.

Gouveia, L, Coutinho, C, Mendonça, E, Batista, AP, Sousa, I, Bandarra, NM, Raymundo, A. 2008. Functional biscuits with PUFA-ω3 from *Isochrysis galbana*. *J Sci Food Agri* 88: 891-896.

Gupta, A, Barrow, CJ, Puri, M. 2012. Omega-3 biotechnology: Thraustochytrids as a novel source of omega-3 oils. *Biotechnol Adv* 30: 1733-1745.

Hastings, N, Agaba, M, Tocher, DR, Leaver, MJ, Dick, JR, Sargent, JR, Teale, AJ. 2001. A vertebrate fatty acid desaturase with Δ5 and Δ6 activities. *Proc Natl Acad Sci USA* 98: 14304-14309.

Heidari, MR, Azad, EM, Mehrabani, M. 2006. Evaluation of the analgesic effect of *Echium amoenum* Fisch & C.A. Mey. extract in mice: Possible mechanism involved. *J Ethnopharm* 103:345-349.

Kaewsuwan, S, Cahoon, EB, Perroud, PF, Wiwat, C, Panvisavas, N, Quatrano, RS, Cove, DJ, Bunyapraphatsara, N. 2006. Identification and functional characterization of the moss *Physcomitrella patens* Δ-desaturase gene involved in arachidonic and eicosapentaenoic acid biosynthesis. *J Biol Chem* 281: 21988-21997.

Kajikawa, M, Yamato, KT, Kohzu, Y, Nojiri, M, Sakuradani, E, Shimizu, S, Sakai, Y, Fukuzawa, H, Ohyama, K. 2004. Isolation and characterization of Δ6-desaturase, an ELO-Like enzyme and Δ5-desaturase from the liverwort *Marchantia Polymorpha* and production of arachidonic and eicosapentaenoic acids in the methylotrophic yeast *Pichia pastoris*. *Plant Mol Biol* 54: 335-352.

Leonard, AE, Kelder, B, Bobik, EG, Chuang, L-T, Parker-Barnes, JM, Thurmond, JM, Kroger, PE, Kopchick, JJ, Huang, Y-S, Mukerji, P. 2000. cDNA cloning and characterization of human Δ5-desaturase involved in the biosynthesis of arachidonic acid. *Biochem J* 347: 719-724.

Liu, H, Guo, Z, Zheng, H, Wang, S, Wang, Y, Liu, W, Zhang, G. 2014. Functional characterization of a Δ5-like fatty acyl desaturase and its expression during early embryogenesis in the noble scallop *Chlamys nobilis* Reeve. *Mol Biol Rep* 41: 7437-7445.

Michaelson, LV, Lazarus, CM, Griffiths, G, Napier, JA, Stobart, AK. 1998. Isolation of a Δ5-fatty acid desaturase gene from *Mortierella alpina*. *J Biol Chem* 273: 19055-19059.

Monroig, Ó, Navarro, JC, Dick, JR, Alemany, F, Tocher, DR. 2012. Identification of a Δ5-like fatty acyl desaturase from the cephalopod *Octopus vulgaris* (Cuvier 1797) involved in the biosynthesis of essential fatty acids. *Mar Biotechnol* 14: 411-422.

Petrie, JR, Liu, Q, Mackenzie, AM, Shrestha, P, Mansour, MP, Robert, SS, Frampton, DF, Blackburn, SI, Nichols, PD, Singh, SP. 2010a. Isolation and characterisation of a high-efficiency desaturase and elongases from microalgae for transgenic LC-PUFA production. *Mar Biotechnol* 12:430-438.

Petrie, JR, Shrestha, P, Belide, S, Kennedy, Y, Lester, G, Liu, Q, Divi, UK, Mulder, RJ, Mansour, MP, Nichols, PD, Singh, SP. 2014. Metabolic engineering *Camelina sativa* with fish oil-like levels of DHA. *Plos One* 9: e85061.

Petrie, JR, Shrestha, P, Mansour, MP, Nichols, PD, Liu, Q, Singh, SP. 2010b. Metabolic engineering of omega-3 long-chain polyunsaturated fatty acids in plants using an acyl-CoA Δ6-desaturase with omega 3-preference from the marine microalga *Micromonas pusilla*. *Metab Eng* 12:233-240.

Petrie, JR, Shrestha, P, Zhou, X-R, Mansour, MP, Liu, Q, Belide, S, Nichols, PD, Singh, SP. 2012. Metabolic engineering plant seeds with fish oil-like levels of DHA. *Plos One* 7: e49165.

Saito, T, Morio, T, Ochiai, H. 2000. A second functional delta5 fatty acid desaturase in the cellular slime mould *Dictyostelium discoideum*. *Eur J Biochem* 267: 1813-1818.

Sakuradani, E, Shimizu, S. 2009. Single cell oil production by *Mortierella alpina*. *J Biotechnol* 144:31-36.

Venegas-Calerón, M, Beaudoin, F, Sayanova, O, Napier, JA. 2007. Co-transcribed genes for long chain polyunsaturated fatty acid biosynthesis in the protozoon *Perkinsus marinus* include a plant-like FAE1 3-ketoacyl coenzyme A synthase. *J Biol Chem* 282: 2996-3003.

Watanabe, K, Oura, T, Sakai, H, Kajiwara, S. 2004. Yeast Δ12 fatty acid desaturase: Gene cloning, expression, and function. *Biosci Biotechnol Biochem* 68:721-727.

Wood, CC, Petrie, JR, Shrestha, P, Mansour, MP, Nichols, PD, Green, AG, Singh, SP. 2009. A leaf-based assay using interchangeable design principles to rapidly assemble multistep recombinant pathways. *Plant Biotechnol J* 7: 914-924.

Zhang, X, Li, M, Wei, D, Xing, L. 2008. Identification and characterization of a novel yeast ω3-fatty acid desaturase acting on long-chain n-6 fatty acid substrates from *Pichia pastoris*. *Yeast* 25:21-27.

Zhou, X-R, Green, AG, Singh, SP. 2011. *Caenorhabditis elegans* Δ12-desaturase FAT-2 is a bifunctional desaturase able to desaturate a diverse range of fatty acid substrates at the Δ12 and Δ15 positions. *J Biol Chem* 286:43644-43650.

Zhou, X-R, Robert, S, Singh, S, Green, A. 2006. Heterologous production of GLA and SDA by expression of an *Echium plantagineum* Δ6-desaturase gene. *Plant Sci* 170:665-673.

Zhou, X-R, Robert, SS, Petrie, JR, Frampton, DMF, Mansour, MP, Blackburn, SI, Nichols, PD, Green, AG, Singh, SP. 2007. Isolation and characterization of genes from the

marine microalga *Pavlova salina* encoding three front-end desaturases involved in docosahexaenoic acid biosynthesis. *Phytochem* 68:785-796.

VIII. UNPUBLISHED REFERENCES

Report Nº 2016-005. [redacted]
Nuseed Pty Ltd.

Report Nº 2016-006. [redacted].
Nuseed Pty Ltd.

Report Nº 2016-007. [redacted].
Nuseed Pty Ltd.

Report Nº 2016-008. [redacted]
Nuseed Pty Ltd.

Report Nº 2016-010. [redacted]
Nuseed Pty Ltd.

Report Nº 2016-011. [redacted]
Nuseed Pty Ltd.