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TITLE:
CHARACTERIZATION OF *LACHANCEA KLUYVERI* Δ12-DESATURASE

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ABBREVIATIONS

BMGY	Buffered glycerol-complex medium
BMMY	Buffered complex medium containing methanol
CoA	Coenzyme A
ALA	α-Linoleic acid, $18:3^{\Delta 9,12,15}$ (ω3)
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
LA	Linoleic acid, $18:2^{\Delta 9,12}$ (ω6)
Lackl-Δ12D	*Lachancea kluyveri* Δ12-desaturase
Micpu-Δ6D	*Micromonas pusilla* Δ6-desaturase
MQ	MilliQ water
OA	Oleic acid, $18:1^{\Delta 9}$
OD_{600}	Optical density at 600 nm wavelength
ω3 LC-PUFA	Omega-3 long-chain (≥C20) polyunsaturated fatty acids
MMT	Million metric ton
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 *Lachancea kluyveri* Δ12-desaturase (Lackl-Δ12D) 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 Lackl-Δ12D was a functional enzyme that desaturating oleic acid (OA) to linoleic acid (LA) in different cells for accumulating more precursor of omega-3 long-chain (≥C20) polyunsaturated fatty acids (ω3 LC-PUFA). Lackl-Δ12D protein contains 416 amino acid residues and shares high homology to other Δ12-desaturases that have been consumed as food, used in food production or in animal feeds. The molecular weight of Lackl-Δ12D is predicted to be 48.2 kDa, with an estimated isoelectric point (pI) of 7.84.

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 the *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 2016-006, 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).

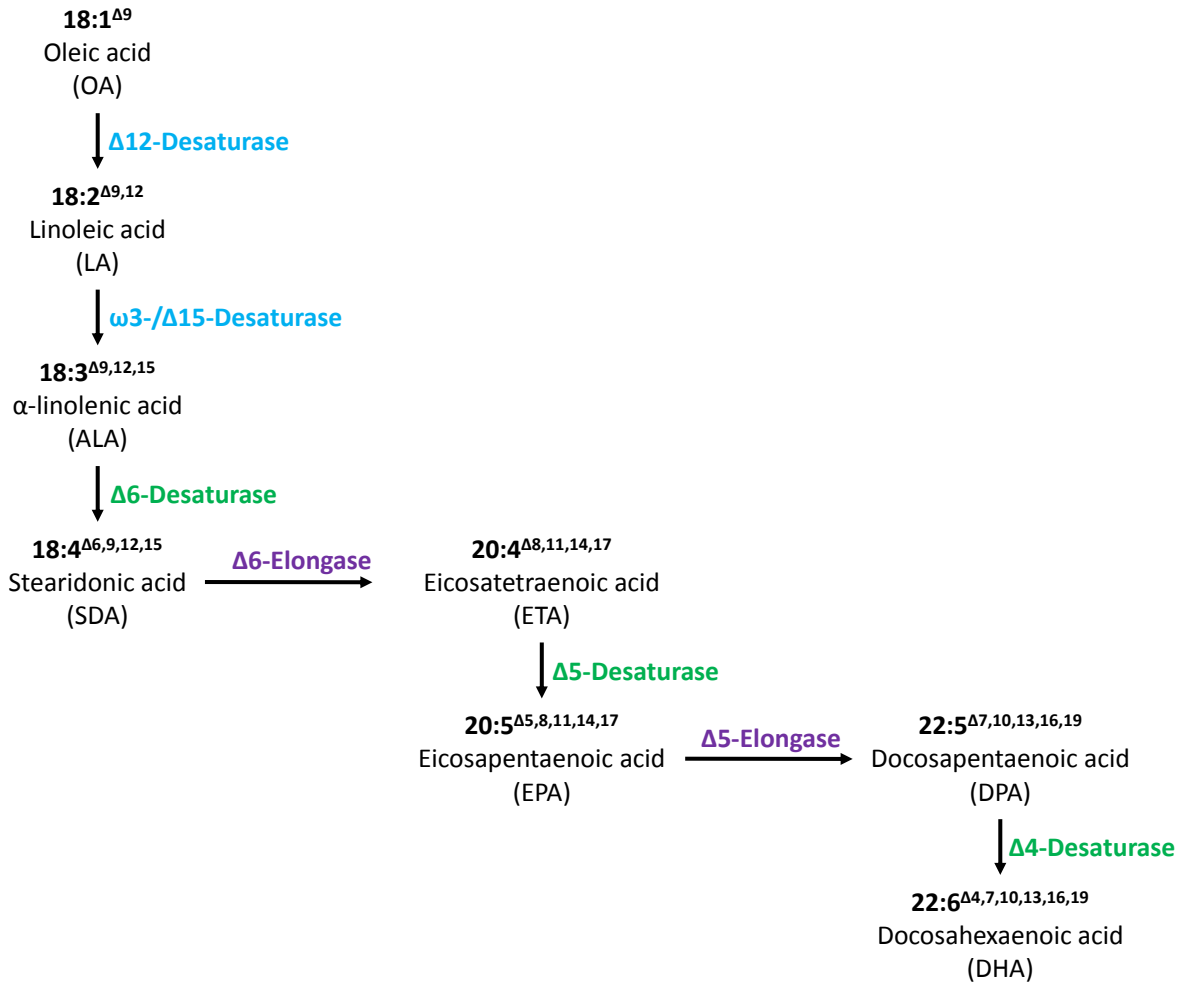

Figure 1. DHA biosynthesis pathway engineered into DHA canola event NS-B50027-4. Seven enzymes introduced in canola to convert oleic acid to final product docosahexaenoic acid were grouped into 3 classes, two fatty acid desaturases from yeast in blue, two elongases from microalgae in purple, and three 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 *Lachancea kluyveri* Δ12-desaturase (Lackl-Δ12D) protein to catalyse the desaturation of oleic acid (OA) at Δ12 position to linoleic acid (LA) ($18:1^{\Delta 9} \rightarrow 18:2^{\Delta 9,12}$).

III. MATERIALS

A. TARGET PROTEIN

The Δ12-desaturase gene used in DHA canola event was previously cloned from yeast *L. kluyveri* (Watanabe et al. 2004). The Lackl-Δ12D protein was expressed as native sequence in 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 a secretion peptide. 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 *Lackl-Δ12D* gene.

B. OTHER MATERIALS

The *Lackl-Δ12D* gene was synthesized at GeneArt (Life Science Technologies, Germany), according to sequence in NCBI database under accession AB115968 as a His-tag fusion with or without Pichia secretion peptide (SP), and cloned into the Pichia expression vector pPinkα-HC (Invitrogen, Carlsbad, CA, USA).

IV. METHODS

A. SEQUENCE COMPARISON

The *Lackl-Δ12D* gene was previously cloned from yeast *L. kluyveri* (Watanabe et al. 2004). The translated amino acid sequence was compared to other published Δ12-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 the published protocol (Chen et al. 2013). Pichia expression vector DNA containing *Lackl-Δ12D* 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 a 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 *Lackl-Δ12D* gene was previously cloned from yeast *L. kluyveri* (Watanabe et al. 2004). *L. kluyveri* was formerly called as *Saccharomyces kluyveri,* and was reclassified (Kurtzman, 2003). The open reading frame (ORF) of *Lackl-Δ12D* gene consisted of 1251 bp, and is shown in Figure 2.

ATGTCTGCAGTTACAGTCACAGGGTCCGACCCAAAGAACCGTGGTTCCTCCTCTAATACC
GAGCAAGAAGTTCCAAAAGTTGCAATTGACACCAATGGTAATGTTTTCAGCGTACCAGAT
TTTACCATCAAGGATATTTTGGGCGCCATTCCCCACGAATGTTACGAAAGAAGACTAGCA
ACATCGTTATACTATGTTTTTAGAGACATCTTCTGCATGCTAACAACCGGTTACCTTACA
CACAAAATCTTATATCCATTGCTGATCTCATACACTTCTAACTCAATAATCAAGTTTACC
TTCTGGGCTTTGTACACATACGTCCAAGGTTTGTTTGGTACTGGTATCTGGGTGTTGGCC
CACGAATGTGGCCATCAAGCCTTCTCAGACTATGGTATTGTCAACGATTTTGTTGGCTGG
ACTCTACACTCTTACTTGATGGTACCATATTTTTCGTGGAAGTATTCCCATGGTAAGCAT
CACAAGGCCACCGGTCACATGACTAGAGACATGGTTTTTGTTCCTGCCACAAAGGAGGAA
TTTAAGAAAAGCAGAAACTTTTTCGGAAATTTGGCAGAATACTCCGAGGATTCCCCATTA
AGAACTTTGTACGAATTGCTGGTACAACAACTAGGAGGTTGGATTGCATATCTTTTTGTC
AACGTTACTGGTCAACCGTATCCAGATGTTCCTTCCTGGAAATGGAACCACTTCTGGCTA
ACTTCTCCATTATTTGAACAAAGGGATGCTTTGTACATTTTTTTGAGTGATCTAGGTATC
TTGACCCAAGGCATTGTTTTGACCTTGTGGTACAAGAAGTTTGGTGGCTGGTCTCTGTTC
ATCAATTGGTTTGTTCCATACATTTGGGTTAACCACTGGTTGGTTTTTATCACTTTTTTG
CAACACACCGACCCAACTATGCCCCATTACAATGCTGAGGAATGGACTTTTGCCAAGGGT
GCTGCCGCCACCATTGATAGAAAATTCGGGTTTATTGGTCCTCACATTTTCCATGACATT
ATTGAAACCCATGTGCTACACCACTACTGTAGCAGAATTCCATTCTATAACGCTCGTCCA
GCAAGCGAGGCTATTAAGAAAGTGATGGGCAAGCATTATAGATCTAGTGACGAAAACATG
TGGAAGTCCTTATGGAAGTCTTTTAGATCTTGTCAGTATGTTGATGGAGACAATGGTGTT
TTAATGTTCAGAAACATCAACAACTGTGGTGTTGGCGCCGCTGAGAAA**TGA**

Figure 2. Nucleotide sequence of native Lackl-Δ12D gene.
Start codon (ATG) and stop codon (TGA) are in bold.

B. PROTEIN SEQUENCE

The translated *L. kluyveri* Δ12-desaturase (Lackl-Δ12D) contained 416 amino acid residues (Figure 3). The molecular weight of Lackl-Δ12D is predicted as 48.2 kDa, with an estimated pI of 7.84.

```
MSAVTVTGSDPKNRGSSSNTEQEVPKVAIDTNGNVFSVPDFTIKDILGAIPHECYERRLA
TSLYYVFRDIFCMLTTGYLTHKILYPLLISYTSNSIIKFTFWALYTYVQGLFGTGIWVLA
HECGHQAFSDYGIVNDFVGWTLHSYLMVPYFSWKYSHGKHHKATGHMTRDMVFVPATKEE
FKKSRNFFGNLAEYSEDSPLRTLYELLVQQLGGWIAYLFVNVTGQPYPDVPSWKWNHFWL
TSPLFEQRDALYIFLSDLGILTQGIVLTLWYKKFGGWSLFINWFVPYIWVNHWLVFITFL
QHTDPTMPHYNAEEWTFAKGAAATIDRKFGFIGPHIFHDIIETHVLHHYCSRIPFYNARP
ASEAIKKVMGKHYRSSDENMWKSLWKSFRSCQYVDGDNGVLMFRNINNCGVGAAEK
```

Figure 3. Amino acid sequence of Lackl-Δ12D.

The fatty acid Δ12-desaturases have been cloned from a wide range of organisms, including traustochytrid (Matsuda et al. 2012), diatom (Domergue et al. 2003), fungus (Sakuradani et al. 1999), plant (Okuley et al. 1994), nematode (Peyou-Ndi et al. 2000), insect (Zhou et al. 2008). The Lackl-Δ12D shared high homology to other Δ12-desaturase proteins as shown in Figure 4.

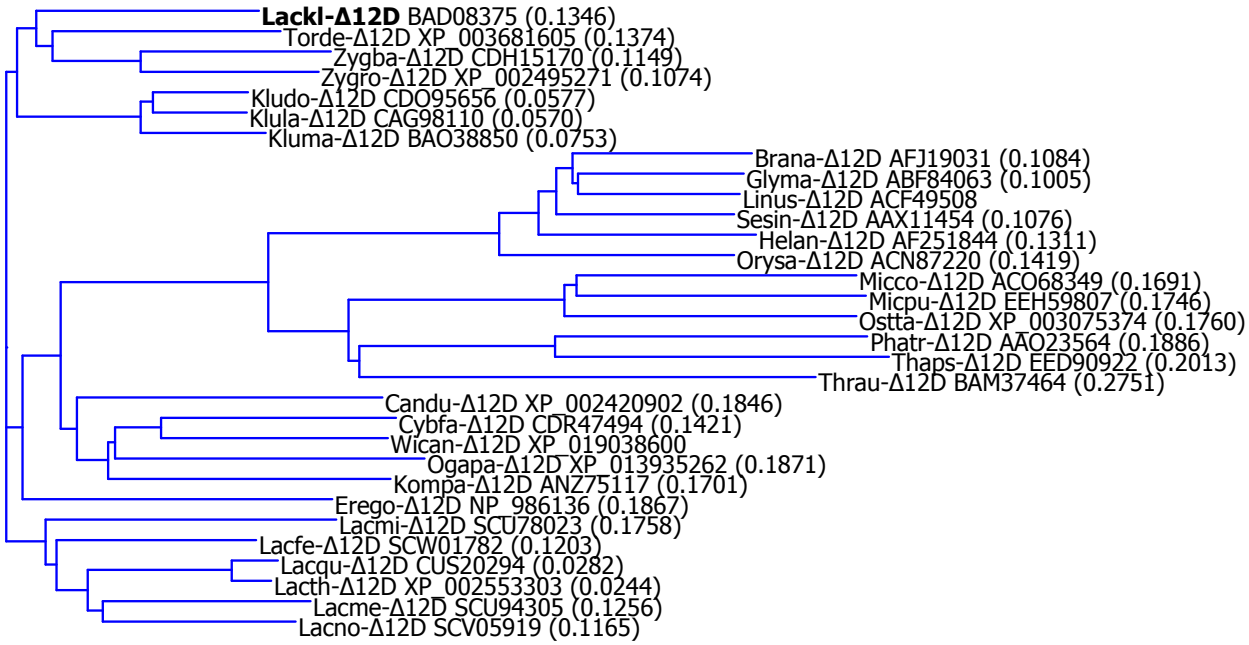

Figure 4. Phylogenetic tree for sequence comparison of Lackl-Δ12D with representative Δ12-desaturases.

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. Brana, *Brassica napus* (canola); Candu, *Candida dubliniensis* (fungus); Cybfa, *Cyberlindnera fabianii* (fungus); Erego, *Eremothecium gossypii* (fungus); Glyma, *Glycine max* (soy bean); Helan, *Helianthus annuus* (sunflower); Linus, *Linum usitatissimum* (flax); Kludo, *Kluyveromyces dobzhanskii* (fungus); Klula, *K. lactis* (fungus); Kluma, *K. marxianus* (fungus); Kompa, *Komagataella pastoris* (fungus); Lacfe, *Lachancea fermentati* (fungus); Lackl, *Lachancea kluyveri* (fungus); Lacme, *L. meyersii* (fungus); Lacmi, *L. mirantina* (fungus); Lacno, *L. nothofagi* (fungus); Lacqu, *L. quebecensis* (fungus); Lacth, *L. thermotolerans* (fungus); Micco, *Micromonas commode* (alga); Micpu, *M. pusilla* (alga); Ogapa, *Ogataea parapolymorpha* (fungus); Orysa, *Oryza sativa* (rice); Ostta, *Ostreococcus tauri* (alga); Phatr, *Phaeodactylum tricornutum* (diatom); Sesin, *Sesamum indicum* (sesame); Thaps, *Thalassiosira pseudonana* (alga); Thrau, *Thraustochytrium aureum* (protist); Torde, *Torulaspora delbrueckii* (fungus); Wican, *Wickerhamomyces anomalus* (fungus); Zybga, *Zygosaccharomyces bailii* (fungus); Zygro, *Z. rouxii* (fungus).

C. SIMILARITY OF LACKL-Δ12D TO OTHER PROTEINS IN CONSUMED FOODS, USED IN FOOD PRODUCTION OR IN ANIMAL FEEDS

Yeasts are essential microorganisms in the production of various foods and drinks such as bread, beer, wine and cider. The yeast strain *L. kluyveri* itself where the gene was cloned is widely used in Emmental, Roquefort, Damietta and Greek cheeses, fermented milk. The close related strain *L. lanzarotensis* naturally present in grape must, contributes to spontaneous alcoholic fermentation during the early phases of wine fermentation, before *Saccharomyces cerevisiae* becomes dominant and completes the process. Another closely related species *Kluyveromyces lactis* can ferment lactose, thus it is mainly used in dairy industry. *K. lactis* is also used for the manufacture of infant nutrition products, the fermented milk drink kefir, single cell protein (Spohner et al. 2016).

Microbial food cultures (MFC) are live bacteria, yeasts or molds used in food production (Bourdichon et al. 2012). At least 69 species of yeasts and molds are listed in present "Inventory of MFC", including *Zygosaccharomyces bailii*, *Z. rouxii*. Among them, *Dekkera bruxellensis* (anamorph *Brettanomyces bruxellensis*), which was formerly regarded as a spoiler of beer (and wine). However, it is used for production of Belgian Lambic-Geuze beer. *D. bruxellensis* produces acetic acid that in moderate amounts gives a unique taste to those beers. Other examples are *Debaryomyces hansenii* and *Yarrowia lipolytica*, which are very important for aroma formation in Munster and Parmesan cheeses. *Saccharomyces cerevisiae*, *Hanseniaspora uvarum*, *Kluyveromyces marxianus* and *Pichia fermentans* are extremely important for the development of the fine aroma of cocoa beans. *Aspergillus oryzae* and *A. sojae* are used in the production of miso and soya sauce fermentations. *A. acidus* is used for fermenting Puerh tea. *Fusarium domesticum* was first identified as *Trichothecium domesticum*, but was later allocated to *Fusarium*. This species has been used for cheese fermentations (cheese smear). Blue-mold cheeses are always fermented with *Penicillium roqueforti*. *K. marxianus* is a dairy yeast that produces β-galactosidase allowing for whey fermentation (Belem and Lee, 1998). It was also traditionally found in kefir grains. Kefir is an alcoholic acid milk drink made from the milk of cows, goats, or sheep, which, in the past, was mainly consumed in Russia and the Caucasian mountains, but now is also being commercialized in North America. *Pichia pastoris* is a species of methylotrophic yeast. *Pichia* is widely used for protein production using recombinant DNA techniques. A number of food proteins and enzymes have been expressed in *P. pastoris* (Batt, 2014).

Lackl-Δ12D shares sequence homology to fatty acid Δ12-desaturases isolated from many of these fungi species (Table 1). Lackl-Δ12D shares 64% sequence identity to *P. pastoris* (*Komagataella pastoris*) Δ12-desaturase (AAX20125), and 58% sequence identity to *P. angusta (Hansenula polymorpha)* Δ12-desaturase (BAN63793). Lackl-Δ12D shares 68%

sequence identity to *K. marxianus* Δ12-desaturase (BAO38850), 69% sequence identity to *Z. bailii* Δ12-desaturase (CDH15170), or 66% sequence identity to *Z. rouxii* Δ12-desaturase (XP_002495271).

Lackl-Δ12D shares 37.5% identity to Δ12-desaturase from soil fungus species *Mortierella alpine* (BAAB1754). *M. alpina* 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).

The Lackl-Δ12D protein also shared 34% to 37% of sequence identities with plant Δ12-desaturases, including those from edible crops like rice (ACN87220), sesame (AAX11454), linseed (ACF49508), soybean (ABF84063), sunflower (AF251844) or canola (AFJ19031). Sesame, linseed, soybean, sunflower and canola are typical oil crops for food application. Specifically, the introduced Lackl-Δ12D protein in DHA canola shared 36% of sequence identity with the endogenous canola Δ12-desaturase.

Table 1. Amino acid sequence identity between Lackl-Δ12D 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	10	11	12	13
1	NS-B50027-4 Lackl-Δ12D			100	64.4	58.1	67.6	69.0	66.4	37.5	36.1	34.6	36.1	33.9	35.5	36.7
2	Picpa-Δ12D	AAX20125	Yeast		100	62.3	59.2	51.5	52.5	38.0	33.9	33.8	33.6	34.1	33.1	35.1
3	Pican-Δ12D	BAN63793	Yeast			100	57.2	53.7	54.7	38.0	37.4	35.9	35.6	35.1	35.9	36.0
4	Kluma-Δ12D	BAO38850	Fungus				100	64.7	65.1	37.8	37.1	36.4	35.3	35.0	34.1	36.0
5	Zygba-Δ12D	CDH15170	Fungus					100	78.0	36.8	35.3	35.8	35.4	34.3	33.7	34.3
6	Zygro-Δ12D	XP_002495271	Fungus						100	38.3	35.2	36.3	35.2	36.6	34.4	36.1
7	Moral-Δ12D	BAAB1754	Fungus							100	39.5	40.1	40.5	41.0	40.7	42.5
8	Brana-Δ12D	AFJ19031	Canola								100	69.3	78.9	77.6	72.7	75.8
9	Orysa-Δ12D	ACN87220	Rice									100	71.6	68.5	66.8	71.9
10	Glyma-Δ12D	ABF84063	Soybean										100	79.4	74.0	79.4
11	Linus-Δ12D	ACF49508	Flax											100	73.7	78.3
12	Helan-Δ12D	AF251844	Sunflower												100	75.0
13	Sesin-Δ12D	AAX11454	Sesame													100

Δ12D, Δ12-desaturase; Brana, *Brassica napus*; Glyma, *Glycine max;* Helan, *Helianthus annus;* Kluma, *Kluyveromyces marxianus*; Lackl, *Lachancea kluyveri;* Linus, *Linum usitatissmum;* Moral, *Mortierella alpina*; Orysa, *Oryza sativa;* Pican, *Pichia angusta*; Picpa, *P. pastoris*; Sesin, *Sesamum indicum;* Zygba, *Zygosaccharomyces bailii*; Zygro, *Z. rouxii.*

D. HETEROLOGOUS EXPRESSION

The enzyme functionality of Lackl-Δ12D has been confirmed in different heterologous expression systems, including yeast (Watanabe et al. 2004), Arabidopsis seed (Petrie et al. 2012) and Camelina seed (Petrie et al. 2014). In this study, Lackl-Δ12D was expressed in

P. pastoris, as fusion proteins designated as SP::His_{10}::Lackl-Δ12D or His_{10}::Lackl-Δ12D. In SP::His_{10}::Lackl-Δ12D, the Lackl-Δ12D sequence was fused to *Saccharomyces cerevisiae* α-mating type signal peptide as secretion peptide (SP), followed by His-tag (His_{10}) and PreScission protease cleavage site (SLEVLFQ↓GP) at its N-terminal (Figure 5). In His_{10}::Lackl-Δ12D, the Lackl-Δ12D sequence was fused to His-tag (His_{10}) and PreScission protease cleavage site (SLEVLFQ↓GP) at its N-terminal (Figure 6). No SP was used in His_{10}::Lackl-Δ12D.

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTN
NGLLFINTTIASIAAKEEGVSLEKRPHHHHHHHHHHSLEVLFQGPMSAVTVTGSDPKNRG
SSSNTEQEVPKVAIDTNGNVFSVPDFTIKDILGAIPHECYERRLATSLYYVFRDIFCMLT
TGYLTHKILYPLLISYTSNSIIKFTFWALYTYVQGLFGTGIWVLAHECGHQAFSDYGIVN
DFVGWTLHSYLMVPYFSWKYSHGKHHKATGHMTRDMVFVPATKEEFKKSRNFFGNLAEYS
EDSPLRTLYELLVQQLGGWIAYLFVNVTGQPYPDVPSWKWNHFWLTSPLFEQRDALYIFL
SDLGILTQGIVLTLWYKKFGGWSLFINWFVPYIWVNHWLVFITFLQHTDPTMPHYNAEEW
TFAKGAAATIDRKFGFIGPHIFHDIIETHVLHHYCSRIPFYNARPASEAIKKVMGKHYRS
SDENMWKSLWKSFRSCQYVDGDNGVLMFRNINNCGVGAAEK

Figure 5. Amino acid sequence of SP::His10::Lackl-Δ12D.
Lackl-Δ12D was expressed in *P. pastoris*, fused to secretion peptide (SP, underlined), followed by His-tag (His_{10}, double underlined) and PreScission protease cleavage site (SLEVLFQ↓GP, dotted underlined) at its N-terminal.

MRPHHHHHHHHHHSLEVLFQGPMSAVTVTGSDPKNRGSSSNTEQEVPKVAIDTNGNVFSV
PDFTIKDILGAIPHECYERRLATSLYYVFRDIFCMLTTGYLTHKILYPLLISYTSNSIIK
FTFWALYTYVQGLFGTGIWVLAHECGHQAFSDYGIVNDFVGWTLHSYLMVPYFSWKYSHG
KHHKATGHMTRDMVFVPATKEEFKKSRNFFGNLAEYSEDSPLRTLYELLVQQLGGWIAYL
FVNVTGQPYPDVPSWKWNHFWLTSPLFEQRDALYIFLSDLGILTQGIVLTLWYKKFGGWS
LFINWFVPYIWVNHWLVFITFLQHTDPTMPHYNAEEWTFAKGAAATIDRKFGFIGPHIFH
DIIETHVLHHYCSRIPFYNARPASEAIKKVMGKHYRSSDENMWKSLWKSFRSCQYVDGDN
GVLMFRNINNCGVGAAEK

Figure 6. Amino acid sequence of His10::Lackl-Δ12D.
Lackl-Δ12D was expressed in P. pastoris, fused to His-tag (His10, double underlined), and PreScission protease cleavage site (SLEVLFQ↓GP, dotted underlined) at its N-terminal.

Table 2 shows the enzyme activity of Lackl-Δ12D expressed as fusion proteins in *P. pastoris* with or without secretion peptide.

Table 2. Activity of Lackl-Δ12D fusion protein in P. pastoris cells.

Sample	Substrate (%)		Product (%)		Conversion (%)	
Vector	18:1	30.9 ± 6.4	18:2	37.3 ± 4.0	58.7 ± 8.0	n=10
SP::His_{10}::Lackl-Δ12D		24.8 ± 5.2		41.9 ± 3.2	66.6 ± 6.5	n=10
Vector		34.1 ± 1.0		26.4 ± 0.9	47.8 ± 1.4	n=3
His_{10}::Lackl-Δ12D		3.3 ± 0.5		56.9 ± 0.7	94.9 ± 0.8	n=3

Substrate and product are shown in percentage of total fatty acid in cells. Conversion rate is based on all the products (including part of 18:2 that have been further desaturated by *P. pastoris* host cell ω3-desaturase) compare to the remaining substrate 18:1. SP, secretion peptide. n = repeats with individual colonies.

Overexpression of Lackl-Δ12D fusion protein in *P. pastoris* substantially increased the desaturation activity of 18:1 to 18:2 compared to vector alone (Table 2). In addition, the His_{10}::Lackl-Δ12D had higher activity than SP::His_{10}::Lackl-Δ12D.

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 Lackl-Δ12D protein, there is one potential glycosylation site within this amino acid sequence derived from the nucleotide sequence of the inserted DNA (Figure 7, highlighted in green).

MSAVTVTGSDPKNRGSSSNTEQEVPKVAIDTNGNVFSVPDFTIKDILGAIPHECYERRLA
TSLYYVFRDIFCMLTTGYLTHKILYPLLISYTSNSIIKFTFWALYTYVQGLFGTGIWVLA
HECGHQAFSDYGIVNDFVGWTLHSYLMVPYFSWKYSHGKHHKATGHMTRDMVFVPATKEE
FKKSRNFFGNLAEYSEDSPLRTLYELLVQQLGGWIAYLFV**NVT**GQPYPDVPSWKWNHFWL
TSPLFEQRDALYIFLSDLGILTQGIVLTLWYKKFGGWSLFINWFVPYIWVNHWLVFITFL
QHTDPTMPHYNAEEWTFAKGAAATIDRKFGFIGPHIFHDIIETHVLHHYCSRIPFYNARP
ASEAIKKVMGKHYRSSDENMWKSLWKSFRSCQYVDGDNGVLMFRNINNCGVGAAEK

Figure 7. Theoretical glycosylation site (NXT/NXS) in Lackl-Δ12D.

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 Lackl-Δ12D in the insert was confirmed to be identical to the original sequence (Figure 8).

```
                        1                                                  50
Lackl-Δ12D_vec     (1)  MSAVTVTGSDPKNRGSSSNTEQEVPKVAIDTNGNVFSVPDFTIKDILGAI
NS-B50027-4        (1)  MSAVTVTGSDPKNRGSSSNTEQEVPKVAIDTNGNVFSVPDFTIKDILGAI
                        51                                                 100
Lackl-Δ12D_vec    (51)  PHECYERRLATSLYYVFRDIFCMLTTGYLTHKILYPLLISYTSNSIIKFT
NS-B50027-4       (51)  PHECYERRLATSLYYVFRDIFCMLTTGYLTHKILYPLLISYTSNSIIKFT
                        101                                                150
Lackl-Δ12D_vec   (101)  FWALYTYVQGLFGTGIWVLAHECGHQAFSDYGIVNDFVGWTLHSYLMVPY
NS-B50027-4      (101)  FWALYTYVQGLFGTGIWVLAHECGHQAFSDYGIVNDFVGWTLHSYLMVPY
                        151                                                200
Lackl-Δ12D_vec   (151)  FSWKYSHGKHHKATGHMTRDMVFVPATKEEFKKSRNFFGNLAEYSEDSPL
NS-B50027-4      (151)  FSWKYSHGKHHKATGHMTRDMVFVPATKEEFKKSRNFFGNLAEYSEDSPL
                        201                                                250
Lackl-Δ12D_vec   (201)  RTLYELLVQQLGGWIAYLFVNVTGQPYPDVPSWKWNHFWLTSPLFEQRDA
NS-B50027-4      (201)  RTLYELLVQQLGGWIAYLFVNVTGQPYPDVPSWKWNHFWLTSPLFEQRDA
                        251                                                300
Lackl-Δ12D_vec   (251)  LYIFLSDLGILTQGIVLTLWYKKFGGWSLFINWFVPYIWVNHWLVFITFL
NS-B50027-4      (251)  LYIFLSDLGILTQGIVLTLWYKKFGGWSLFINWFVPYIWVNHWLVFITFL
                        301                                                350
Lackl-Δ12D_vec   (301)  QHTDPTMPHYNAEEWTFAKGAAATIDRKFGFIGPHIFHDIIETHVLHHYC
NS-B50027-4      (301)  QHTDPTMPHYNAEEWTFAKGAAATIDRKFGFIGPHIFHDIIETHVLHHYC
                        351                                                400
Lackl-Δ12D_vec   (351)  SRIPFYNARPASEAIKKVMGKHYRSSDENMWKSLWKSFRSCQYVDGDNGV
NS-B50027-4      (351)  SRIPFYNARPASEAIKKVMGKHYRSSDENMWKSLWKSFRSCQYVDGDNGV
                        401          416
Lackl-Δ12D_vec   (401)  LMFRNINNCGVGAAEK
NS-B50027-4      (401)  LMFRNINNCGVGAAEK
```

Figure 8. Alignment of protein sequences of Lackl-Δ12D.
Δ12D sequence translated from sequenced T-DNA insert in DHA canola NS-B50027-4 event was identical to the original Δ12D sequence from *L. kluyveri* in binary vector (Lackl-Δ12D_vec).

VI. CONCLUSIONS

The results of this study demonstrated that the cloned yeast Lackl-Δ12D protein has activity in heterologous expression systems, including in DHA canola, event NS-B50027-4. The Lackl-Δ12D protein shares similarity to desaturase proteins present in consumed food, used in food production or in animal feeds. The enzyme functionality of Lackl-Δ12D has been confirmed in several different heterologous expression systems. Data for Lackl-Δ12D expressed in Pichia as fusion proteins confirmed this functionality.

Lackl-Δ12D protein contains 416 amino acid residues. The molecular weight of Lackl-Δ12D is predicted to be 48.2 kDa, with an estimated pI of 7.84. For the Lackl-Δ12D protein, there is one potential glycosylation site 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 Lackl-Δ12D protein sequence.

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