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Good Laboratory Practice	**No GLP**
Confidentiality	**None**

TITLE:
CHARACTERIZATION OF *PICHIA PASTORIS* ω3-DESATURASE

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ABBREVIATIONS

ALA	α-Linoleic 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
LA	Linoleic acid, $18:2^{\Delta 9,12}$ (ω6)
Lackl-Δ12D	*Lachancea kluyveri* Δ12-desaturase
Micpu-Δ6D	*Micromonas pusilla* Δ6-desaturase
MQ	MilliQ water
MUFA	Mono unsaturated fatty acid
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 *Pichia pastoris* ω3/Δ15-desaturase (Picpa-ω3D) 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 Picpa-ω3D was a functional enzyme that desaturating linoleic acid (LA) to α-linolenic acid (ALA) in different cells for accumulating more precursor of omega-3 long-chain (≥C20) polyunsaturated fatty acids (ω3 LC-PUFA). Picpa-ω3D protein contains 415 amino acid residues and shares high homology to other Δ15-desaturases that have been consumed as food, used in food production or in animal feeds. The molecular weight of Picpa-ω3Dis predicted to be 47.8 kDa, with an estimated isoelectric point (pI) of 7.67.

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 2016-005, 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).

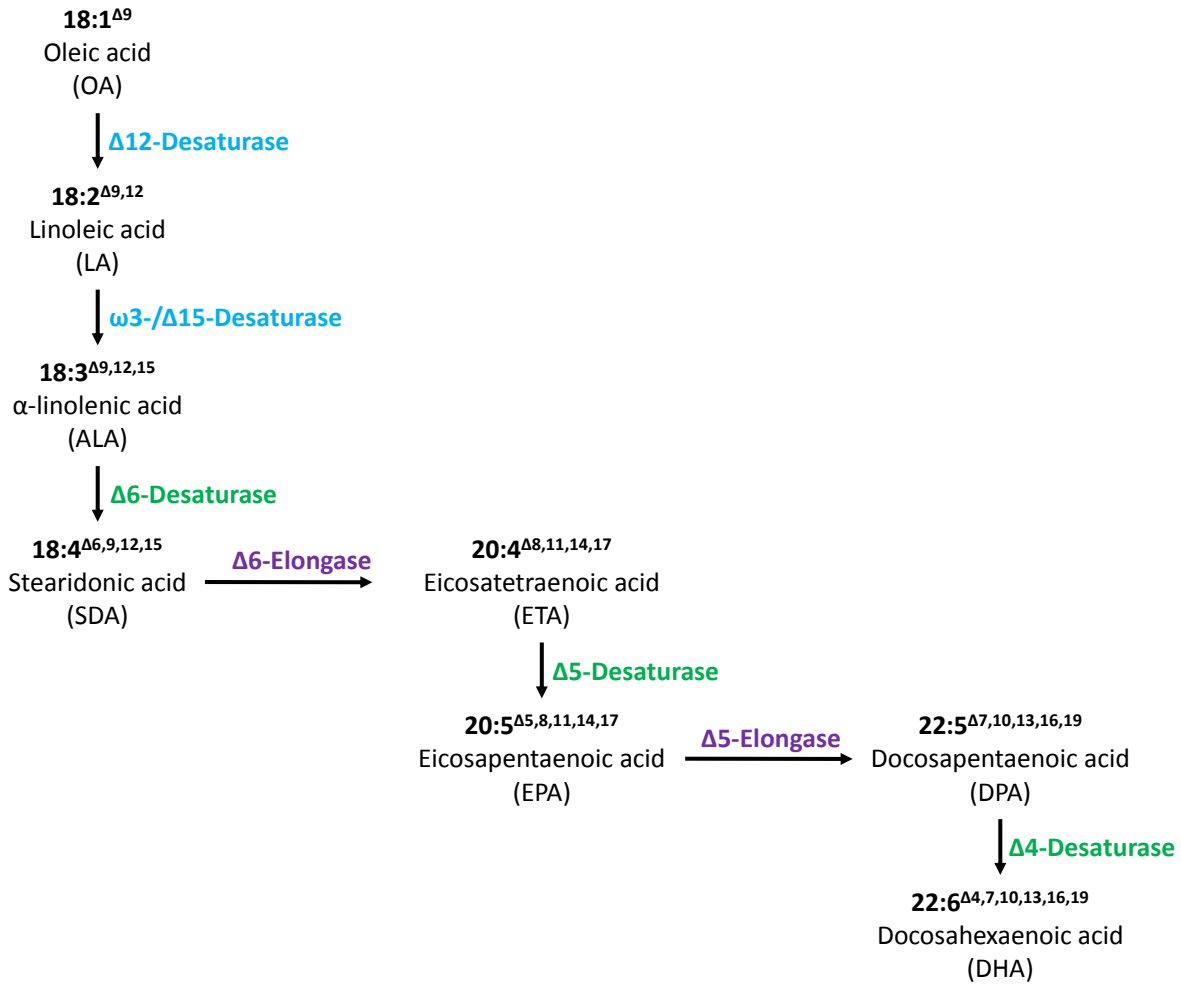

Figure 1. DHA biosynthesis pathway engineered into DHA canola event NS-B50027-4. Seven enzymes introduced in canola to convert oleic acid to final product docosahexaenoic acid were grouped into 3 classes, two fatty acid desaturases from yeast in blue, two elongases from microalgae in purple, and three 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 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. pastoris* ω3-/Δ15-desaturase (Picpa-ω3D) protein to catalyse the desturation of LA at Δ15 position to ALA ($18:2^{\Delta 9,12} \rightarrow 18:3^{\Delta 9,12,15}$).

III. MATERIALS

A. TARGET PROTEIN

The ω3-desaturase gene used in DHA canola event was previously cloned from yeast *P. pastoris* (Zhang et al. 2008). The Picpa-ω3D 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 *Saccharomyces cerevisiae* α-mating type signal peptide as secretion peptide (SP). The His-tag fusion vectors contained a coding sequences encoding a His-tag (His_{10}) and a PreScission protease cleavage site (SLEVLFQ$^{\downarrow}$GP) fused to the codon optimized *Picpa-ω3D* gene.

B. OTHER MATERIALS

The *Picpa-ω3D* gene was synthesized at GeneArt (Life Science Technologies, Germany), according to sequence in NCBI database under accession EF116884 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 *Picpa-ω3D* gene was previously cloned from yeast *P. pastoris* (Zhang et al. 2008). The translated amino acid sequence was compared to other published ω3/Δ15-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 *Picpa-ω3D* 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 -20°C. 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 28 °C for 3 to 4 days. Single colony was inoculated in 10 mL of YPD medium and cultured at 28°C 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 28°C 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 28°C without shaking for 4 hrs, then plated out on Pichia Adenine Dropout Dextrose (Invitrogen) plate and incubate at 28°C 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 28°C 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 28°C 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 80°C 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 *Picpa-ω3D* gene was previously cloned from yeast *P. pastoris* (Zhang et al. 2008). *P. pastoris* was also called as *Komagataella pastoris.* The open reading frame (ORF) of *Picpa-ω3D* gene consisted of 1248 bp, and is shown in Figure 2.

ATGTCAAAAGTCACTGTTTCGGGTTCAGAGATCCTAGAAGGGTCCACCAAGACCGTCAG
ACGTTCAGGAAACGTTGCTTCTTTCAAGCAACAGAAAACTGCCATCGATACGTTTGGTA
ATGTTTTCAAAGTGCCAGATTACACCATCAAAGATATCCTAGACGCCATTCCAAAACAC
TGCTATGAAAGATCTTTGGTCAAGTCGATGTCCTACGTCGTCAGGGATATTGTTGCTAT
TTCTGCTATTGCCTACGTGGGCCTAACTTACATCCCTCTGCTGCCAAACGAGTTTCTTC
GTTTTGCTGCTTGGAGTGCGTATGTGTTCAGTATTTCTTGTTTTGGATTCGGAATTTGG
ATTCTTGGACACGAATGCGGTCACTCTGCCTTCAGCAACTATGGCTGGGTTAACGACAC
GGTCGGCTGGGTTCTTCATTCCCTGGTAATGGTTCCATATTTTTCTTGGAAGTTCTCCC
ATGCCAAGCATCATAAGGCAACAGGTCATATGACCAGAGACATGGTCTTTGTTCCATAC
ACCGCTGAGGAGTTCAAGGAAAAACACCAAGTTACTAGTCTGCACGATATTGCAGAGGA
AACTCCTATTTATTCAGTTTTTGCCCTTTTGTTTCAACAGCTCGGAGGACTCAGTTTGT
ATCTTGCCACGAATGCTACTGGTCAACCGTATCCCGGGGTGTCCAAGTTCTTCAGAAGT
CATTACTGGCCATCCTCTCCTGTTTTTGATAAGAAGGACTATTGGTACATTGTTCTGAG
TGACCTGGGAATCTTGGCAACCCTGACTAGTGTTTACACTGCTTACAAAGTGTTCGGAT
TTTGGCCCACATTCATTACTTGGTTTTGCCCTTGGATTTTGGTCAACCACTGGCTGGTA
TTTGTCACCTTCCTACAACACACAGACTCGTCCATGCCTCACTACGATGCCCAAGAATG
GACTTTCGCCAAAGGTGCCGCCGCCACAATTGATAGAGAATTTGGTATCTTAGGAATTA
TATTTCACGACATTATCGAAACCCACGTTTTGCACCACTATGTTAGTAGAATTCCATTC
TACCATGCTAGAGAGGCTACTGAGTGCATTAAGAAGGTTATGGGCGAACATTACCGTCA
CACTGACGAGAACATGTGGGTCAGTCTCTGGAAAACCTGGAGGTCGTGCCAGTTTGTTG
AGAACCATGATGGTGTGTACATGTTCAGAAACTGCAACAATGTTGGTGTTAAACCTAAG
GATACC**TAA**

Figure 2. Nucleotide sequence of native Picpa-ω3D gene.
Start codon (ATG) and stop codon (TAA) are in bold.

B. PROTEIN SEQUENCE

The translated *P. pastoris* ω3/Δ15-desaturase (Picpa–ω3D) contained 415 amino acid residues (Figure 3). The molecular weight of Picpa–ω3D is predicted as 47.8 kDa, with estimated pI of 7.67.

MSKVTVSGSEILEGSTKTVRRSGNVASFKQQKTAIDTFGNVFKVPDYTIKDILDAIPKHC
YERSLVKSMSYVVRDIVAISAIAYVGLTYIPLLPNEFLRFAAWSAYVFSISCFGFGIWIL
GHECGHSAFSNYGWVNDTVGWVLHSLVMVPYFSWKFSHAKHHKATGHMTRDMVFVPYTAE
EFKEKHQVTSLHDIAEETPIYSVFALLFQQLGGLSLYLATNATGQPYPGVSKFFKSHYWP
SSPVFDKKDYWYIVLSDLGILATLTSVYTAYKVFGFWPTFITWFCPWILVNHWLVFVTFL
QHTDSSMPHYDAQEWTFAKGAAATIDREFGILGIIFHDIIETHVLHHYVSRIPFYHAREA
TECIKKVMGEHYRHTDENMWVSLWKTWRSCQFVENHDGVYMFRNCNNVGVKPKDT

Figure 3. Amino acid sequence of Picpa-ω3D.

The fatty acid ω3/Δ15-desaturases have been cloned from a wide range of organisms, including cynobacteria (Sakamoto et al. 1994), protozoon (Sayanova et al. 2006), thraustochytrid (Meesapyodsuk and Qiu 2016), nematode (Spychalla et al. 1997), plant (Arondel et al. 1992) and fungus (Pereira et al. 2004). The Picpa-ω3D shared high homology to other Δ15-desaturase proteins as shown in Figure 4.

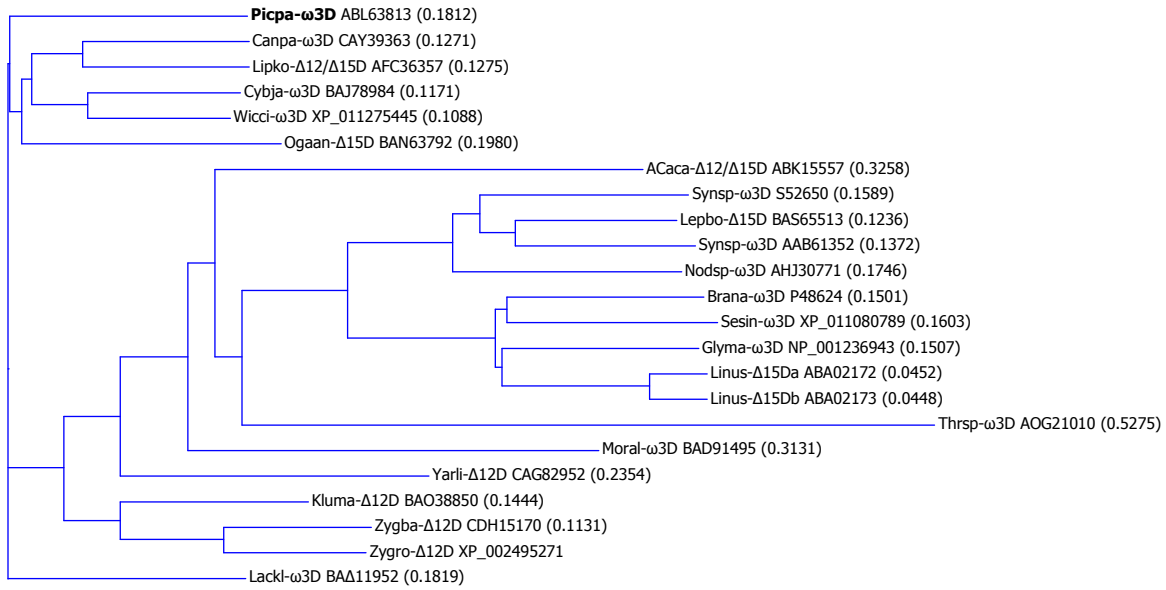

Figure 4. Phylogenetic tree for sequence comparison of Picpa-ω3D with other fatty acid desaturases. The phylogenetic tree was generated with Vector NTI software (Invitrogen, Carlsbad, USA). The protein sequences were named as 5-letter initial of species name, followed by NCBI accession numbers. Acaca, *Acathamoeba castellanii* (protozoon); Brana, *Brassica napus* (canola); Canpa, *Candida parapsilosis* (fungus); Cybja, *Cyberlindernera jadinii* (fungus); Glyma, *Glycine max* (soybean); Kluma, *Kluyveromyces marxianus* (fungus); Lackl, *Lachancea kluyveri* (fungus); Lepbo,

Leptolyngbya boryana (cynobacteria); Linus, *Linum usitatissmum* (flax)*;* Lipko, *Lipomyces kononenkoae* (fungus); Moral, *Mortierella alpine* (fungus); Nodsp, *Nodularia spunigena* (cynobacteria); Ogaan, *Ogataea angusta* (fungus); Picpa, *Pichia pastoris* (fungus); Sesin, *Sesamum indicum* (sesame)*;* Synsp, *Synechococcus* sp. (cynobacteria); Thrsp, *Thraustochytrium* sp. (Thraustochytrid); Wicci, *Wickerhamomyces ciferrii* (fungus); Yarli, *Yarrowia lipolytica* (fungus); Zygba, *Zygosaccharomyces bailii* (fungus);. Zygro, *Z. rouxii* (fungus). Δ12D, Δ12-desaturase; Δ15D, Δ15-desaturase; ω3D, ω3-desaturase.

C. Similarity of PICPA-ω3D 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. *P. pastoris* is a species of methylotrophic yeast. *Pichia* itself is widely used for protein production using recombinant DNA techniques (Ahmad et al. 2014; Cregg et al. 2000). A number of food proteins and enzymes have been expressed in *P. pastoris* (Batt, 2014). Number of products obtained by heterologous expression in *P. pastoris* have already found their way to the market (Ahmad et al. 2014), including phytase used as animal feed additive to cleave plant derived phytate, trypsin as proteinase in proteomics research, phospholipase C used for degumming of vegetable oils, collagen used for medical research and as dermal filler, Jetrea® as a drug for treatment of symptomatic vitreomacular adhesion, and recombinant human insulin. Some of them have been approved by the FDA for market release.

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 ß-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.

Wickerhamomyces ciferrii, formerly described as *Hansenula ciferrii* and *Pichia ciferrii* recently reassigned to the *Wickerhamomyces* genus, produces and secretes acetylated sphingoid long-chain bases, mainly as tetraacetyl phytosphingosine (1,3,4-trihydroxy-2-amino-octadecane). This remarkable capability is currently exploited as bioindustrial platform for the production of high-purity sphingosines. These sphingolipids are valuable active ingredients used mainly for cosmetic applications, e.g., for moisture retention and for protection and repair of the skin lipid barrier, and their effectiveness has been demonstrated in several cosmetic and clinical studies (ter Veld et al. 2013).

Candida utilis (anamorph of *Cyberlindernera jadinii*, also misnomer *Lindnera jadinii*) is a yeast stain very important for the food and feed industry. Its industrial utilization started in World War I, when common protein sources became scarce. This yeast can efficiently assimilate pentoses including xylose to use waste hardwood hydrolysates from the pulp industry. The obtained single-cell protein (SCP) had excellent nutritional properties regarding protein content, nucleotide concentration, and vitamins and had an agreeable odor and flavor (the so-called *umami* taste due to a high content of glutamate). Besides pentoses, *C. utilis* also assimilates other carbon sources such as organic acids, alcohols, propionaldehyde, and acetaldehyde, as well as various nitrogen sources including nitrate, nitrite, urea, ammonium hydroxide, and amino acids, which allows its growth on many biomass-derived waste substrates, e.g., spent sulfite liquor wheat, molasses, and ryegrass straw. Following its initial use as a dietary supplement, different endogenous compounds were isolated from *C. utilis* including invertase, glutathione, ribonucleic acids, glucomannan, phospholipase B, or biotin (Buerth et al. 2016). Because of its long and safe history in the food industry *C. utilis*, as *S. cerevisiae*, has been classified as generally recognized as safe (GRAS) by the US Food and Drug Administration.

The yeast *Lipomyces starkeyi* and *L. kononenkoae* have been used in food related applications especially due to their ability to produce α-amylase and/or endo-dectranase, and are not known to produce antibiotics or toxic metabolites (Kang et al. 2004). The dextranase has been demonstrated to be an effective agent for removing dextran during sugar processing. *Ogataea angusta* is another methylotrophic yeast used as a protein factory for pharmaceuticals. *L. kluyveri* 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 diary 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).

Mortierella 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).

Picpa-ω3D shares sequence homology to fatty acid Δ15-desaturases isolated from many of above fungal species. Picpa-ω3D shares 63% identity to *W. ciferrii* ω3-desaturase (XP_011275445) or *L. kluyveri* ω3-desaturase (BAD11952), 60% identity to *C. jadinii* ω3-desaturase (BAJ78984), 59% identity to *L. kononenkoae* bifunctional Δ12/ω3-desaturase (AFC36357), 35% identity to *M. alpine* ω3-desaturase (BAD91495).

Picpa-ω3D also shares sequence homology to fatty acid Δ12-desaturases isolated from many of above fungi species. Picpa-ω3D shares 55% identity to *K. marxianus* Δ12-desaturase (BAO38850), 50% identity to Δ12-desaturases of *Z. bailii* (ACF49508) or *Z. rouxii*. (XP_002495271), 49% identity to *Y. lipolytica* Δ12-desaturase (CAG82952). Picpa-ω3D shares 59% identity to *L. kononenkoae* bifunctional Δ12/Δ15-desaturase (ACF36357).

The Picpa-ω3D protein also shared 26% to 28% of sequence identities with plant Δ15-desaturases, including those from edible like canola (P48624), soybean (NP_001236943), linseed (ABA02172, ABA02173) or sesame (XP_011080789). Canola, soybean, linseed and sesame are typical oil crops for food application. Particularly, the introduced Picpa-ω3D protein in DHA canola shared 28% of sequence identity with the endogenous canola Δ15-desaturase.

Table 1. Amino acid sequence identity between Picpa- ω3D 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 Picpa-ω3D			100	63.1	63.4	59.8	58.9	48.8	35.5	50.2	55.2	27.6	25.6	25.6	25.9
2	Wicci-ω3D	XP_011275445	Fungus		100	65.2	72.1	64.0	49.2	33.9	51.1	58.0	24.8	25.6	23.5	25.6
3	Lackl-ω3D	BAD11952	Fungus			100	57.4	61.5	51.5	33.3	52.2	54.8	24.5	23.4	23.9	23.6
4	Cybjan-ω3D	BAJ78984	Fungus				100	62.1	44.9	30.9	46.8	53.4	24.9	23.8	22.6	24.0
5	Lipko-Δ12D/Δ15D	ACF36357	Fungus					100	46.7	31.8	49.8	56.6	24.4	25.9	25.6	23.7
6	Yarli-Δ12D	CAG82952	Yeast						100	39.3	47.3	52.5	28.1	28.1	27.4	26.7
7	Moral-ω3D	BAD91495	Fungus							100	32.6	32.6	29.3	29.5	30.5	26.4
8	Zygba-Δ12D	CDH15170	Fungus								100	64.7	26.7	26.6	26.2	25.2
9	Kluma-Δ12D	BAO38850	Fungus									100	26.5	26.7	25.9	26.9
10	Brana-ω3D	P48624	Canola										100	68.7	66.3	65.5
11	Glyma-ω3D	NP_001236943	Soybean											100	67.9	62.2
12	Linus-Δ15D	ABA02172	Flax												100	65.0
13	Sesin-ω3D	XP_011080789	Sesame													100

Δ12D, Δ12-desaturase; Δ15D, Δ15-desaturase; ω3D, ω3-desaturase; Brana, *Brassica napus*; Cybji, *Cyberlindernera jadinii*; Glyma, *Glycine max;* Kluma, *Kluyveromyces marxianus*; Lackl, *Lachancea kluyveri*; Linus, *Linum usitatissmum;* Lipko, *Lipomyces kononenkoae*; Moral, *Mortierella alpine*; Picpa, *Picia pastoris*; Sesin, *Sesamum indicum;* Wicci, *Wickerhamomyces ciferrii* ; Yarli, *Yarrowia lipolytica*; Zygba, *Zygosaccharomyces bailii.*

D. HETEROLOGOUS EXPRESSION

The enzyme functionality of Picpa-ω3D have been confirmed in different heterologous expression systems, including yeast (Zhang et al. 2008), Arabidopsis seed (Petrie et al. 2012) and Camelina seed (Petrie et al. 2014). In this study, Picpa-ω3D was expressed in *P. pastoris*, as fusion proteins designated as SP::His_{10}::Picpa-ω3D or His_{10}::Picpa-ω3D. In SP::His_{10}::Picpa-ω3D, the Picpa-ω3D 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}::Picpa-ω3D, the Picpa-ω3D sequence was fused to His-tag (His_{10}) and PreScission protease cleavage site (SLEVLFQ$^{\downarrow}$GP) at its N-terminal (Figure 6). No signal peptide was used in His_{10}::Picpa-ω3D.

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTN
NGLLFINTTIASIAAKEEGVSLEKRPHHHHHHHHHHSLEVLFQGPMSKVTVSGSEILEGS
TKTVRRSGNVASFKQQKTAIDTFGNVFKVPDYTIKDILDAIPKHCYERSLVKSMSYVVRD
IVAISAIAYVGLTYIPLLPNEFLRFAAWSAYVFSISCFGFGIWILGHECGHSAFSNYGWV
NDTVGWVLHSLVMVPYFSWKFSHAKHHKATGHMTRDMVFVPYTAEEFKEKHQVTSLHDIA
EETPIYSVFALLFQQLGGLSLYLATNATGQPYPGVSKFFKSHYWPSSPVFDKKDYWYIVL
SDLGILATLTSVYTAYKVFGFWPTFITWFCPWILVNHWLVFVTFLQHTDSSMPHYDAQEW

TFAKGAAATIDREFGILGIIFHDIIETHVLHHYVSRIPFYHAREATECIKKVMGEHYRHT
DENMWVSLWKTWRSCQFVENHDGVYMFRNCNNVGVKPKDT

Figure 5. Amino acid sequence of SP::His10::Picpa- ω3D.
Picpa-ω3D 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.

MRPHHHHHHHHHHSLEVLFQGPMSKVTVSGSEILEGSTKTVRRSGNVASFKQQKTAIDTF
GNVFKVPDYTIKDILDAIPKHCYERSLVKSMSYVVRDIVAISAIAYVGLTYIPLLPNEFL
RFAAWSAYVFSISCFGFGIWILGHECGHSAFSNYGWVNDTVGWVLHSLVMVPYFSWKFSH
AKHHKATGHMTRDMVFVPYTAEEFKEKHQVTSLHDIAEETPIYSVFALLFQQLGGLSLYL
ATNATGQPYPGVSKFFKSHYWPSSPVFDKKDYWYIVLSDLGILATLTSVYTAYKVFGFWP
TFITWFCPWILVNHWLVFVTFLQHTDSSMPHYDAQEWTFAKGAAATIDREFGILGIIFHD
IIETHVLHHYVSRIPFYHAREATECIKKVMGEHYRHTDENMWVSLWKTWRSCQFVENHDG
VYMFRNCNNVGVKPKDT

Figure 6. Amino acid sequence of His10::Picpa- ω3D.
Picpa-ω3D 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.

Table 2 shows the enzyme activity of Picpa-ω3D expressed as fusion proteins in *P. pastoris* with or without secretion peptide. Overexpression of Picpa-ω3D fusion protein substantially increased the desaturation of 18:2 to 18:3 compared to vector alone. In addition, the His_{10}::Picpa-ω3D had higher activity than SP::His_{10}::Picpa-ω3D.

Table 2. Activity of Picpa- ω3D fusion protein in P. pastoris cells.

Sample	Substrate (%)		Product (%)		Conversion (%)	
Vector	18:2	26.0 ± 1.1	18:3	4.2 ± 0.2	14.0 ± 0.8	n=3
SP::His_{10}::Picpa-ω3D		12.7 ± 1.7		11.2 ± 2.4	46.5± 8.1	n=10
Vector		26.4 ± 0.9		2.0 ± 0.2	15.3 ± 0.8	n=3
His_{10}::Picpa-ω3D		3.8 ± 0.5		21.9 ± 1.8	85.4 ± 0.9	n=3

Substrate and product are shown in percentage of total fatty acid in cells. Conversion rate is based on the product 18:3 compared to the total of product 18:3 and remaining substrate 18:2. SP, secretion peptide. n = repeats with individual colonies.

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 Picpa-ω3D 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).

MSKVTVSGSEILEGSTKTVRRSGNVASFKQQKTAIDTFGNVFKVPDYTIKDILDAIPKHC
YERSLVKSMSYVVRDIVAISAIAYVGLTYIPLLPNEFLRFAAWSAYVFSISCFGFGIWIL
GHECGHSAFSNYGWVNDTVGWVLHSLVMVPYFSWKFSHAKHHKATGHMTRDMVFVPYTAE
EFKEKHQVTSLHDIAEETPIYSVFALLFQQLGGLSLYLATNATGQPYPGVSKFFKSHYWP
SSPVFDKKDYWYIVLSDLGILATLTSVYTAYKVFGFWPTFITWFCPWILVNHWLVFVTFL
QHTDSSMPHYDAQEWTFAKGAAATIDREFGILGIIFHDIIETHVLHHYVSRIPFYHAREA
TECIKKVMGEHYRHTDENMWVSLWKTWRSCQFVENHDGVYMFRNCNNVGVKPKDT

Figure 7. Theoretical glycosylation sites (NXT/NXS) in Picpa- ω3D.

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 Picpa-ω3D in the insert was confirmed to be identical to the original sequence (Figure 8).

```
                      1                                                  50
Picpa-ω3D_vec     (1) MSKVTVSGSEILEGSTKTVRRSGNVASFKQQKTAIDTFGNVFKVPDYTIK
NS-B50027-4       (1) MSKVTVSGSEILEGSTKTVRRSGNVASFKQQKTAIDTFGNVFKVPDYTIK
                      51                                                 100
Picpa-ω3D_vec    (51) DILDAIPKHCYERSLVKSMSYVVRDIVAISAIAYVGLTYIPLLPNEFLRF
NS-B50027-4      (51) DILDAIPKHCYERSLVKSMSYVVRDIVAISAIAYVGLTYIPLLPNEFLRF
                      101                                                150
Picpa-ω3D_vec   (101) AAWSAYVFSISCFGFGIWILGHECGHSAFSNYGWVNDTVGWVLHSLVMVP
NS-B50027-4     (101) AAWSAYVFSISCFGFGIWILGHECGHSAFSNYGWVNDTVGWVLHSLVMVP
                      151                                                200
Picpa-ω3D_vec   (151) YFSWKFSHAKHHKATGHMTRDMVFVPYTAEEFKEKHQVTSLHDIAEETPI
NS-B50027-4     (151) YFSWKFSHAKHHKATGHMTRDMVFVPYTAEEFKEKHQVTSLHDIAEETPI
                      201                                                250
Picpa-ω3D_vec   (201) YSVFALLFQQLGGLSLYLATNATGQPYPGVSKFFRSHYWPSSPVFDKKDY
NS-B50027-4     (201) YSVFALLFQQLGGLSLYLATNATGQPYPGVSKFFRSHYWPSSPVFDKKDY
                      251                                                300
Picpa-ω3D_vec   (251) WYIVLSDLGILATLTSVYTAYKVFGFWPTFITWFCPWILVNHWLVFVTFL
NS-B50027-4     (251) WYIVLSDLGILATLTSVYTAYKVFGFWPTFITWFCPWILVNHWLVFVTFL
                      301                                                350
Picpa-ω3D_vec   (301) QHTDSSMPHYDAQEWTFAKGAAATIDREFGILGIIFHDIIETHVLHHYVS
NS-B50027-4     (301) QHTDSSMPHYDAQEWTFAKGAAATIDREFGILGIIFHDIIETHVLHHYVS
                      351                                                400
Picpa-ω3D_vec   (351) RIPFYHAREATECIKKVMGEHYRHTDENMWVSLWKTWRSCQFVENHDGVY
NS-B50027-4     (351) RIPFYHAREATECIKKVMGEHYRHTDENMWVSLWKTWRSCQFVENHDGVY
                      401         415
Picpa-ω3D_vec   (401) MFRNCNNVGVKPKDT
NS-B50027-4     (401) MFRNCNNVGVKPKDT
```

Figure 8. Alignment of protein sequences of Picpa- ω3D.
ω3D sequence translated from sequenced T-DNA insert in DHA canola NS-B50027-4 event was identical to the original ω3D sequence from *P. pastoris* in binary vector (Picpa-ω3D_vec).

VI. CONCLUSIONS

The results of this study demonstrated that the cloned yeast Picpa-ω3D protein has activity in heterologous expression systems, including in DHA canola, event NS-B50027-4. The Picpa-ω3D protein shares similarity to desaturase proteins present in consumed food, used in food production or in animal feeds. The enzyme functionality of Picpa-ω3D has been confirmed in several different heterologous expression systems. Data for Picpa-ω3D expressed in Pichia as fusion proteins confirmed this functionality.

Picpa-ω3D protein contains 415 amino acid residues. The molecular weight of Picpa-ω3D is predicted to be 47.8 kDa, with an estimated pI of 7.67. For the Picpa-ω3D 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 Picpa-ω3D protein sequence.

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