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(54) Title: A PROCESS TO CONCENTRATE Ω 3-PUFA FROM FISH OIL BY SELECTIVE HYDROLYSIS USING CUTINASE IMMOBILIZED ON ZEOLITE NAY

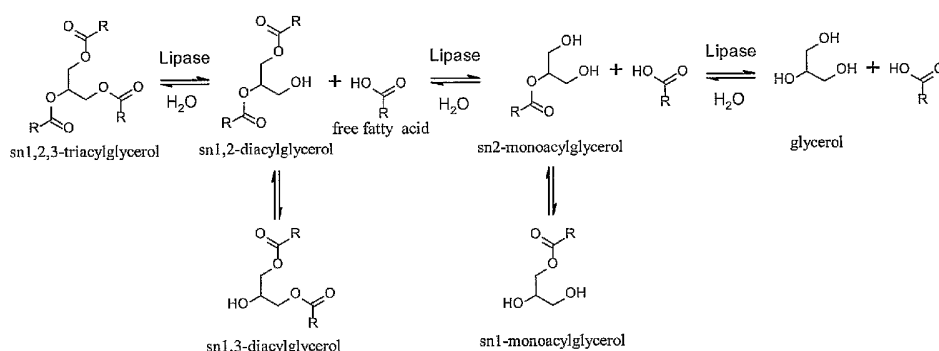


Figure: Simplified reaction scheme of Lipase-catalysed hydrolysis of triacylglycerols. Ultimately, the complete hydrolysis of one molecule of triacylglycerol will yield three molecules of free fatty acids and one molecule of glycerol. The full reaction will have consumed three molecules of water. In standard hydrolytic conditions, the reaction is not complete, because equilibria are reached and lipases are *sn*1 or *sn*3 stereospecific. Cutinase is essentially *sn*3 specific. Acyl migration can occur in certain conditions (aqueous conditions at basic pH), where fatty acids migrate from the *sn*2 position to the *sn*1 or *sn*3 positions. R: any type of fatty acid alkyl chain.

(57) Abstract: The present invention relates to a process for concentrating the content of ω 3 -polyunsaturated fatty acids (PUFA) in a natural oil source such as fish oil using a cutinase catalysed selective hydrolysis of the triglycerides, wherein the cutinase is immobilised to a solid support comprising zeolite NaY.

A process to concentrate ω 3-PUFA from fish oil by selective hydrolysis using cutinase immobilized on zeolite NaY

FIELD OF THE INVENTION

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The present invention relates to a process in which immobilized cutinase is used to obtain concentrates of ω 3-PUFA (EPA and DHA) from a natural oil source, such as fish oil, by selective hydrolysis. The solid phase to which the cutinase is immobilized would be a zeolite, such as zeolite NaY or a modified form thereof. The invention further relates to a genetically engineered cutinase having improved zeolite binding properties. A fish oil product obtained by said process and wherein the original content of ω 3-PUFA is enriched and which is optionally in addition detoxified and/or dearomatized is also encompassed by the invention.

15 **BACKGROUND OF THE INVENTION**

Enzymatic methods for polyunsaturated fatty acid enrichment of oils, such as fish oil is described in US pat. appl. No. 20030054509 and in US Patent 6,537,787, and in Jonzo et al. (2000). Most lipases from microorganisms generally used in these processes show strong stereospecificity for the sn1 and sn3 positions on the glycerol backbone, which leads to an enrichment in EPA and DHA in acylglycerols. Microbial lipases are in general relatively unspecific for chain length. *Rhizomucor miehei* lipase has been shown to be very selective against EPA and DHA and is used in industrial methods to make concentrates of ω 3-PUFA from fish-oil. However, this lipase enriches acylglycerols only in DHA. Other active lipases show the same trend as has been described by Halldorsson et al. 2004.

Schmitt et al. (2002) studied the catalytic site of various microbial lipases and mentioned that they have been assigned to different classes: (i) lipases with a hydrophobic, crevice-like binding site located near the protein surface (lipases from *Rhizomucor* and *Rhizopus*); and (ii) lipases with a funnel-like binding site (lipases from *Candida antarctica*, *Pseudomonas* and cutinase). Cutinase is grouped by these authors with some lipases, in that it presents a funnel-like binding site and the same catalytic triad,

but mainly differs by the lack of a “lid” structure, which normally covers the active site. Instead, cutinase presents two small loop regions, partially covering the active site (Longhi et al., 1996).

5 Cutinase from *Fusarium solani* pisi is an esterase with lipolytic activity, capable of hydrolysing acylglycerols. The gene coding the enzyme has been cloned and the protein expressed in recombinant form in *E. coli* first in 1991 (Lauwereys et al, 1991). Since then, the protein has been extensively studied to unravel structure/function relationships. Several crystal structures have been solved and a great deal of its catalytic
10 mechanism is already described. In fact, cutinase has been used as a model protein to understand the mode of action of lipases. Cutinase is considered as an “intermediate” between esterases and lipases in the way that it can both hydrolyse water soluble esters and lipids (it can work at the interface of water/lipids). Catalytic properties of cutinase have been used extensively in biotechnological applications for hydrolytic or synthetic
15 reactions in organic media. Cutinase has potential use in the dairy industry for the hydrolysis of milk fat, in house hold detergents, in the oleochemical industry, in the synthesis of structured triglycerides, polymers and surfactants, in the synthesis of ingredients for personal-care products, and the synthesis of pharmaceuticals and agrochemicals containing one or more chiral centers. At low water activities transesterification of
20 fats and oils or (stereo)selective esterification of alcohols can be achieved.

Cutinase hydrolyses cutin, a polyester composed of hydroxy and epoxy fatty acids (typically with 16 or 18 carbon atoms), its supposed natural substrate. As mentioned earlier, cutinase also hydrolyses triacylglycerols and the chain length specificity of
25 cutinase is for short-chain fatty acids, typically C4:0/C6:0 (Melo et al, 1995; Manesse et al, 1995). The activity of the enzyme decreases with increasing chain length, a phenomenon attributed to the size of its binding pocket which cannot accommodate long-chain fatty acids (Longhi et Cambillau, 1999). Despite this short-chain specificity described above, the enzyme is also capable of hydrolysing long-chain fatty acids like
30 oleic acid (C18:1, where 18 is the number of carbon atoms and 1 the number of double bonds), with significant lower catalytic rates. The enzyme will hydrolyse first short-chain fatty acids before those of longer chain, which concentrate in the acylglycerol fraction. The concentration factor reaches a maximum value over time, after which equilibrium is obtained. Thus, it is assumed that cutinase would be an interesting

lipolytic enzyme to perform concentration of ω 3-PUFAs from fish-oil, as these fatty acids are of the long-chain type (C20:5 and C22:6). Fish oil consists of 70% to 80% fatty acids with chain length equal or below C18.

5 It has been observed that the activity of enzymes during the hydrolysis of fish-oil decreases rapidly over time, leading to poor yields in hydrolysis products overall. Ideally, the reaction should be pushed as much as possible towards the formation of di- and monoacylglycerols enriched in PUFAs, such as EPA+DHA. In addition, for industrial applications, the volumes of oil to treat are considerable, meaning that the
10 amounts of enzyme to be used are consequently large. As enzymes can be expensive and may be the most expensive part of the process, it is necessary to be able to recycle the biocatalyst as many times as possible to reduce costs. Enzymes are very rarely used in free form in such processes, but are rather bound to solid supports, that can be easily recovered from the oil after one cycle of processing. In addition, immobilization
15 of enzymes, including lipases, is known to increase dramatically their stability over time. This is also the case with cutinase which has been successfully immobilized on diverse solid materials, cf. Carvalho et al, (1998) for a review.

An attractive solid support for an enzyme to be used in a selective hydrolysis reaction
20 is zeolite which is a microcrystalline material composed of silicate and aluminate (Goncalves et al, 1996; Goncalves et al, 1998). In addition, zeolite can sequester ions and small molecules in its microporous structure. This is especially beneficial in the case of concentrating fish oils where traces of heavy metals and undesired aromas need to be removed. However, the affinity of zeolite for an enzyme, such as cutinase,
25 is through adsorption by electrostatic attraction between the negative charges of zeolite and the positive charges of numerous lysine and arginine residues at the surface of the enzyme. However, due to the non-covalent nature of cutinase interaction with zeolite, a proportion of the enzyme is lost during the hydrolysis process and cannot be recycled.

30

Enzymatic selective hydrolysis to concentrate ω 3-PUFAs from fish-oil using lipase generally results in a selective DHA enrichment or at best an unbalanced EPA/DHA enrichment in the final product. Also the process is costly because of the expense in

providing a large amount of enzyme which, when using zeolite as the solid support, can only be partially recycled. The present invention reduces these problems by providing a process which enriches acylglycerols in both EPA and DHA. The present invention also provides a process where the amount of recycled enzyme is increased
5 and where toxic compounds and unwanted aromas can be removed.

BRIEF DESCRIPTION OF THE PRESENT INVENTION

The present invention relates in a first aspect to a process for concentrating the content
10 of ω 3-poly-unsaturated fatty acids (PUFA) in a natural oil source such as fish oil using a cutinase catalysed selective hydrolysis, wherein the cutinase is immobilised to a solid support comprising zeolite NaY. The natural oil source may be any other natural oils such as oil from marine mammals and vegetable oils having a relatively high content of PUFA, e.g. rape seed oil.

15 In a second aspect the present invention relates to a process for concentrating the content of both the ω 3-polyunsaturated fatty acids EPA and DHA in a natural oil source such as fish oil using a cutinase catalysed selective hydrolysis of the triglycerides, wherein the cutinase is immobilised to a solid support comprising zeolite NaY.

20 In a third aspect the present invention relates to a process for concentrating the content of the ω 3-PUFAs EPA and DHA in a natural oil source such as fish oil comprising the following steps:

- (a) adding cutinase immobilised to a zeolite NaY solid support to a neutralised fish oil
25 containing TAGs to form a first reaction mixture wherein said cutinase is allowed to catalyse hydrolysis of the TAGs,
- (b) separating saturated, mono-, and di-saturated FFAs from the first reaction mixture by molecular distillation and formation of Mg^{2+} and Ca^{2+} salt complexes to obtain a first PUFAGE product comprising essentially DAGs, and MAGs wherein the com-
30 bined amount of EPA +DHA is about 50%,
- (c) reforming TAGs and further increasing the PUFA content in acylglycerols, by addition of PUFAEE preferably containing 70% to 80% EPA+DHA obtained from a

urea crystallization to said first PUFA product in a second reaction mixture catalysed by said immobilised cutinase, and

(d) applying vacuum to said second reaction mixture to remove ethanol and obtain a second PUFA product comprising TAGs, DAGs, and MAGs wherein the combined amount of EPA+DHA is about 60%.

In another aspect the present invention relates to a genetically modified cutinase for use in a process according to the above-mentioned aspects comprising a cutinase wild-type amino acid sequence having at its C-terminus 5 to 7 and preferably about 6 arginine residues

In a further aspect the present invention relates to a genetically modified cutinase selected from the group consisting of the amino acid sequence as set out in SEQ ID:1 and said sequence wherein the N-terminal PhoA signal peptide sequence has been deleted.

In yet a further aspect the present invention relates to an isolated nucleic acid sequence, which comprises a nucleic acid sequence which encodes the cutinase according to claim 8 or 9 and which is operably linked to one or more control sequences, such as the PhoA signal peptide sequence, that direct the production of the cutinase in a suitable expression host.

A still further aspect according to the present invention relates to a recombinant expression vector comprising the above nucleic acid construct.

Yet another aspect according to the present invention relates to a recombinant host cell comprising the above-mentioned nucleic acid construct and/or the above-mentioned expression vector.

Yet a still further aspect according to the present invention relates to a method for producing a recombinant cutinase, the method comprising (a) cultivating an E. coli recombinant host cell of the above type to produce the polypeptide in the periplasmic space; and (b) recovering the recombinant cutinase.

Finally, another aspect according to the present invention relates to a process of preparing an immobilised recombinant cutinase comprising mixing a solution of said cutinase with zeolite NaY in a ratio of enzyme:solid support of 10 to 40 mg:g in a phosphate buffer or carbonate-bicarbonate buffer in a pH range from 7.0 to 9.0, incubated
5 at room temperature for 5 minutes under mechanical agitation, dried and stored at 4 °C in the presence of a desiccant until use.

BRIEF DESCRIPTION OF THE DRAWINGS

10 **Figure 1** is a reaction scheme of lipase catalysed hydrolysis of triacylglycerols.

Figure 2 illustrates the principle of PUFA enrichment on the glycerol backbone by selective hydrolysis by lipases.

15 **Figure 3** is a flow chart showing the various process steps of a process for concentrating the PUFA content of fish oil. this embodiment uses immobilized genetically engineered cutinase for PUFA production from fish-oil. The following abbreviations are used in the chart and/or the present description: PUFA: polyunsaturated fatty acids (typically EPA and DHA); EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid;
20 FFA: free fatty acids; FAEE: fatty acid ethyl esters; FAGE: fatty acid glyceryl esters; PUFAGE: polyunsaturated fatty acid glyceryl esters; PUFAEE: polyunsaturated fatty acid ethyl esters; TAG: triacylglycerols; DAG: diacylglycerols; MAG: monoacylglycerols.

25 **Figure 4** shows the amino-acid and nucleotide sequences of the wild-type cutinase and the 6xArg mutant with PhoA signal peptide. The sequence of wild-type cutinase corresponds to the construct where the coding region of the native protein has been fused at its N-terminus to the transit peptide *PhoA* of *E. coli* alkaline phosphatase, for periplasmic sorting, as described in Lauwereys et al (1991). The modification in the
30 mutant is shown in bold: It consists of a C-terminal tag of 6 consecutive arginines with a proline + glycine spacer. The PhoA signal peptide for targeting cutinase in the periplasmic space of *Escherichia coli* is shown in italics on the wild type sequence. This peptide is cleaved-off in the mature protein.

Figure 5 is a cartoon representation of *Fusarium solani* pisi cutinase in complex with the substrate analogue N-undecyl O-methyl chlorophosphonate ester (Longhi et al, 1996). The C-terminus of the protein is shown by the antepenultimate residue S213, in space-filling representation, at the bottom of the picture (arrowhead). The catalytic site is situated at the top of the picture, where the substrate analogue is represented in space-filling representation (arrow). A 6xArg tag added to the C-terminus would orientate the enzyme in a favorable position on the Zeolite NaY support for optimal catalytic activity. In the original publication (Goncalves et al, 1998), the authors used wild type cutinase for adsorption onto Zeolite NaY. They already noticed the enhanced stability of cutinase for catalytic reactions in organic media. They also mentioned that a significant proportion of enzyme was lost in recycling the catalyst. The present invention proposes a solution to obtain a better catalyst.

Figure 6A and 6B demonstrate enhanced adsorption of genetically modified cutinase (Cutinase_6xR) on Zeolite NaY (Fig 6B) compared to wild-type cutinase (Fig. 6A). Wild-type cutinase (Fig. 6A) and cutinase tagged with a 6xArg C-terminal sequence (Fig. 6B) were applied on a column containing 0.5ml Zeolite NaY. The initial elution conditions consisted of buffer A: phosphate 20mM at pH 8.0. A linear gradient of buffer B (same as buffer A with NaCl 1M) was applied 2.5 ml after injection. The proportion of buffer B was 100% after 7.5 ml and maintained constant until 12.5 ml. Protein elution was monitored by UV absorption at 280nm (bold black curve) and 215nm (bold grey curve). Buffer B gradient (thin black curve) and conductivity (thin grey curve) are also shown. The peaks appearing between 1 and 1.5 ml on the graph of Fig. 6B are chromatographic artefacts due to air bubbles.

DETAILED DESCRIPTION OF THE PRESENT INVENTION

The present invention relates in a first aspect to a process for concentrating the content of ω 3-poly-unsaturated fatty acids (PUFA) in a natural oil source such as fish oil using a cutinase catalysed selective hydrolysis, wherein the cutinase is immobilised to a solid support comprising zeolite NaY.

The natural oil source may be any other natural oils such as oil from marine mammals and vegetable oils having a relatively high content of PUFA, e.g. rapeseed oil.

5 In a second aspect the present invention relates to a process for concentrating the content of both the ω 3-polyunsaturated fatty acids EPA and DHA in a natural oil source such as fish oil using a cutinase catalysed selective hydrolysis of the triglycerides, wherein the cutinase is immobilised to a solid support comprising zeolite NaY.

10 It should be emphasized that in the processes according to the present invention which comprise a cutinase catalysed selective hydrolysis it is understood that the hydrolysis of the triglycerides is performed by removing non- ω 3-poly-unsaturated fatty acid moieties originally bonded to the glyceryl backbone of the fatty acid glycidyl esters of the starting natural oil, thereby during the process, as far as possible retaining bonded
15 to said glyceryl backbone, the ω 3-poly-unsaturated fatty acid moieties which are originally bonded to the glycidyl backbone of the fatty acid glycidyl esters of the starting natural oil.

In the present description and the appended claims the term “...as far as possible retaining bonded to said glyceryl backbone, the ω 3-poly-unsaturated fatty acid moieties which are originally bonded to the glyceryl backbone of the fatty acid glycidyl esters of the starting natural oil...” and the term “...as far as possible retaining bonded to
20 said glyceryl backbone, the EPA as well as DHA fatty acid moieties which are originally bonded to the glyceryl backbone of the fatty acid glycidyl esters of the starting natural oil...” is to be interpreted as “to the extent possible” or “as far as the cutinase allows”.

In the processes according to the present invention involving cutinase catalysed selective hydrolysis a key feature is accordingly to retain bonded to the glyceryl backbone,
30 the PUFA moieties originally bonded to said glycidyl backbone and to remove non-PUFA during the hydrolysis. Hence, it is undesirable during the hydrolysis reactions to obtain released PUFAs as free fatty acids. However, the release of free fatty acid PUFAs during the hydrolysis reaction will inevitably happen to a small extent. The

nature of the cutinase determines to what extent such release of free PUFA fatty acids will take place.

Optionally, the hydrolysed fish oil having an increased content of PUFA moieties
5 and/or EPA as well as DHA moieties may furthermore be subjected to a transesterification in order to further increase the content of such PUFA moieties.

Properties of cutinase

10 Cutinase is a serine esterase containing the classical Ser, His, Asp triad of serine hydrolases (Ettinger et al, 1987). The protein belongs to the alpha-beta class, with a central beta-sheet of 5 parallel strands covered by 5 helices on either side of the sheet. The active site cleft is partly covered by 2 thin bridges formed by amino acid side chains, by contrast with the hydrophobic lid possessed by other lipases (Martinez et al, 1992).
15 The protein also contains 2 disulphide bridges, which are essential for activity, their cleavage resulting in complete loss of enzymatic activity (Ettinger et al, 1987). Two cutinase-like proteins (MtCY39.35 and MtCY339.08c) have been found in the genome of the bacterium *Mycobacterium tuberculosis*. Cf. the INTERPRO database (<http://www.ebi.ac.uk/interpro/IEntry?ac=IPR000675>).

20 Dissing (2004) has shown that cutinase activity towards diverse natural oils shows the following order: olive oil (75% of C18:1) > thistle oil (75% of C18:2) > linseed oil (57% of C18:3) > fish oil ω 3-PUFA concentrate (60% C20:5 + C22:6). This strongly suggests that cutinase activity decreases with an increasing number of double bonds in
25 fatty acids. Alternatively, cutinase activity may be sensitive to the position of the double bonds in the acyl chain.

The net result is that cutinase can be used to concentrate long-chain ω 3-PUFA on the glycerol backbone by kinetic resolution. We also assumed that cutinase may present
30 an advantage over existing lipases used in processes to concentrate ω 3-PUFA from fish-oil. Most lipases from microorganisms generally used in these processes show strong stereospecificity for the sn1 and sn3 position on the glycerol backbone, which lead to an enrichment in EPA and DHA in acylglycerols (as EPA and DHA mainly

occur at the sn2 position). Cutinase is also stereospecific for the sn1 and sn3 position and at the same time shows preference for short chain fatty acids. This may confer to cutinase additional discriminating power against EPA and DHA, over microbial li-
pases which are in general less specific for chain length. This holds to that fatty acids
5 can migrate from the sn2 position to sn1 or sn3 positions, in the presence of water at basic pH values, which can in turn be attacked by the enzyme.

Preliminary experiment No. 1

10 Hydrolysis of fish oil with cutinase leads to concentration of EPA and DHA on the glycerol backbone

To test whether cutinase would hydrolyse preferentially other fatty acids than EPA and DHA from fish oil triacylglycerols, we analyzed the fatty acid content of the frac-
15 tions resulting from hydrolysis: tri-, di- and monoacylglycerols. Results showed that the contents in EPA and DHA in the di- and monoacylglycerol fractions increase significantly, cf. Table 1 below. In accordance with our first observations and our as-
sumptions regarding cutinase fatty acid selectivity, it appears that cutinase cleaves-off monounsaturated fatty acids first. In comparison, we used a lipase from *Rhizomucor*
20 *miehei* in the same reaction conditions. This lipase has been shown to be very selective against EPA and DHA and is used in industrial methods to make concentrates of ω 3-PUFA from fish-oil. This lipase gives the same concentration levels in combined EPA+DHA, app. 38% in the MAG, see Table 1, as compared to cutinase, although in somewhat different ratios. Cutinase appears to enrich acylglycerols in both EPA and
25 DHA, whereas *Rhizomucor miehei* lipase enriches only in DHA.

Table 1: Fatty-acid composition of triacylglycerol (TAG), diacylglycerol (DAG) and monoacylglycerol (MAG) fractions from fish-oil after hydrolysis with lipases.

30 A 5% emulsion of fish-oil in 50mM Tris-Cl buffer pH7.5 was hydrolyzed for 24 h with 4 μ g wt cutinase or *Rhizomucor miehei* lipase. The hydrolysis product was fractionated by liquid chromatography into TAG, DAG, MAG and free fatty acids. Fatty acid profiles of TAG, DAG and MAG were analyzed by gas chromatography.

	Fish oil	Cutinase			R. miehei lipase		
		TAG	DAG	MAG	TAG	DAG	MAG
Sum of FAs above C20:0	44,5	45,7	48,1	49,8	45,8	48,8	47,9
Sum of N-3 FAs	26,9	28,4	41,7	45,7	28,8	37,2	46,9
Sum of N-6 FAs	3,4	3,5	3,8	3,3	3,5	3,4	2,2
Sum of N-9 FAs	20,7	20,6	15,7	13,7	20,4	16,8	9,4
Sum of saturated FAs	22,2	21,7	18,1	21,7	20,8	21,5	12,6
Sum of monounsaturated FAs	40,6	40,2	30,0	24,2	40,1	32,8	15,4
Sum of PUFAs	33,1	32,2	46,4	49,0	32,7	41,0	49,6
EPA	7,7	8,4	13,0	12,3	8,2	8,6	6,9
DHA	11,3	12,3	16,3	25,7	12,2	19,2	30,7
Conc. Fold EPA			1,7	1,6		1,1	0,9
Conc. Fold DHA			1,4	2,3		1,7	2,7

Table 1

5

Preliminary experiment No.2

In order to test whether cutinase would further enrich acylglycerols in EPA+DHA, we used as a substrate the diacylglycerol fractions obtained in the first experiment instead of the triacylglycerols from fish oil. The results show that cutinase still discriminates against EPA and DHA as the combined proportion of EPA+DHA was 54.4% in the resulting monoacylglycerols compared to 29.3% in the initial diacylglycerols, cf. Table 2 below. It also appears that in these experimental conditions DHA is preferred over EPA by cutinase, as the enrichment level is higher for EPA.

15

Table 2: Fatty-acid composition of monoacylglycerols (MAG) fraction obtained from diacylglycerols hydrolysis with cutinase

A 5% emulsion of fish-oil in 50mM Tris-Cl buffer pH7.5 was hydrolyzed for 24 h with 4 µg wt cutinase. The hydrolysis product was fractionated by liquid chromatography into DAG, MAG and free fatty acids. Fatty acids profiles from MAG were analyzed by gas chromatography.

20

	DAG (substrate)	MAG (product)
Sum of FAs above C20:0	48,1	67,2
Sum of N-3 FAs	41,7	65,6
Sum of N-6 FAs	3,8	4,8
Sum of N-9 FAs	15,7	12,1
Sum of saturated FAs	18,1	4,5
Sum of monounsaturated FAs	30,0	20,1
Sum of PUFAs	46,4	70,8
EPA	13,0	35,9
DHA	16,3	18,5
Conc. Fold EPA		2,8
Conc. Fold DHA		1,1

Table 2

5

Cutinase properties

The Tables 3 and 4 which follow and Figures 1 and 2 are there to illustrate that cutinase can be used to concentrate EPA and DHA on the glycerol backbone of fish oils. The major fatty acids found in fish oil are presented in Table 3. Cutinase is essentially sn3 specific, which will naturally lead to an enrichment of EPA and DHA in diacylglycerols and mono-acylglycerols, due to their sn2 position (Table 4). This is a stereo-specific effect which all other microbial lipases also exhibit, that is to say that all microbial lipases have the potential to concentrate EPA and DHA on the glycerol backbone. The general scheme for acylglycerols hydrolysis by lipases is given on Figure 1. What could make one particular lipase more attractive than another for this purpose, is the relative rate at which the sn2 position is also attacked. Cutinase is very poor at attacking the sn2 position, which makes it attractive. Another feature making any particular lipase attractive for the purpose of concentrating EPA and DHA is its selectivity for the acyl chain itself (the fatty acid). Cutinase prefers short-chain

lengths, with a maximum typically around 4 to 6 carbons. This means that EPA and DHA being respectively 20 and 22 carbon chains, they should be the last to be cleaved-off (hence a concentrating effect). The principle of this concentration is illustrated on Figure 2.

5

A last feature making some lipases more attractive than others is their selectivity according to double bonds in the acyl-chain (number and position). In general, data available in the literature concerning lipases selectivity for double bonds is scarce. Authors working with lipases and fish-oil have been more interested in the final product, like factors and yield of enrichment in EPA and DHA, than in understanding why lipases may achieve this. In this respect, there is merely any rational approach in selecting lipases for PUFA concentrates manufacture, essentially empirical knowledge (final yields). In conclusion, up till now nothing is known for cutinase as for its selectivity for or against double bonds, thus cutinase preference in terms of double bond position or number is not predictable. Table 3 and Figure 2 illustrate the principle of selective hydrolysis, applicable to cutinase.

10

15

Table: Major fatty acids in fish-oils: structure and molar ratios. The common name of the fatty acids is given along with an abbreviated nomenclature "Ca:bn-c", where "a", "b" and "c" represent respectively the number of carbon atoms, the number of double bonds and the position of the first double bond, starting from the terminal methyl group. The typical fatty acid composition of fish oils is given in molar fractions, as determined by gas-chromatography.

Common name	Structure	Typical molar fraction (%)		
		Menhaden	Herring	Sprat (FF)
Myristic acid (C14:0)		7-12	5-8	4
Palmitic acid (C16:0)		15-26	10-19	18
Stearic acid (C18:0)		2-4	1-2	2
cis-9-Palmitoleic acid (C16:1n-7)		9-16	6-12	5
Oleic acid (C18:1n-9)		8-14	9-25	25
cis-Vaccenic acid (C18:1n7)				2
Linoleic acid (C18:2n-6)				4
α-linolenic acid (C18:3n-3)				3
Moroctic acid (C18:4n-3)				3
Eicosapentaenoic acid / EPA (C20:5n-3)		11-16	4-15	9
Docosahexaenoic acid / DHA (C22:6n-3)		5-14	2-8	15

Table 3

Table 4: Typical position-specific distribution of fatty-acids in fish tri-acylglycerols

The three carbon atoms on the glycerol backbone are numbered sn1, sn2 and sn3, the sn3 position being occupied by the less substituted acyl-chain by convention. Fatty acids are presented in abbreviated nomenclature (see Table 3). Long-chain PUFA like EPA (20:5) and DHA (22:6) are in general found on the sn2 position.

	sn	14:0	16:0	16:1	18:0	18:1	18:2	20:1	22:1	20:5	22:5	22:6
h	1	6	12	13	1	16	3	25	14	3	1	1
	2	10	17	10	1	10	3	6	5	18	3	13
	3	4	7	5	1	8	1	20	50	4	1	1
c	1	6	15	14	6	28	2	12	6	2	1	1
	2	8	16	12	1	9	2	7	5	12	3	20
	3	4	7	14	1	23	2	17	7	13	1	6

h: herring; c: cod

10 Table 4

Immobilization of cutinase on zeolite NaY

15 Zeolites are a family of natural or synthetic earths with a well-defined crystalline structure, forming a microporous network with pore openings of 3Å to 8Å. Zeolites are commercially available in bulk quantities, relatively inexpensive, and presented in the form of inert superfine powders (typically 0.5 to few micrometers). They have found applications in many industrial processes, in particular in the oleochemistry industry as cracking catalysts, in laundry detergents, etc. They are well accepted in food processing technologies as their toxicity has been shown to be null.

25 The advantage of zeolites resides in their huge surface area (up to 650 m² g⁻¹), their strong cation-exchange capacity due to AlO₄ which confers buffering properties (useful for controlled enzymatic reactions), their defined structure which has been exploited for molecular sieving, their physical and chemical stability (most can be heated

to 500 °C) which makes them easily recyclable. In addition, being essentially hydrophilic their water retention capacity is excellent which is useful to perform hydrolytic reactions in organic medium. This is important for the cutinase catalysed hydrolysis of fish oil which requires water.

5

Amongst zeolites, the basic form Zeolite NaY has been shown to be the most effective to immobilize cutinase for performing hydrolytic reactions of tri-acylglycerols in organic medium (Goncalves et al, 1996). This efficiency was attributed to the basic nature of Zeolite NaY which ensures that the protein works at optimal pH (around pH 9.0), compared to more acidic forms. The mode of adsorption of cutinase on zeolite at pH 9.0 (experimental conditions used by the authors) is believed to be essentially through electrostatic attraction between the negative charges of zeolite and the positive charges of numerous lysine and arginine residues at the surface of cutinase.

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We have shown in a preliminary experiment that cutinase immobilized on zeolite NaY according to a previously published protocol (Goncalves et al, 1998) is useful for the hydrolysis of fish oil. Our experimental conditions were essentially the same as those described by Goncalves et al (1998) using 4% of aqueous buffer directly dispersed in the oil which is enabled by the zeolite, instead of using a solution of tri-acylglycerol in hexane. With 10 mg of zeolite material having app. 150 µg immobilized unmodified cutinase we achieved almost complete hydrolysis of 1ml triacylglycerols in 7 days of reaction at 25 °C, resulting in a mixture of essentially free fatty acids (FFA), di- and monoacylglycerols (DAG + MAG). The immobilized cutinase presents a considerable improvement over the free form, which in the same experimental conditions did show little activity (probably due to denaturation of the enzyme in the presence of such proportions of oil). The solid material (zeolite + cutinase) could easily be recovered from the reaction medium by sedimentation, for eventual recycling.

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Description of the process (Preferred embodiment)

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The process of PUFA concentration with cutinase is illustrated in Figure 3. The first step consists in the hydrolysis of purified fish-oil by immobilized cutinase on Zeolite NaY in a ratio of 10 to 40mg protein per gram of solid material, preferably 20mg/g. In

a typical experiment, 1 L of fish-oil is treated by 50 to 200g of catalyst at 40 degrees Celsius for 5h to 12h, until an appropriate equilibrium is reached (between PUFA enrichment and yield of PUFA recovery). The hydrolytic reaction is done in the presence of a phosphate or carbonate buffer 20 to 100mM, at pH 7.0 to pH 9.0. The water content is between 1 and 10% (v/v) of the volume of oil. In these conditions, 50 to 70% of the tri-acylglycerols are converted in free-fatty acids, di- and mono-acylglycerols. The EPA+DHA content on the glycerol backbone is comprised between 40 and 50%. Free fatty acids are removed by complexation with divalent cations like Ca^{2+} or Mg^{2+} , by addition of 50g to 80g of CaCl_2 for example. The PUFA-enriched acylglycerols are extracted with 1L hexane and the free fatty acids recovered in the aqueous phase in the form of soaps. The di- and mono-acylglycerols resulting from the reaction (approximately 600ml) are subjected to a second reaction to reform tri-acylglycerols by transfer of PUFA from high-PUFA ethyl esters onto the acylglycerols. The PUFAEE (70 to 80% EPA+DHA) are obtained by standard urea crystallization methods. The reaction is achieved in water-free conditions under reduced pressure (0.1 bars), by mixing 1 L of acylglycerols to 0.5 L to 1 L of PUFAEE. The reaction is catalyzed by 50 to 200g immobilized cutinase at 40 degrees Celsius. The reaction is done under reduced pressure (0.1 bar) to remove the ethanol released during the alcoholysis of PUFAEE, in order to displace the equilibrium towards the formation of acylglycerols (tri-acylglycerols). Unreacted PUFAEE can be removed by short-path distillation. Typically, a mixture of tri-, di- and mono-acylglycerols containing approximately 60% EPA+DHA is obtained in 8 to 12h.

Improving cutinase immobilization

Due to the non-covalent nature of cutinase interaction with zeolite, a proportion of the enzyme is lost in the buffer/oil and cannot be recycled. This problem was mentioned by Goncalves et al (1998). In order to improve the yields in recovery of the biocatalyst, we envisage the following solution. The first solution consists in modifying genetically the enzyme to carry an additional stretch of positively charged residues (Arg, Lys) at its C-terminus (Figure 4). The C-terminus of the protein is roughly situated at the opposite side of the hydrophobic cavity responsible for the substrates binding and catalysis (Figure 5). Hence the modification is not expected to hinder the catalytic reaction. A poly Lys/Arg stretch would confer an excess of charges on the surface of

the enzyme to bind even more strongly the zeolite. Eventually, it would also confer cutinase a better orientation for interaction with the substrate, for enhanced catalytic rates.

5 **Zeolite can be made super-paramagnetic**

Adjunction of ferric and ferrous iron to zeolite NaY and subsequent treatment with sodium hydroxide can render this material magnetic, without affecting dramatically its adsorption capacity (Oliviera et al, 2004). Combining such a material with cutinase
10 immobilization could yield an easy recovery method of the biocatalyst from the reaction medium, without time/energy consuming sedimentation/filtration techniques. A disadvantage we foresee using this material with fish oil, is the susceptibility of fish oil to oxidation (due to its natural high content in PUFA). Iron is a well-known metal catalyst for the formation of reactive forms of oxygen involved in lipids peroxidation
15 (via the Fenton chemistry). Ultimately, this problem could be counteracted by the addition of water-soluble efficient scavengers of oxygen radical species, ascorbate for instance, in addition to the liposoluble antioxidant added to the oil, such as tocopherols.

20 **Zeolite can sequester ions and small molecules in its microporous structure**

The molecular sieving properties of zeolites can be exploited to remove contaminants from complex macromolecular mixtures. In the case of fish oil, the pore opening cavities of zeolite NaY is around 7Å, which is too small to accommodate bulky lipids.
25 Tri-, di- and even monoacylglycerols or free fatty acids above a certain chain length are not expected to enter these cavities. The hydrophilic nature of zeolite neither would favor contacts with lipids. On the contrary, toxic heavy metals like lead or cadmium would easily enter these cavities and be sequestered, participating in the detoxification of the oil. The same could well apply to highly toxic dioxins and polychlorinated biphenyls (PCBs), present in trace amounts, but known to be a major problem
30 with fish oils. In fact zeolites have already been used successfully for such detoxification purposes. Besides toxic chemicals, zeolite would also be useful in trapping small aromatic molecules like short-chain aldehydes and ketones, responsible for the unde-

sirable “fishy” taste of fish oil. These molecules are products of oxidative degradation of PUFAs.

Genetically engineered Cutinase from *Fusarium solani* pisi

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Cutinase from *Fusarium solani* pisi is genetically modified to show enhanced adsorption onto Zeolite NaY, the solid support chosen for immobilization of the catalyst. A tag consisting of six arginine residues is added to the C-terminus of the wild-type sequence (Figure 1). The mutant enzyme presents an excess of positive charges stemming from the added arginines on its surface, with its calculated pI changing from 6.8 (wild-type) to 9.5. The optimum pH for catalytic activity of cutinase is around pH 9.0. At this pH, the wild type enzyme is globally negatively charged. Zeolite NaY is also negatively charged due to AlO_4^- , acts as a cation exchanger, thus enzyme and support are experiencing electrostatic repulsions. This is not desirable for recycling purposes, as most of the enzyme will be lost in the aqueous phase whilst recovering the zeolite. The mutant enzyme Cutinase_6xR still exhibits positive charges at pH 9.0, and experience electrostatic attraction for Zeolite NaY. This modification of the wild-type cutinase (stronger adsorption) presents several advantages:

- Higher charge of enzyme onto the support which means higher specific activity
- Adsorption to support in basic buffers where the enzyme works best
- Potentially higher stability of the enzyme
- Better recovery of the enzyme during recycling

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Production of cutinase by recombinant expression

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Recombinant cutinase is produced in *Escherichia coli* according to the original method published by Lauwereys et al. (1991), with the following modifications. A nucleic acid construct comprising the coding region of cutinase from *Fusarium solani* pisi, fused to a transit signal peptide PhoA at its N-terminus (Figure 1) and a 5'-region coding for the PGRRRRRR peptide extension insert immediately prior to the double taa termination signal, is cloned into the pET11a expression vector (Novagen) and

expressed in *Escherichia coli* strain BL21(DE3). This will ensure that the recombinant cutinase accumulates in the periplasmic space of the cell. Typically, cells are grown at 25 °C in M9 minimal medium containing 200mg/l ampicillin until OD_{600nm} reaches 0.6-0.8. Protein expression is induced by addition of 0.5mM IPTG. Production is stopped when OD_{600nm} reaches 6.0-8.0. After harvesting of the cells, the periplasmic content is recovered by disruption of the outer membrane in TES buffer (50mM TRIS-HCl, 10mM EDTA, 20% sucrose). The crude protein extract containing cutinase is dialysed against a 20mM sodium acetate buffer pH 5.0. Cutinase is purified by a one-step cation-exchange chromatography on SOURCE 15S material (Amersham Biosciences). The chromatographic fractions contain cutinase in essentially pure form and are dialysed against a 10mM sodium acetate buffer pH 5.0 to remove salts from the chromatography. Typical production yields range from 0.1 to 0.5 g of purified cutinase per liter of culture. The same protocol can be applied for the production of the mutant cutinase.

An aspect of the present invention is the recombinant cutinase of SEQ ID:1 and a recombinant expression vector comprising the cDNA sequence of SEQ ID:2. Further aspects of the invention relate to an isolated nucleic acid sequence, which comprises a nucleic acid sequence which encodes the recombinant cutinase described above, a nucleic acid construct comprising the nucleic acid sequence which encodes the recombinant cutinase described above operably linked to one or more control sequences that direct the production of the polypeptide in a suitable expression host. Preferably said control sequence is the PhoA signal peptide sequence which directs the cutinase into the periplasmic space of an *E. coli* host. Further aspects of the invention are a recombinant expression vector comprising said nucleic acid construct, a recombinant host cell comprising said nucleic acid construct, and a method for producing a recombinant cutinase, the method comprising (a) cultivating said recombinant host cell to produce said recombinant cutinase in the periplasmic space; and (b) disrupting the host cells to recover said cutinase.

It will be obvious to a person skilled in the art to envisage and produce other genetically modified cutinase variants having increased content of positively charged amino

acids, e.g. a cutinase_7xR mutant or a cutinase_6K mutant having a different spacer or without a spacer. Such variants are within the scope of the present invention.

Immobilisation of cutinase on Zeolite NaY

5

Cutinase solution and Zeolite NaY (CBV100, Zeolyst International) are blended with a ratio enzyme/solid support of 20mg/g in a 50mM phosphate buffer pH8.0 (Goncalves et al, 1998). The mixture is incubated at room temperature for 5 min, under mechanic agitation. The water is removed by freeze-drying. Immobilized cutinase is stored at 4degC in the presence of a desiccant until use. This protocol may be applied for immobilization of both the 6xArg cutinase mutant and the wild-type cutinase.

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Enhanced adsorption of cutinase genetically modified with a C-terminal sequence containing 6 arginines (Cutinase_6xR).

15

The cutinase mutant, Cutinase_6xR, was expressed in *E. coli* and purified by ion-exchange chromatography. In order to test for the claimed enhanced adsorption on Zeolite NaY, the purified protein was loaded on a column containing 0.5 ml of Zeolite NaY, and compared to the wild-type cutinase not containing the 6xR tag. The column was equilibrated with a 20 mM phosphate buffer at pH 8.0 in initial conditions. In these conditions, it is seen (Figure 6A) that wild-type cutinase is poorly retained by Zeolite NaY, as the peak of protein (bold grey and bold black curves) elutes essentially after 1ml of elution. On the contrary, Cutinase_6xR is retained by Zeolite NaY and does not elute from the column in initial chromatographic conditions (Figure 6B). Most of Cutinase_6xR is eluted from the column after 8.2 ml of elution (Figure 6B, bold grey and bold black curves), as the concentration of NaCl is increased with buffer B, displacing the bound protein. Activity assays were performed on the chromatographic fractions and showed that cutinase activity was associated with the protein-containing fractions. Identical chromatographic experiments were also performed with arginine alone and showed that this amino-acid elutes after 9.5ml.

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The results show that the isoelectric point of wild-type cutinase, which is 6.8, has been increased to over 8.0 by adjunction of 6 arginine residues at the C-terminus of the protein in Cutinase_6xR. This change in isoelectric point allows for better binding of cutinase to Zeolite NaY (through salt bridges) at pH 8.0, which is the preferred pH value for performing hydrolysis of fish-oil.

Example 1

Cutinase immobilized on Zeolite NaY hydrolyses fish-oil efficiently and concentrates ω 3-PUFAs on the monoacyl- and diacyl-glycerol products

In order to study the fatty-acid selectivity of wild-type cutinase immobilized on Zeolite NaY, fatty acids profiles of the acylglycerols formed during the reaction were determined over time. The reaction mixture consisted of fish-oil and phosphate buffer 20 mM pH 8.0. The reaction mixture was incubated at 30 °C under reduced oxygen pressure (to protect PUFAs from oxidation) and vigorous shaking. In these conditions, Zeolite particles are well dispersed, ensuring efficient hydrolysis of the oil. Table 5 shows such an experiment where, free fatty acids are released over the course of reaction. After 24h of reaction, almost 50% of the fatty acids are released from the glycerol backbone. In the experimental conditions, the net release of free fatty acids is stopped after 24h. In similar conditions, no hydrolysis of fatty acids could be recorded with free cutinase (without Zeolite) or by Zeolite itself, indicating that the reaction is enzymatically catalysed and that cutinase is strongly stabilized by Zeolite NaY.

The analysis of free fatty acid profiles of the products of reaction, with combined liquid chromatography and gas chromatography, clearly show that ω 3-PUFAs, EPA and DHA, are hydrolysed at significant lower rates than other fatty acids (Table 6). For instance, after 4h of hydrolysis there is a 1.7-fold and 2-fold increase, in EPA and DHA respectively, on the diacyl- and monoacylglycerol products (analysed as a whole), as compared to the initial triacylglycerols. The concentration of EPA and DHA appears to reach a maximum after 12h of hydrolysis in these reaction conditions, and even decreases slightly after 48h of reaction (Table 6). The profile of fatty acids in the triacylglycerol fraction does not change significantly over time. This experiment

exemplifies how cutinase immobilized on Zeolite NaY is used to produce a mixture of diacyl- and monoacylglycerols with PUFA contents over 50% and a combined content in EPA and DHA of about 45%.

5 Table 5: Fish-oil hydrolysis by cutinase immobilized on Zeolite NaY: progression of reaction.

10 One millilitre of fish-oil was hydrolysed with 200 µg of wild-type cutinase immobilized on 10 mg of Zeolite NaY at 30 °C. Formation of free fatty acids was monitored by FTIR, from the appearance of the absorption band at 1710 cm⁻¹, typical of free carboxylic acids. The amount of free fatty acids is expressed as molar ratio over the total amount of free fatty acids in the initial volume of oil.

Reaction time (h)	FFA released (%)
1	2,1
2	4,0
3	8,7
4	14,8
6	29,2
12	35,7
24	47,2
48	48,9

15 Table 5

Table 6: Recovery of ω3-PUFA, EPA and DHA, in the diacyl- and monoacyl-glycerol fraction after hydrolysis with immobilized cutinase

20

One millilitre of fish-oil was hydrolysed with 200µg of wild-type cutinase immobilized on 10mg of Zeolite NaY at 30°C. At regular time intervals, samples were taken from the reaction mixture for analysis of the products formed. Reaction products were separated and purified by liquid chromatography. Analysis of fatty acid composition

in the acyl-glycerols was achieved by gas chromatography, following standard procedures. Fatty acid contents are expressed as % of total fatty acids, on the basis of peak area in the chromatograms

5

	Fish oil (initial)	TAG	DAG +	TAG	DAG +	TAG	DAG +	TAG	DAG +
			MAG		MAG		MAG		MAG
Time of reaction (h)	0	4		12		24		48	
Sum of FAs above C20:0	44.5	44.1	47.1	45.0	49.2	44.6	49.7	44.3	46.1
Sum of N-3 FAs	26.9	26.3	43.8	27.4	47.4	28.2	48.1	27.8	41.9
Sum of N-6 FAs	3.4	3.5	3.8	3.4	3.4	3.4	3.6	3.3	3.7
Sum of N-9 FAs	20.7	20.8	16.3	20.6	15.4	20.5	14.3	20.5	17.0
Sum of saturated FAs	22.2	21.9	19.1	21.3	20.6	22.0	21.0	21.9	22.7
Sum of monounsaturated FAs	40.6	40.5	35.0	40.7	31.2	42.2	30.7	40.7	36.3
Sum of PUFAs	33.1	32.5	46.2	32.5	53.5	33.2	55.6	31.0	48.7
EPA	7.7	7.9	13.0	7.7	15.2	8.0	15.4	7.8	11.4
DHA	11.3	11.7	25.3	11.9	29.4	12.3	29.3	11.9	24.3
Conc. Fold EPA			1.7		2.0		2.0		1.5
Conc. Fold DHA			2.2		2.6		2.6		2.2

Table 6

10 Example 2

Cutinase immobilized on Zeolite NaY re-esterifies acylglycerols from fatty acid ethyl-esters to yield acyl glycerols with high EPA and DHA contents.

15 The acylglycerol fraction resulting from selective hydrolysis of fish oil with cutinase can be further enriched in ω 3-PUFAs. In the following experiment, cutinase immobilized on Zeolite NaY was used in an essentially water-free reaction medium to perform trans-esterification reaction between a mixture of purified diacyl- and monoacyl-glycerols and ethyl-esters of concentrated ω 3-PUFAs. The mixture of purified diacyl-
20 and monoacylglycerols resulted from the selective hydrolysis of fish-oil with cutinase,

where the combined EPA and DHA content was about 45%. Fatty acid ethyl esters with high content in EPA and DHA resulted from urea crystallization of a mixture of fish oil fatty acids trans-esterified with ethanol, where the combined content in EPA and DHA was about 75%.

5

The reaction mixture consisted of 1 volume of acylglycerols (purified fraction obtained after 4 hours of the reaction of example 1) and 1 volume of ethyl-esters to which 20 mg of immobilized cutinase was added. The reaction was performed at 40 °C under reduced pressure to evaporate the ethanol produced during the reaction and, to push the reaction towards formation of acylglycerols. After 24h, the reaction was stopped and the acylglycerols formed were separated from unreacted ethyl esters by liquid chromatography on silica gel. Table 7 shows the fatty acid profile of the resulting mixture of acylglycerols. In these experimental conditions, the combined content of EPA and DHA in the acylglycerol mixture is 63%. This result shows that cutinase immobilized on Zeolite NaY can also be used as bio-catalyst in a process where, the goal is to produce acylglycerols (mixture of triacyl, diacyl- and monoacyl glycerols) with an EPA and DHA content of about 60%.

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15

Table 7: ω 3-PUFAs enrichment of an acylglycerol mixture by immobilized cutinase from ethyl-esters with high EPA and DHA content, by trans-esterification

20

Equal amount (vol:vol) of acylglycerols and ethyl-esters were mixed and subjected to trans-esterification reaction catalyzed by immobilized cutinase on Zeolite NaY. The reaction was performed under vacuum for 24h. The resulting acyl glycerols (TAG + DAG + MAG) were purified by liquid chromatography and the fatty acid profile of the resulting mixture analyzed by gas chromatography. Acylglycerols (Reactant 1) were obtained from selective hydrolysis of fish-oil by cutinase and, high-EPA/DHA ethyl-esters (Reactant 2) were obtained from urea crystallization of fish-oil fatty acid ethyl-esters.

25

	Reactant 1 (initial)	Reactant 2 (FA ethyl-ester)	Product (TAG + DAG + MAG)
Sum of FAs above C20:0	47.1	79.2	62.0
Sum of N-3 FAs	43.8	87.6	69.2
Sum of N-6 FAs	3.8	3.1	3.5
Sum of N-9 FAs	16.3	4.4	8.6
Sum of saturated FAs	19.1	0.4	8.7
Sum of monounsaturated FAs	35.0	2.2	16.9
Sum of PUFAs	46.2	90.8	75.1
EPA	13.0	34.2	26.3
DHA	25.3	42.0	37.3

Table 7

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The references mentioned herein all have particular relevance for the understanding of the invention and they are all incorporated in the specification by reference.

CLAIMS

1. A process for concentrating the content of ω 3-poly-unsaturated fatty acids (PUFA) in a natural oil source such as fish oil using a cutinase catalysed selective hydrolysis of the triglycerides, wherein the cutinase is immobilised to a solid support comprising zeolite NaY.

2. A process according to claim 1, wherein the cutinase catalysed selective hydrolysis of the triglycerides is performed by removing non- ω 3-poly-unsaturated fatty acid moieties originally bonded to the glyceryl backbone of the fatty acid glycidyl esters of the starting natural oil, thereby during the process, as far as possible retaining bonded to said glyceryl backbone, the ω 3-poly-unsaturated fatty acid moieties which are originally bonded to the glyceryl backbone of the fatty acid glycidyl esters of the starting natural oil.

3. A process for concentrating the content of both the ω 3-polyunsaturated fatty acids EPA and DHA in a natural oil source such as fish oil using a cutinase catalysed selective hydrolysis of the triglycerides, wherein the cutinase is immobilised to a solid support comprising zeolite NaY.

4. A process according to claim 3, wherein the cutinase catalysed selective hydrolysis of the triglycerides is performed by removing non-EPA fatty acid moieties as well as non-DHA fatty acid moieties originally bonded to the glyceryl backbone of the fatty acid glycidyl esters of the starting natural oil, thereby during the process, as far as possible retaining bonded to said glyceryl backbone, the EPA as well as DHA fatty acid moieties which are originally bonded to the glyceryl backbone of the fatty acid glycidyl esters of the starting natural oil.

5. A process for concentrating the content of the ω 3-PUFAs EPA and DHA in a natural oil source such as fish oil comprising the following steps:
(a) adding cutinase immobilised to a zeolite NaY solid support to a neutralised fish oil containing TAGs to form a first reaction mixture wherein said cutinase is allowed to catalyse hydrolysis of the TAGs,

(b) separating saturated, mono-, and di-saturated FFAs from the first reaction mixture by molecular distillation and formation of Mg^{2+} and Ca^{2+} salt complexes to obtain a first PUFAGE product comprising essentially DAGs, and MAGs wherein the combined amount of EPA +DHA is about 50%,

(c) reforming TAGs and further increasing the PUFA content in acylglycerols, by addition of PUFAEE preferably containing 70% to 80% EPA+DHA obtained from a urea crystallization to said first PUFAGE product in a second reaction mixture catalysed by said immobilised cutinase, and

(d) applying vacuum to said second reaction mixture to remove ethanol and obtain a second PUFAGE product comprising TAGs, DAGs, and MAGs wherein the combined amount of EPA+DHA is about 60%.

15

6. A process according to any of claims 1 to 5, wherein the cutinase is modified to show enhanced adsorption onto zeolite NaY by providing additional positive charges at its surface preferably resulting in a calculated pI at about 9.5.

20

7. A process according to claim 6, wherein the cutinase carries a poly-arginine tag C-terminally.

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8. A genetically modified cutinase for use in the process of claims 1 to 7 comprising a cutinase wild-type amino acid sequence having at its C-terminus 5 to 7 and preferably about 6 arginine residues.

30

9. A genetically modified cutinase according to the preceding claim further comprising a cleavage site, such as a PG sequence, immediately preceding the terminal arginine residues.

10. A genetically modified cutinase selected from the group consisting of the amino acid sequence of SEQ ID:1 and said sequence wherein the N-terminal PhoA signal peptide sequence has been deleted.

11. An isolated nucleic acid sequence, which comprises a nucleic acid sequence which encodes the cutinase according to claim 8 or 9 and which is operably linked to one or more control sequences, such as the PhoA signal peptide sequence, that direct the production of the cutinase in the a suitable expression host.

5

12. A recombinant expression vector comprising the nucleic acid construct of claim 11.

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13. A recombinant host cell comprising the nucleic acid construct of claim 11 and/or the expression vector of claim 12.

14. A method for producing a recombinant cutinase, the method comprising (a) cultivating an E. coli recombinant host cell according to claim 13 to produce the polypeptide in the periplasmic space; and (b) recovering the recombinant cutinase.

15

15. A process of preparing an immobilised recombinant cutinase comprising mixing a solution of said cutinase with zeolite NaY in a ratio of enzyme:solid support of 10 to 40 mg:g in a phosphate buffer or carbonate-bicarbonate buffer in a pH range from 7.0 to 9.0, incubated at room temperature for 5 minutes under mechanical agitation, dried and stored at 4 °C in the presence of a desiccant until use.

20

16. A process according to claim 15, wherein the ratio of enzyme:solid support is 20 mg:g, and wherein the pH of the phosphate buffer is 8.0.

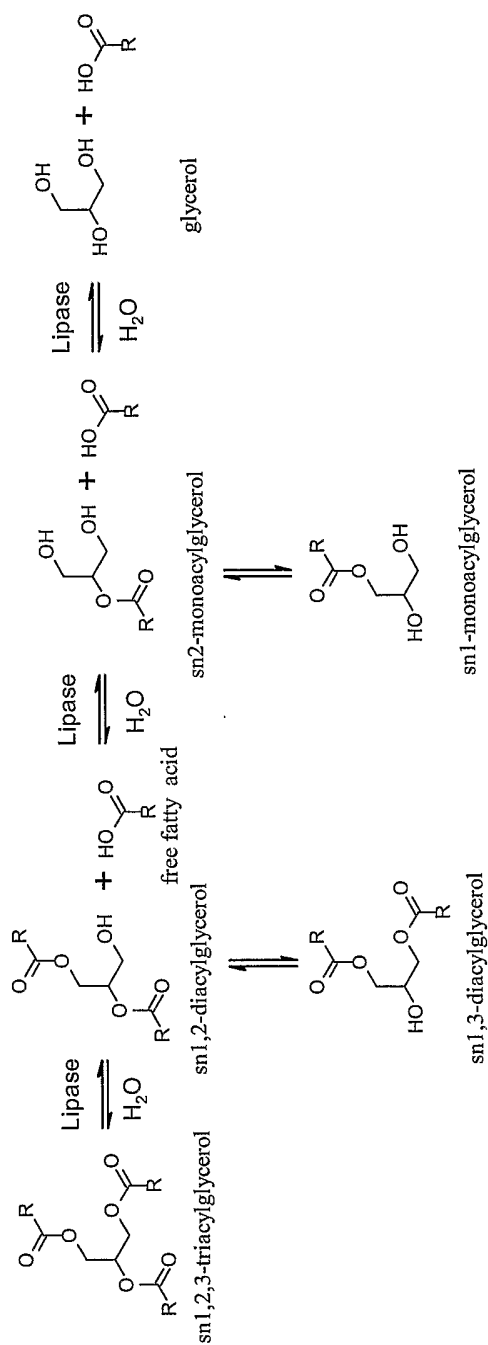


Figure: Simplified reaction scheme of Lipase-catalysed hydrolysis of triacylglycerols. Ultimately, the complete hydrolysis of one molecule of triacylglycerol will yield three molecules of free fatty acids and one molecule of glycerol. The full reaction will have consumed three molecules of water. In standard hydrolytic conditions, the reaction is not complete, because equilibria are reached and lipases are sn1 or sn3 stereospecific. Cutinase is essentially sn3 specific. Acyl migration can occur in certain conditions (aqueous conditions at basic pH), where fatty acids migrate from the sn2 position to the sn1 or sn3 positions. R: any type of fatty acid alkyl chain.

Fig. 1

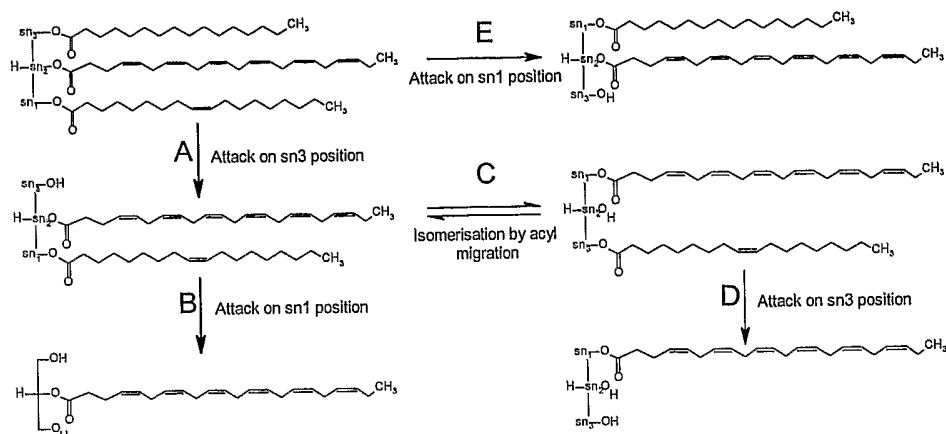


Figure: Hydrolysis of a typical triacylglycerol found in fish-oil: glycerol 1-palmitate 2-docosahexaenoate 3-oleate (*all-Z*)-form. The figure illustrates the principle of long-chain PUFA enrichment on the glycerol backbone. The carbon atoms on the glycerol backbone are numbered sn1, sn2 and sn3, where the sn1 position is by convention occupied by the most substituted acyl chain (oleate, the longest in this case). Cutinase, being specific for short-chain fatty acids, attacks preferentially the sn3 position. In hydrolytic conditions, the sn3 position of TAG will be preferred giving glycerol 1-oleate 2-docosahexaenoate (A). Then, the sn1 position of DAG will be attacked to release glycerol 2-docosahexaenoate (B). If docosahexaenoic acid migrates from the sn2 position to the sn1 position (C), the sn3 position will be attacked to give glycerol 1-docosahexaenoate (D). Eventually, the sn1 position of TAG is also attacked to give glycerol 1-palmitate 2-docosahexaenoate (E).

Fig. 2

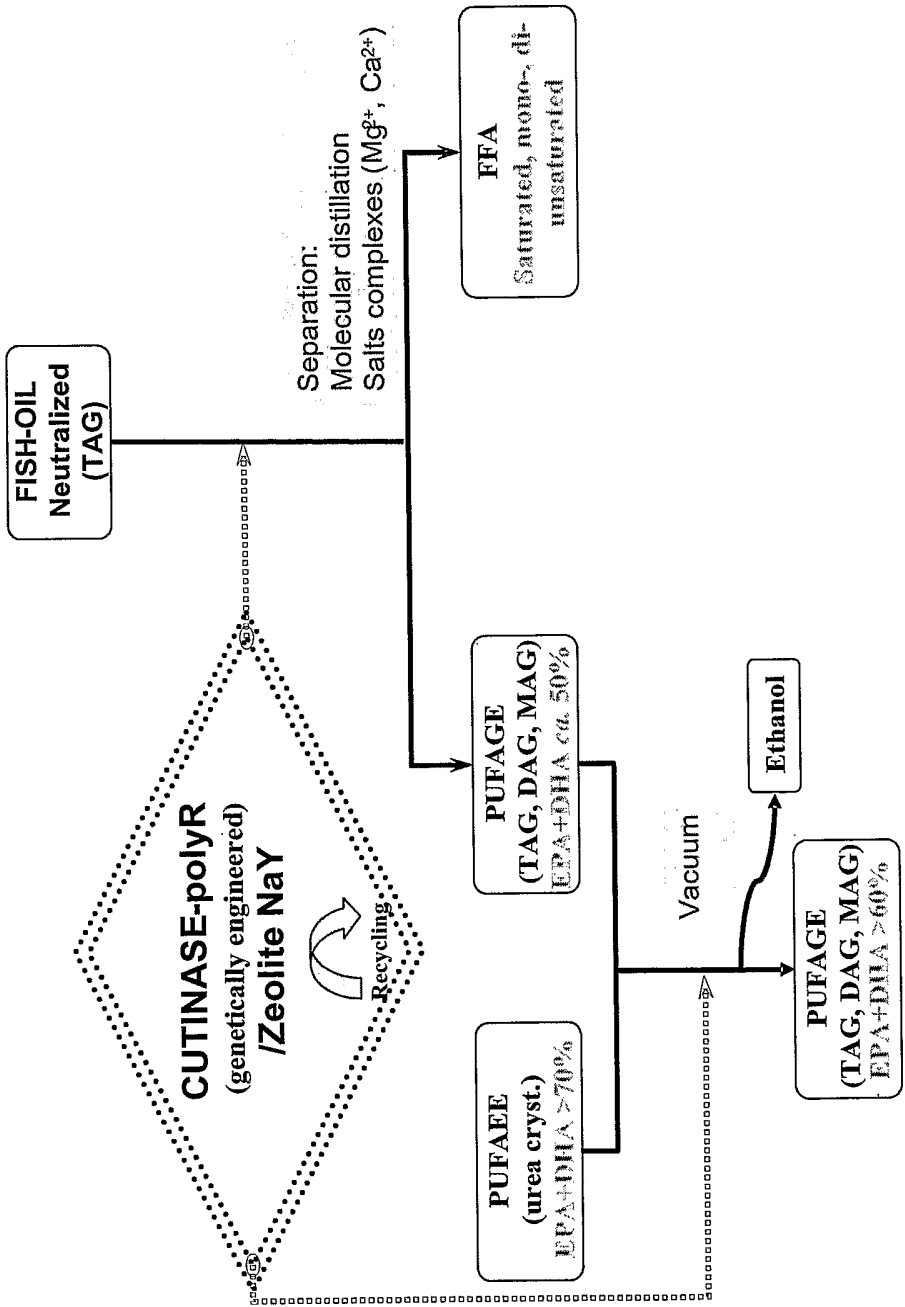


Fig. 3

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Cutinase 6xR mutant

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Fig. 4

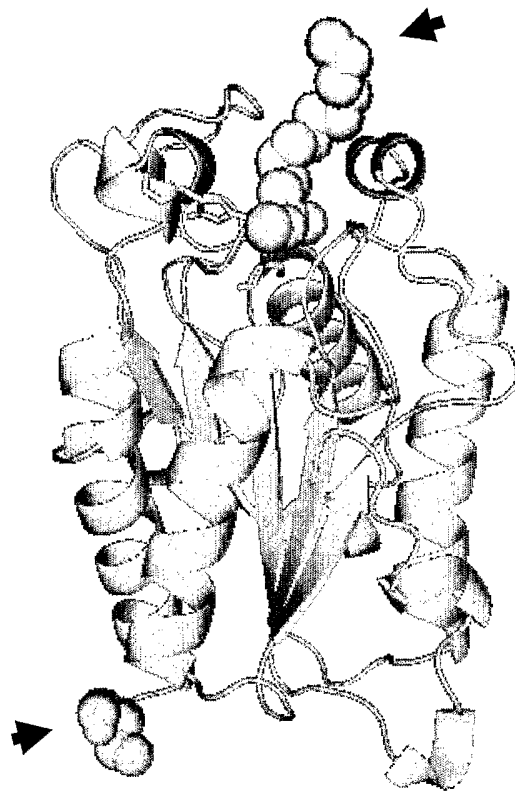
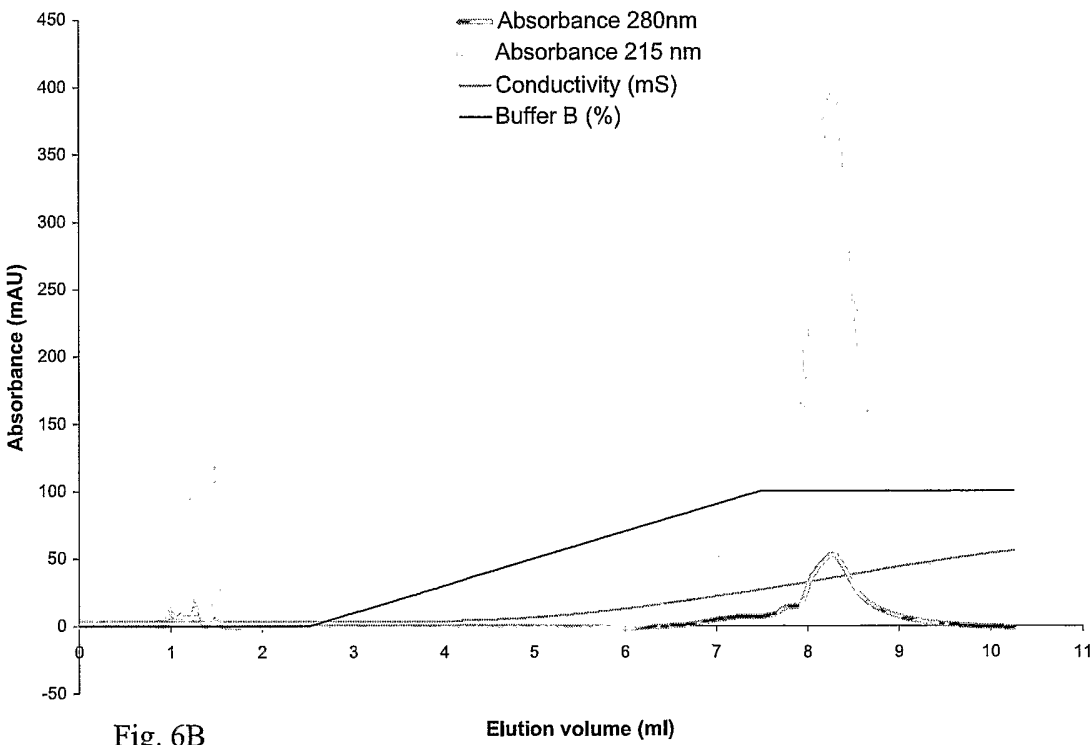
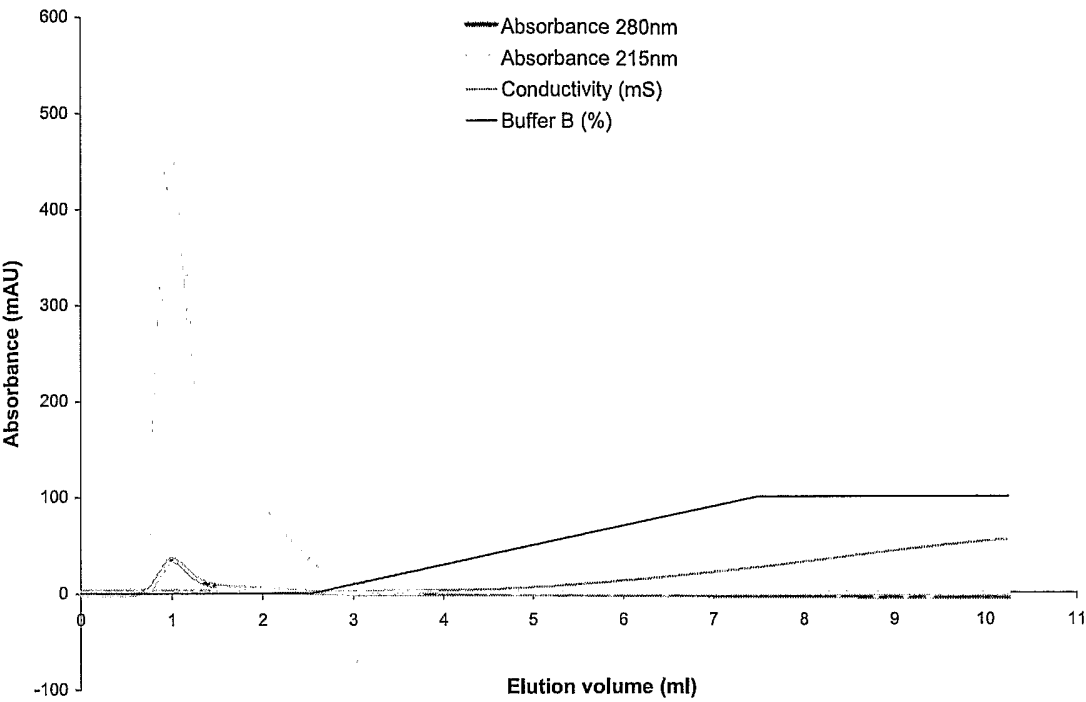


Fig. 5



SEQUENCE LISTING

Organization Applicant

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Street : Fredrik Bajers Vej 5

City : Aalborg Ø

State :

Country : Denmark

PostalCode : 9220

PhoneNumber : 9635 8080

10

FaxNumber :

EmailAddress : info@aua.auc.dk

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<110> OrganizationName : Aalborg Universitet
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Application Project

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<120> Title : A process to concentrate 3-PUFA from fish oil by selective hydrolysis using cu-tinase immobilized on zeolite NaY

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Custom Codon

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