METHOD

REFERENCE TO RELATED APPLICATIONS

Reference is made to the following related applications: United States Application Serial Number 09/750,990 filed on 20 July 1999; United States Application Serial Number 10/409,391; United States Application Serial Number 60/489,441 filed on 23 July 2003; United Kingdom Application Number GB 0301117.8 filed on 17 January 2003; United Kingdom Application Number GB 0301118.6 filed on 17 January 2003; United Kingdom Application Number GB 0301119.4 filed on 17 January 2003; United Kingdom Application Number GB 0301120.2 filed on 17 January 2003; United Kingdom Application Number GB 0301121.0 filed on 17 January 2003; United Kingdom Application Number GB 0301122.8 filed on 17 January 2003; United Kingdom Application Number GB 0330016.7 filed on 24 December 2003 and International Patent Application Number PCT/IB2004/000655 filed on 15 January 2004. Each of these applications and each of the documents cited in each of these applications ("application cited documents"), and each document referenced or cited in the application cited documents, either in the text or during the prosecution of those applications, as well as all arguments in support of patentability advanced during such prosecution, are hereby incorporated herein by reference. Various documents are also cited in this text ("herein cited documents"). Each of the herein cited documents, and each document cited or referenced in the herein cited documents, is hereby incorporated herein by reference.

FIELD OF INVENTION

The present invention relates to a novel method for the enzymatic removal and/or reduction of diglyceride (preferably 1, 2-diacylglyceride) from an edible oil.

TECHNICAL BACKGROUND

Oils and fats consist of complex mixtures of triacylglycerols (TAGs), diacylglycerols (DAGs), free fatty acids and other minor components. The crystallisation of these mixtures depends on the characteristics of the TAGs (structure, chain length, saturation compared with unsaturation, and the like) and the interaction of these TAGs with each other. Regarding the presence of DAGs, previous studies have shown that they have significant effect on the physical properties of oils and fats. These vary from rate of crystallisation, polymorphism changes, melting point, crystal size and habits (Siew, 2001).

In most oils that are extracted from oilseeds, the effect of DAGs is less pronounced, as DAGs are only present in small quantities. In primarily palm oil and olive oil, which are oils containing high natural amounts of DAGs, however, the quality of these oils suffers if DAGs are present therein.

Palm oil obtained from oil palm (*Elaeis guineensis*) is commercially important edible oil. Palm oil has been a prominent fat and oil resource for the food industry due to several advantageous properties, such as high productivity, low price, high thermal and oxidative stability and plasticity at room temperature. In addition, compared with other vegetable oils, palm oil is a rich source of the anti-oxidant vitamin E.

A typical chemical composition of refined palm oil is about 93% triglycerides, 6% diglycerides and 1% monoglycerides (MAGs) (Okiy, 1977).

When palm oil crystallizes, a complex 3-dimensional network of the present components is formed. In the theory it is described that the bigger the diversity of the building blocks (TAGs, DAGs and MAGs) in the network, the more complicated the network will be and the slower the crystallization will happen (Jacobsberg & Ho, 1976). This theory was confirmed by Drozdowski (1994). Furthermore, his studies showed that the more the fatty acid composition variation in the triacylglycerol molecule, the more difficult was the transition between the different crystal phases.

As previously mentioned, a high content of diglycerides in palm oil affects its crystallization properties (Okiy *et al.*, 1978, Okiy, 1978).

The presence of diglycerides in such oils is disadvantageous. In particular, diglycerides in edible oils (in particular palm oil) can lead to a low quality oil.

The problems relating to the diglyceride content in palm oil and other edible oils and fat have been the subject of many studies and different solutions to attempt to overcome the problem of too much diglyceride can be found in the literature.

The Japanese enzyme producer Amano on their home page (Amano Enzyme Inc., 2004), recommend an enzymatic process to remove diglyceride in fats and oils. This process is based on the use of an enzyme LIPASE G "AMANO" 50 which is able to degrade diglycerides to free fatty acids and glycerol. This enzyme is a diglyceride (DAG) and/or monoglyceride (MAG) hydrolyzing. The free fatty acids produced are removed by vacuum distillation or fractional crystallisation.

EP 0 558 112 describes a process for the enzymatic hydrolysis of residual diglycerides in triglyceride preparations in emulsions. The process is based on the hydrolysis of diglyceride with Lipase G from Amano, Japan (*supra*). The process was enhanced by making the enzymatic reaction in an emulsion for the degradation of diglyceride to fatty acids and glycerol. The water phase is separated after reaction and the enzyme is partly reused.

JP 62061590 teaches a hard butter containing low amounts of diglyceride, which is manufactured by treating oils or fats with a partially glyceride-specific enzyme (e.g., a lipase) in the presence of a catalytic amount of water and by a lipase that is a 1,3-specific enzyme in the presence of fatty acids, fatty acid esters, or other glyceride oils or fats. The product is hard butter especially suitable for use as a cacao butter substitute. Thus, lipase G and *Rhizopus deremer* lipase (1,3-specific enzyme) were mixed with diatomaceous earth and granulated. The granules was mixed with palm

medium melting point fraction (5.7% diglyceride, acid value 0.25) and water (10% with regard to the partial glyceride-specific enzyme). The mixture was stirred at room temp. for 1 h, and the enzymes and water removed to give a hard butter containing 1.2% diglyceride (acid value 10.5).

The prior art thus teaches ways to reduce or remove the content of diglyceride in palm oil and other edible oils by enzymatic reactions. These processes rely on the hydrolysis of diglyceride with a specific diglyceride hydrolysing lipase during formation of free fatty acids and glycerol. The free fatty acids can then be removed by means of different processes like vacuum distillation or fractionation.

The disadvantage of using a specific diglyceride hydrolysing enzyme is the disadvantageous formation of free fatty acids. These free fatty acids have to be removed from the palm oil. Thus, the formation of free fatty acids is often considered as loss of product.

To overcome the problems with the removal of free fatty acid and the loss of product caused by the free fatty acid formation we have found a new method to overcome the problems with high diglyceride content in palm oil and other vegetable oils.

Enzymatic removal of diglycerides from palm oil has been taught by use of lipases, which are typically 1,3 specific triacylglycerol hydrolyzing enzymes (E.C. 3.1.1.3) (for example see JP 62061590 or EP 0 652 289). WO00/05396 teaches *inter alia* treatment of a food material which may comprise glycerol with a lipase to effect glycerolysis in a low water environment.

However, both 1,3 specific triacylglycerol hydrolyzing enzymes (lipases) and DAG/MAG hydrolyzing enzymes result in a significant increase in free fatty acid in the oil, and also result in the hydrolysis of monoglycerides.

However, in some vegetable oils for some applications for example it may be desirable to increase the monoglyceride content of the oil as this provides emulsifier

functionality. Thus, in one aspect it is preferable to reduce diglyceride content without decreasing the monoglyceride content. In another aspects it may preferably to reduce both the diglyceride and monoglyceride content.

Lipase enzymes can also result in a detrimental increase in DAG due to the hydrolysis of triacylglycerol (TAG), the bulk lipid present in food oils.

In WO2000/36114, US2003/0028923 and US2003/0074695 the transformation of a plant with a nucleic acid having a sequence which encodes a diacylglycerol acyltransferase (DGAT) enzyme or an antisense sequence thereto is taught. The DGAT enzyme taught in these documents catalyses the final step in the "Kennedy pathway" where a diacylglycerol (DAG) is combined with the acyl groups of acyl CoA to form a triglyceride (TAG). Thus, these documents teach the production of transgenic plants with modified TAG compositions and/or contents. The DGAT enzymes taught in these documents are not lipid acyl transferases and/or diglyceride:glycerol acyltransferases in accordance with the present invention.

In particular, DGAT's require the presence of acyl CoA or fatty acid CoA to function. Acyl CoA is not suitable for use commercially for treating edible oils as it is prohibitively expensive. However, these enzymes will not work without the present of acyl CoA. In addition enzyme reactions relying on fatty acid-CoA are very difficult to control industrially.

In addition, all of these documents teach that it is often desirable to reduce the expression of DGAT enzymes in a plant, thus to reduce the amount of TAG in the plant. This contrasts sharply with the present invention which ultimately requires the preservation and/or production of TAG whilst reducing diglycerides (DAG) in an edible oil.

WO03/100044 teaches a phospholipids:diacylglycerol acyltransferase (PDAT) which catalyses the formation of triglycerides (TAGs) by an acyltransfer from phospholipids (lecithin) to *inter alia* diacylglycerols (DAGs). PDATs require phospholipid as the

acyl donor. This contrasts sharply with the present invention where the acyl donor is DAG. This document does not teach the removal of DAG from edible oils using a lipid acyltransferase in accordance with the present invention.

SUMMARY OF THE INVENTION

It has been found that the use of fatty-acid CoA independent lipid acyltransferases as defined herein, specifically fatty-acid CoA independent diglyceride:glycerol acyltransferases, results in the selective reduction and/or removal of diglycerides (preferably 1,2-diglycerides) from edible oils.

The term "selective" as used herein means that in an edible oil environment the enzyme utilizes diglycerides (DAGs), preferably 1,2-diglycerides, as a substrate preferentially to either triacylglycerides (TAGs) or monoglycerides (MAGs). Thus, diglycerides can be removed and/or reduced from the edible oil whilst leaving the amount of triglyceride in the oil unchanged (or substantially unchanged). The amount of monoglycerides in the oil either remains unchanged (or substantially unchanged) or may increase. In some applications, the amount of monoglyceride in the oil may be reduced.

In one aspect of the present invention there is provided a method of reducing and/or removing diglyceride from a foodstuff, comprising a) admixing a foodstuff or a portion thereof with an acyl acceptor substrate and a fatty-acid CoA independent diglyceride:glycerol acyltransferase, wherein the fatty-acid CoA independent diglyceride:glycerol acyltransferase is characterized as an enzyme which in an edible oil can transfer an acyl group from a diglyceride to glycerol.

In a further aspect of the present invention there is provided a method of reducing and/or removing diglyceride from an edible oil, comprising a) admixing an edible oil with an acyl acceptor substrate and a fatty-acid CoA independent diglyceride:glycerol acyltransferase, wherein the fatty-acid CoA independent diglyceride:glycerol

acyltransferase is characterized as an enzyme which in an edible oil can transfer an acyl group from a diglyceride to glycerol.

Suitably, the method according to the present invention may further comprise adding the treated edible oil or a portion thereof to one or more food constituents to formulate a foodstuff, such as margarine or spread for example.

The present invention yet further provides the use of a fatty-acid CoA independent diglyceride:glycerol acyltransferase characterized as an enzyme which in an edible oil can transfer an acyl group from a diglyceride to glycerol, in the manufacture of a foodstuff, for reducing and/or removing (preferably selectively reducing and/or removing) diglyceride from said foodstuff.

In a further aspect, the present invention yet further provides the use of a fatty-acid CoA independent diglyceride:glycerol acyltransferase characterized as an enzyme which in an edible oil can transfer an acyl group from a diglyceride to glycerol, in the manufacture of a foodstuff, for improving the crystallization properties of the foodstuff.

In another aspect the present invention provides the use of a fatty-acid CoA independent diglyceride:glycerol acyltransferase characterized as an enzyme which in an edible oil can transfer an acyl group from a diglyceride to glycerol, in the manufacture of an edible oil, for reducing and/or removing (preferably selectively reducing and/or removing) diglyceride from said edible oil.

In another aspect the present invention provides the use of a fatty-acid CoA independent diglyceride:glycerol acyltransferase characterized as an enzyme which in an edible oil can transfer an acyl group from a diglyceride to glycerol, in the manufacture of an edible oil, for improving the crystallization properties of the edible oil.

DETAILED DISCLOSURE OF INVENTION

The terms "fatty-acid CoA independent lipid acyltransferase" and "fatty-acid CoA independent diglyceride:glycerol acyltransferase" as used herein means an enzyme which has acyltransferase activity (generally classified as E.C. 2.3.1.x in accordance with the Enzyme Nomenclature Recommendations (1992) of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology), whereby the enzyme is capable of transferring an acyl group from a diglyceride to one or more acceptor substrates).

Thus, the "fatty-acid CoA independent lipid acyltransferase" or "fatty-acid CoA independent diglyceride:glycerol acyltransferase" according to the present invention is an enzyme which has acyltransferase activity (generally classified as E.C. 2.3.1.x), but which is not a diacylglycerol acyltransferase (DGAT) or a phospholipid:diacylglycerol acyltransferase (PDAT). DGATs are typically classified as E.C. 2.3.1.20. PDATs are typically classified as E.C. 2.3.1.158. Thus, the fatty-acid CoA independent lipid acyltransferase or fatty-acid CoA independent diglyceride:glycerol acyltransferase according to the present invention is an enzyme which has acyltransferase activity, but which is not any enzyme classified as E.C. 2.3.1.20 or E.C. 2.3.1.158.

The fatty-acid CoA independent lipid acyltransferase enzyme or fatty-acid CoA independent diglyceride:glycerol acyltransferase enzyme according to the present invention is one which is capable in an edible oil of transferring an acyl group from DAG to glycerol. Thus, the reaction catalysed by the enzyme according to the present invention is:

Diglyceride (DAG) + glycerol $\rightarrow$ 2 monoglycerides (MAGs)

This contrasts sharply with enzymes known as diacylglycerol acyltransferases (or diacylglycerol *O*-acyltransferase) (DGATs) (such enzymes are classified as E.C. 2.3.1.20) which catalyse the final step in the Kennedy process, namely:

1,2-DAG + acyl CoA → CoA + triacylglycerol (TAG)

For the avoidance of doubt, DGATs are not fatty-acid CoA independent lipid acyltransferases or fatty-acid CoA independent diglyceride:glycerol acyltransferases in accordance with the present invention.

The reaction catalysed by the enzyme according to the present invention also contrasts sharply with that catalysed by enzymes known as phospholipids:diacylglycerol acyltransferase (PDAT), namely:

Phospholipids (such as lecithin) + 1,2-DAG → triacylglycerol (TAG) + lysophospholipid

For the avoidance of doubt, PDATs are not fatty-acid CoA independent lipid acyltransferases or fatty-acid CoA independent diglyceride:glycerol acyltransferases in accordance with the present invention.

Preferably, the fatty-acid CoA independent lipid acyltransferase or fatty-acid CoA independent diglyceride:glycerol acyltransferase according to the present invention is an enzyme classified as E.C. 2.3.1.73.

Suitably, the fatty-acid CoA independent diglyceride:glycerol acyltransferase may be membrane independent, i.e. may be a protein that is not, in its natural environment, associated with a membrane by the presence of a membrane anchor or membrane spanning domain.

For the avoidance of doubt, the fatty-acid CoA independent lipid acyltransferase according to the present invention is not an enzyme taught in any one of WO03/100044, WO2000/36114, US2003/0028923 or US2003/0074695.

As well as having acyltransferase activity the enzyme may have lipase activity, e.g. phospholipase activity, (generally classified as E.C. 3.1.1.x).

The fatty-acid CoA independent lipid acyltransferase in accordance with the present invention is a fatty-acid CoA independent diglyceride:glycerol acyltransferase. These terms may be used interchangeably herein.

Preferably the fatty-acid CoA independent diglyceride:glycerol acyltransferase according to the present invention is an acyltransferase which comprises the amino acid sequence motif GDSX, wherein X is one or more of the following amino acid residues L, A, V, I, F, Y, H, Q, T, N, M or S.

The term "diglyceride:glycerol acyltransferase" as used herein is synonymous with the term "fatty-acid CoA independent diglyceride:glycerol acyltransferase".

For the avoidance of doubt, the term "fatty-acid CoA" is the same as the terms "Acyl-CoA", "fatty-acid enzyme CoA" and "Acyl-Co enzyme A". These terms may be used interchangeably herein.

The edible oil used in a process or use according to the present invention may be in the form of a crude oil or may be a refined oil.

In one embodiment, preferably the amount of diglyceride is reduced rather than completely removed.

The term "reduced" as used herein means that the amount of diglyceride in an edible oil treated with the enzyme in accordance with the present invention is less than the amount of diglyceride in the edible oil before enzyme treatment.

Preferably, diglycerides are not completely removed from the edible oil.

In some applications, such as the use of the treated oil in margarines and/or shortening, the amount of diglycerides, particularly 1,2 diglycerides, should be reduced to a point where the crystallisation speed of the fat blend produces small beta-crystals. The

amount of 1,2-diglyceride to the amount of total diglyceride in the palm oil depends on the age and storage conditions of the oil. For commercial oils the ratio of 1,3 diglyceride: 1,2 diglyceride is 1.8:3.3. The removal of 1,2 diglycerides will have the most impact on the crystallisation properties.

As will be readily apparent to the skilled person the reduction in the amount of diglyceride can be controlled by the reaction time and temperature of the reaction. In a flow reactor with an immobilised enzyme the reaction may be controlled by the flow rate.

Preferably, the lipid acyltransferase for use in the methods and/or uses of the present invention is capable of transferring an acyl group from a diglyceride to an acyl acceptor, wherein the acyl acceptor is any compound comprising a hydroxy group (-OH).

Suitably, the term "diglyceride" as used herein means one or more of 1,2-diglyceride or 1,3-diglyceride. Preferably, the diglyceride is a 1,2-diglyceride.

The terms "diglyceride" and "diacylglycerol" are used herein interchangeably.

The term "diglyceride" does not encompass digalactosyldiglyceride (DGDG) and/or lecithin, e.g. phosphatidylcholine.

Preferably the acyl acceptor is one which is soluble in an edible oil.

Suitably, the acyl acceptor may be an alcohol such as for example ethanol or polyvalent alcohols, including glycerol and mixtures and derivatives thereof. Suitably, the acyl acceptor may be one or more of a sterol, a stanol, a hydroxy acid, sorbitol, sorbitan or other carbohydrate.

In one preferred embodiment the acyl acceptor is glycerol.

Thus, in one embodiment the present invention provides a method of reducing and/or removing diglyceride from an edible oil, comprising a) admixing an edible oil with both glycerol and a fatty-acid CoA independent diglyceride:glycerol acyltransferase, wherein the fatty-acid CoA independent diglyceride:glycerol acyltransferase is characterized as an enzyme which possesses acyl transferase activity and which comprises the amino acid sequence motif GDSX, wherein X is one or more of the following amino acid residues L, A, V, I, F, Y, H, Q, T, N, M or S.

Preferably, the acyl acceptor according to the present invention is not water.

Preferably the acyl acceptor is not a monoglyceride and/or a diglyceride.

Preferably, the fatty-acid CoA independent lipid acyltransferase according to the present invention is capable of cleaving the acyl bond between a fatty acid residue(s) and a glycerol backbone of a lipid substrate, wherein preferably the lipid substrate is a diglyceride, preferably 1,2-diglyceride.

Preferably, the fatty-acid CoA independent lipid acyltransferase according to the present invention does not act on triglycerides and/or monoglycerides. In other words, preferably the fatty-acid CoA independent lipid acyltransferase is selective for diglycerides, preferably selective for 1,2-diglycerides. This lipid substrate may be referred to herein as the "lipid acyl donor".

Thus in accordance with the present invention, one or more of the following advantageous properties can be achieved: reduction in diglyceride content of an edible oil; a reduction in the diglyceride content of an edible oil without a reduction in the triglyceride content of the edible oil; a reduction in the diglyceride content of the edible oil without an increase in the monoglyceride content; a reduction in the diglyceride content of the edible oil with an increase in the monoglyceride content; a reduction of diglyceride and a reduction in monoglyceride content of an edible oil; a reduction in the diglyceride content of the edible oil without a significant increase in the fatty acid content in the edible oil.

Preferably, the fatty-acid CoA independent lipid acyltransferase in accordance with the present invention performs an alcoholysis reaction (preferably glycerolysis) by transferring a fatty acid acyl group from the diglyceride (preferably the 1,2-DAG) to an alcohol (preferably glycerol) thereby producing two monoglyceride molecules, i.e. one from the diglyceride and one from the glycerol together with the accepted acyl group.

Preferably, X of the GDSX motif is L. Thus, preferably the enzyme according to the present invention comprises the amino acid sequence motif GSDL.

The GDSX motif is comprised of four conserved amino acids. Preferably, the serine within the motif is a catalytic serine of the lipid acyltransferase enzyme. Suitably, the serine of the GDSX motif may be in a position corresponding to Ser-16 in *Aeromonas hydrophila* lipolytic enzyme taught in Brumlik & Buckley (Journal of Bacteriology Apr. 1996, Vol. 178, No. 7, p 2060-2064).

To determine if a protein has the GDSX motif according to the present invention, the sequence is preferably compared with the hidden markov model profiles (HMM profiles) of the pfam database.

Pfam is a database of protein domain families. Pfam contains curated multiple sequence alignments for each family as well as profile hidden Markov models (profile HMMs) for identifying these domains in new sequences. An introduction to Pfam can be found in Bateman A *et al.* (2002) Nucleic Acids Res. **30**; 276-280. Hidden Markov models are used in a number of databases that aim at classifying proteins, for review see Bateman A and Haft DH (2002) Brief Bioinform **3**; 236-245.

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&list_uids=12230032&dopt=Abstract

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&list_uids=11752314&dopt=Abstract

For a detailed explanation of hidden Markov models and how they are applied in the Pfam database see Durbin R, Eddy S, and Krogh A (1998) Biological sequence analysis; probabilistic models of proteins and nucleic acids. Cambridge University Press, ISBN 0-521-62041-4. The Hammer software package can be obtained from Washington University, St Louis, USA.

Alternatively, the GDSX motif can be identified using the Hammer software package, the instructions are provided in Durbin R, Eddy S, and Krogh A (1998) Biological sequence analysis; probabilistic models of proteins and nucleic acids. Cambridge University Press, ISBN 0-521-62041-4 and the references therein, and the HMMER2 profile provided within this specification.

The PFAM database can be accessed, for example, through several servers which are currently located at the following websites.

http://www.sanger.ac.uk/Software/Pfam/index.shtml

http://pfam.wustl.edu/

http://pfam.jouy.inra.fr/

http://pfam.cgb.ki.se/

The database offers a search facility where one can enter a protein sequence. Using the default parameters of the database the protein sequence will then be analysed for the presence of Pfam domains. The GDSX domain is an established domain in the database and as such its presence in any query sequence will be recognised . The database will return the alignment of the Pfam00657 consensus sequence to the query sequence.

A multiple alignment, including *Aeromonas salmonicida* or *Aeromonas hydrophila* can be obtained by:

a) manual

obtain an alignment of the protein of interest with the Pfam00657 consensus sequence and obtain an alignment of P10480 with the Pfam00657 consensus sequence following the procedure described above;

or

b) through the database

After identification of the Pfam00657 consensus sequence the database offers the option to show an alignment of the query sequence to the seed alignment of the Pfam00657 consensus sequence. P10480 is part of this seed alignment and is indicated by GCAT_AERHY. Both the query sequence and P10480 will be displayed in the same window.

The *Aeromonas hydrophila* reference sequence:

The residues of *Aeromonas hydrophila* GDSX lipase are numbered in the NCBI file P10480, the numbers in this text refer to the numbers given in that file which in the present invention is used to determine specific amino acids residues which, in a preferred embodiment are present in the lipid acyltransferase enzymes of the invention.

<u>The Pfam alignment was performed (Figure 33 and 34):</u>

The following conserved residues can be recognised and in a preferable embodiment may be present in the enzymes for use in the compositions and methods of the invention;

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Block 1 - GDSX block
hid hid hid hid Gly Asp Ser hid
 28  29  30  31  32  33  34  35

Block 2 - GANDY block
hid Gly hid Asn Asp hid
130 131 132 133 134 135
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Block 3 - HPT block
His
309

Where 'hid' means a hydrophobic residue selected from Met, Ile, Leu, Val, Ala, Gly, Cys, His, Lys, Trp, Tyr, Phe.

Preferably the lipid acyltransferase enzyme for use in the compositions/methods of the invention can be aligned using the Pfam00657 consensus sequence.

Preferably, a positive match with the hidden markov model profile (HMM profile) of the pfam00657 domain family indicates the presence of the GDSL or GDSX domain according to the present invention.

Preferably when aligned with the Pfam00657 consensus sequence the lipid acyltransferase for use in the compositions/methods of the invention have at least one, preferably more than one, preferably more than two, of the following, a GDSx block, a GANDY block, a HPT block. Suitably, the lipid acyltransferase may have a GDSx block and a GANDY block. Alternatively, the enzyme may have a GDSx block and a HPT block. Preferably the enzyme comprises at least a GDSx block.

Preferably, when aligned with the Pfam00657 consensus sequence the enzyme for use in the compositions/methods of the invention have at least one, preferably more than one, preferably more than two, preferably more than three, preferably more than four, preferably more than five, preferably more than six, preferably more than seven, preferably more than eight, preferably more than nine, preferably more than ten, preferably more than eleven, preferably more than twelve, preferably more than thirteen, preferably more than fourteen, of the following amino acid residues when compared to the reference *A.hydrophilia* polypeptide sequence, namely SEQ ID No. 32: 28hid, 29hid, 30hid, 31hid, 32gly, 33Asp, 34Ser, 35hid, 130hid, 131Gly, 132Hid, 133Asn, 134Asp, 135hid, 309His

The pfam00657 GDSX domain is a unique identifier which distinguishes proteins possessing this domain from other enzymes.

The pfam00657 consensus sequence is presented in Figure 1 as SEQ ID No. 1. This is derived from the identification of the pfam family 00657, database version 6, which may also be referred to as pfam00657.6 herein.

The consensus sequence may be updated by using further releases of the pfam database.

For example, Figures 33 and 34 show the pfam alignment of family 00657, from database version 11, which may also be referred to as pfam00657.11 herein.

The presence of the GDSx, GANDY and HPT blocks are found in the pfam family 00657 from both releases of the database. Future releases of the pfam database can be used to identify the pfam family 00657.

Preferably, the fatty-acid CoA independent lipid acyltransferase enzyme according to the present invention may be characterised using the following criteria:

(i) the enzyme possesses acyl transferase activity which may be defined as ester transfer activity whereby the acyl part of an original ester bond of a lipid acyl donor is transferred to acyl acceptor to form a new ester;

(ii) the enzyme comprises the amino acid sequence motif GDSX, wherein X is one or more of the following amino acid residues L, A, V, I, F, Y, H, Q, T, N, M or S.;

(iii) the enzyme comprises His-309 or comprises a histidine residue at a position corresponding to His-309 in the *Aeromonas hydrophila* lipolytic enzyme shown in Figure 2 (SEQ ID No. 2 or SEQ ID No. 32).

Preferably, the amino acid residue of the GDSX motif is L.

In SEQ ID No. 2 or SEQ ID No. 32 the first 18 amino acid residues form a signal sequence. His-309 of the full length sequence, that is the protein including the signal sequence, equates to His-291 of the mature part of the protein, i.e. the sequence without the signal sequence.

Preferably, the fatty-acid CoA independent lipid acyltransferase enzyme according to the present invention comprises the following catalytic triad: Ser-34, Asp-134 and His-309 or comprises a serine residue, an aspartic acid residue and a histidine residue, respectively, at positions corresponding to Ser-34, Asp-134 and His-309 in the *Aeromonas hydrophila* lipolytic enzyme shown in Figure 2 (SEQ ID No. 2) or Figure 28 (SEQ ID No. 32). As stated above, in the sequence shown in SEQ ID No. 2 or SEQ ID No. 32 the first 18 amino acid residues form a signal sequence. Ser-34, Asp-134 and His-309 of the full length sequence, that is the protein including the signal sequence, equate to Ser-16, Asp-116 and His-291 of the mature part of the protein, i.e. the sequence without the signal sequence. In the pfam00657 consensus sequence, as given in Figure 1 (SEQ ID No. 1) the active site residues correspond to Ser-7, Asp-157 and His-348.

Preferably, the fatty-acid CoA independent lipid acyltransferase enzyme according to the present invention may be characterised using the following criteria:

(i) the enzyme possesses acyl transferase activity which may be defined as ester transfer activity whereby the acyl part of an original ester bond of a first lipid acyl donor is transferred to an acyl acceptor to form a new ester; and

(ii) the enzyme comprises at least Gly-32, Asp-33, Ser-34, Asp-134 and His-309 or comprises glycine, aspartic acid, serine, aspartic acid and histidine residues at positions corresponding to Gly-32, Asp-33, Ser-34, Asp-134 and His-309, respectively, in the *Aeromonas hydrophila* lipolytic enzyme shown in Figure 2 (SEQ ID No. 2) or Figure 28 (SEQ ID No. 32).

Suitably, the fatty-acid CoA independent lipid acyltransferase enzyme according to the present invention may be obtainable, preferably obtained, from organisms from one or more of the following genera: *Aeromonas*, *Streptomyces*, *Saccharomyces*, *Lactococcus*, *Mycobacterium*, *Streptococcus*, *Lactobacillus*, *Desulfitobacterium*, *Bacillus*, *Campylobacter*, *Vibrionaceae*, *Xylella*, *Sulfolobus*, *Aspergillus*, *Schizosaccharomyces*, *Listeria*, *Neisseria*, *Mesorhizobium*, *Ralstonia*, *Xanthomonas* and *Candida*.

Suitably, the fatty-acid CoA independent lipid acyltransferase enzyme according to the present invention may be obtainable, preferably obtained, from one or more of the following organisms: *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Streptomyces coelicolor*, *Streptomyces rimosus*, *Mycobacterium*, *Streptococcus pyogenes*, *Lactococcus lactis*, *Streptococcus pyogenes*, *Streptococcus thermophilus*, *Lactobacillus helveticus*, *Desulfitobacterium dehalogenans*, *Bacillus sp*, *Campylobacter jejuni*, *Vibrionaceae*, *Xylella fastidiosa*, *Sulfolobus solfataricus*, *Saccharomyces cerevisiae*, *Aspergillus terreus*, *Schizosaccharomyces pombe*, *Listeria innocua*, *Listeria monocytogenes*, *Neisseria meningitidis*, *Mesorhizobium loti*, *Ralstonia solanacearum*, *Xanthomonas campestris*, *Xanthomonas axonopodis* and *Candida parapsilosis*.

In one aspect, preferably the fatty-acid CoA independent lipid acyltransferase enzyme according to the present invention is obtainable, preferably obtained, from one or more of *Aeromonas hydrophila* or *Aeromonas salmonicida*.

Suitably, the fatty-acid CoA independent lipid acyltransferase enzyme according to the present invention comprises one or more of the following amino acid sequences:

(i) the amino acid sequence shown as SEQ ID No. 2 (see Figure 2)

(ii) the amino acid sequence shown as SEQ ID No. 3 (see Figure 3)

(iii) the amino acid sequence shown as SEQ ID No. 4 (see Figure 4)

(iv) the amino acid sequence shown as SEQ ID No. 5 (see Figure 5)

(v) the amino acid sequence shown as SEQ ID No. 6 (see Figure 6)

(vi) the amino acid sequence shown as SEQ ID No. 12 (see Figure 14)

(vii) the amino acid sequence shown as SEQ ID No. 20 (Figure 16)

(viii) the amino acid sequence shown as SEQ ID No. 22 (Figure 18)

(ix) the amino acid sequence shown as SEQ ID No. 24 (Figure 20)

(x) the amino acid sequence shown as SEQ ID No. 26 (Figure 22)

(xi) the amino acid sequence shown as SEQ ID No. 28 (Figure 24)

(xii) the amino acid sequence shown as SEQ ID No. 30 (Figure 26)

(xiii) the amino acid sequence shown as SEQ ID No. 32 (Figure 28)

(xiv) the amino acid sequence shown as SEQ ID No. 34 (Figure 30)

(xv) the amino acid sequence shown as SEQ ID No. 55 (Figure 52)

(xvi) the amino acid sequence shown as SEQ ID No. 58

(xvii) the amino acid sequence shown as SEQ ID No. 60

(xviii) the amino acid sequence shown as SEQ ID No. 61

(xix) the amino acid sequence shown as SEQ ID No. 63

(xx) the amino acid sequence shown as SEQ ID No. 65

(xxi) the amino acid sequence shown as SEQ ID No. 67

(xxii) the amino acid sequence shown as SEQ ID No. 70 or

(xxiii) an amino acid sequence which has 75% or more identity with any one of the sequences shown as SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 12, SEQ ID No. 20, SEQ ID No. 22, SEQ ID No. 24, SEQ ID No. 26, SEQ ID No. 28, SEQ ID No. 30, SEQ ID No. 32, SEQ ID No. 34, SEQ ID No. 55, SEQ ID No. 58, SEQ ID No. 60, SEQ ID No. 61, SEQ ID No. 63, SEQ ID No. 65, SEQ ID No. 67 or SEQ ID No. 70.

Suitably, the fatty-acid CoA independent lipid acyltransferase enzyme according to the present invention comprises either the amino acid sequence shown as SEQ ID No. 2 or as SEQ ID No. 3 or SEQ ID No. 32 or SEQ ID No. 34 or comprises an amino acid sequence which has 75% or more, preferably 80% or more, preferably 85% or more, preferably 90% or more, preferably 95% or more, identity with the amino acid sequence shown as SEQ ID No. 2 or the amino acid sequence shown as SEQ ID No. 3 or the amino acid sequence shown as SEQ ID No. 32 or the amino acid sequence shown as SEQ ID No. 34.

For the purposes of the present invention, the degree of identity is based on the number of sequence elements which are the same. The degree of identity in accordance with the present invention may be suitably determined by means of computer programs known in the art, such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, US53711) (Needleman & Wunsch (1970), J. of Molecular Biology 48, 443-45) using the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

Suitably the fatty-acid CoA independent lipid acyltransferase enzyme according to the present invention comprises an amino acid sequence which has 80% or more, preferably 85% or more, more preferably 90% or more and even more preferably 95% or more identity with any one of the sequences shown as SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 12, SEQ ID No. 20, SEQ ID No. 22, SEQ ID No. 24, SEQ ID No. 26, SEQ ID No. 28, SEQ ID No. 30, SEQ ID No. 32, SEQ ID No. 34, SEQ ID No. 55, SEQ ID No. 58, SEQ ID No. 60, SEQ ID No. 61, SEQ ID No. 63, SEQ ID No. 65, SEQ ID No. 67 or SEQ ID No. 70.

Suitably, the fatty-acid CoA independent lipid acyltransferase enzyme according to the present invention comprises one or more of the following amino acid sequences:

(a) an amino acid sequence shown as amino acid residues 1-100 of SEQ ID No. 2 or SEQ ID No. 32;

(b) an amino acid sequence shown as amino acids residues 101-200 of SEQ ID No. 2 or SEQ ID No. 32;

(c) an amino acid sequence shown as amino acid residues 201-300 of SEQ ID No. 2 or SEQ ID No. 32; or

(d) an amino acid sequence which has 75% or more, preferably 85% or more, more preferably 90% or more, even more preferably 95% or more identity to any one of the amino acid sequences defined in (a)-(c) above.

Suitably, the fatty-acid CoA independent lipid acyltransferase enzyme according to the present invention comprises one or more of the following amino acid sequences:

(a) an amino acid sequence shown as amino acid residues 28-39 of SEQ ID No. 2 or SEQ ID No. 32;

(b) an amino acid sequence shown as amino acids residues 77-88 of SEQ ID No. 2 or SEQ ID No. 32;

(c) an amino acid sequence shown as amino acid residues 126-136 of SEQ ID No. 2 or SEQ ID No. 32;

(d) an amino acid sequence shown as amino acid residues 163-175 of SEQ ID No. 2 or SEQ ID No. 32;

(e) an amino acid sequence shown as amino acid residues 304-311 of SEQ ID No. 2 or SEQ ID No. 32; or

(f) an amino acid sequence which has 75% or more, preferably 85% or more, more preferably 90% or more, even more preferably 95% or more identity to any one of the amino acid sequences defined in (a)-(e) above.

Suitably, the fatty-acid CoA independent lipid acyltransferase enzyme according to the present invention may comprise an amino acid sequence produced by the expression or one or more of the following nucleotide sequences:

(a) the nucleotide sequence shown as SEQ ID No. 7 (see Figure 9);

(b) the nucleotide sequence shown as SEQ ID No. 8 (see Figure 10);

(c) the nucleotide sequence shown as SEQ ID No. 9 (see Figure 11);

(d) the nucleotide sequence shown as SEQ ID No. 10 (see Figure 12);

(e) the nucleotide sequence shown as SEQ ID No. 11 (see Figure 13);

(f) the nucleotide sequence shown as SEQ ID No. 13 (see Figure 15);

(g) the nucleotide sequence shown as SEQ ID No. 21 (see Figure 17);

(h) the nucleotide sequence shown as SEQ ID No. 23 (see Figure 19);

(i) the nucleotide sequence shown as SEQ ID No. 25 (see Figure 21);

(j) the nucleotide sequence shown as SEQ ID No. 27 (see Figure 23);

(k) the nucleotide sequence shown as SEQ ID No. 29 (see Figure 25);

(l) the nucleotide sequence shown as SEQ ID No. 31 (see Figure 27);

(m) the nucleotide sequence shown as SEQ ID No. 33 (see Figure 29);

(n) the nucleotide sequence shown as SEQ ID No. 35 (see Figure 31);

(o) the nucleotide sequence shown as SEQ ID No. 54 (Figure 51);

(p) the nucleotide sequence shown as SEQ ID No. 59;

(q) the nucleotide sequence shown as SEQ ID No. 62;

(r) the nucleotide sequence shown as SEQ ID No. 64;

(s) the nucleotide sequence shown as SEQ ID No. 66;

(t) the nucleotide sequence shown as SEQ ID No. 68

(u) the nucleotide sequence shown as SEQ ID No. 69 or

(v) a nucleotide sequence which has 75% or more identity with any one of the sequences shown as SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 13, SEQ ID No. 21, SEQ ID No. 23, SEQ ID No. 25, SEQ ID No. 27, SEQ ID No. 29, SEQ ID No. 31, SEQ ID No. 33, SEQ ID No. 35, SEQ ID No. 54, SEQ ID No. 59, SEQ ID No. 62, SEQ ID No. 64, SEQ ID No. 66, SEQ ID No. 68 or SEQ ID No. 69.

Suitably the nucleotide sequence may have 80% or more, preferably 85% or more, more preferably 90% or more and even more preferably 95% or more identity with any one of the sequences shown as SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 13, SEQ ID No. 21, SEQ ID No. 23, SEQ ID No. 25, SEQ ID No. 27, SEQ ID No. 29, SEQ ID No. 31, SEQ ID No. 33 or SEQ ID No. 35, SEQ ID No. 54, SEQ ID No. 59, SEQ ID No. 62, SEQ ID No. 64, SEQ ID No. 66, SEQ ID No. 68 or SEQ ID No. 69.

In one aspect, the fatty-acid CoA independent lipid acyltransferase according to the present invention may be a lecithin:cholesterol acyltransferases (LCAT) or variant thereof (for example a variant made by molecular evolution)

Suitable LCATs are known in the art and may be obtainable from one or more of the following organisms for example: mammals, rat, mice, chickens, *Drosophila melanogaster*, plants, including *Arabidopsis* and *Oryza sativa*, nematodes, fungi and yeast.

In one embodiment the fatty-acid CoA independent lipid acyltransferase enzyme according to the present invention may be the lipid acyltransferase obtainable, preferably obtained, from the *E. coli* strains TOP 10 harbouring pPet12aAhydro and pPet12aASalmo deposited by Danisco A/S of Langebrogade 1, DK-1001 Copenhagen K, Denmark under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the purposes of Patent Procedure at the National Collection of Industrial, Marine and Food Bacteria (NCIMB) 23 St. Machar Street, Aberdeen Scotland, GB on 22 December 2003 under accession numbers NICMB 41204 and NCIMB 41205, respectively.

Preferably, when carrying out a method according to the present invention the product is produced without increasing or substantially increasing the free fatty acids in the foodstuff.

The term "transferase" as used herein is interchangeable with the term "lipid acyltransferase".

Suitably, the fatty-acid CoA independent lipid acyltransferase as defined herein catalyses one or more of the following reactions: interesterification, transesterification, alcoholysis, hydrolysis.

The term "interesterification" refers to the enzymatic catalysed transfer of acyl groups between a lipid donor and lipid acceptor, wherein the lipid donor is not a free acyl group.

The term "transesterification" as used herein means the enzymatic catalysed transfer of an acyl group from a lipid donor (other than a free fatty acid) to an acyl acceptor (other than water).

As used herein, the term "alcoholysis" refers to the enzymatic cleavage of a covalent bond of an acid derivative by reaction with an alcohol ROH so that one of the products

combines with the H of the alcohol and the other product combines with the OR group of the alcohol.

As used herein, the term "alcohol" refers to an alkyl compound containing a hydroxyl group.

As used herein, the term "hydrolysis" refers to the enzymatic catalysed transfer of an acyl group from a lipid to the OH group of a water molecule. Acyl transfer which results from hydrolysis requires the separation of the water molecule.

The term "without increasing or without substantially increasing the free fatty acids" as used herein means that preferably the lipid acyl transferase according to the present invention has 100% transferase activity (i.e. transfers 100% of the acyl groups from an acyl donor onto the acyl acceptor, with no hydrolytic activity) in an edible oil environment; however, the enzyme may transfer less than 100% of the acyl groups present in the lipid acyl donor to the acyl acceptor. In which case, preferably the acyltransferase activity accounts for at least 30%, more preferably at least 40%, more preferably at least 50%, more preferably at least 70%, more preferably at least 80%, more preferably at least 90% and more preferably at least 98% of the total enzyme activity. The % transferase activity (i.e. the transferase activity as a percentage of the total enzymatic activity) may be determined by the following protocol:

Protocol for the determination of % acyltransferase activity:

An edible oil to which a fatty-acid CoA independent lipid acyltransferase according to the present invention has been added may be extracted following the enzymatic reaction with $CHCl_3$:CH_3OH 2:1 and the organic phase containing the lipid material is isolated and analysed by GLC according to the procedure detailed herein below. From the GLC analyses the amount of free fatty acids and diglycerides are determined. A control edible oil to which no enzyme according to the present invention has been added, is analysed in the same way.

Calculation:

From the results of the GLC analyses the increase in free fatty acids and the decrease in diglycerides can be calculated:

Δ % fatty acid = % Fatty acid(enzyme) - % fatty acid(control);

Mv Fa = average molecular weight of the fatty acids;

Δ % diglyceride = % Diglyceride(control) - % Diglyceride(enzyme);

Mv Di= average molecular weight of diglyceride;

The transferase activity is calculated as a percentage of the total enzymatic activity:

$$\% \text{ transferase activity} = \frac{((\Delta \% \text{ Di}/(\text{Mv Di}) - \Delta \% \text{ Fa}/(\text{Mv FA})) \times 100}{\Delta \% \text{ Di}/(\text{Mv Di}) - \Delta \% \text{ Fa}/(\text{Mv Fa}) + \Delta \% \text{ Fa}/(\text{Mv Fa})}$$

$$\% \text{ transferase activity} = = \frac{((\Delta \% \text{ Di}/(\text{Mv Di}) - \Delta \% \text{ Fa}/(\text{Mv FA})) \times 100}{\Delta \% \text{ Di}/(\text{Mv Di})}$$

If the free fatty acids are increased in the edible oil they are preferably not increased substantially, i.e. to a significant degree. By this we mean, that the increase in free fatty acid does not adversely affect the quality of the edible oil.

In some aspects of the present invention, the term "without substantially increasing free fatty acids" as used herein means that the amount of free fatty acid in an edible oil treated with an lipid acyltransferase according to the present invention is less than the amount of free fatty acid produced in an edible oil or composition when an enzyme other than a lipid acyltransferase according to the present invention had been used, such as for example as compared with the amount of free fatty acid produced when a conventional lipase e.g. *Pseudomonas cepacia* lipase (Lipase PS, Amano Japan) or *Rhizopus oryzae* lipase (LipaseF, Amano Japan).

Suitably, any glycerol remaining in the edible oil after the reaction has taken place may be removed, e.g. by centrifugation or vacuum distillation.

Optionally, the enzyme may be removed from the edible oil after the enzymatic reaction has taken place. Alternatively, the enzyme may simply be deactivated and left in the edible oil. Suitably, the enzyme may be deactivated by heating for example.

In one embodiment the fatty-acid CoA independent lipid acyltransferase for use in the methods of the present invention may be immobilised. When it is the case that the enzyme is immobilised an admixture comprising an acyl acceptor and the edible oil may be passed through a column for example comprising the immobilised enzyme. By immobilising the enzyme it is possible to easily reuse it.

Suitably the immobilised enzyme may be used in a flow reactor or in a batch reactor containing a reaction mixture which comprises an acyl acceptor and an edible oil as a two-phase system. The reaction mixture may be optionally stirred or sonicated. Once the reaction has reached equilibrium for example, the reaction mixture and the immobilised enzyme may be separated. Suitably, excess acyl acceptor (such as excess glycerol) may be removed after the reaction, e.g. by centrifugation or vacuum distillation.

Immobilised lipid acyltransferase can be prepared using immobilisation techniques known in the art. There are numerous methods of preparing immobilised enzymes, which will be apparent to a person skilled in the art (for example the techniques referred to in EP 0 746 608; or Balcao V.M., Paiva A.L., Malcata F.X., Enzyme Microb Technol. 1996 May 1;18(6):392-416; or Retz M.T., Jaeger K.E. Chem Phys Lipids. 1998 Jun;93(1-2):3-14; Bornscheuer U.T., Bessler C, Srinivas R, Krishna S.H. Trends Biotechnol. 2002 Oct; 20(10):433-7; Plou et al, J. Biotechnology 92 (2002) 55-66; Warmuth et al., 1992. Bio Forum 9, 282-283; Ferrer et al., 2000. J. Chem. Technol. Biotechnol. 75, 1-8; or Christensen et al., 1998. Nachwachsende Rohstoff 10, 98-105; Petersen and Christenen, 2000, Applied Biocatalysis. Harwood Academic Publishers, Amsterdam. (each of which is incorporated herein by reference). Techniques which may be used herein include covalent coupling to Eupergit C, adsorption on polypropylene and silica-granulation for example.

Preferably, the edible oil is any edible oil containing a diglyceride, preferably a significant amount of diglyceride.

Preferably, the edible oil is one or more of the following oils: oils extracted from or derived from palm oil, palm olein, palm stearin, palm mid fraction or any palm oil fraction or olive oil.

More preferably, the edible oil is palm oil and/or palm olein and/or palm stearin.

With regard to the admixing of the edible oil, the acyl acceptor substrate and the lipid acyltransferase, as a person skilled in the art would readily appreciate this can be done in any combination and/or order. By way of example only, the acyl acceptor substrate may be admixed with the edible oil followed by addition of the lipid acyltransferase. Alternatively, the edible oil may be admixed with the lipid acyltransferase followed by addition of the acyl acceptor substrate. Alternatively, the lipid acyltransferase and acyl acceptor substrate may be admixed followed by admixing of the enzyme/substrate mixture with the edible oil. Alternatively, of course, all three substances (namely the edible oil, the acyl acceptor substrate and the lipid acyltransferase) may be admixed simultaneously.

Preferably the method is carried out at a temperature above the melting point of the edible oil.

Suitably the method may be carried out at a temperature of between 30-50°C, preferably between 35-45°C, preferably between 40-45°C.

Preferably, the enzyme is added to crude or refined edible oil. Preferably the present invention does not encompass the enzyme treatment of an edible oil when admixed with water containing constituents. Thus, the present invention envisages the treatment of an edible oil *per se*, i.e. in a low water environment.

Although water may be added to the oil prior or during the method of the present invention, in a most preferable embodiment no water is added. If water is present in the edible oil, preferably there is less than 10% water present, more preferably less than 7.5%, less than 5%, less than 1%, less than 0.5%, less than 0.4%, less than 0.3%, less than 0.2% or less than 0.1% water present. Suitably, between 0.1. and 1% water may be present in the edible oil.

In one embodiment the edible oil used in a process according to the present invention may be supplemented with one or more acyl donors and/or may be supplemented with one or more acyl acceptors and/or may be supplemented with both one or more acyl acceptors and one or more acyl donors. By "supplemented with" means that either the acyl donor and/or the acyl acceptor is not naturally present in the edible oil and is added at the same time, immediately after and/or immediately before bringing the edible into contact with the lipid acyltransferase in accordance with the present invention.

Suitably, the supplementary acyl donor may be other than a DAG. Suitably the supplemental acyl donor may be for example a phospholipid (e.g. lecithin).

Suitably, the supplementary acyl acceptor may be glycerol. Suitably, however it may be an acyl acceptor other than glycerol, for example a plant sterol and/or plant stanol for instance. Suitably, the supplementary acyl acceptor may be a combination of glycerol and one or more further acyl acceptors.

The use of an oil supplemented with a phospholipid allows for an additional emulsifier (lyso-phospholipid) to be produced in the oil. The use of an oil supplemented with a plant sterol and/or plant stanol allows for a plant sterol ester and/or a plant stanol ester to be produced in the edible both. Both plant sterol esters and plant stanol esters have been reported to have blood serum cholesterol reducing effects when incorporated into the diet.

Enzymatic interesterification using immobilised lipases, such as Lipozyme® TL IM, a 1,3 specific lipase (Novozymes, Denmark), is used to reduce the amount of trans fatty acids in oils for dietary use and/or for modifying the melting characteristics of edible oils and fats.

During interesterification, one or two of the polyunsaturated fatty acids in the triglycerides of the food oil can be replaced with a fatty acid from another oil low in trans fatty acids, such as palm oil. This transfer of fatty acids from palm oil allows the modification of the melting point of the food oil without introduction of the trans fatty acids. The immobilisation of lipases is described in US5776741, US4798793 and US5156963. US6284501 describes the interesterification of phospholipids.

An immobilised transferase can be produced using the same technology as used for lipases.

In one embodiment, the fatty-acid CoA independent lipid acyl transferase as described herein can be used in combination with a interesterification lipase. The lipase interesterification and the acyl transferase steps are preferably carried out separately.

In a further aspect, the fatty-acid CoA independent lipid acyl transferase as described herein can be used in combination with conventional crystallisation inhibitors.

In a further aspect, the present invention provides a method of improving the crystallization properties in a foodstuff comprising an edible oil, comprising admixing an edible oil with an acyl acceptor substrate and a fatty-acid CoA independent diglyceride:glycerol acyltransferase as described herein and optionally a further crystallization inhibitor.

In one embodiment the present invention provides a fatty-acid CoA independent diglyceride:glycerol acyltransferase in the preparation of a foodstuff comprising an edible oil for improving the crystallization properties of the foodstuff.

Suitably one or more further crystallization inhibitors may optionally be added.

The present invention yet further provides the use of a fatty-acid CoA independent diglyceride:glycerol acyltransferase as described herein in the manufacture of a foodstuff comprising an edible oil for improving the crystallization properties of said foodstuff.

In another aspect, the present invention provides the use of a fatty-acid CoA independent diglyceride:glycerol acyltransferase as described herein in combination with a further crystallization inhibitor in the manufacture of a foodstuff comprising an edible oil for improving the crystallization properties of said foodstuff.

The further crystallization inhibitor may be any conventional crystallization inhibitor such as one or more of sorbitan tristearates, lecithins, PGE or polysorbates for example.

ADVANTAGES

In the present invention by use of the method taught herein, and in particular by selection of the enzymes taught herein for use in the claimed method, for removing or reducing diglyceride from or in an edible oil, a selective reduction in diglyceride over mono- and di-glyceride can be effected, without a decrease in triglycerides and/or a significant increase in free fatty acids (FFAs).

The fact that the present inventive method can be carried out without substantially increasing free fatty acid content of the edible oil overcomes the problem of loss of product.

If a crude palm oil is treated with a conventional lipase it is necessary to remove the free fatty acid during the oil refining, but if a refined palm oil is treated with the lipid acyltransferase according to the present invention (thus negating the need to treat the

crude palm oil with a conventional lipase) it is not necessary to remove the fatty acids because the content of free fatty acid does not increase substantially.

In addition or alternatively, the present invention results in the removal and/or reduction of diglycerides from or in an edible oil, with no significant decrease in monoglyceride levels.

In addition or alternatively, the present invention results in the removal and/or reduction of diglycerides from or in an edible oil, whilst increasing monoglyceride levels in the edible oil. This contrasts prior art enzymes which utilise monoglyceride as a substrate and therefore reduce the amount of monoglyceride in the edible oil.

The present invention is advantageous as it does not require the addition of fatty acid CoA. This contrasts sharply with the acyl CoA dependent DGATs. Rather the enzyme according to the present invention relies on the presence (and addition if necessary) of glycerol – which is cheap commodity.

COMMERCIAL RELEVANCE OF REMOVING/REDUCING DIGLYCERIDE IN PALM OIL

Diglycerides retard the crystallisation in palm based margarines and shortenings

For many years, we have seen a steady increase in the growth of palm oil consumption, partially because of localised availability, partially because of economics, and primarily because of performance. Presence of Palm oil in Margarine / shortening type products is found to enhance the β' quality of an oil blend. However, the use of palm oil was previously restricted to the use of palm stearine, and palm olein, with some use of palm kernels and its fractions. Today, this is more diverse, because of confectionery and food processors wanting very specific melting profiles.

With customers trying to increase the use of palm oil in formulations, the industry is seeing more crystallization issues than ever before. Where it is necessary to add "pro-

crystal" promoters to initiate crystallization in low trans or no-trans formulations thereby preventing or delaying post crystal formation before point of use. Also increased interest in anti-crystallisers, such as Sorbitan Tristearates, lecithins, PGE, Polysorbates, which will slow, delay post-crystal growth has been seen. This is a symptom of high diglyceride presence.

The commercial benefits of the present invention may be one or more of the following:

a) reduces the need to add additional monoglyceride to the edible oil during processing and/or later during use. Monoglyceride is a widely used emulsifier in food systems.

b) decreases in diglyceride from about 6 - 8% to about 4-5%, and even more.

c) increases the flexibility on plant (factory) capacity - hence reduction in production overheads.

d) allows for major decreases in post-crystallisation issues.

e) in some formulations, it would be possible to remove some fully saturated oil triglycerides, because we reduce the need for crystal promotion. In this regard manufacturers conventionally add fully saturated triglycerides to formulations in order to "promote" crystal formation and overcome undesirable delayed crystal formation in the product post-sale. Such crystal formation is a symptom of having a significant amount of palm oil or its components in a formulation, which would contain concentrations of diglycerides sufficient to delay crystal growth to the desired crystal type (i.e. preferably beta-prime in the case of margarines and shortenings) during manufacture. However, by use of palm oil treated in accordance with the present invention, the diglyceride content is sufficiently reduced within a given blend or formulation so that the need for additional fully saturated triglyceride addition to aid crystal development becomes less important.

f) allows greater diversification towards palm based blends, with increased liquid oil costs, and without major process changes.

g) allows for replacement of anti-crystallisers and/or partial replacement of anti-crystallisers.

B Trans fatty acid

Today, we see legislation and public opinion against the use of trans acids in food products (Fødevareministeriet, Denmark 2003). So, this has led to performance problems. Manufacturers where possible may wish to switch to palm based solutions because of the high levels of C:16:0 and C:18:0 isomers found within this oil type. The result is that we are beginning to see increased consumption of palm in economies that previously did not use palm.

C Crystallisation inhibition of diglycerides in confectionery products

Cocoa Butter Equivalents (CBEs) are formulated from speciality fats such as fractionated palm oil, in particular palm mid fractions (PMF), fractionated shea fat as well as many other exotic fats. For cost reasons it is favourable to include as much as possible PMF in the CBE. In fact many CBEs may only contain palm fractions. The most delicate aspect of chocolate manufacture is the crystallisation of the fat. Diglycerides will have a negative impact on the crystallisation, for example demoulding can be a serious problem (Siew, 2001).

D Crystal quality

Triglyceride quality greatly affects performance, and this is evident, from examples of a typical trans acid containing oil formula for standard 82% fat table margarine, against trans free version containing a typical interesterified palm stearine fraction and palm kernel as hard stock, both having similar SFC profile. Reduced speed and quality of crystallization can result in a symptom known as "oiling out." In turn this calls for the need of crystal promoters, such as hard MAGs. Further, delay in crystal stabilized crystal formation leads to symptom known as "sandiness" whereby transformation of desired crystal type eventually reverts to the more stable Beta form, and so gives sandy texture. This symptom is particular problem for Industrial type products.

SOME USES

Fat Modification

During the chemical production of fats for use in high fat solid food products such as margarines, spreads, confectionery/chocolates, trans fatty acids are introduced into the food oil during a process of hydrogenation (hardening). This allows for the modification of the melting temperature of the food oil to ensure a suitable consistency of the final food product, e.g. a table margarine, which is solid but spreadable at room temperature, or a chocolate which is solid during storage, but melts in the mouth.

However, chemical production uses large quantities of solvents such as hexane which are considered hazardous to the environment and health and requires complete removal of the solvent prior to use of the modified oil/fat as a food ingredient.

In many countries the use of partial hydrogenation for food production is being limited by legislation and regulatory controls, and many major food producers are now switching to alternative low-trans alternatives.

The oil prepared by the process of the invention can be used as an ingredient for margarine and/or spreads.

Cocoa Butter Replacements/Equivalents

Cocoa butter contains a unique composition which provides a solid confectionery which melts in the mouth. In order to substitute cocoa butter using cheaper alternatives, plant oils have been hydrogenated to produce trans fatty acids, thereby raising the melting point of the food oil to provide a similar solid confectionery which melts at body temperature. Such modified plant oils are known as cocoa butter equivalents (CBEs) or, in the case where the modified fats enhance the characteristics of the chocolate product, cocoa butter replacements (CBRs). One major problem with

CBEs and CBRs is that the hydrogenation of plant oils produces trans fats which are considered to be detrimental to health and temperature. This has lead to the use of low trans plant oils, or fractions thereof, which have a higher melting temperature. Palm oil and palm oil fractions are considered advantageous in this respect as a major component low in trans CBRs/CBEs.

Cocoa butter replacement (CBR) fats are used to provide preferable characteristics to chocolate products such as non-tempering, reduced post hardening, stability, bloom resistant. It is particularly desirable to use non-lauric/non-trans fats such as palm oil. The crystallisation properties of the fats used in CBRs play a key role in ensuring a suitable balance between the product melting in the mouth whilst retaining the above preferable characteristics. The use of low-trans fats, such as palm oil, in CBR blends is particularly desirable for health reasons. The use of palm oil in CBRs is described in EP0293194.

The oil prepared in accordance with a process of the present invention can be used as an ingredient for chocolate, for example within a fat blend as a cocoa butter replacement and/or equivalent.

SEQUENCES OF SOME DIGLYCERIDE:GLYCEROL ACYLTRANSFERASE ENZYMES FOR USE IN ACCORDANCE WITH THE PRESENT INVENTION

Suitable diglyceride:glycerol acyltransferase enzymes for use in accordance with the present invention and/or in the methods of the present invention may comprise any one of the following amino acid sequences and/or be encoded by the following nucleotide sequences:

Termobifida\fusca GDSx 548 aa

SEQ ID No. 58

ZP_00058717

```
   1 mlphpagerg evgaffallv gtpqdrrlrl echetrplrg rcgcgerrvp pltlpgdgvl
  61 cttsstrdae tvwrkhlqpr pdggfrphlg vgcllagqgs pgvlwcgreg crfevcrrdt
 121 pglsrtrngd ssppfragws lppkcgeisq sarktpavpr yslirtdrpd gprgrfvgsg
 181 praatrrrlf lgipalvlvt altlvlavpt gretlwrmwc eatqdwclgv pvdsrgqpae
 241 dgeflllspv qaatwgnyya lgdsyssgdg ardyypgtav kggcwrsana ypelvaeayd
 301 faghlsflac sgqrgyamld aidevgsqld wnsphtslvt igiggndlgf stvlktcmvr
 361 vplldskact dqedairkrm akfettfeel isevrtrapd arilvvgypr ifpeeptgay
 421 ytltasnqrw lnetiqefnq qlaeavavhd eeiaasggvg svefvdvyha ldgheigsde
 481 pwvngvqlrd latgvtvdrs tfhpnaaghr avgervieqi etgpgrplya tfavvagatv
 541 dtlagevg
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SEQ ID No. 59

nt

```
   1 ggtggtgaac cagaacaccc ggtcgtcggc gtgggcgtcc aggtgcaggt gcaggttctt
  61 caactgctcc agcaggatgc cgccgtggcc gtgcacgatg gccttgggca ggcctgtggt
 121 ccccgacgag tacagcaccc atagcggatg gtcgaacggc agcggggtga actccagttc
 181 cgcgccttcg cccgcggctt cgaactccgc ccaggacagg gtgtcggcga cagggccgca
 241 gcccaggtac ggcaggacga cggtgtgctg caggctgggc atgccgtcgc gcagggcttt
 301 gagcacgtca cggcggtcga agtccttacc gccgtagcgg tagccgtcca cggccagcag
 361 cactttcggt tcgatctgcg cgaaccggtc gaggacgctg cgcaccccga agtcggggga
 421 acaggacgac caggtcgcac cgatcgcggc gcaggcgagg aatgcggccg tcgcctcggc
 481 gatgttcggc aggtaggcca cgacccggtc gccggggccc accccgaggc tgcggagggc
 541 cgcagcgatc gcggcggtgc gggtccgcag ttctccccag gtccactcgg tcaacggccg
 601 gagttcggac gcgtgccgga tcgccacggc tgatgggtca cggtcgcgga agatgtgctc
 661 ggcgtagttg agggtggcgc cggggaacca gacggcgccg ggcatggcgt cggaggcgag
 721 cactgtggtg tacggggtgg cggcgcgcac ccggtagtac tcccagatcg cggaccagaa
 781 tccttcgagg tcggttaccg accagcgcca cagtgcctcg tagtccggtg cgtccacacc
 841 gcggtgctcc cgcacccagc gggtgaacgc ggtgaggttg gcgcgttctt tgcgctcctc
 901 gtcgggactc cacaggatcg gcggctgcgg cttgagtgtc atgaaacgcg acccccttcgt
 961 ggacggtgcg gatgcggtga gcgtcgggtg cctcccctaa cgctccccgg tgacggagtg
1021 ttgtgcacca catctagcac gcgggacgcg gaaaccgtat ggagaaaaca cctacaaccc
1081 cggccggacg gtgggtttcg gccacactta ggggtcgggt gcctgcttgc cgggcagggc
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1201 actccgggcc tcagccgtac ccgcaacggg gacagttctc ctcccttccg ggctggatgg
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1321 aggtactctt tgcttcgaac agacaggccg gacggtccac gggggaggtt tgtgggcagc
1381 ggaccacgtg cggcgaccag acgacggttg ttcctcggta tccccgctct tgtacttgtg
1441 acagcgctca cgctggtctt ggctgtcccg acggggcgcg agacgctgtg gcgcatgtgg
1501 tgtgaggcca cccaggactg gtgcctgggg gtgccggtcg actcccgcgg acagcctgcg
1561 gaggacggcg agtttctgct gctttctccg gtccaggcag cgacctgggg gaactattac
1621 gcgctcgggg attcgtactc ttcgggggac ggggcccgcg actactatcc cggcaccgcg
1681 gtgaagggcg gttgctggcg gtccgctaac gcctatccgg agctggtcgc cgaagcctac
1741 gacttcgccg gacacttgtc gttcctggcc tgcagcggcc agcgcggcta cgccatgctt
1801 gacgctatcg acgaggtcgg ctcgcagctg gactggaact cccctcacac gtcgctggtg
1861 acgatcggga tcggcggcaa cgatctgggg ttctccacgg ttttgaagac ctgcatggtg
1921 cgggtgccgc tgctggacag caaggcgtgc acggaccagg aggacgctat ccgcaagcgg
1981 atggcgaaat tcgagacgac gtttgaagag ctcatcagcg aagtgcgcac ccgcgcgccg
2041 gacgcccgga tccttgtcgt gggctacccc cggatttttc cggaggaacc gaccggcgcc
2101 tactacacgc tgaccgcgag caaccagcgg tggctcaacg aaaccattca ggagttcaac
2161 cagcagctcg ccgaggctgt cgcggtccac gacgaggaga ttgccgcgtc gggcggggtg
2221 ggcagcgtgg agttcgtgga cgtctaccac gcgttggacg gccacgagat cggctcggac
```

2281 gagccgtggg tgaacggggt gcagttgcgg gacctcgcca ccggggtgac tgtggaccgc
2341 agtaccttcc accccaacgc cgctgggcac cgggcggtcg gtgagcgggt catcgagcag
2401 atcgaaaccg gcccgggccg tccgctctat gccactttcg cggtggtggc gggggcgacc
2461 gtggacactc tcgcgggcga ggtggggtga cccggcttac cgtccggccc gcaggtctgc
2521 gagcactgcg gcgatctggt ccactgccca gtgcagttcg tcttcggtga tgaccagcgg
2581 cggggagagc cggatcgttg agccgtgcgt gtctttgacg agcacacccc gctgcaggag
2641 ccgttcgcac agttctcttc cggtggccag agtcgggtcg acgtcgatcc cagcccacag
2701 gccgatgctg cgggccgcga ccacgccgtt gccgaccagt tggtcgaggc gggcgcgcag
2761 cacgggggcg agggcgcgga catggtccag gtaagggccg tcgcggacga ggctcaccac
2821 ggcagtgccg accgcgcagg cgagggcgtt gccgccgaag gtgctgccgt gctggccggg
2881 gcggatcacg tcgaagactt ccgcgtcgcc taccgccgcc gccacgggca ggatgccgcc
2941 gcccagcgct ttgccgaaca ggtagatatc ggcgtcgact ccgctgtggt cgcaggcccg
//

Termobifida\fusca\ - GDSx

SEQ ID No. 60

1 vgsgpraatr rrlflgipal vlvtaltlvl avptgretlw rmwceatqdw clgvpvdsrg
61 qpaedgefll lspvqaatwg nyyalgdsys sgdgardyyp gtavkggcwr sanaypelva
121 eaydfaghls flacsgqrgy amldaidevg sqldwnspht slvtigiggn dlgfstvlkt
181 cmvrvpllds kactdqedai rkrmakfett feelisevrt rapdarilvv gyprifpeep
241 tgayytltas nqrwlnetiq efnqqlaeav avhdeeiaas ggvgsvefvd vyhaldghei
301 gsdepwvngv qlrdlatgvt vdrstfhpna aghravgerv ieqietgpgr plyatfavva
361 gatvdtlage vg

Corynebacterium\effciens\ GDSx 300 aa

SEQ ID No. 61

1 mrttviaasa llllagcadg areetagapp gessggiree gaeastsitd vyialgdsya
61 amggrdqplr gepfclrssg nypellhaev tdltcqgavt gdlleprtlg ertlpaqvda
121 ltedttlvtl siggndlgfg evagcireri agenaddcvd llgetigeql dqlppqldrv
181 heairdragd aqvvvtgylp lvsagdcpel gdvseadrrw aveltgqine tvreaaerhd
241 alfvlpddad ehtscappqq rwadiqgqqt dayplhptsa gheamaaavr dalglepvqp
//

SEQ ID No. 62
nt

1 ttctggggtg ttatggggtt gttatcggct cgtcctgggt ggatcccgcc aggtggggta
61 ttcacggggg acttttgtgt ccaacagccg agaatgagtg ccctgagcgg tgggaatgag
121 gtgggcgggg ctgtgtcgcc atgagggggc ggcgggctct gtggtgcccc gcgacccccg
181 gccccggtga gcggtgaatg aaatccggct gtaatcagca tcccgtgccc accccgtcgg
241 ggaggtcagc gcccggagtg tctacgcagt cggatcctct cggactcggc catgctgtcg
301 gcagcatcgc gctcccgggt cttggcgtcc ctcggctgtt ctgcctgctg tccctggaag
361 gcgaaatgat caccggggag tgatacaccg gtggtctcat cccggatgcc cacttcggcg
421 ccatccggca attcgggcag ctccgggtgg aagtaggtgg catccgatgc gtcggtgacg
481 ccatagtggg cgaagatctc atcctgctcg agggtgctca ggccactctc cggatcgata
541 tcgggggcgt ccttgatggc gtccttgctg aaaccgaggt gcagcttgtg ggcttccaat
601 ttcgcaccac ggagcgggac gaggctggaa tgacggccga agagcccgtg gtggacctca

661 acgaaggtgg gtagtcccgt gtcatcattg aggaacacgc cctccaccgc acccagcttg
721 tggccggagt tgtcgtaggc gctggcatcc agaagggaaa cgatctcata tttgtcggtg
781 tgctcagaca tgatcttcct ttgctgtcgg tgtctggtac taccacggta gggctgaatg
841 caactgttat ttttctgtta ttttaggaat tggtccatat cccacaggct ggctgtggtc
901 aaatcgtcat caagtaatcc ctgtcacaca aaatgggtgg tgggagccct ggtcgcggtt
961 ccgtgggagg cgccgtgccc cgcaggatcg tcggcatcgg cggatctggc cggtaccccg
1021 cggtgaataa aatcattctg taaccttcat cacggttggt tttaggtatc cgccccttc
1081 gtcctgaccc cgtccccggc gcgcgggagc ccgcgggttg cggtagacag gggagacgtg
1141 gacaccatga ggacaacggt catcgcagca agcgcattac tccttctcgc cggatgcgcg
1201 gatggggccc gggaggagac cgccggtgca ccgccgggtg agtcctccgg gggcatccgg
1261 gaggaggggg cggaggcgtc gacaagcatc accgacgtct acatcgccct cggggattcc
1321 tatgcggcga tgggcgggcg ggatcagccg ttacggggtg agccgttctg cctgcgctcg
1381 tccggtaatt acccggaact cctccacgca gaggtcaccg atctcacctg ccaggggcg
1441 gtgaccgggg atctgctcga acccaggacg ctgggggagc gcacgctgcc ggcgcaggtg
1501 gatgcgctga cggaggacac caccctggtc accctctcca tcgggggcaa tgacctcgga
1561 ttcggggagg tggcgggatg catccgggaa cggatcgccg gggagaacgc tgatgattgc
1621 gtggacctgc tgggggaaac catcggggag cagctcgatc agcttccccc gcagctggac
1681 cgcgtgcacg aggctatccg ggaccgcgcc ggggacgcgc aggttgtggt caccggttac
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1801 cgttgggcgg ttgagctgac cgggcagatc aacgagaccg tgcgcgaggc ggccgaacga
1861 cacgatgccc tctttgtcct gcccgacgat gccgatgagc acaccagttg tgcacccccа
1921 cagcagcgct gggcggatat ccagggccaa cagaccgatg cctatccgct gcacccgacc
1981 tccgccggcc atgaggcgat ggccgccgcc gtccgggacg cgctgggcct ggaaccggtc
2041 cagccgtagc gccgggcgcg cgcttgtcga cgaccaaccc atgccaggct gcagtcacat
2101 ccgcacatag cgcgcgcggg cgatggagta cgcaccatag aggatgagcc cgatgccgac
2161 gatgatgagc agcacactgc cgaagggttg ttccccgagg gtgcgcagag ccgagtccag
2221 acctgcggcc tgctccggat catgggccca accggcgatg acgatcaaca cccccaggat
2281 cccgaaggcg ataccacggg cgacataacc ggctgttccg gtgatgatga tcgcggtccc
2341 gacctgccct gaccccgcac ccgcctccag atcctcccgg aaatcccggg tggccccctt
2401 ccagaggttg tagacacccg cccccagtac caccagcccg gcgaccacaa ccagcaccac
2461 accccagggt tgggatagga cggtggcggt gacatcggtg gcggtctccc catcggaggt
2521 gctgccgccc cgggcgaagg tggaggtggt caccgccagg gagaagtaga ccatggccat
2581 gaccgccccc ttggcccttt ccttgaggtc ctcgcccgcc agcagctggc tcaattgcca
2641 gagtcccagg gccgccaggg cgatgacggc aacccacagg aggaactgcc cacccggagc
2701 ctccgcgatg gtggccaggg cacctgaatt cgaggcctca tcacccgaac cgccggatcc
2761 agtggcgatg cgcaccgcga tccacccgat gaggatgtgc agtatgccca ggacaatgaa
2821 accacctctg gccagggtgg tcagcgcggg gtggtcctcg gcctggtcgg cagcccgttc
2881 gatcgtccgt ttcgcggatc tggtgtcgcc cttatccata gctcccattg aaccgccttg
2941 aggggtgggc ggccactgtc agggcggatt gtgatctgaa ctgtgatgtt ccatcaaccc
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Novosphingobium\aromaticivorans\ GDSx 284 aa

SEQ ID No. 63

ZP_00094165

1 mgqvklfarr capvllalag lapaatvare aplaegaryv algssfaagp gvgpnapgsp
61 ercgrgtlny phllaealkl dlvdatcsga tthhvlgpwn evppqidsvn gdtrlvtlti
121 ggndvsfvgn ifaaacekma spdprcgkwr eiteeewqad eermrsivrq iharaplarv
181 vvvdyitvlp psgtcaamai spdrlaqsrs aakrlarita rvareegasl lkfshisrrh
241 hpcsakpwsn glsapaddgi pvhpnrlgha eaaaalvklv klmk
//

SEQ ID No. 64

1 tgccggaact caagcggcgt ctagccgaac tcatgcccga aagcgcgtgg cactatcccg
61 aagaccaggt ctcggacgcc agcgagcgcc tgatggccgc cgaaatcacg cgcgaacagc
121 tctaccgcca gctccacgac gagctgccct atgacagtac cgtacgtccc gagaagtacc
181 tccatcgcaa ggacggttcg atcgagatcc accagcagat cgtgattgcc cgcgagacac
241 agcgtccgat cgtgctgggc aagggtggcg cgaagatcaa ggcgatcgga gaggccgcac
301 gcaaggaact ttcgcaattg ctcgacacca aggtgcacct gttcctgcat gtgaaggtcg
361 acgagcgctg ggccgacgcc aaggaaatct acgaggaaat cggcctcgaa tgggtcaagt
421 gaagctcttc gcgcgccgct gcgccccagt acttctcgcc cttgccgggc tggctccggc
481 ggctacggtc gcgcgggaag caccgctggc cgaaggcgcg cgttacgttg cgctgggaag
541 ctccttcgcc gcaggtccgg gcgtggggcc caacgcgccc ggatcgcccg aacgctgcgg
601 ccggggcacg ctcaactacc cgcacctgct cgccgaggcg ctcaagctcg atctcgtcga
661 tgcgacctgc agcggcgcga cgacccacca cgtgctgggc ccctggaacg aggttccccc
721 tcagatcgac agcgtgaatg gcgacacccg cctcgtcacc ctgaccatcg gcggaaacga
781 tgtgtcgttc gtcggcaaca tcttcgccgc cgcttgcgag aagatggcgt cgcccgatcc
841 gcgctgcggc aagtggcggg agatcaccga ggaagagtgg caggccgacg aggagcggat
901 gcgctccatc gtacgccaga tccacgcccg cgcgcctctc gcccgggtgg tggtggtcga
961 ttacatcacg gtcctgccgc catcaggcac ttgcgctgcc atggcgattt cgccggaccg
1021 gctggcccag agccgcagcg ccgcgaaacg gcttgcccgg attaccgcac gggtcgcgcg
1081 agaagagggt gcatcgctgc tcaagttctc gcatatctcg cgccggcacc atccatgctc
1141 tgccaagccc tggagcaacg gcctttccgc cccggccgac gacggcatcc cggtccatcc
1201 gaaccggctc ggacatgctg aagcggcagc ggcgctggtc aagcttgtga aattgatgaa
1261 gtagctactg cactgatttc aaatagtatt gcctgtcagc tttccagccc ggattgttgc
1321 agcgcaacag aaacttgtcc gtaatggatt gatggtttat gtcgctcgca aattgccgtc
1381 gaagggaacg ggcgcgtcgc tcgttaacgt cctgggtgca gcagtgacgg agcgcgtgga
1441 tgagtgatac tggcggtgtc atcggtgtac gcgccgccat tcccatgcct gtacgcgccg
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S.coelicolor\ GDSx 268 aa

SEQ ID No. 65

NP_625998.

1 mrrfrlvgfl sslvlaagaa ltgaataqaa qpaaadgyva lgdsyssgvg agsyisssgd
61 ckrstkahpy lwaaahspst fdftacsgar tgdvlsgqlg plssgtglvs isiggndagf
121 adtmttcvlq sessclsria taeayvdstl pgkldgvysa isdkapnahv vvigyprfyk
181 lgttciglse tkrtainkas dhlntvlaqr aaahgftfgd vrttftghel csgspwlhsv
241 nwlnigesyh ptaagqsggy lpvlngaa
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SEQ ID No. 66

1 cccggcggcc cgtgcaggag cagcagccgg cccgcgatgt cctcgggcgt cgtcttcatc
61 aggccgtcca tcgcgtcggc gaccggcgcc gtgtagttgg cccggacctc gtcccaggtg
121 cccgcggcga tctggcgggt ggtgcggtgc gggccgcgcc gaggggagac gtaccagaag
181 cccatcgtca cgttctccgg ctgcggttcg ggctcgtccg ccgctccgtc cgtcgcctcg
241 ccgagcacct tctcggcgag gtcggcgctg gtcgccgtca ccgtgacgtc ggcgccccgg
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361 tcgtcgcggg cgatccgcag cacgcgcgcg ccgggcggca gcagcgtggc gccggaccgt
421 acgcggtcga tgttcgccgc gtgcgagtac ggctgctcac ccgtggcgaa acggccgagg
481 aacagcgcgt cgacgacgtc ggacggggag tcgctgtcgt ccacgttgag ccggatcggc
541 agggcttcgt gcgggttcac ggacatgtcg ccatgatcgg gcacccggcc gccgcgtgca
601 cccgctttcc cgggcacgca cgacaggggc tttctcgccg tcttccgtcc gaacttgaac

661 gagtgtcagc catttcttgg catggacact tccagtcaac gcgcgtagct gctaccacgg
721 ttgtggcagc aatcctgcta agggaggttc catgagacgt ttccgacttg tcggcttcct
781 gagttcgctc gtcctcgccg ccggcgccgc cctcaccggg gcagcgaccg cccaggcggc
841 ccaacccgcc gccgccgacg gctatgtggc cctcggcgac tcctactcct ccggggtcgg
901 agcgggcagc tacatcagct cgagcggcga ctgcaagcgc agcacgaagg cccatcccta
961 cctgtgggcg gccgcccact cgccctccac gttcgacttc accgcctgtt ccggcgcccg
1021 tacgggtgat gttctctccg gacagctcgg cccgctcagc tccggcaccg gcctcgtctc
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1141 gtccgagagc tcctgcctgt cgcggatcgc caccgccgag gcgtacgtcg actcgacgct
1201 gcccggcaag ctcgacggcg tctactcggc aatcagcgac aaggcgccga acgcccacgt
1261 cgtcgtcatc ggctacccgc gcttctacaa gctcggcacc acctgcatcg gcctgtccga
1321 gaccaagcgg acggcgatca acaaggcctc cgaccacctc aacaccgtcc tcgcccagcg
1381 cgccgccgcc cacggcttca ccttcggcga cgtacgcacc accttcaccg gccacgagct
1441 gtgctccggc agcccctggc tgcacagcgt caactggctg aacatcggcg agtcgtacca
1501 ccccaccgcg gccggccagt ccggtggcta cctgccggtc ctcaacggcg ccgcctgacc
1561 tcaggcggaa ggagaagaag aaggagcgga gggagacgag gagtgggagg ccccgcccga
1621 cggggtcccc gtccccgtct ccgtctccgt cccggtcccg caagtcaccg agaacgccac
1681 cgcgtcggac gtggcccgca ccggactccg cacctccacg cgcacggcac tctcgaacgc
1741 gccggtgtcg tcgtgcgtcg tcaccaccac gccgtcctgg cgcgagcgct cgccgcccga
1801 cgggaaggac agcgtccgcc accccggatc ggagaccgac ccgtccgcgg tcacccaccg
1861 gtagccgacc tccgcgggca gccgcccgac cgtgaacgtc gccgtgaacg cgggtgcccg
1921 gtcgtgcggc ggcggacagg cccccgagta gtgggtgcgc gagcccacca cggtcacctc
1981 caccgactgc gctgcggggc

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S.avermitilis\ GDSx 269 aa

SEQ ID No. 67

NP_827753.

1 mrrsritayv tslllavgca ltgaataqas paaaatgyva lgdsyssgvg agsylsssgd
61 ckrsskaypy lwqaahspss fsfmacsgar tgdvlanqlg tlnsstglvs ltiggndagf
121 sdvmttcvlq sdsaclsrin takayvdstl pgqldsvyta istkapsahv avlgyprfyk
181 lggsclagls etkrsainda adylnsaiak raadhgftfg dvkstftghe icssstwlhs
241 ldllnigqsy hptaagqsgg ylpvmnsva

//

SEQ ID No. 68

1 ccaccgccgg gtcggcggcg agtctcctgg cctcggtcgc ggagaggttg gccgtgtagc
61 cgttcagcgc ggcgccgaac gtcttcttca ccgtgccgcc gtactcgttg atcaggccct
121 tgcccttgct cgacgcggcc ttgaagccgg tgcccttctt gagcgtgacg atgtagctgc
181 ccttgatcgc ggtgggggag ccggcggcga gcaccgtgcc ctcggccggg gtggcctggg
241 cgggcagtgc ggtgaatccg cccacgaggg cgccggtcgc cacggcggtt atcgcggcga
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421 gcgtcgtgtt ccgccctggg ttgagtaaag cctcggccat ctacgggggt ggctcaaggg
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541 ggggcgaccc cggaaagtgc cgagaagtct tggcatggac acttcctgtc aacacgcgta
601 gctggtacga cggttacggc agagatcctg ctaaagggag gttccatgag acgttcccga
661 attacggcat acgtgacctc actcctcctc gccgtcggct gcgccctcac cggggcagcg
721 acggcgcagg cgtccccagc cgccgcggcc acgggctatg tggccctcgg cgactcgtac
781 tcgtccggtg tcggcgccgg cagctacctc agctccagcg gcgactgcaa gcgcagttcg
841 aaggcctatc cgtacctctg gcaggccgcg cattcaccct cgtcgttcag tttcatggct
901 tgctcgggcg ctcgtacggg tgatgtcctg gccaatcagc tcggcaccct gaactcgtcc

961 accggcctgg tctccctcac catcggaggc aacgacgcgg gcttctccga cgtcatgacg
1021 acctgtgtgc tccagtccga cagcgcctgc ctctcccgca tcaacacggc gaaggcgtac
1081 gtcgactcca ccctgcccgg ccaactcgac agcgtgtaca cggcgatcag cacgaaggcc
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1321 ttcaccggcc atgagatctg ctccagcagc acctggctgc acagtctcga cctgctgaac
1381 atcggccagt cctaccaccc gaccgcggcc ggccagtccg gcggctatct gccggtcatg
1441 aacagcgtgg cctgagctcc cacggcctga atttttaagg cctgaatttt taaggcgaag
1501 gtgaaccgga agcggaggcc ccgtccgtcg gggtctccgt cgcacaggtc accgagaacg
1561 gcacggagtt ggacgtcgtg cgcaccgggt cgcgcacctc gacggcgatc tcgttcgaga
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1681 tcgccgggaa ggacagcgtc ttccagcccg gatccgggac ctcgcccttc ttggtcaccc
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1861 ccttcacgga ctgggccggc ggggtcgtcg taccgccgcc gccaccgccg cctcccggag
1921 tggagcccga gctgtggtcg cccccgccgt cggcgttgtc gtcctcgggg gttttcgaac
//

Streptomyces diglyceride:glycerol acyltransferase

SEQ ID No. 69

ACAGGCCGATGCACGGAACCGTACCTTTCCGCAGTGAAGCGCTCTCCCCCCATCGTTCGC
CGGGACTTCATCCGCGATTTTGGCATGAACACTTCCTTCAACGCGCGTAGCTTGCTACAA
GTGCGGCAGCAGACCCGCTCGTTGGAGGCTCAGTGAGATTGACCCGATCCCTGTCGGCCG
CATCCGTCATCGTCTTCGCCCTGCTGCTCGCGCTGCTGGGCATCAGCCCGGCCCAGGCAG
CCGGCCCGGCCTATGTGGCCCTGGGGGATTCCTATTCCTCGGGCAACGGCGCCGGAAGTT
ACATCGATTCGAGCGGTGACTGTCACCGCAGCAACAACGCGTACCCCGCCCGCTGGGCGG
CGGCCAACGCACCGTCCTCCTTCACCTTCGCGGCCTGCTCGGGAGCGGTGACCACGGATG
TGATCAACAATCAGCTGGGCGCCCTCAACGCGTCCACCGGCCTGGTGAGCATCACCATCG
GCGGCAATGACGCGGGCTTCGCGGACGCGATGACCACCTGCGTCACCAGCTCGGACAGCA
CCTGCCTCAACCGGCTGGCCACCGCCACCAACTACATCAACACCACCCTGCTCGCCCGGC
TCGACGCGGTCTACAGCCAGATCAAGGCCCGTGCCCCCAACGCCCGCGTGGTCGTCCTCG
GCTACCCGCGCATGTACCTGGCCTCGAACCCCTGGTACTGCCTGGGCCTGAGCAACACCA
AGCGCGCGGCCATCAACACCACCGCCGACACCCTCAACTCGGTGATCTCCTCCCGGGCCA
CCGCCCACGGATTCCGATTCGGCGATGTCCGCCCGACCTTCAACAACCACGAACTGTTCT
TCGGCAACGACTGGCTGCACTCACTCACCCTGCCGGTGTGGGAGTCGTACCACCCCACCA
GCACGGGCCATCAGAGCGGCTATCTGCCGGTCCTCAACGCCAACAGCTCGACCTGATCAA
CGCACGGCCGTGCCCGCCCCGCGCGTCACGCTCGGCGCGGGCGCCGCAGCGCGTTGATCA
GCCCACAGTGCCGGTGACGGTCCCACCGTCACGGTCGAGGGTGTACGTCACGGTGGCGCC
GCTCCAGAAGTGGAACGTCAGCAGGACCGTGGAGCCGTCCCTGACCTCGTCGAAGAACTC
CGGGGTCAGCGTGATCACCCCTCCCCCGTAGCCGGGGGCGAAGGCGGCGCCGAACTCCTT
GTAGGACGTCCAGTCGTGCGGCCCGGCGTTGCCACCGTCCGCGTAGACCGCTTCCATGGT
CGCCAGCCGGTCCCCGCGGAACTCGGTGGGGATGTCCGTGCCCAAGGTGGTCCCGGTGGT
GTCCGAGAGCACCGGGGGCTCGTACCGGATGATGTGCAGATCCAAAGAATT

Streptomyces diglyceride:glycerol acyltransferase

SEQ ID No. 70

MRLTRSLSAASVIVFALLLALLGISPAQAAGPAYVALGDSYSSGNGAGSYIDSSGDCHRSN
NAYPARWAAANAPSSFTFAACSGAVTTDVINNQLGALNASTGLVSITIGGNDAGFADAMTT
CVTSSDSTCLNRLATATNYINTTLLARLDAVYSQIKARAPNARVVVLGYPRMYLASNPWYC
LGLSNTKRAAINTTADTLNSVISSRATAHGFRFGDVRPTFNNHELFFGNDWLHSLTLPVWE
SYHPTSTGHQSGYLPVLNANSST

IDENTIFICATION OF A DIGLYCERIDE:GLYCEROL ACYLTRANSFERASE ACCORDING TO THE PRESENT INVENTION

Assay for enzymatic reduction of diglyceride in palm oil.

1 gram of palm oil containing 7% diglyceride is scaled in a glass with lid.
50 mg glycerol and 10 µl enzyme solution is added. The reaction mixture is agitated with a magnetic stirrer in a heating chamber at 40°C for 20 hours. The enzyme reaction is stopped by heating to 100°C for 10 minutes. A reference sample added 10µl water instead of enzyme solution is treated in the same way. The samples are analysed by GLC according to standard procedures (see hereinbelow) and the amount of fatty acids, monoglyceride and diglyceride are calculated.

Calculation:

From the results of the GLC analyses the increase in free fatty acids and the decrease in diglycerides can be calculated:

Δ % fatty acid = % Fatty acid(enzyme) - % fatty acid(control);

Mv Fa = average molecular weight of the fatty acids;

Δ % diglyceride = % Diglyceride(control) - % Diglyceride(enzyme);

Mv Di= average molecular weight of diglyceride;

The transferase activity is calculated as a percentage of the total enzymatic activity:

$$\% \text{ transferase activity} = = \frac{((\Delta\ \%\ Di/(Mv\ Di) - \Delta\ \%\ Fa/(Mv\ FA)) \times 100}{\Delta\ \%\ Di/(Mv\ Di)}$$

GLC analysis

Perkin Elmer Autosystem 9000 Capillary Gas Chromatograph equipped with WCOT fused silica column 12.5 m x 0.25 mm ID x 0.1 µ film thickness 5% phenyl-methyl-silicone (CP Sil 8 CB from Chrompack).

Carrier gas: Helium.

Injector. PSSI cold split injection (initial temp 50°C heated to 385°C), volume 1.0µl

Detector FID: 395°C

Oven program:	1	2	3
Oven temperature, °C.	90	280	350
Isothermal, time, min.	1	0	10
Temperature rate, °C/min.	15	4	

Sample preparation: 30 mg of sample was dissolved in 9 ml Heptane:Pyridin, 2:1 containing internal standard heptadecane, 0.5 mg/ml. 300µl sample solution was transferred to a crimp vial, 300 µl MSTFA (N-Methyl-N-trimethylsilyl-trifluoraceamid) was added and reacted for 20 minutes at 60°C.

ISOLATED

In one aspect, preferably the polypeptide or protein for use in the present invention is in an isolated form. The term "isolated" means that the sequence is at least substantially free from at least one other component with which the sequence is naturally associated in nature and as found in nature.

PURIFIED

In one aspect, preferably the polypeptide or protein for use in the present invention is in a purified form. The term "purified" means that the sequence is in a relatively pure state – e.g. at least about 51% pure, or at least about 75%, or at least about 80%, or at least about 90% pure, or at least about 95% pure or at least about 98% pure.

CLONING A NUCLEOTIDE SEQUENCE ENCODING A POLYPEPTIDE ACCORDING TO THE PRESENT INVENTION

A nucleotide sequence encoding either a polypeptide which has the specific properties as defined herein or a polypeptide which is suitable for modification may be isolated from any cell or organism producing said polypeptide. Various methods are well known within the art for the isolation of nucleotide sequences.

For example, a genomic DNA and/or cDNA library may be constructed using chromosomal DNA or messenger RNA from the organism producing the polypeptide. If the amino acid sequence of the polypeptide is known, labelled oligonucleotide probes may be synthesised and used to identify polypeptide-encoding clones from the genomic library prepared from the organism. Alternatively, a labelled oligonucleotide probe containing sequences homologous to another known polypeptide gene could be used to identify polypeptide-encoding clones. In the latter case, hybridisation and washing conditions of lower stringency are used.

Alternatively, polypeptide-encoding clones could be identified by inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming enzyme-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing an enzyme inhibited by the polypeptide, thereby allowing clones expressing the polypeptide to be identified.

In a yet further alternative, the nucleotide sequence encoding the polypeptide may be prepared synthetically by established standard methods, e.g. the phosphoroamidite method described by Beucage S.L. *et al* (1981) Tetrahedron Letters 22, p 1859-1869, or the method described by Matthes *et al* (1984) EMBO J. 3, p 801-805. In the phosphoroamidite method, oligonucleotides are synthesised, e.g. in an automatic DNA synthesiser, purified, annealed, ligated and cloned in appropriate vectors.

The nucleotide sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin, or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate) in accordance with standard techniques. Each ligated fragment corresponds to various parts of the entire nucleotide sequence. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or in Saiki R K *et al* (Science (1988) 239, pp 487-491).

NUCLEOTIDE SEQUENCES

The present invention also encompasses nucleotide sequences encoding polypeptides having the specific properties as defined herein. The term "nucleotide sequence" as used herein refers to an oligonucleotide sequence or polynucleotide sequence, and variant, homologues, fragments and derivatives thereof (such as portions thereof). The nucleotide sequence may be of genomic or synthetic or recombinant origin, which may be double-stranded or single-stranded whether representing the sense or antisense strand.

The term "nucleotide sequence" in relation to the present invention includes genomic DNA, cDNA, synthetic DNA, and RNA. Preferably it means DNA, more preferably cDNA for the coding sequence.

In a preferred embodiment, the nucleotide sequence *per se* encoding a polypeptide having the specific properties as defined herein does not cover the native nucleotide sequence in its natural environment when it is linked to its naturally associated sequence(s) that is/are also in its/their natural environment. For ease of reference, we shall call this preferred embodiment the "non-native nucleotide sequence". In this regard, the term "native nucleotide sequence" means an entire nucleotide sequence that is in its native environment and when operatively linked to an entire promoter with which it is naturally associated, which promoter is also in its native environment. Thus, the polypeptide of the present invention can be expressed by a nucleotide sequence in its native organism but

wherein the nucleotide sequence is not under the control of the promoter with which it is naturally associated within that organism.

Preferably the polypeptide is not a native polypeptide. In this regard, the term "native polypeptide" means an entire polypeptide that is in its native environment and when it has been expressed by its native nucleotide sequence.

Typically, the nucleotide sequence encoding polypeptides having the specific properties as defined herein is prepared using recombinant DNA techniques (i.e. recombinant DNA). However, in an alternative embodiment of the invention, the nucleotide sequence could be synthesised, in whole or in part, using chemical methods well known in the art (see Caruthers MH *et al* (1980) Nuc Acids Res Symp Ser 215-23 and Horn T *et al* (1980) Nuc Acids Res Symp Ser 225-232).

MOLECULAR EVOLUTION

Once an enzyme-encoding nucleotide sequence has been isolated, or a putative enzyme-encoding nucleotide sequence has been identified, it may be desirable to modify the selected nucleotide sequence, for example it may be desirable to mutate the sequence in order to prepare an enzyme in accordance with the present invention.

Mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites.

A suitable method is disclosed in Morinaga *et al* (Biotechnology (1984) 2, p646-649). Another method of introducing mutations into enzyme-encoding nucleotide sequences is described in Nelson and Long (Analytical Biochemistry (1989), 180, p 147-151).

Instead of site directed mutagenesis, such as described above, one can introduce mutations randomly for instance using a commercial kit such as the GeneMorph PCR mutagenesis kit from Stratagene, or the Diversify PCR random mutagenesis kit from Clontech. EP 0 583 265 refers to methods of optimising PCR based mutagenesis,

which can also be combined with the use of mutagenic DNA analogues such as those described in EP 0 866 796. Error prone PCR technologies are suitable for the production of variants of lipid acyl transferases with preferred characteristics. WO0206457 refers to molecular evolution of lipases.

A third method to obtain novel sequences is to fragment non-identical nucleotide sequences, either by using any number of restriction enzymes or an enzyme such as Dnase I, and reassembling full nucleotide sequences coding for functional proteins. Alternatively one can use one or multiple non-identical nucleotide sequences and introduce mutations during the reassembly of the full nucleotide sequence. DNA shuffling and family shuffling technologies are suitable for the production of variants of lipid acyl transferases with preferred characteristics. Suitable methods for performing 'shuffling' can be found in EP0 752 008, EP1 138 763, EP1 103 606. Shuffling can also be combined with other forms of DNA mutagenesis as described in US 6,180,406 and WO 01/34835.

Thus, it is possible to produce numerous site directed or random mutations into a nucleotide sequence, either *in vivo* or *in vitro*, and to subsequently screen for improved functionality of the encoded polypeptide by various means. Using in silico and exo mediated recombination methods (see WO 00/58517, US 6,344,328, US 6,361,974), for example, molecular evolution can be performed where the variant produced retains very low homology to known enzymes or proteins. Such variants thereby obtained may have significant structural analogy to known transferase enzymes, but have very low amino acid sequence homology.

As a non-limiting example, In addition, mutations or natural variants of a polynucleotide sequence can be recombined with either the wild type or other mutations or natural variants to produce new variants. Such new variants can also be screened for improved functionality of the encoded polypeptide.

The application of the above-mentioned and similar molecular evolution methods allows the identification and selection of variants of the enzymes of the present

invention which have preferred characteristics without any prior knowledge of protein structure or function, and allows the production of non-predictable but beneficial mutations or variants. There are numerous examples of the application of molecular evolution in the art for the optimisation or alteration of enzyme activity, such examples include, but are not limited to one or more of the following: optimised expression and/or activity in a host cell or in vitro, increased enzymatic activity, altered substrate and/or product specificity, increased or decreased enzymatic or structural stability, altered enzymatic activity/specificity in preferred environmental conditions, e.g. temperature, pH, substrate

As will be apparent to a person skilled in the art, using molecular evolution tools an enzyme may be altered to improve the functionality of the enzyme.

Suitably, the lipid acyltransferase used in the invention may be a variant, i.e. may contain at least one amino acid substitution, deletion or addition, when compared to a parental enzyme. Variant enzymes retain at least 20%, 30%, 40%, 50 %, 60%, 70%, 80%, 90%, 95%, 97%, 99% homology with the parent enzyme. Suitable parent enzymes may include any enzyme with esterase or lipase activity. Preferably, the parent enzyme aligns to the pfam00657 consensus sequence.

In a preferable embodiment a variant lipid acyltransferase enzyme retains or incorporates at least one or more of the pfam00657 consensus sequence amino acid residues found in the GDSx, GANDY and HPT blocks.

Enzymes, such as lipases with no or low lipid acyltransferase activity in an aqueous environment may be mutated using molecular evolution tools to introduce or enhance the transferase activity, thereby producing a lipid acyltransferase enzyme with significant transferase activity suitable for use in the compositions and methods of the present invention.

Suitably, the lipid acyltransferase for use in the invention may be a variant with enhanced enzyme activity on polar lipids, preferably phospholipids and/or glycolipids

when compared to the parent enzyme. Preferably, such variants also have low or no activity on lyso polar lipids. The enhanced activity on polar lipids, phospholipids and/or glycolipids may be the result of hydrolysis and/or transferase activity or a combination of both.

Variant lipid acyltransferases for use in the invention may have decreased activity on triglycerides, and/or monoglycerides and/or diglycerides compared with the parent enzyme.

Suitably the variant enzyme may have no activity on triglycerides and/or monoglycerides and/or diglycerides.

Alternatively, the variant enzyme for use in the invention may have increased activity on triglycerides, and/or may also have increased activity on one or more of the following, polar lipids, phospholipids, lecithin, phosphatidylcholine, glycolipids, digalactosyl monoglyceride, monogalactosyl monoglyceride.

Variants of lipid acyltransferases are known, and one or more of such variants may be suitable for use in the methods and uses according to the present invention and/or in the enzyme compositions according to the present invention. By way of example only, variants of lipid acyltransferases are described in the following references may be used in accordance with the present invention: Hilton & Buckley J Biol. Chem. 1991 Jan 15: 266 (2): 997-1000; Robertson *et al* J. Biol. Chem. 1994 Jan 21; 269(3):2146-50; Brumlik *et al* J. Bacteriol 1996 Apr; 178 (7): 2060-4; Peelman *et al* Protein Sci. 1998 Mar; 7(3):587-99.

AMINO ACID SEQUENCES

The present invention also encompasses amino acid sequences of polypeptides having the specific properties as defined herein.

As used herein, the term "amino acid sequence" is synonymous with the term "polypeptide" and/or the term "protein". In some instances, the term "amino acid sequence" is synonymous with the term "peptide".

The amino acid sequence may be prepared/isolated from a suitable source, or it may be made synthetically or it may be prepared by use of recombinant DNA techniques.

Suitably, the amino acid sequences may be obtained from the isolated polypeptides taught herein by standard techniques.

One suitable method for determining amino acid sequences from isolated polypeptides is as follows:

Purified polypeptide may be freeze-dried and 100 µg of the freeze-dried material may be dissolved in 50 µl of a mixture of 8 M urea and 0.4 M ammonium hydrogen carbonate, pH 8.4. The dissolved protein may be denatured and reduced for 15 minutes at 50°C following overlay with nitrogen and addition of 5 µl of 45 mM dithiothreitol. After cooling to room temperature, 5 µl of 100 mM iodoacetamide may be added for the cysteine residues to be derivatized for 15 minutes at room temperature in the dark under nitrogen.

135 µl of water and 5 µg of endoproteinase Lys-C in 5 µl of water may be added to the above reaction mixture and the digestion may be carried out at 37°C under nitrogen for 24 hours.

The resulting peptides may be separated by reverse phase HPLC on a VYDAC C18 column (0.46x15cm;10µm; The Separation Group, California, USA) using solvent A: 0.1% TFA in water and solvent B: 0.1% TFA in acetonitrile. Selected peptides may be re-chromatographed on a Develosil C18 column using the same solvent system, prior to N-terminal sequencing. Sequencing may be done using an Applied Biosystems 476A sequencer using pulsed liquid fast cycles according to the manufacturer's instructions (Applied Biosystems, California, USA).

SEQUENCE IDENTITY OR SEQUENCE HOMOLOGY

The present invention also encompasses the use of sequences having a degree of sequence identity or sequence homology with amino acid sequence(s) of a polypeptide having the specific properties defined herein or of any nucleotide sequence encoding such a polypeptide (hereinafter referred to as a "homologous sequence(s)"). Here, the term "homologue" means an entity having a certain homology with the subject amino acid sequences and the subject nucleotide sequences. Here, the term "homology" can be equated with "identity".

The homologous amino acid sequence and/or nucleotide sequence should provide and/or encode a polypeptide which retains the functional activity and/or enhances the activity of the enzyme.

In the present context, a homologous sequence is taken to include an amino acid sequence which may be at least 75, 85 or 90% identical, preferably at least 95 or 98% identical to the subject sequence. Typically, the homologues will comprise the same active sites etc. as the subject amino acid sequence. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express homology in terms of sequence identity.

In the present context, a homologous sequence is taken to include a nucleotide sequence which may be at least 75, 85 or 90% identical, preferably at least 95 or 98% identical to a nucleotide sequence encoding a polypeptide of the present invention (the subject sequence). Typically, the homologues will comprise the same sequences that code for the active sites etc. as the subject sequence. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express homology in terms of sequence identity.

Homology comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate % homology between two or more sequences.

% homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence is directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues.

Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalising unduly the overall homology score. This is achieved by inserting "gaps" in the sequence alignment to try to maximise local homology.

However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. "Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

Calculation of maximum % homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (Devereux *et al* 1984 Nuc. Acids Research 12 p387). Examples of other software that can perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel *et al* 1999 Short Protocols in Molecular Biology, 4th Ed – Chapter 18), FASTA (Altschul *et al* 1990 J. Mol. Biol. 403-410) and the GENEWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching (see Ausubel *et al* 1999, pages 7-58 to 7-60). However, for some applications, it is preferred to use the GCG Bestfit program. A new tool, called BLAST 2 Sequences is also available for comparing protein and nucleotide sequence (see FEMS Microbiol Lett 1999 174(2): 247-50; FEMS Microbiol Lett 1999 177(1): 187-8 and tatiana@ncbi.nlm.nih.gov).

Although the final % homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table if supplied (see user manual for further details). For some applications, it is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

Alternatively, percentage homologies may be calculated using the multiple alignment feature in DNASIS™ (Hitachi Software), based on an algorithm, analogous to CLUSTAL (Higgins DG & Sharp PM (1988), *Gene* **73**(1), 237-244).

Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

The sequences may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent substance. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the secondary binding activity of the substance is retained. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, and tyrosine.

Conservative substitutions may be made, for example according to the Table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar – uncharged	C S T M
		N Q
	Polar – charged	D E
		K R
AROMATIC		H F W Y

The present invention also encompasses homologous substitution (substitution and replacement are both used herein to mean the interchange of an existing amino acid residue, with an alternative residue) that may occur i.e. like-for-like substitution such as basic for basic, acidic for acidic, polar for polar etc. Non-homologous substitution may also occur i.e. from one class of residue to another or alternatively involving the inclusion of unnatural amino acids such as ornithine (hereinafter referred to as Z),

diaminobutyric acid ornithine (hereinafter referred to as B), norleucine ornithine (hereinafter referred to as O), pyriylalanine, thienylalanine, naphthylalanine and phenylglycine.

Replacements may also be made by unnatural amino acids.

Variant amino acid sequences may include suitable spacer groups that may be inserted between any two amino acid residues of the sequence including alkyl groups such as methyl, ethyl or propyl groups in addition to amino acid spacers such as glycine or β-alanine residues. A further form of variation, involves the presence of one or more amino acid residues in peptoid form, will be well understood by those skilled in the art. For the avoidance of doubt, "the peptoid form" is used to refer to variant amino acid residues wherein the α-carbon substituent group is on the residue's nitrogen atom rather than the α-carbon. Processes for preparing peptides in the peptoid form are known in the art, for example Simon RJ et al., PNAS (1992) 89(20), 9367-9371 and Horwell DC, Trends Biotechnol. (1995) 13(4), 132-134.

Nucleotide sequences for use in the present invention or encoding a polypeptide having the specific properties defined herein may include within them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones and/or the addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the nucleotide sequences described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or life span of nucleotide sequences.

The present invention also encompasses the use of nucleotide sequences that are complementary to the sequences discussed herein, or any derivative, fragment or derivative thereof. If the sequence is complementary to a fragment thereof then that sequence can be used as a probe to identify similar coding sequences in other organisms etc.

Polynucleotides which are not 100% homologous to the sequences of the present invention but fall within the scope of the invention can be obtained in a number of ways. Other variants of the sequences described herein may be obtained for example by probing DNA libraries made from a range of individuals, for example individuals from different populations. In addition, other viral/bacterial, or cellular homologues particularly cellular homologues found in mammalian cells (e.g. rat, mouse, bovine and primate cells), may be obtained and such homologues and fragments thereof in general will be capable of selectively hybridising to the sequences shown in the sequence listing herein. Such sequences may be obtained by probing cDNA libraries made from or genomic DNA libraries from other animal species, and probing such libraries with probes comprising all or part of any one of the sequences in the attached sequence listings under conditions of medium to high stringency. Similar considerations apply to obtaining species homologues and allelic variants of the polypeptide or nucleotide sequences of the invention.

Variants and strain/species homologues may also be obtained using degenerate PCR which will use primers designed to target sequences within the variants and homologues encoding conserved amino acid sequences within the sequences of the present invention. Conserved sequences can be predicted, for example, by aligning the amino acid sequences from several variants/homologues. Sequence alignments can be performed using computer software known in the art. For example the GCG Wisconsin PileUp program is widely used.

The primers used in degenerate PCR will contain one or more degenerate positions and will be used at stringency conditions lower than those used for cloning sequences with single sequence primers against known sequences.

Alternatively, such polynucleotides may be obtained by site directed mutagenesis of characterised sequences. This may be useful where for example silent codon sequence changes are required to optimise codon preferences for a particular host cell in which the polynucleotide sequences are being expressed. Other sequence changes may be desired

in order to introduce restriction polypeptide recognition sites, or to alter the property or function of the polypeptides encoded by the polynucleotides.

Polynucleotides (nucleotide sequences) of the invention may be used to produce a primer, e.g. a PCR primer, a primer for an alternative amplification reaction, a probe e.g. labelled with a revealing label by conventional means using radioactive or non-radioactive labels, or the polynucleotides may be cloned into vectors. Such primers, probes and other fragments will be at least 15, preferably at least 20, for example at least 25, 30 or 40 nucleotides in length, and are also encompassed by the term polynucleotides of the invention as used herein.

Polynucleotides such as DNA polynucleotides and probes according to the invention may be produced recombinantly, synthetically, or by any means available to those of skill in the art. They may also be cloned by standard techniques.

In general, primers will be produced by synthetic means, involving a stepwise manufacture of the desired nucleic acid sequence one nucleotide at a time. Techniques for accomplishing this using automated techniques are readily available in the art.

Longer polynucleotides will generally be produced using recombinant means, for example using a PCR (polymerase chain reaction) cloning techniques. This will involve making a pair of primers (e.g. of about 15 to 30 nucleotides) flanking a region of the lipid targeting sequence which it is desired to clone, bringing the primers into contact with mRNA or cDNA obtained from an animal or human cell, performing a polymerase chain reaction under conditions which bring about amplification of the desired region, isolating the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector.

HYBRIDISATION

The present invention also encompasses sequences that are complementary to the sequences of the present invention or sequences that are capable of hybridising either to the sequences of the present invention or to sequences that are complementary thereto.

The term "hybridisation" as used herein shall include "the process by which a strand of nucleic acid joins with a complementary strand through base pairing" as well as the process of amplification as carried out in polymerase chain reaction (PCR) technologies.

The present invention also encompasses the use of nucleotide sequences that are capable of hybridising to the sequences that are complementary to the subject sequences discussed herein, or any derivative, fragment or derivative thereof.

The present invention also encompasses sequences that are complementary to sequences that are capable of hybridising to the nucleotide sequences discussed herein.

Hybridisation conditions are based on the melting temperature (Tm) of the nucleotide binding complex, as taught in Berger and Kimmel (1987, Guide to Molecular Cloning Techniques, Methods in Enzymology, Vol. 152, Academic Press, San Diego CA), and confer a defined "stringency" as explained below.

Maximum stringency typically occurs at about Tm-5°C (5°C below the Tm of the probe); high stringency at about 5°C to 10°C below Tm; intermediate stringency at about 10°C to 20°C below Tm; and low stringency at about 20°C to 25°C below Tm. As will be understood by those of skill in the art, a maximum stringency hybridisation can be used to identify or detect identical nucleotide sequences while an intermediate (or low) stringency hybridisation can be used to identify or detect similar or related polynucleotide sequences.

Preferably, the present invention encompasses sequences that are complementary to sequences that are capable of hybridising under high stringency conditions or

intermediate stringency conditions to nucleotide sequences encoding polypeptides having the specific properties as defined herein.

More preferably, the present invention encompasses sequences that are complementary to sequences that are capable of hybridising under high stringent conditions (e.g. 65°C and 0.1xSSC {1xSSC = 0.15 M NaCl, 0.015 M Na-citrate pH 7.0}) to nucleotide sequences encoding polypeptides having the specific properties as defined herein.

The present invention also relates to nucleotide sequences that can hybridise to the nucleotide sequences discussed herein (including complementary sequences of those discussed herein).

The present invention also relates to nucleotide sequences that are complementary to sequences that can hybridise to the nucleotide sequences discussed herein (including complementary sequences of those discussed herein).

Also included within the scope of the present invention are polynucleotide sequences that are capable of hybridising to the nucleotide sequences discussed herein under conditions of intermediate to maximal stringency.

In a preferred aspect, the present invention covers nucleotide sequences that can hybridise to the nucleotide sequences discussed herein, or the complement thereof, under stringent conditions (e.g. 50°C and 0.2xSSC).

In a more preferred aspect, the present invention covers nucleotide sequences that can hybridise to the nucleotide sequences discussed herein, or the complement thereof, under high stringent conditions (e.g. 65°C and 0.1xSSC).

EXPRESSION OF POLYPEPTIDES

A nucleotide sequence for use in the present invention or for encoding a polypeptide having the specific properties as defined herein can be incorporated into a recombinant

replicable vector. The vector may be used to replicate and express the nucleotide sequence, in polypeptide form, in and/or from a compatible host cell. Expression may be controlled using control sequences which include promoters/enhancers and other expression regulation signals. Prokaryotic promoters and promoters functional in eukaryotic cells may be used. Tissue specific or stimuli specific promoters may be used. Chimeric promoters may also be used comprising sequence elements from two or more different promoters described above.

The polypeptide produced by a host recombinant cell by expression of the nucleotide sequence may be secreted or may be contained intracellularly depending on the sequence and/or the vector used. The coding sequences can be designed with signal sequences which direct secretion of the substance coding sequences through a particular prokaryotic or eukaryotic cell membrane.

EXPRESSION VECTOR

The term "expression vector" means a construct capable of *in vivo* or *in vitro* expression.

Preferably, the expression vector is incorporated in the genome of the organism. The term "incorporated" preferably covers stable incorporation into the genome.

The nucleotide sequence of the present invention or coding for a polypeptide having the specific properties as defined herein may be present in a vector, in which the nucleotide sequence is operably linked to regulatory sequences such that the regulatory sequences are capable of providing the expression of the nucleotide sequence by a suitable host organism, i.e. the vector is an expression vector.

The vectors of the present invention may be transformed into a suitable host cell as described below to provide for expression of a polypeptide having the specific properties as defined herein.

The choice of vector, e.g. plasmid, cosmid, virus or phage vector, will often depend on the host cell into which it is to be introduced.

The vectors may contain one or more selectable marker genes – such as a gene which confers antibiotic resistance e.g. ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Alternatively, the selection may be accomplished by co-transformation (as described in WO91/17243).

Vectors may be used *in vitro*, for example for the production of RNA or used to transfect or transform a host cell.

Thus, in a further embodiment, the invention provides a method of making nucleotide sequences of the present invention or nucleotide sequences encoding polypeptides having the specific properties as defined herein by introducing a nucleotide sequence into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector.

The vector may further comprise a nucleotide sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

REGULATORY SEQUENCES

In some applications, a nucleotide sequence for use in the present invention or a nucleotide sequence encoding a polypeptide having the specific properties as defined herein may be operably linked to a regulatory sequence which is capable of providing for the expression of the nucleotide sequence, such as by the chosen host cell. By way of example, the present invention covers a vector comprising the nucleotide sequence of the present invention operably linked to such a regulatory sequence, i.e. the vector is an expression vector.

The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

The term "regulatory sequences" includes promoters and enhancers and other expression regulation signals.

The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase binding site.

Enhanced expression of the nucleotide sequence encoding the enzyme having the specific properties as defined herein may also be achieved by the selection of heterologous regulatory regions, e.g. promoter, secretion leader and terminator regions.

Preferably, the nucleotide sequence of the present invention may be operably linked to at least a promoter.

Examples of suitable promoters for directing the transcription of the nucleotide sequence in a bacterial, fungal or yeast host are well known in the art.

CONSTRUCTS

The term "construct" - which is synonymous with terms such as "conjugate", "cassette" and "hybrid" - includes a nucleotide sequence encoding a polypeptide having the specific properties as defined herein for use according to the present invention directly or indirectly attached to a promoter. An example of an indirect attachment is the provision of a suitable spacer group such as an intron sequence, such as the Sh1-intron or the ADH intron, intermediate the promoter and the nucleotide sequence of the present invention. The same is true for the term "fused" in relation to the present invention which includes

direct or indirect attachment. In some cases, the terms do not cover the natural combination of the nucleotide sequence coding for the protein ordinarily associated with the wild type gene promoter and when they are both in their natural environment.

The construct may even contain or express a marker which allows for the selection of the genetic construct.

For some applications, preferably the construct comprises at least a nucleotide sequence of the present invention or a nucleotide sequence encoding a polypeptide having the specific properties as defined herein operably linked to a promoter.

HOST CELLS

The term "host cell" - in relation to the present invention includes any cell that comprises either a nucleotide sequence encoding a polypeptide having the specific properties as defined herein or an expression vector as described above and which is used in the recombinant production of a polypeptide having the specific properties as defined herein.

Thus, a further embodiment of the present invention provides host cells transformed or transfected with a nucleotide sequence of the present invention or a nucleotide sequence that expresses a polypeptide having the specific properties as defined herein. The cells will be chosen to be compatible with the said vector and may for example be prokaryotic (for example bacterial), fungal, yeast or plant cells. Preferably, the host cells are not human cells.

Examples of suitable bacterial host organisms are gram negative bacterium or gram positive bacteria.

Depending on the nature of the nucleotide sequence encoding a polypeptide having the specific properties as defined herein, and/or the desirability for further processing of the expressed protein, eukaryotic hosts such as yeasts or other fungi may be preferred.

In general, yeast cells are preferred over fungal cells because they are easier to manipulate. However, some proteins are either poorly secreted from the yeast cell, or in some cases are not processed properly (e.g. hyperglycosylation in yeast). In these instances, a different fungal host organism should be selected.

The use of suitable host cells, such as yeast, fungal and plant host cells – may provide for post-translational modifications (e.g. myristoylation, glycosylation, truncation, lapidation and tyrosine, serine or threonine phosphorylation) as may be needed to confer optimal biological activity on recombinant expression products of the present invention.

The host cell may be a protease deficient or protease minus strain.

ORGANISM

The term "organism" in relation to the present invention includes any organism that could comprise a nucleotide sequence according to the present invention or a nucleotide sequence encoding for a polypeptide having the specific properties as defined herein and/or products obtained therefrom.

Suitable organisms may include a prokaryote, fungus, yeast or a plant.

The term "transgenic organism" in relation to the present invention includes any organism that comprises a nucleotide sequence coding for a polypeptide having the specific properties as defined herein and/or the products obtained therefrom, and/or wherein a promoter can allow expression of the nucleotide sequence coding for a polypeptide having the specific properties as defined herein within the organism. Preferably the nucleotide sequence is incorporated in the genome of the organism.

The term “transgenic organism” does not cover native nucleotide coding sequences in their natural environment when they are under the control of their native promoter which is also in its natural environment.

Therefore, the transgenic organism of the present invention includes an organism comprising any one of, or combinations of, a nucleotide sequence coding for a polypeptide having the specific properties as defined herein, constructs as defined herein, vectors as defined herein, plasmids as defined herein, cells as defined herein, or the products thereof. For example the transgenic organism can also comprise a nucleotide sequence coding for a polypeptide having the specific properties as defined herein under the control of a heterologous promoter.

TRANSFORMATION OF HOST CELLS/ORGANISM

As indicated earlier, the host organism can be a prokaryotic or a eukaryotic organism. Examples of suitable prokaryotic hosts include *E. coli* and *Bacillus subtilis*.

Teachings on the transformation of prokaryotic hosts is well documented in the art, for example see Sambrook *et al* (Molecular Cloning: A Laboratory Manual, 2nd edition, 1989, Cold Spring Harbor Laboratory Press). If a prokaryotic host is used then the nucleotide sequence may need to be suitably modified before transformation - such as by removal of introns.

In another embodiment the transgenic organism can be a yeast.

Filamentous fungi cells may be transformed using various methods known in the art – such as a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known. The use of *Aspergillus* as a host microorganism is described in EP 0 238 023.

Another host organism can be a plant. A review of the general techniques used for transforming plants may be found in articles by Potrykus (*Annu Rev Plant Physiol Plant Mol Biol* [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27). Further teachings on plant transformation may be found in EP-A-0449375.

General teachings on the transformation of fungi, yeasts and plants are presented in following sections.

TRANSFORMED FUNGUS

A host organism may be a fungus - such as a filamentous fungus. Examples of suitable such hosts include any member belonging to the genera Thermomyces, Acremonium, Aspergillus, Penicillium, Mucor, Neurospora, Trichoderma and the like.

Teachings on transforming filamentous fungi are reviewed in US-A-5741665 which states that standard techniques for transformation of filamentous fungi and culturing the fungi are well known in the art. An extensive review of techniques as applied to *N. crassa* is found, for example in Davis and de Serres, *Methods Enzymol* (1971) **17A**: 79-143.

Further teachings on transforming filamentous fungi are reviewed in US-A-5674707.

In one aspect, the host organism can be of the genus *Aspergillus*, such as *Aspergillus niger*.

A transgenic *Aspergillus* according to the present invention can also be prepared by following, for example, the teachings of Turner G. 1994 (Vectors for genetic manipulation. In: Martinelli S.D., Kinghorn J.R. (Editors) *Aspergillus*: 50 years on. Progress in industrial microbiology vol 29. Elsevier Amsterdam 1994. pp. 641-666).

Gene expression in filamentous fungi has been reviewed in Punt *et al.* (2002) Trends Biotechnol 2002 May; 20(5):200-6, Archer & Peberdy Crit Rev Biotechnol (1997) 17(4):273-306.

TRANSFORMED YEAST

In another embodiment, the transgenic organism can be a yeast.

A review of the principles of heterologous gene expression in yeast are provided in, for example, *Methods Mol Biol* (1995), 49:341-54, and *Curr Opin Biotechnol* (1997) Oct;8(5):554-60

In this regard, yeast – such as the species *Saccharomyces cerevisi or Pichia pastoris* (see FEMS Microbiol Rev (2000 24(1):45-66), may be used as a vehicle for heterologous gene expression.

A review of the principles of heterologous gene expression in *Saccharomyces cerevisiae* and secretion of gene products is given by E Hinchcliffe E Kenny (1993, "Yeast as a vehicle for the expression of heterologous genes", *Yeasts*, Vol **5**, Anthony H Rose and J Stuart Harrison, eds, 2nd edition, Academic Press Ltd.).

For the transformation of yeast, several transformation protocols have been developed. For example, a transgenic Saccharomyces according to the present invention can be prepared by following the teachings of Hinnen *et al.*, (1978, *Proceedings of the National Academy of Sciences of the USA* **75**, 1929); Beggs, J D (1978, *Nature,* London, **275**, 104); and Ito, H *et al* (1983, J Bacteriology 153, 163-168).

The transformed yeast cells may be selected using various selective markers – such as auxotrophic markers dominant antibiotic resistance markers.

A suitable yeast host organism can be selected from the biotechnologically relevant yeasts species such as, but not limited to, yeast species selected from *Pichia* spp., *Hansenula* spp., *Kluyveromyces, Yarrowinia* spp., *Saccharomyces* spp., including *S. cerevisiae,* or *Schizosaccharomyce* spp. including *Schizosaccharomyce pombe*.

A strain of the methylotrophic yeast species *Pichia pastoris* may be used as the host organism.

In one embodiment, the host organism may be a *Hansenula* species, such as *H. polymorpha* (as described in WO01/39544).

TRANSFORMED PLANTS/PLANT CELLS

A host organism suitable for the present invention may be a plant. A review of the general techniques may be found in articles by Potrykus (*Annu Rev Plant Physiol Plant Mol Biol* [1991] **42**:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27), or in WO01/16308. The transgenic plant may produce enhanced levels of phytosterol esters and phytostanol esters, for example.

Therefore the present invention also relates to a method for the production of a transgenic plant with enhanced levels of phytosterol esters and phytostanol esters, comprising the steps of transforming a plant cell with a lipid acyltransferase as defined herein (in particular with an expression vector or construct comprising a lipid acyltransferase as defined herein), and growing a plant from the transformed plant cell.

SECRETION

Often, it is desirable for the polypeptide to be secreted from the expression host into the culture medium from where the enzyme may be more easily recovered. According to the present invention, the secretion leader sequence may be selected on the basis of the desired expression host. Hybrid signal sequences may also be used with the context of the present invention.

Typical examples of heterologous secretion leader sequences are those originating from the fungal amyloglucosidase (AG) gene (*gla*A - both 18 and 24 amino acid versions e.g. from *Aspergillus*), the a-factor gene (yeasts e.g. *Saccharomyces, Kluyveromyces* and *Hansenula*) or the α-amylase gene (*Bacillus*).

DETECTION

A variety of protocols for detecting and measuring the expression of the amino acid sequence are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS).

A wide variety of labels and conjugation techniques are known by those skilled in the art and can be used in various nucleic and amino acid assays.

A number of companies such as Pharmacia Biotech (Piscataway, NJ), Promega (Madison, WI), and US Biochemical Corp (Cleveland, OH) supply commercial kits and protocols for these procedures.

Suitable reporter molecules or labels include those radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles and the like. Patents teaching the use of such labels include US-A-3,817,837; US-A-3,850,752; US-A-3,939,350; US-A-3,996,345; US-A-4,277,437; US-A-4,275,149 and US-A-4,366,241.

Also, recombinant immunoglobulins may be produced as shown in US-A-4,816,567.

FUSION PROTEINS

A polypeptide having the specific properties as defined herein may be produced as a fusion protein, for example to aid in extraction and purification thereof. Examples of fusion protein partners include glutathione-S-transferase (GST), 6xHis, GAL4 (DNA binding and/or transcriptional activation domains) and β-galactosidase. It may also be convenient to include a proteolytic cleavage site between the fusion protein partner and the protein sequence of interest to allow removal of fusion protein sequences. Preferably the fusion protein will not hinder the activity of the protein sequence.

Gene fusion expression systems in *E. coli* have been reviewed in Curr. Opin. Biotechnol. (1995) 6(5):501-6.

In another embodiment of the invention, the amino acid sequence of a polypeptide having the specific properties as defined herein may be ligated to a heterologous sequence to encode a fusion protein. For example, for screening of peptide libraries for agents capable of affecting the substance activity, it may be useful to encode a chimeric substance expressing a heterologous epitope that is recognized by a commercially available antibody.

The invention will now be described, by way of example only, with reference to the following Figures and Examples.

Figure 1 shows a pfam00657 consensus sequence from database version 6 (SEQ ID No. 1);

Figure 2 shows an amino acid sequence (SEQ ID No. 2) obtained from the organism *Aeromonas hydrophila* (P10480; GI:121051);

Figure 3 shows an amino acid sequence (SEQ ID No. 3) obtained from the organism *Aeromonas salmonicida* (AAG098404; GI:9964017);

Figure 4 shows an amino acid sequence (SEQ ID No. 4) obtained from the organism *Streptomyces coelicolor* A3(2) (Genbank accession number NP_631558);

Figure 5 shows an amino acid sequence (SEQ ID No. 5) obtained from the organism *Streptomyces coelicolor* A3(2) (Genbank accession number: CAC42140);

Figure 6 shows an amino acid sequence (SEQ ID No. 6) obtained from the organism *Saccharomyces cerevisiae* (Genbank accession number P41734);

Figure 7 shows an alignment of selected sequences to pfam00657 consensus sequence;

Figure 8 shows a pairwise alignment of SEQ ID No. 3 with SEQ ID No. 2 showing 93% amino acid sequence identity. The signal sequence is underlined. + denotes differences. The GDSX motif containing the active site serine 16, and the active sites aspartic acid 116 and histidine 291 are highlighted (see shaded regions). Numbers after the amino acid is minus the signal sequence;

Figure 9 shows a nucleotide sequence (SEQ ID No. 7) encoding a lipid acyl transferase according to the present invention obtained from the organism *Aeromonas hydrophila;*

Figure 10 shows a nucleotide sequence (SEQ ID No. 8) encoding a lipid acyl transferase according to the present invention obtained from the organism *Aeromonas salmonicida*;

Figure 11 shows a nucleotide sequence (SEQ ID No. 9) encoding a lipid acyl transferase according to the present invention obtained from the organism *Streptomyces coelicolor* A3(2) (Genbank accession number NC_003888.1:8327480..8328367);

Figure 12 shows a nucleotide sequence (SEQ ID No. 10) encoding a lipid acyl transferase according to the present invention obtained from the organism *Streptomyces coelicolor* A3(2) (Genbank accession number AL939131.1:265480..266367);

Figure 13 shows a nucleotide sequence (SEQ ID No. 11) encoding a lipid acyl transferase according to the present invention obtained from the organism *Saccharomyces cerevisiae* (Genbank accession number Z75034);

Figure 14 shows an amino acid sequence (SEQ ID No. 12) obtained from the organism *Ralstonia* (Genbank accession number: AL646052);

Figure 15 shows a nucleotide sequence (SEQ ID No. 13) encoding a lipid acyl transferase according to the present invention obtained from the organism *Ralstonia*;

Figure 16 shows SEQ ID No. 20. Scoe1 NCBI protein accession code CAB39707.1 GI:4539178 conserved hypothetical protein [Streptomyces coelicolor A3(2)];

Figure 17 shows a nucleotide sequence shown as SEQ ID No. 21 encoding NCBI protein accession code CAB39707.1 GI:4539178 conserved hypothetical protein [Streptomyces coelicolor A3(2)];

Figure 18 shows an amino acid shown as SEQ ID No.22. Scoe2 NCBI protein accession code CAC01477.1 GI:9716139 conserved hypothetical protein [Streptomyces coelicolor A3(2)];

Figure 19 shows a nucleotide sequence shown as SEQ ID No. 23 encoding Scoe2 NCBI protein accession code CAC01477.1 GI:9716139 conserved hypothetical protein [Streptomyces coelicolor A3(2)];

Figure 20 shows an amino acid sequence (SEQ ID No.24) Scoe3 NCBI protein accession code CAB88833.1 GI:7635996 putative secreted protein. [Streptomyces coelicolor A3(2)];

Figure 21 shows a nucleotide sequence shown as SEQ ID No. 25 encoding Scoe3 NCBI protein accession code CAB88833.1 GI:7635996 putative secreted protein. [Streptomyces coelicolor A3(2)];

Figure 22 shows an amino acid sequence (SEQ ID No.26) Scoe4 NCBI protein accession code CAB89450.1 GI:7672261 putative secreted protein. [Streptomyces coelicolor A3(2)];

Figure 23 shows an nucleotide sequence shown as SEQ ID No. 27 encoding Scoe4 NCBI protein accession code CAB89450.1 GI:7672261 putative secreted protein. [Streptomyces coelicolor A3(2)];

Figure 24 shows an amino acid sequence (SEQ ID No.28) Scoe5 NCBI protein accession code CAB62724.1 GI:6562793 putative lipoprotein [Streptomyces coelicolor A3(2)];

Figure 25 shows a nucleotide sequence shown as SEQ ID No. 29, encoding Scoe5 NCBI protein accession code CAB62724.1 GI:6562793 putative lipoprotein [Streptomyces coelicolor A3(2)];

Figure 26 shows an amino acid sequence (SEQ ID No.30) Srim1 NCBI protein accession code AAK84028.1 GI:15082088 GDSL-lipase [Streptomyces rimosus];

Figure 27 shows a nucleotide sequence shown as SEQ ID No. 31 encoding Srim1 NCBI protein accession code AAK84028.1 GI:15082088 GDSL-lipase [Streptomyces rimosus];

Figure 28 shows an amino acid sequence (SEQ ID No.32)A lipid acyl transferase from *Aeromonas hydrophila* (ATCC #7965);

Figure 29 shows a nucleotide sequence (SEQ ID No. 33) encoding a lipid acyltransferase from *Aeromonas hydrophila* (ATCC #7965);

Figure 30 shows an amino acid sequence (SEQ ID No.34) of a lipid acyltransferase from *Aeromonas salmonicida* subsp. *Salmonicida* (ATCC#14174);

Figure 31 shows a nucleotide sequence (SEQ ID No 35) encoding a lipid acyltransferase from *Aeromonas salmonicida* subsp. *Salmonicida* (ATCC#14174);

Figure 32 shows that homologues of the *Aeromonas* genes can be identified using the basic local alignment search tool service at the National Center for Biotechnology Information, NIH, MD, USA and the completed genome databases. The GDSX motif was used in the database search and a number of sequences/genes potentially encoding enzymes with lipolytic activity were identified. Genes were identified from the genus Streptomyces, Xanthomonas and Ralstonia. As an example below, the Ralstonia solanacearum was aligned to the Aeromonas salmonicida (satA) gene. Pairwise alignment showed 23% identity. The active site serine is present at the amino terminus and the catalytic residues histidine and aspartic acid can be identified;

Figure 33 shows the Pfam00657.11 [family 00657, database version 11] consensus sequence (hereafter called Pfam consensus) and the alignment of various sequences to the Pfam consensus sequence. The arrows indicate the active site residues, the underlined boxes indicate three of the homology boxes indicated by [Upton C and Buckley JT (1995) Trends Biochem Sci **20**; 179-179]. Capital letters in the Pfam consensus indicate conserved residues in many family members. The – symbol indicates a position where the hidden Markov model of the Pfam consensus expected to find a residue but did not, so a gap is inserted. The . symbol indicates a residue without a corresponding residue in the Pfam consensus. The sequences are the amino acid sequences listed in Figures 16, 18, 20, 22, 24, 26, 28 and 30.

Figure 34 shows the Pfam00657.11 [family 00657, database version 11] consensus sequence (hereafter called Pfam consensus) and the alignment of various sequences to the Pfam consensus sequence. The arrows indicate the active site residues, the underlined boxes indicate three of the homology boxes indicated by [Upton C and Buckley JT (1995) Trends Biochem Sci **20**; 179-179]. Capital letters in the Pfam consensus indicate conserved residues in many family members. The – symbol indicates a position where the hidden Markov model of the Pfam consensus expected to find a residue but did not, so a gap is inserted. The . symbol indicates a residue without a corresponding residue in the Pfam consensus. The sequences are the amino acid sequences listed in Figures 2, 16, 18, 20, 26, 28 and 30. All these proteins were found to be active against lipid substrates.

Figure 35 shows a expression vector pet12-AsalGCAT= pSM containing the C-terminal His-tagged *Aeromonas salmonicida* lipid acyltransferase gene;

Figure 36 shows the results of testing cell extracts in a NEFA Kit Assay, which depicts the activity of a recombinant, *A. salmonicida* lipid acyltransferase, towards lecithin. The wells from left to right indicate: a positive control, a negative control (i.e. extracts from empty plasmid) and samples collected after 0, 1, 2 and 3 hours cultivation after IPTG induction;

Figure 37 shows growth optimisation of BL21(DE3)pLysS harboring the expression vector pet12-AsalGCAT= pSM showing cultivation at 30 °C resulted in the production of enzyme with high activity towards lecithin. Cell extracts were tested for phospholipase activity using the NEFA kit assay. Wells from left to right: positive control; negative control; 20°C; 30°C;

Figure 38 shows crude cell extracts from BL21(DE3)pLysS expressing active lipid acyltransferase incubated with the substrate lecithin and reaction mixture was analyzed using thin layer chromatography showing the presence of degradation products. Lanes: 1. No enzyme; 2. + A.sal –10ul 37°C; 3. + A. sal –20ul 37°C; 4. + A.sal – 10ul 24°C; 5. + A. sal –20u 24°C;

Figure 39 shows partial purification of the *Aeromonas salmonicida* Acyl Transferase showing the phospholipase activity associated with purified His-tag protein. SE = Sonicated extracts, His = Purified with Ni-NTA spin-kit from Qiagen;

Figure 40 shows the expression vector pet12-A.h. GCAT=pSMa containing the C-terminal His-tagged *Aeromonas hydrophila* Glycerolipid Acyl Transferase (GCAT) gene was used to transform *E.coli* strain BL21(DE3)pLysS;

Figure 41 shows the activity of the crude extracts (5 & 10ul) containing the recombinant *Aeromonas hydrophila* GCAT enzyme was tested towards lecithin using

Non-Esterified Fatty Acid (NEFA) kit (Roche, Switzerland), showing the presence of active enzyme towards the phospholipid, lecithin;

Figure 42 shows growth optimisation of BL21(DE3)pLysS harboring the expression vector pet12-AsalGCAT= pSM showing cultivation at 30 °C resulted in the production of enzyme with high activity towards lecithin. Cell extracts were tested for phospholipase activity using the NEFA kit assay;

Figure 43 shows the partial purification of the *Aeromonas hydrophila* & *A. salmonicida* Acyl Transferases showing the phospholipase activity associated with purified His-tag protein. SE = Sonicated extracts,
His = Purified with Ni-NTA spin-kit from Qiagen);

Figure 44 shows the expression of the *Aeromonas* genes in *Bacillus subtilis* 163 showing the production of secreted enzyme with activity towards both lecithin and DGDG. pUB-AH= construct containing the *A. hydrophila* gene and pUB-AS, construct with the *A. salmonicida* gene, Culture filtrate was incubated with the substrates for 60 minutes.

Figure 45 shows an amino acid sequence (SEQ ID No. 36) of the fusion construct used for mutagenesis of the *Aeromonas hydrophila* lipid acyltransferase gene in Example 17. The underlined amino acids is a xylanase signal peptide;

Figure 46 shows a nucleotide sequence (SEQ ID No. 45) encoding an enzyme from *Aeromonas hydrophila* including a xylanase signal peptide;

Figure 47 shows the result of the HPTLC analysis in Experiment I;

Figure 48 shows the result of the HPTLC analysis in Experiment II;

Figure 49 shows a calibration curve for monoglyceride standard solutions;

Figure 50 shows a calibration curve for diglyceride standard solutions.

Figure 51 shows a nucleotide sequence encoding a lipid acyltransferase enzyme according to the present invention from *Streptomyces* (SEQ ID No. 54);

Figure 52 shows a polypeptide sequence of a lipid acyltransferase enzyme according to the present invention from *Streptomyces* (SEQ ID No. 55);

EXAMPLES

For the avoidance of doubt, the following abbreviations may be used herein:
MONO = monoglyceride
MAG = monoacylglycerol = monoglyceride
MAG and MONO are interchangeable herein.
DAG = diacylglycerol
FFA = free fatty acid

EXAMPLE 1: The cloning, sequencing and heterologous expression of a transferase from *Aeromonas salmonicida* subsp. *Salmonicida*

Strains used:

Aeromonas salmonicida subsp. Salmonicida (ATCC 14174) was obtained from ATCC and grown overnight at 30°C in Luria-Bertani medium (LB). The cells were centrifuged and genomic DNA was isolated using the procedures for genomic DNA isolation from Qiagen Ltd. Genomic DNA buffer set (cat.19060), protease K (cat. 19131) and RNAse A (cat. 19101) were all obtained from Qiagen Ltd. (Boundary court Gatwick Court, West Sussex, RH10 2AX).

Host bacterial strain BL21(DE3)pLysS (Novagen) was used for production of the recombinant *Aeromonas* enzymes. Competent cells of BL21(DE3)pLysS were used as host for transformation with the expression vector **pet12-AsalGCAT=pSM.**

Transformants containing the appropriate plasmid were grown at 37 °C in LB agar medium containing 100-ug ampicillin/ml.

Construction of expression vector pet12-AsalGCAT- pSM:

For all DNA amplifications of the transferase genes from *Aeromonas*, genomic DNA (0.2-1 ul) was used as template and *pfu* DNA polymerase (2.5 units) was used with 10ul of 10x pfu buffer, 1ul each primer (50pmol/ul), 200 uMdNTP in a total reaction volume of 100ul. PCR reactions were performed in a programmable thermal cycler using the following conditions: 95 °C for 30 seconds, 30 cycles of 95 °C for 30 seconds, 60 °C for 1 minute and 68 °C for 2 minutes. An additional extension of 5 minutes at 72 °C was applied.

The PCR amplification of the transferase gene from *A. salmonicida* was carried in 2 separate PCR reactions. PCR reaction 1 was performed using primer pairs, as1USNEW(5'AGCATATGAAAA AATGGTTTGT TTGTTTATTG GGG 3' [SEQ ID No. 56]) and asls950new (5' GTG ATG GTG GGC GAG GAA CTC GTA CTG3' [SEQ ID No. 37]). A second PCR reaction was performed to incorporate a C-terminal Histidine tag using the PCR product from the first reaction and the primers: as1USNEW(5'AGCATATGAAAA AATGGTTTGT TTGTTTATTG GGG 3' [SEQ ID No. 38]) and AHLS1001(5'TTGGATCC GAATTCAT CAATG GTG ATG GTG ATG GTG GGC3' [SEQ ID No. 39]). The PCR product from the second reaction was purified and digested with restriction enzymes Nde1 and BamHI. 2 ug of pET 12a vector DNA was also digested with restriction enzymes Nde1 and BamHI and treated with phosphatase. The restriction enzyme-treated pet12a and PCR product from reaction 2 were purified and ligated using the Rapid Ligation Kit (Roche, Switzerland). The ligation mix was used to transform *E. coli* TOP10 cells. Transformants were plated on LB agar medium containing 100ug/ml ampicillin.

The T7 promoter primer (5'TAATACGACTCACTATAG3' [SEQ ID No. 40]) and the T7 terminator primer (5'CTAGTTATTGCTCAGCGG3' [SEQ ID No. 41]) were used to verify the sequences and the orientation of the cloned transferase genes in pET12a

vector. DNA sequencing was performed using ABI Prism® BigDye™ Terminators Cycle sequencing kit with 500ng plasmid DNA as template and 3.2pmol T7 promoter and terminator primers.

The construct shown in Figure 35 was used to transform competent bacterial host strain BL21(DE3)pLysS (Novagen) and ampicillin resistant transformants were picked and used for expression analysis.

Expression of the recombinant *Aeromonas salmonicida* lipid acyltransferase

Quantification of enzyme activity towards lecithin was determined on cell extracts using Non-Esterified Fatty Acid (NEFA) kit (Roche, Switzerland).

In Figure 36, BL21(DE3)pLysS harboring the expression vector pet12-AsalGCAT= pSM was grown in LB medium + 100ug/ml ampicillin and incubated with shaking at 37°C until OD_{600} = 0.6 to1.0 is reached. The cultures are then induced using IPTG (0.4mM) and incubation was continued for the next 3 hours. Samples where taken at 0 hour, 1, 2, and 3 hours after IPTG induction. Enzyme Activity was tested using the NEFA kit and lecithin as substrate.

Growth Optimisation for the production of more active enzymes

BL21(DE3)pLysS harboring the expression vector pet12-AsalGCAT= pSM was grown in LB medium + 100ug/ml ampicillin and incubated with shaking at different growth temperatures (37°C, 30 °C, & 20 °C). The optimal condition for the production of active lipid acyltransferase enzyme was when cultures are grown at 30°C as shown in Figure 37.

Partial purification of recombinant *Aeromonas salmonicida* transferase

Strain BL21(DE3)pLysS harboring the expression vector pet12-AsalGCAT=pSM was grown at 37°C & crude cell extracts were prepared by sonication. The recombinant enzyme was further purified from the sonicated crude cell extracts using

the Ni-NTA spin kit from Qiagen. Phospholipase activity using the NEFA kit & Lecithin as substrate. Crude cell extracts from BL21(DE3)pLysS expressing active transferase incubated with the substrate lecithin and reaction mixture was analysed using thin layer chromatography showing the presence of degradation products (see Figure 38).

Partial Purification of recombinant *Aeromonas salmonicidae* transferase. Strain BL21(DE3)pLysS harbouring the expression vector pet12-AsalGCAT=pSM was grown at 37°C and crude cell extracts were prepared by sonication. The recombinant enzyme ware further purified from the sonicated crude cell extract using the Ni-NTA spin kit from Qiagen. Phospholipase activity using the NEFA kit and lecithin as substrate was tested (see Figure 39).

EXAMPLE 2 Cloning and Expression of *Aeromonas hydrophila* transferase in *E. coli*

Aeromonas hydrophila (ATCC # 7965) was obtained from ATCC and grown overnight at 30°C in Luria-Bertani medium (LB). The cells were centrifuged and genomic DNA was isolated using the procedures for genomic DNA isolation from Qiagen Ltd. Genomic DNA buffer set (cat.19060), protease K (cat. 19131) and RNAse A (cat. 19101) were all obtained from Qiagen Ltd. (Boundary court Gatwick Court, West Sussex, RH10 2AX).

Host bacterial strain BL21(DE3)pLysS (Novagen) was used for production of the recombinant *Aeromonas* enzymes. Competent cells of BL21(DE3)pLysS were used as host for transformation with the expression vector pet12a-A.h.GCAT=pSMa. Transformants containing the appropriate plasmid were grown at 37 °C in LB agar medium containing 100-ug ampicillin/ml.

Construction of expression vector pet12a-A.h.GCAT- pSMa:

For all DNA amplifications of the transferase gene from *Aeromonas*, genomic DNA (0.2-1 ul) was used as template and *pfu* DNA polymerase (2.5 units) was used with

10ul of 10x pfu buffer, 1ul each primer (50pmol/ul), 200 uMdNTP in a total reaction volume of 100ul. PCR reactions were performed in a programmable thermal cycler using the following conditions: 95 °C for 30 seconds, 30 cycles of 95 °C for 30 seconds, 60 °C for 1 minute and 68 °C for 2 minutes. An additional extension of 5 minutes at 72 °C was applied.

The PCR amplification of the transferase gene from *A. hydrophila* (ATCC # 7965) was carried out in 2 separate PCR reactions.

PCR reaction 1 was performed using primer pairs, AHUS1 (5'GTCATATGAAAAAATGGTTTGTGTGTTTATTGGGATTGGTC3', SEQ ID No. 42) and ahls950 (5'ATGGTGATGGTGGGCGAGGAACTCGTACTG3', SEQ ID No. 43).

A second PCR reaction was performed to incorporate a C-terminal Histidine tag using the PCR product from the first reaction and the primer pairs:

AHUS1(5'GTCATATGAAAAAATGGTTTGTGTGTTTATTGGGATTGGTC3' SEQ ID No. 44,) and AHLS1001(5'TTGGATCCGAATTCATCAATGGTGATGGTGATGGTGGGC3' SEQ ID No. 57).

The PCR product from the second reaction was purified and digested with restriction enzymes Nde1 and BamHI. 2 ug of pET 12a vector DNA was also digested with restriction enzymes Nde1 and BamHI and treated with phosphatase. The restriction enzyme-treated pet12a and PCR product from reaction 2 were purified and ligated using the Rapid Ligation Kit (Roche, Switzerland). The ligation mix was used to transform *E. coli* TOP10 cells. Transformants were plated on LB agar medium containing 100ug/ml ampicillin.

The T7 promoter primer (5'TAATACGACTCACTATAG3') and the T7 terminator primer (5'CTAGTTATTGCTCAGCGG3') were used to verify the sequences and the

orientation of the cloned GCAT genes in pET12a vector. DNA sequencing was performed using ABI Prism® BigDye™ Terminators Cycle sequencing kit with 500ng plasmid DNA as template and 3.2pmol T7 promoter and terminator primers.

The construct shown in Figure 40 was used to transform competent bacterial host strain BL21 (DE3)pLysS (Novagen) and ampicillin resistant transformants were picked and used for expression analysis.

Expression of the *Aeromonas hydrophila* transferase in BL21(DE3)pLysS

The *E. coli* strain BL21(DE3)pLysS harboring the expression vector pet12a-A.h.GCAT= pSMa was grown in LB medium + 100ug/ml ampicillin and incubated with shaking at 37°C until OD_{600} = 0.6 to1.0 is reached. The cultures are then induced using IPTG (0.4mM) and incubation was continued for the next 3 hours. Samples where taken at 0hour, 1, 2, and 3 hours after IPTG induction. Enzyme Activity was tested using the NEFA kit and lecithin as substrate (Figure 41).

Growth Optimisation for the production of more active enzymes

BL21(DE3)pLysS harboring the expression vector pet12a-A.h.GCAT= pSMa was grown in LB medium + 100ug/ml ampicillin and incubated with shaking at different growth temperatures (37°C, 30 °C, & 20 °C). The optimal condition for the production of active GCAT enzyme was when cultures are grown at 30°C as shown in Figure 42.

Partial purification of recombinant *A.hydrophila* transferase (GCAT)

Strain BL21(DE3)pLysS harboring the expression vector pet12a-A.h.GCAT=pSMa was grown at 37°C & crude cell extracts were prepared by sonication. The recombinant enzyme was further purified from the sonicated crude cell extracts using the Ni-NTA spin kit from Qiagen. Phospholipase activity assay using the NEFA kit & Lecithin as substrate. (Figure 43).

EXAMPLE 3: Expression of *Aeromonas* transferases in *Bacillus subtilis* 163

Plasmid Construction

Two different *Bacillus subtilis* expression vectors (pUB 110 & pBE5) were used for the heterologous expression of the *Aeromonas* genes in *Bacillus subtilis*. The pUB110 vector contains the alpha amylase promoter while the pBE vector has the P32 promoter as the regulatory region for the expression of the fused *Aeromonas* genes. In pUB110, the first amino acid of the mature GCAT genes of *Aeromonas* were fused in frame with the last amino acid of the xylanase signal peptide sequence from *Bacillus subtilis* via the restriction site Nhe1, creating an additional 2 amino acids in front of the mature proteins. pBE5 contains the cgtase signal sequence fusion at the Nco1 site for secretion of the recombinant proteins into the culture filtrate.

PCR reactions were carried out to obtain the *Aeromonas* genes fuse in frame to the signal sequences of the pUB 110 and the pBE5 vectors. PCRs were performed using the following primer pairs for *A. hydrophila* gene:

PCR reaction 1: usAHncol (5'ATGCCATGGCCGACAGCCGTCCCGCC3', SEQ ID No. 46) and lsAH (5'TTGGATCCGAATTCATCAATGGTGATG3', SEQ ID No. 47)

PCR reaction 2: US-AhnheI (5'TTGCTAGCGCCGACAGCCGTCCCGCC3', SEQ ID No. 48.) and lsAH (5'TTGGATCCGAATTCATCAATGGTGATG3, SEQ ID No. 49)

PCRs were performed using the following primer pairs for *A. salmonicida* gene:

PCR reaction 3: US-Asncol (5'TTGCCATGGCCGACACTCGCCCCGCC3', SEQ ID No. 50) and lsAH (5'TTGGATCCGAATTCATCAATGGTGATG3', SEQ ID No. 51)

PCR reaction 4: US-ASnhe1 (5'TTGCTAGCGCCGACACTCGCCCCGCC3', SEQ ID No. 52) and lsAH (5'TTGGATCCGAATTCATCAATGGTGATG3', SEQ ID No. 53).

All the PCR products were cloned into PCR blunt II (TOPO vector) and sequenced with reverse & forward sequencing primers.

Clones from PCR reactions 1 & 3 were cut with Nco1 & Bam HI and used as inserts for ligation to the pBE5 vector cut with Nco1/BamH1/phosphatase. Clones from PCR reactions 2 & 4 were cut with Nhe1 & Bam H1 and used as inserts for ligation to the pUB vector that was cut with Nhe1/BamH1/phosphatase.

Expression of the *Aeromonas* transferase genes in *Bacillus subtilis* and characterization of the enzyme activity.

The acyl transferases from the two *Aeromonas* species have been successfully expressed in *E. coli* (results above). The *Bacillus* pUB110 & pBE5 gene fusion constructs were used to transform *Bacillus subtilis* and transformants were selected by plating on kanamycin plates. The kanamycin resistant transformants isolated and grown in 2xYT are capable of heterologous expression of the *Aeromonas* genes in *Bacillus*. The culture filtrates have digalactosyldiacylglycerol (DGDG) galactolipase activity, in addition to having both acyl transferase and phospholipase activities. The activity towards digalactosyldiacylglycerol (DGDG) was measured after 60 minutes of incubation of culture supernatant with the substrate, DGDG from wheat flour (obtainable form Sigma) as well as the activity towards lecithin as shown in Figure 44. *Bacillus* produced the enzyme after overnight (20-24 hours) to 48 hours of cultivation in the culture medium as a secreted protein. In some instances, the expression of the *Aeromonas* genes has been shown to interfere with cell viability and growth in *Bacillus & E. coli*, it is therefore necessary to carefully select expression strains and optimise the growth conditions to ensure expression. For example, several *Bacillus* host strains (B.s 163, DB104 and OS 21) were transformed with the expression vectors for growth comparison. B.s163 is transformable with the 2 *Aeromonas* genes and is capable of expressing active protein. DB104 is transformable with all the constructs but is only able to express *A. salmonicida* transferase.

EXAMPLE 4: Fermentation and Purification of *Aeromonas* lipid acyltransferases produced in *E.coli*

***E.coli* Fermentations:**

Microorganisms

Two strains of *Eschericia coli, one* containing an *Aeromonas hydrophila* (Example 2) lipid acyltransferase and two containing *Aeromonas salmonicida* lipid acyltransferases, (Example 1) were used in this study.

The *E. coli* strain containing the *A. hydrophila* gene was named DIDK0124 , and the *E. coli* strain containing the *A. salmonicida* gene was named DIDK0125. The fermentation with DIDK0124 was named HYDRO0303 and the fermentation with DIDK0125 was named SAL0302. The purified protein from HYDRO025 was named REF#138. The purified protein from HYDRO0303 was named REF#135.

Growth media and culture conditions

LB-agar

The LB agar plates used for maintaining the strains contained: 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 15 g/L agar, 100 mg/L ampicillin and 35 mg/L chloramphenicol. The agar plates were incubated at 30°C.

LB shake flask

The LB medium (50 mL pr shake flask) used for production of inoculum material for the bioreactor cultivations contained: 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 100 mg/L ampicillin and 35 mg/L chloramphenicol. The shake flasks were inoculated from the LB agar plates, and incubated at 30°C and 200 rpm.

Bioreactor cultivation

The bioreactor cultivations were carried out in 6 L in-house built bioreactors filled with 4 L medium containing: 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 8 g/L

KH_2PO_4, 0.9 g/L $MgSO_4,7H_2O$, 40 g/L glucose monohydrate, 0.4 mL/ ADD APT® Foamstop Sin 260 (ADD APT Chemicals AG, Helmond, The Netherlands), 10 mg/L $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$, 0.7 mg/L $CuSO_4 \cdot 5H_2O$, 3 mg/L $ZnSO_4 \cdot 7H_2O$, 3 mg/L $MnSO_4 \cdot H_2O$, 10 mg/L EDTA, 0.1 mg/L $NiSO_4 \cdot 6H_2O$, 0.1 mg/L $CoCl_2$, 0.1 mg/L H_3BO_4, 0.1 mg/L KI, 0.1 mg/L $Na_2MoO_4 \cdot 2H_2O$, 1 g/L ampicillin and 35 mg/L chloramphenicol.

The bioreactors were inoculated with an amount of LB culture ensuring end of growth after approximately 20 hours of cultivation (calculated from the maximum specific growth rate of 0.6 h^{-1}, the OD_{600} of the LB shake flask and the final OD_{600} in the bioreactor of approximately 20).

SAL0302 was inoculated with 10 mL of LB culture, and HYDRO0303 was inoculated with 4 mL of LB culture.

The bioreactors were operated at the following conditions: temperature 30°C, stirring 800-1000 rpm (depending on experiment), aeration 5 L/min, pH 6.9, pH control 8.75% (w/v) NH_3-water and 2 M H_2SO_4. Induction was achieved by addition of isopropyl β-D-thiogalactoside to a final concentration of 0.6 mM, when 0.4 moles (HYDRO0303) and 0.7 moles CO_2 was produced respectively.

Harvest

The following procedure was used for harvest and homogenisation of the biomass:

1) The fermentation broth from the fermentations was centrifuged at 5000 × g and 4°C for 10 minutes, and the supernatant was discharged. The biomass was stored at –20°C until use. The biomass was thawed and resuspended in 500 mL of 20 mM NaH_2PO_4, pH 7.4, 500 mM NaCl, 10 mM Imidazole and Complete (EDTA-free) protease inhibitor (Roche, Germany).
2) The suspended biomass was homogenized at 2 kbar and 4°C in a cell disrupter from Constant Systems Ltd (Warwick, UK).
3) The cell debris was removed by centrifugation at 10.000 × g and 4°C for 30 minutes followed by collection of the supernatant.

4) The supernatant was clarified further by centrifugation at 13.700× g and 4°C for 60 minutes, followed by collection of the supernatant.
5) The supernatant was filtered through 0.2 µm Vacu Cap filters (Pall Life Sciences, UK) and the filtrate was collected for immediate chromatographic purification.

Chromatographic purification of the Transferases

A column (2.5 x 10 cm) was packed with 50 ml of Chelating Sepharose ff. gel and charged with Ni-sulphate (according to the method described by manufacturer, Amersham Biosciences). The column was equilibrated with 200 ml of 20 mM NaH_2PO_4, pH 7.4, 500 mM NaCl, 10 mM Imidazole. 400 ml of crude was applied to the column at a flow rate of 5 ml/min. The column was then washed with 20 mM NaH_2PO_4, pH 7.4, 500 mM NaCl, 10 mM Imidazole until the UV_{280} reached the base line. The GCAT was then eluted with 40 ml of 20 mM NaH_2PO_4, pH 7.4, 500 mM NaCl and 500 mM Imidazole.

EXAMPLE 5: Fermentation and Purification of *Aeromonas* lipid acyltransferases produced in *Bacillus subtilis*.

Fermentations

BAC0318-19, BAC0323-24

Microorganism

The microorganisms used in this study originate from transformation of a *Bacillus subtilis* host strain, #163 with a plasmid containing the gene encoding the *Aeromonas salmonicida* transferase inserted in the vector pUB110OIS. The expression of the gene is controlled by an alpha-amylase promoter, and the secretion of the transferase is mediated by the *B. subtilis* xylanase signal sequence (Example 3). The strains were named DIDK0138 (fermentation BAC0318-19) and DIDK0153 (fermentation BAC0323-24).

Growth media and culture conditions

Pre culture medium

A shake flask (500 mL total volume, with baffles) was added 100 mL of a medium containing:

NaCl	5 g/L
K_2HPO_4	10 g/L
Soy flour	20 g/L
Yeast extract, BioSpringer 106	20 g/L
Antifoam, SIN260	5 mL/L

pH was adjusted to 7.0 before autoclaving

After autoclaving 6 mL 50% (w/w) Nutriose were added pr flask. Kanamycin was added at a concentration of 50 mg/L after autoclaving.

Inoculation

A pre culture shake flask was inoculated with frozen culture directly from a 25% (w/v) glycerol stock. The shake flask was incubated at 33°C and 175 rpm for approximately 16 hours, whereupon 50 mL was used to inoculate the fermentor.

Fermentations

The fermentations were carried out in 6 L in house built fermentors.

The batch medium (3 L) contained:

Corn steep liquor (50% dw)	40 g/L
Yeast extract BioSpringer 153 (50% dw)	10 g/L
NaCl	5 g/L
$CaCl_2, 2H_2O$	0.25 g/L
$Mn(NO_3)_2, H_2O$	0.2 g/L
Antifoam SIN260	1 mL/L
Kanamycin (filter sterilised to the fermentor after autoclaving	50 mg/L

The feed contained:

Glucose monohydrate	540 g/kg
$MgSO_4$, $7H_2O$	4.8 g/kg
Antofoam SIN260	4 mL/kg
Yeast extract, BioSpringer 153 (50% dw) (autoclaved separately)	150 g/kg

The feed in fermentation BAC0318 and BAC0323 was started based on the accumulated CO_2, according to the equations below:

$$\text{Feed} - \text{flow}[\text{g/h}] = 0, \text{AcCO}_2 < 0.15$$
$$\text{Feed} - \text{flow}[\text{g/h}] = 2.85 + t \cdot 1.54, \text{AcCO}_2 \geq 0.15 \text{ and } t < 12$$
$$\text{Feed} - \text{flow}[\text{g/h}] = 21.3, t > 12$$

t: time (hours) from the point when the accumulated CO_2 ($AcCO_2$) reached 0.15 moles.

The feed in fermentation BAC0319 and BAC0324 was started based on the accumulated CO_2, according to the equations below:

$$\text{Feed} - \text{flow}[\text{g/h}] = 0, \text{AcCO}_2 < 0.15$$
$$\text{Feed} - \text{flow}[\text{g/h}] = 2.0 + t \cdot 1.08, \text{AcCO}_2 \geq 0.15 \text{ and } t < 12$$
$$\text{Feed} - \text{flow}[\text{g/h}] = 15, t > 12$$

t: time (hours) from the point when the accumulated CO_2 ($AcCO_2$) reached 0.15 moles.

The pH was controlled at 7.0 by adding 12.5% (w/v) NH_3-water or 2M phosphoric acid.

The aeration was 3 L/min corresponding to 1 vvm.

The temperature was 33°C.

The fermentor was equipped with two 8 cm Ø Rushton impellers placed with a distance of 10 cm.

Harvest

The biomass was removed by centrifugation at 16,000× g for 10 minutes at room temperature. The supernatant was filter sterilized, and the filtrate was used for purification and application tests.

EXAMPLE 6: Enzymatic removal of DAGs in palm oil catalysed by a lipid acyltransferase from *Aeromonas salmonicidae.*

SUMMARY

The enzymatic glycerolysis experiments were initiated by the addition of the glycerol/transferase solution to the reactor containing palm oil and glycerol/water in varying concentrations. All the reactions were conducted at 43°C, using magnetic stirring for 24 hours. After reaction, the samples were analyzed using HPTLC and in some experiments the result was confirmed by GC analysis. The conducted trials showed that there was a good correlation between the water concentration and levels of DAGs and MAGs, respectively: the lowest water concentrations tested gave the highest MAG level and the lowest DAG level.

Based on the trials, it can be concluded that it was possible to reduce the amount of DAGs in palm oil by a transferase-catalysed reaction where the enzyme used DAGs as donor molecules and glycerol as acceptor molecule - in a glycerolysis reaction in which monoglycerides were synthesized. This was in contrast to the glycerolysis reaction with conventional triglyceride hydrolyzing lipases where the amount of DAG increases.

Furthermore, it can be concluded that the water concentration has a significant impact on the synthesized yield of monoglycerides and amount of DAG. The following correlation was found: Low water concentration (<1%) and 5% glycerol gives increased MAG yield and decreased concentration of DAGs. The obtained results also show that primarily 1,2 isomer of diglyceride tend to be reduced.

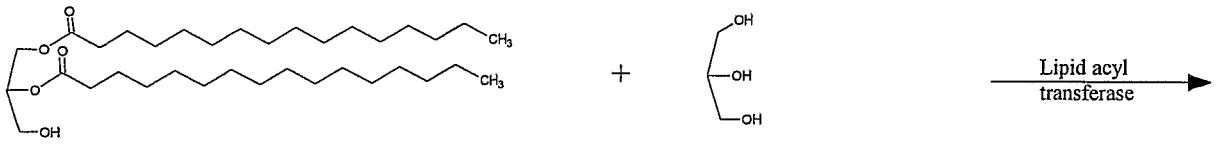
It is known that diglycerides, especially 1,2 isomers delay the crystallization of fat. This effect causes post-crystallization of fat products like margarine and shortenings, which favors formation of large crystals (sandiness). In certain fat blends it was observed that diglycerides improve the stability of β'crystals. A reduction but not a complete removal of diglycerides in palm oil would therefore be preferable. It can thus be concluded that a reduction of diglyceride will result in a better and more uniform oil quality; a fact, which must not be underestimated.

INTRODUCTION

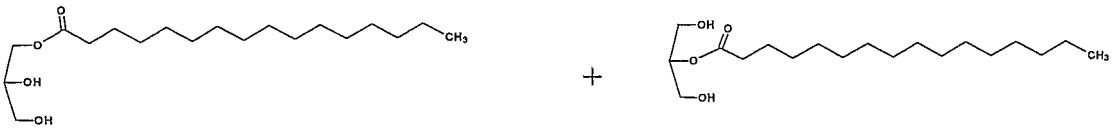
Problems concerning diglycerides depend to some extent on type of product, e.g. whether it is in margarine and shortenings. Thus, depending on the product the presence of diglycerides both have negative and positive effects. For margarine production, only fat product of a required crystal structure can be used in order to obtain proper consistency and plasticity. The β'-crystal form is the most required structure, showing small crystals and a high ability for maintaining the liquid fraction. The most stable crystal form (β-form) is undesirable, as having big crystals causes grained structure of the margarine. According to Hernquist & Anjou (1993) and Wahnelt *et al.* (1991) the presence of diglycerides in fat retard the transition from β' to β-crystal. But as diglycerides retard the transition from β' to β, they also retard the transition from α form to β' form (Walnet *et al.*, 1991). For this reason it can be defined that the presence of diglycerides retards the whole crystallization process. Slow crystallization has a crucial impact on margarine application. The effect of retarding crystallization in margarine blends containing a high portion of palm oil is that after conventional processing the product may be somewhat soft, causing packing difficulties, and subsequent crystallization may lead to a firmer texture than desired (Berger, 1990). In spite of advantages and disadvantages, it may be important to find the right balance between reduced and total removal of diglycerides.

It has surprisingly been found that it is possible to reduce the amount of diglyceride in palm oil by enzymatic glycerolysis of palm oil using a lipid acyltransferase, for

example the GDSx lipid acyl transferase from *Aeromonas salmonicida.* Without wishing to be bound by theory, in this process the enzyme is added to palm oil together with small amount of glycerol and the enzyme catalyses the following reaction:



1,2 diglyceride glycerol



1- monoglyceride 2- monoglyceride

After the reaction the surplus glycerol can, if deemed necessary, easily be separated from the reaction mixture by centrifugation or other processes.

The advantage of this process is that the amount of diglyceride (preferably 1,2 diglyceride) is reduced by a glycerolysis reaction catalysed by an enzyme which is specific for diglyceride without using the triglyceride as donor for the transferase reaction.

The reaction products monoglycerides do not have to be removed from the reaction mixture, but can advantageously be used as an efficient emulsifier for the production of food products like margarine and shortening. The process thus solves two problems. First of all the amount of diglyceride is reduced and preferable 1,2 diglyceride is removed, which has a negative impact on the crystallisation properties of the triglycerides. Secondly the reaction product monoglyceride can be used as an efficient emulsifier in the production of food products like margarine and shortening.

In one embodiment, it is preferably to remove the monoglycerides produced (if any) by the transferase reaction.

Suitably, if the transferase reaction has been carried out in the crude palm oil, the monoglyceride and/or glycerol and/or residues of water (if any) may be removed by a deodorisation process during the edible oil refining process.

Another very interesting advantage of the use of the lipid acyl transferase is that this enzyme is less dependent of the water content in the reaction mixture and because this enzyme is a transferase low or no hydrolytic activity takes place which means that the amount of free fatty acids do not increase significantly.

It is well known the water content in glycerolysis reactions are very important when lipolytic enzymes like lipases are use in this process (Kristensen, 2004). Even small amount of water, which is necessary for most lipolytic reactions will cause the formation of significant amount of free fatty acids. This problem can be overcome by using the lipid acyl transferase in the glycerolysis reaction.

Another aspect is that lipases known in the art to catalyse glycerolysis reaction, mainly uses triglyceride as donor during formation of diglyceride instead of reducing the amount of diglyceride.

Materials:

Palm oil: Palmotex, Aarhus United, Denmark

Glycerol: J. T. Baker, (7044)

DIMODAN® P: Danisco A/S, Denmark

Enzyme: Lipid acyltransferase GCAT from *Aeromonas salmonicida* expressed in *B. subtilis* and fermented in lab scale (Transferase #196).

Methods

Lyophilization of enzyme

The enzymes were desalted (PD-10 Desalting columns, Amersham Biosciences) before lyophilization. The desalted enzyme were mixed with glycerol (Enzyme:Glycerol ratio 3,5:1). The sample was lyophilised and added 10% water. The sample contained approximately 20 U (phospholipase units) per gram.

Determination of phospholipase activity (phospholipase activity assay):

Substrate

0.6% L-α Phosphatidylcholine 95% Plant (Avanti #441601), 0.4% Triton-X 100 (Sigma X-100) and 5 mM $CaCl_2$ was dissolved in 0.05M HEPES buffer pH 7.

Assay procedure:

400 µL substrate was added to an 1.5 mL Eppendorf tube and placed in an Eppendorf Thermomixer at 37°C for 5 minutes. At time t= 0 min, 50 µL enzyme solution was added. Also a blank with water instead of enzyme was analyzed. The sample was mixed at 10*100 rpm in an Eppendorf Thermomixer at 37°C for 10 minutes. At time t=10 min the Eppendorf tube was placed in another thermomixer at 99°C for 10 minutes to stop the reaction.

Free fatty acid in the samples was analyzed by using the NEFA C kit from WAKO GmbH.

Enzyme activity PLU-7 at pH 7 was calculated as micromole fatty acid produced per minute under assay conditions

Enzyme reaction

Palm oil was reacted with the glycerol-enzyme solution according to the following recipes in Table 1 and Table 2

Table 1: Experiment I

Enz. no.	Transferase #196			
Sample no.	1	2	3	4

Gl (%)	5	5	5	10
Water (%)	0	1	5	1
Oil phase (%)	95	94	90	89
Water in the reaction mixture	0.5	1.5	5.5	1.5

Table 2: Experiment II

Enz. No.	Transferase #196					
Sample no.	1	2	3	4	5	6
Gl (%)	5	5	5	10	10	10
Water (%)	1	5	7.5	1	5	7.5
Oil phase (%)	94	90	87,5	89	85	82,5
Water in the reaction mixture	1	5	7.5	1	5	7.5

The palm oil was scaled in a 20 ml Wheaton glass and the glycerol/enzyme and optional water was added. The sample was placed in heating block (Multitherm HP 15 Stheating block heated with differential stirring (15 wells) controlled by a VARIOMAG – Thermomodul 40 ST thermostat) and reacted under the following conditions:

Reaction temperature : 43°C

Magnetic Stirring : 650 rpm

Reaction time : 20 hours

The enzyme in the reaction mixture was inactivated at 97.5°C in 10 min. After reaction the sample were homogenized (Ultra Turrax) for 20 sec. and a homogeneous sample was taken out for further analysis.

HPTLC

Applicator: LINOMAT 5, CAMAG applicator.
HPTLC plate: 10 x 10 cm (Merck no. 1.05633)

The plate was activated before use by drying in an oven at 180°C for 20-30 minutes.

Application: 2,0µl of a 2,0% solution of reacted palm oil dissolved in Chloroform:Methanol (2:1) was applied to the HPTLC plate using LINOMAT 5 applicator.

Running-buffer: P-ether:MTBE:Acetic acid (50:50:1)
Application/Elution time: 8 minutes.

Developing fluid: 6% Cupriacetate in 16% H_3PO_4

After elution the plate was dried in an oven at 180°C for 10 minutes, cooled and immersed in the developing fluid and then dried additional in 20 minutes at 180°C. The plate was evaluated visually and scanned (ScanWizard 5) directly.

In Experiment II the components are quantified by Adobe photoshop 6,0 and the amount of MAG and DAG was calculated from calibrations curves of DAG and MAG standard solutions (see Figures 49 & 50).

Gas chromatography

Perkin Elmer 8420 Capillary Gas Chromatography equipped with WCOT fused silica column 12.5 m x 0,25 mm ID x 0,1μm 5%phenyl-methyl-silicone (CP Sil 8 CB from Crompack).

Carrier: Helium.
Injection: 1.5 μl with split.
Detector: FID. 385 °C.

Oven program	:	1	2	3	4
Oven temperature, °C	:	80	200	240	360
Isothermal, time, min.	:	2	0	0	10
Temperature rate, °C. /min.	:	20	10	12	

Sample preparation: 50 mg lipid was dissolved in 12ml heptane:pyridine (2:1) containing an internal standard heptadecane, 2 mg/ml. 500 μl of the sample was transferred to a crimp vial. 100 μl MSTFA (N-Methyl-N-trimethylsilyl-trifluoracetamid) was added and the reaction was incubated for 15 minutes at 60 °C.

Calculation: Response factors for mono-di-triglycerides, free fatty acids were determined from reference mixtures of these components. Based on these response factors the lipids in the samples were calculated.

Results

Experiment I:

The aim of this experiment was to examine the impact of a diglyceride:glycerol acyltransferase from *Aeromonas salmonicida* on DAGs in palm oil by glycerolysis reaction. It is known that a GCAT is able to transfer fatty acid from lecithin the cholesterol during formation of cholesterolester and lysolecithin. In this study we have investigated the possibility of the enzyme to use DAGs as donor and glycerol as

acceptor in order to reduce the amount of DAGs in palm oil and produce monoglyceride.

It is well known in the literature to use enzyme like lipases to catalyze glycerolysis reactions. In these reactions triglycerides is the main substrate and mono-and diglycerides are the reactions products. In these processes the amount of diglycerides increases significantly and the amount of diglycerides produced is on level or high than the amount of monoglyceride produced (Kristensen, 2004).

Four different sample compositions were tested and compared to a reference of palm oil mixed with 5% glycerol but without enzyme and treated in the same way as the samples. Figure 47 shows the result of the HPTLC analysis of the sample from table 1.

The results obtained from the HPTLC analysis shows that the amount of DAGs varies according to the reaction conditions (referring to ratio between GL:H_2O). Equal to all the reactions was that the 1,3-isomers of diglyceride were in higher portion than the 1,2-(2,3-) isomers of diglyceride. This observation can be confirmed by theory, which says that the ratio of 1,3-isomer to 1,2- (2,3-) isomer in crude palm oil is 7:3 (Siew & Ng, 1999; Timms, 2004). Furthermore, analyzing the result, the HPTLC plate shows that the transferease successfully reduces the amount of DAGs. The reduction can to some extent correlate to the amount of synthesized MONO.

If we compare the 1,2 isomer with the 1,3 isomer DAGs in figure 1 it appears that the 1,2 DAGs are primarily reduced by the enzyme-catalyzed reaction. This is in agreement with the transferease has a preferred specificity for fatty acids in sn2-position.

In experiments conducted different water concentrations in range of 0.5-5.5% were used. It appears from the result that the water concentration has a significant impact on the synthesized amount of MONO combined to the reduced amount of DAGs. In this instance transferase #196 shows that if the system contains low water concentration increased concentration of MONO is formed and corresponding decreased amount of

DAG is observed. The experiment also investigates whether the amount of glycerol has an effect on the equilibrium in the system (Lane 5: 10% GL:1% H_2O). Transferease #196 shows that higher concentration of glycerol – also meaning double dosage of enzyme activity - dose not result in higher MONO concentration.

Preferably the present invention is carried out in a low water environment i.e. less than about 1%.

One of the advantages working with transfereases instead of lipases is that the synthesis of MONO dose not correlates to excessive increased in the concentration of FFA. This fact is confirmed in present experiment. Figure 1 shows that none of the reactions shows clear band of FFA (FFA band was expected to be visible between 1,3 DAGs and TAGs).

From this experiment it can be summarized that the optimal concentration of glycerol and water for Transferase #196 is as following:

Transferase #196: 5%GL:0.5% H_2O

The sample procedure in HPTLC analysis did not include specific weighing, because of that it was not possible to calculate the concentrations of the different components in the reaction mixtures. Because of that the observation is solely based on visual evaluation.

Result GC

Based on the HPTLC results sample No. 2 was selected for GC analysis. To find out whether the visual evaluation of the HPTLC plates could be confirmed by quantitative analyses of DAG. Before analysis glycerol was removed from the sample by

centrifugation and only the lipid phase was exposed to GC analysis The GC results are presented in Table 3 below.

Table 3: Result of GC on selected reaction in Experiment I

	Ref.	#196
Sample No.	1	2
Gl (%)*	5	5
Water (%)	0	0
Oil phase (%)	95	95
GC results		
MONO	<0,01	0.34
DAG	6.21	5.77

* Glycerol contains 10% water

Analyzing the result of the GC study it was found that the selected sample differ from the reference in higher MONO content and lower DAG yield. This observation supports the obtained results in the HPTLC analysis.

Conclusion: Experiment I

Concerning content of monoglycerides and diglycerides in the product of glycerolysis, the results of HPTLC analysis indicated that there is a correlation between the concentration of water and the amounts of MONO and DAGs, respectively: The lower concentration of water, the lower DAGs and higher MONO.

GC analysis confirmed degree of reduction in DAG. In this experiment the reduced amount of DAGs counts for 7.1% of the total amount of diglycerides palm oil (Table 4).

Table 4: Degree of reduction of DAGs

Enz. No.	#196
Sample no.	2
Reduction of DAGs (%)	7,1

Experiment II:

The aim of this part was to continue the optimization of DAG reduction in palm oil and analyze the transferase-catalysed glycerolysis using Transferease #196. The primarily purpose is to reach a well-balanced reaction in which the amount of DAG has been reduced in time with increase concentration of MONO.

In this part of the experimental work six different sample compositions were tested and compared to a reference of palm oil mixed with 5% glycerol (Table 2). The reference was exposed to the same heating profile as the enzyme reactions, which makes it possible to observe and determine the degree of thermal degradation during reduction. Figure 48 shows the result of the HPTLC analysis.

It appears from the results in figure 48 that especially the amount of MAG is varying. The HPTLC analysis shows that in samples containing 5% glycerol (Lane: 1-3) there is continuous relation between water concentration and MAG yield: Decreased amount of water in the reaction mixture is followed by increased MAG yield. The same tendency is observed in the reactions containing 10% glycerol (Lane: 5-7).

According to the amount of diglycerides, it was not possible to differentiate the samples solely based on visual evaluation of the HPTLC plate. Therefore, to be able to distinguish between the samples, components were quantified by analyzing standard materials with known composition of MAG and DAG. The HPTLC plates were scanned and handle by Adobe Photoshop 6.0 and further calculated by help from a constructed calculation macro (Microsoft Excel 2000). The quantitative analysis of the components analyzed by HPTLC is presented in Table 5 below.

Table 5:

Enz. No.	Transferase #196						
Run order: HPTLC	1	2	3	4	5	6	7
MONO (%)	0,38	0.01	Nm*.	0.01	0.15	0.07	0.03
DAG (%)	7.24	8.13	Nm*.	8.35	8.26	7.88	7.62
Reduction of DAG (%)	13,29	2,63	-	-	1,08	5,63	8,74

Nm* Not measured

- not calculated.

The investigation supports the visual evaluation and gives a fine picture of the varying level of diglycerides. It is seen from the results that the highest degree of reduction in the amount of DAGs was achieved in sample no. 1 (5% GL:1.5% H_2O). Further it is observed that this sample also contains the highest amount of MAG.

Conclusion: Experiment II

Experiment II confirmed the observations that a lipid acyltransferase GCAT from *Aeromonas salmonicida* was able to reduce the amount of diglycerides in palm oil. For the experiments with 5% glycerol a correlation was found between the concentration of water in the reaction mixture and the amounts of MAG and DAGs, respectively: The lower concentration of water, the lower DAGs and higher MAG.

HPTLC quantified the degree of reduction (see Table 5). It appears from the results, that the reduced amount of DAGs counts for 13,29% of the total amount of diglycerides in palm oil and the amount of synthesized MONO raised from 0,01% (ref.) to 0.38% (sample No. 1).

From this experiment it can be summarized that the optimal concentration of glycerol and water for Transferase #196 is as following:

Transferase #196: 5%GL:1.5% H_2O

Comparing these observations with the result gained in Experiment I, it is confirmed that good consistency in the results is obtained. The optimal concentration of water is presumably between 0-1% H_2O depending on enzyme activity and content of glycerol.

Based on the two experiments conducted, it can be concluded that it is possible to reduce the amount of DAGs in palm oil by a transferase-catalysed reaction, where the enzyme uses DAGs as donor molecules and glycerol as acceptor molecule, in a glycerolysis reaction in which monoglycerides is synthesized. This is in strong contrast to the glycerolysis reaction with conventional triglycerides hydrolyzing lipases where the amount of DAG increases due to the partial hydrolysis activity of the triglycerides hydrolyzing lipase.

Based on the fact that the structure of diglycerides is a mixture 1,2- and 1,3-isomers and that the transferase is specific to the sn2-position, even a small reduction in the amount of diglyceride will have a huge physical impact on palm-based products.

EXAMPLE 7: Immobilisation of a diacylglycerol:glycerol transferase (an lipid acyltransferase according to the present invention) from *Aeromonas salmonicida.*

The diacylglycerol:glycerol transferase is immobilised on Celite by acetone precipitation. 10 ml enzyme solution in 20 mM TEA buffer pH 7 is agitated slowly with 0,1 gram Celite 535 (from Fluka) for 2 hours at room temperature.
50ml cool acetone is added during continued agitation.
The precipitate is isolated by centrifugation 5000 g for 1 minute.
The precipitate is washed 2 times with 20ml cold acetone.
The Celite is tried at ambient temperature for about 1 hour

The immobilised transferase is tested in palm oil (see table below):

Table

	%
Palm oil	92.5
Glycerol	5
Immobilised diacylglycerol:glycerol On Celite, #178, 45 U/g	2.0
Water	0.5

Palm oil and glycerol is heated to 42°C. The immobilised transferase was added.

The transferase reaction continued at 42°C during gentle agitation with a magnetic stirrer. Samples are taken out for analyses after ½, 1, 3, 6 and 24 hours and analysed by HPTLC. The reaction is stopped after 24 hours reaction time and the immobilised enzyme was filtered off.

The HPTLC analysis clearly shows the effect of the immobilised diacylglycerol:glycerol from *A. salmonicida* by the formation of monoglyceride and reduction of diglyceride in palm oil.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the present invention will be apparent to those skilled in the art without departing from the scope and spirit of the present invention. Although the present invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in biochemistry and biotechnology or related fields are intended to be within the scope of the following claims.

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BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

Danisco A/S
Langebrogade 1
DK-1001 Copenhagen
Denmark

NAME AND ADDRESS OF DEPOSITOR

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:

Escherichia coli TOP10pPet12aAhydro

Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:

NCIMB 41204

II. SCIENTI FIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

[ ] a scientific description

[X] a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 22 December 2003 (date of the original deposit)[1]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on

(date of receipt of request for conversion)

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: NCIMB Ltd.,

Address: 23 St Machar Drive
Aberdeen
AB24 3RY
Scotland, UK.

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorised official(s):

Terence Dando

Date: 9 January 2004

[1] Where Rule 6/4(d) applies, such date is the date on which the status of International Depositary Authority was acquired.

Form BP/4 (sole page)

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the INTERNATIONAL DEPOSITARY AUTHORITY identified on the following page

Danisco A/S
Langebrogade 1
DK-1001 Copenhagen
Denmark

NAME AND ADDRESS OF THE PARTY TO WHOM THE VIABILITY STATEMENT IS ISSUED

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: AS ABOVE Address:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCIMB 41204 Date of the deposit or of the transfer[1]: 22 December 2003

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 22 December 2003 [2]. On that date, the said microorganism was:

[3] [X] viable

[ ] no longer viable

1 Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

2 In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

3 Mark with a cross the applicable box.

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: NCIMB Ltd., Address: 23 St Machar Drive Aberdeen AB24 3RY Scotland	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorised official(s): Terence Dando Date: 9 January 2004

[4] Fill in if the information has been requested and if the results of the test were negative.

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

Danisco A/S
Langebrogade 1
DK-1001 Copenhagen
Denmark

NAME AND ADDRESS OF DEPOSITOR

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Escherichia coli TOP10pPet12aAsalmo	NCIMB 41205

II. SCIENTI FIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

[ ] a scientific description

[X] a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 22 December 2003 (date of the original deposit)[1]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on

(date of receipt of request for conversion)

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: NCIMB Ltd.,

Address: 23 St Machar Drive
Aberdeen
AB24 3RY
Scotland, UK.

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorised official(s):

Date: 9 January 2004

[1] Where Rule 6/4(d) applies, such date is the date on which the status of International Depositary Authority was acquired.

Form BP/4 (sole page)

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

Danisco A/S
Langebrogade 1
DK-1001 Copenhagen
Denmark

INTERNATIONAL FORM

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the INTERNATIONAL DEPOSITARY AUTHORITY identified on the following page

NAME AND ADDRESS OF THE PARTY TO WHOM THE VIABILITY STATEMENT IS ISSUED

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: AS ABOVE Address:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCIMB 41205 Date of the deposit or of the transfer[1]: 22 December 2003

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 22 December 2003 [2]. On that date, the said microorganism was:

[3] [X] viable

[3] [ ] no longer viable

1 Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

2 In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

3 Mark with a cross the applicable box.

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: NCIMB Ltd., Address: 23 St Machar Drive Aberdeen AB24 3RY Scotland	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorised official(s): Terence Dandy Date: 9 January 2004

[4] Fill in if the information has been requested and if the results of the test were negative.

CLAIMS

1. A method of reducing and/or removing diglyceride from an edible oil, comprising a) admixing an edible oil with an acyl acceptor substrate and a fatty-acid CoA independent diglyceride:glycerol acyltransferase, wherein the fatty-acid CoA independent diglyceride:glycerol acyltransferase is characterized as an enzyme which in an edible oil transfers an acyl group from a diglyceride to glycerol.

2. A method according to claim 1 wherein the fatty-acid CoA independent diglyceride:glycerol acyltransferase comprises the amino acid sequence motif GDSX, wherein X is one or more of the following amino acid residues L, A, V, I, F, Y, H, Q, T, N, M or S.

3. A method according to claim 1 wherein the amount of diglyceride in the edible oil is reduced.

4. A method according to claim 1 or claim 2 wherein the fatty-acid CoA independent diglyceride:glycerol acyltransferase transfers an acyl group from a diglyceride to an acyl acceptor, wherein the acyl acceptor is any compound comprising a hydroxy group (-OH).

5. A method according any one of claims 1 to 4, wherein the diglyceride is a 1,2-diglyceride.

6. A method according to any one of the preceding claims wherein acyl acceptor is one which is soluble in an edible oil.

7. A method according to any one of the preceding claims wherein the acyl acceptor is an alcohol.

8. A method according to claim 7 wherein the acyl acceptor is an alcohol.

9. A method according to claim 8 wherein the acyl acceptor is glycerol.

10. A method according to any one of the preceding claims wherein the fatty-acid CoA independent diglyceride:glycerol acyltransferase enzyme comprises H-309 or comprises a histidine residue at a position corresponding to His-309 in the amino acid sequence of the *Aeromonas hydrophila* lipolytic enzyme shown as SEQ ID No. 2 or SEQ ID No. 32.

11. A method according to any one of the preceding claims wherein the fatty-acid CoA independent diglyceride:glycerol acyltransferase is obtainable from an organism from one or more of the following genera: *Aeromonas, Streptomyces, Saccharomyces, Lactococcus, Mycobacterium, Streptococcus, Lactobacillus, Desulfitobacterium, Bacillus, Campylobacter, Vibrionaceae, Xylella, Sulfolobus, Aspergillus, Schizosaccharomyces, Listeria, Neisseria, Mesorhizobium, Ralstonia, Xanthomonas and Candida.*

12. A method according to any one of the preceding claims wherein the fatty-acid CoA independent diglyceride:glycerol acyltransferase comprises one or more of the following amino acid sequences: (i) the amino acid sequence shown as SEQ ID No. 2; (ii) the amino acid sequence shown as SEQ ID No. 3; (iii) the amino acid sequence shown as SEQ ID No. 4; (iv) the amino acid sequence shown as SED ID No. 5; (v) the amino acid sequence shown as SEQ ID No. 6; (vi) the amino acid sequence shown as SEQ ID No. 12, (vii) the amino acid sequence shown as SEQ ID No. 20, (viii) the amino acid sequence shown as SEQ ID No. 22, (ix) the amino acid sequence shown as SEQ ID No. 24, (x) the amino acid sequence shown as SEQ ID No. 26, (xi) the amino acid sequence shown as SEQ ID No. 28, (xii) the amino acid sequence shown as SEQ ID No. 30, (xiii) the amino acid sequence shown as SEQ ID No. 32, (xiv) the amino acid sequence shown as SEQ ID No. 34, (xv) the amino acid sequence shown as SEQ ID no. 55; (xvi) the amino acid sequence shown as SEQ ID No. 58*; (xvii)* the amino acid sequence shown as SEQ ID No. 60*; (xviii)* the amino acid sequence shown as SEQ ID No. 61; (xix) the amino acid sequence shown as SEQ ID No. 63; (xx) the amino acid sequence shown as SEQ ID No. 65; (xxi) the amino acid sequence shown as SEQ ID No. 67; (xxii) the amino acid sequence shown as SEQ ID No. 70 or (xxiii) an amino acid sequence which has 75% or more identity with any one of the sequences shown as SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 12, SEQ ID No. 20, SEQ ID No. 22, SEQ ID No. 24, SEQ ID No. 26, SEQ ID No. 28, SEQ ID No. 30, SEQ ID No. 32, SEQ ID No. 34, SEQ ID No. 55, SEQ ID No. 58, SEQ ID No. 60, SEQ ID No. 61, SEQ ID No. 63, SEQ ID No. 65, SEQ ID No. 67 or SEQ ID No. 70.

13. A method according to any one of the preceding claims wherein the fatty-acid CoA independent diglyceride:glycerol acyltransferase comprises an amino acid sequence encoded by one or more of the following nucleotide sequences:

(a) the nucleotide sequence shown as SEQ ID No. 7;

(b) the nucleotide sequence shown as SEQ ID No. 8;

(c) the nucleotide sequence shown as SEQ ID No. 9;

(d) the nucleotide sequence shown as SEQ ID No. 10;

(e) the nucleotide sequence shown as SEQ ID No. 11;

(f) the nucleotide sequence shown as SEQ ID No. 13;

(g) the nucleotide sequence shown as SEQ ID No. 21;

(h) the nucleotide sequence shown as SEQ ID No. 23;

(i) the nucleotide sequence shown as SEQ ID No. 25;

(j) the nucleotide sequence shown as SEQ ID No. 27;

(k) the nucleotide sequence shown as SEQ ID No. 29;

(l) the nucleotide sequence shown as SEQ ID No. 31;

(m)the nucleotide sequence shown as SEQ ID No. 33;

(n) the nucleotide sequence shown as SEQ ID No. 35;

(o) the nucleotide sequence shown as SEQ ID No. 54;

(p) the nucleotide sequence shown as SEQ ID No. 59;

(q) the nucleotide sequence shown as SEQ ID No. 62;

(r) the nucleotide sequence shown as SEQ ID No. 64;

(s) the nucleotide sequence shown as SEQ ID No. 66;

(t) the nucleotide sequence shown as SEQ ID No. 68

(u) the nucleotide sequence shown as SEQ ID No. 69 or

(v) a nucleotide sequence which has 75% or more identity with any one of the sequences shown as SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 13, SEQ ID No. 21, SEQ ID No. 23, SEQ ID No. 25, SEQ ID No. 27, SEQ ID No. 29, SEQ ID No. 31, SEQ ID No. 33, SEQ ID No. 35, SEQ ID No. 54, SEQ ID No. 59, SEQ ID No. 62, SEQ ID No. 64, SEQ ID No. 66, SEQ ID No. 68 or SEQ ID No. 69.

14. A method according to any one of the preceding claims comprising admixing the treated edible oil with one or more food constituents to formulate a foodstuff.

15. Use of a fatty-acid CoA independent diglyceride:glycerol acyltransferase characterized as an enzyme which in an edible oil transfers an acyl group from a diglyceride to glycerol, in the manufacture of an edible oil, for reducing and/or removing (preferably selectively reducing and/or removing) diglyceride from said edible oil.

16. Use of a fatty-acid CoA independent diglyceride:glycerol acyltransferase characterized as an enzyme which in an edible oil transfers an acyl group from a diglyceride to glycerol, in the manufacture of a foodstuff comprising an edible oil for improving the crystallization properties of said foodstuff.

17. Use according claim 15 or claim 16 wherein the fatty-acid CoA independent diglyceride:glycerol acyltransferase comprises the amino acid sequence motif GDSX, wherein X is one or more of the following amino acid residues L, A, V, I, F, Y, H, Q, T, N, M or S.

18. Use according to any one of claims 15, 16 or 17 wherein the amount of diglyceride in the edible oil is reduced.

19. Use according to any one of claims 15 to 18 wherein the fatty-acid CoA independent diglyceride:glycerol acyltransferase transfers an acyl group from a diglyceride to an acyl acceptor, wherein the acyl acceptor is any compound comprising a hydroxy group (-OH).

20. Use according any one of claims 15 to 19, wherein the diglyceride is a 1,2-diglyceride.

21. Use according to any one of claims 15 to 20 wherein acyl acceptor is one which is soluble in an edible oil.

22. Use according to any one of claims 14-19 wherein the acyl acceptor is an alcohol.

23. Use according to claim 22 wherein the acyl acceptor is an alcohol.

24. Use according to claim 23 wherein the acyl acceptor is glycerol.

25. Use according to any one of claims 15-24 wherein the fatty-acid CoA independent diglyceride:glycerol acyltransferase enzyme comprises H-309 or comprises a histidine residue at a position corresponding to His-309 in the amino acid sequence of the *Aeromonas hydrophila* lipolytic enzyme shown as SEQ ID No. 2 or SEQ ID No. 32.

26. Use according to any one of claims 15-25 wherein the fatty-acid CoA independent diglyceride:glycerol acyltransferase is obtainable from an organism from one or more of the following genera: *Aeromonas, Streptomyces, Saccharomyces, Lactococcus, Mycobacterium, Streptococcus, Lactobacillus, Desulfitobacterium, Bacillus, Campylobacter, Vibrionaceae, Xylella, Sulfolobus, Aspergillus, Schizosaccharomyces, Listeria, Neisseria, Mesorhizobium, Ralstonia, Xanthomonas and Candida.*

27. Use according to any one of claims 15-26 wherein the fatty-acid CoA independent diglyceride:glycerol acyltransferase comprises one or more of the following amino acid sequences: (i) the amino acid sequence shown as SEQ ID No. 2; (ii) the amino acid sequence shown as SEQ ID No. 3; (iii) the amino acid sequence shown as SEQ ID No. 4; (iv) the amino acid sequence shown as SED ID No. 5; (v) the amino acid sequence shown as SEQ ID No. 6; (vi) the amino acid sequence shown as SEQ ID No. 12, (vii) the amino acid sequence shown as SEQ ID No. 20, (viii) the amino acid sequence shown as SEQ ID No. 22, (ix) the amino acid sequence shown as SEQ ID No. 24, (x) the amino acid sequence shown as SEQ ID No. 26, (xi) the amino acid sequence shown as SEQ ID No. 28, (xii) the amino acid sequence shown as SEQ ID No. 30, (xiii) the amino acid sequence shown as SEQ ID No. 32, (xiv) the amino acid sequence shown as SEQ ID No. 34, (xv) the amino acid sequence shown as SEQ ID No. 55; (xvi) the amino acid sequence shown as SEQ ID No. 58*; (xvii)* the amino acid sequence shown as SEQ ID No. 60*; (xviii)* the amino acid sequence shown as SEQ ID No. 61; (xix) the amino acid sequence shown as SEQ ID No. 63; (xx) the amino acid sequence shown as SEQ ID No. 65; (xxi) the amino acid sequence shown as SEQ ID No. 67; (xxii) the amino acid sequence shown as SEQ ID No. 70 or (xxiii) an amino acid sequence which has 75% or more identity with any one of the sequences shown as SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 12, SEQ ID No. 20, SEQ ID No. 22, SEQ ID No. 24, SEQ ID No. 26, SEQ ID No. 28, SEQ ID No. 30, SEQ ID No. 32, SEQ ID No. 34, SEQ ID No. 55, SEQ ID No. 58, SEQ ID No. 60, SEQ ID No. 61, SEQ ID No. 63, SEQ ID No. 65, SEQ ID No. 67 or SEQ ID No. 70.

28. Use according to any one of claims 15-27 wherein the fatty-acid CoA independent diglyceride:glycerol acyltransferase comprises an amino acid sequence encoded by one or more of the following nucleotide sequences:

(a) the nucleotide sequence shown as SEQ ID No. 7;
(b) the nucleotide sequence shown as SEQ ID No. 8;
(c) the nucleotide sequence shown as SEQ ID No. 9;
(d) the nucleotide sequence shown as SEQ ID No. 10;
(e) the nucleotide sequence shown as SEQ ID No. 11;
(f) the nucleotide sequence shown as SEQ ID No. 13;
(g) the nucleotide sequence shown as SEQ ID No. 21;
(h) the nucleotide sequence shown as SEQ ID No. 23;
(i) the nucleotide sequence shown as SEQ ID No. 25;
(j) the nucleotide sequence shown as SEQ ID No. 27;
(k) the nucleotide sequence shown as SEQ ID No. 29;
(l) the nucleotide sequence shown as SEQ ID No. 31;
(m)the nucleotide sequence shown as SEQ ID No. 33;
(n) the nucleotide sequence shown as SEQ ID No. 35;
(o) the nucleotide sequence shown as SEQ ID No. 54;
(p) the nucleotide sequence shown as SEQ ID No. 59;
(q) the nucleotide sequence shown as SEQ ID No. 62;
(r) the nucleotide sequence shown as SEQ ID No. 64;
(s) the nucleotide sequence shown as SEQ ID No. 66;
(t) the nucleotide sequence shown as SEQ ID No. 68
(u) the nucleotide sequence shown as SEQ ID No. 69 or
(v) a nucleotide sequence which has 75% or more identity with any one of the sequences shown as SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10; SEQ ID No. 11, SEQ ID No. 13, SEQ ID No. 21, SEQ ID No. 23, SEQ ID No. 25, SEQ ID No. 27, SEQ ID No. 29, SEQ ID No. 31, SEQ ID No. 33, SEQ ID No. 35, SEQ ID No. 54, SEQ ID No. 59, SEQ ID No. 62, SEQ ID No. 64, SEQ ID No. 66, SEQ ID No. 68 or SEQ ID No. 69.

29. Use according to any one of claims 15-28 wherein the fatty-acid CoA independent diglyceride:glycerol acyltransferase is used in combination with a crystallization inhibitor.

30. A method substantially as hereinbefore described with reference to the accompanying description and the figures.

31. A use substantially as hereinbefore described with reference to the accompanying description and figures.