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(54) Titre : PROCEDE POUR LE RAFFINAGE D'HUILES COMESTIBLES AU MOYEN D'UNE LIPIDE ACYLTRANSFERASE

(54) Title: PROCESS FOR EDIBLE OIL REFINING USING A LIPID ACYLTRANSFERASE

(57) Abrégé/Abstract:
A process of water degumming an edible oil (preferably a crude edible oil) comprising the steps of: a) admixing approximately 0.1-5% w/w water with an edible oil (preferably a crude edible oil) and a lipid acyltransferase, b) agitating the admixture for between about 10 minutes and 180 minutes at about 45 to about 900C, and c) separating the oil phase and the gum phase. Preferably said lipid acyltranferase is a polypeptide having lipid acyltransferase activity which polypeptide is obtained by expression of the nucleotide sequence shown as SEQ ID No. 49 or a nucleotide sequence which as has 70% or more identity therewith; and/or is obtained by expression of a nucleic acid which hybridises under medium stringency conditions to a nucleic probe comprising the nucleotide sequence shown as SEQ ID No. 49; and/or is a polypeptide having lipid acyltranferase activity which polypeptide comprises the amino acid sequence shown as SEQ ID No. 68 or an amino acid sequence which as has 70% or more identity therewith. In one embodiment the lipid acyltransferase is preferably used in combination with a phospholipase C enzyme. A process for modifying the gum phase of a degummed oil using a lipid acyltransferase is also taught herein.

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(54) Title: PROCESS

(57) Abstract: A process of water degumming an edible oil (preferably a crude edible oil) comprising the steps of: a) admixing approximately 0.1-5% w/w water with an edible oil (preferably a crude edible oil) and a lipid acyltransferase, b) agitating the admixture for between about 10 minutes and 180 minutes at about 45 to about 900C, and c) separating the oil phase and the gum phase. Preferably said lipid acyltranferase is a polypeptide having lipid acyltransferase activity which polypeptide is obtained by expression of the nucleotide sequence shown as SEQ ID No. 49 or a nucleotide sequence which as has 70% or more identity therewith; and/or is obtained by expression of a nucleic acid which hybridises under medium stringency conditions to a nucleic probe comprising the nucleotide sequence shown as SEQ ID No. 49; and/or is a polypeptide having lipid acyltranferase activity which polypeptide comprises the amino acid sequence shown as SEQ ID No. 68 or an amino acid sequence which as has 70% or more identity therewith. In one embodiment the lipid acyltransferase is preferably used in combination with a phospholipase C enzyme. A process for modifying the gum phase of a degummed oil using a lipid acyltransferase is also taught herein.

PROCESS FOR EDIBLE OIL REFINING USING A LIPID ACYLTRANSFERASE

FIELD OF THE PRESENT INVENTION

The present invention relates to a process for edible oil (preferably vegetable oil) refining using a lipid acyltransferase. The present invention further relates to a process for treating an edible oil (preferably a crude edible oil) (e.g. a vegetable oil) and/or a gum phase of an edible oil (preferably vegetable oil) using a lipid acyltransferase.

BACKGROUND OF THE PRESENT INVENTION

Lipid acyltransferases are known to be advantageous in food applications. Lipid acyltransferases have been found to have significant acyltransferase activity in foodstuffs. This activity has surprising beneficial applications in methods of preparing foodstuffs.

For instance, WO 2004/064537 discloses a method for the *in* situ production of an emulsifier by use of a lipid acyltransferase and the advantages associated therewith.

International Patent Application No. PCT/IB2001/000558 teaches the expression of lipid acyltransferases in (heterologous) host cell.

The purpose of edible oil refining is to remove undesirable impurities that affect quality (taste, smell and appearance for example)) and storability.

Due to the wide variety of these impurities - free fatty acids, metal ions, colour compounds, odours, gums etc. – a series of processes of chemical and physical nature are conventionally employed for refining (see for example Bailey's Industrial Oil and Fat Products – 2006 John Wiley & Sons – Sixth Edition).

Traditionally two processes have been used for degumming of oil which are the physical degumming and the chemical degumming processes.

In the so-called chemical refining, almost all free fatty acid content is removed by initial treatment with a large excess of NaOH. Also the phospholipids content is decreased to a phosphorus level typically below 10 ppm. The oil is subsequently bleached and deodorised.

The so-called physical refining generally consists of a water-degumming step followed by acid degumming, neutralisation, bleaching, steam stripping to remove free fatty acids and deodorisation.

Instead of using acid degumming during physical refinement developments were made to use enzymatic degumming.

The enzymatic degumming process was developed based on the use of pancreatic phospholipase. Because this enzyme was non-kosher the phospholipase was eventually substituted by a microbial phospholipase A1 (Lecitase Ultra™ - Novozymes, Denmark) (Oil Mill Gazetteer, Vol 111 July 2005 pp2-4).

The enzymatic process has several advantages over the chemical or the physical degumming processes including cost savings, higher yield and a more environmentally friendly process.

The enzymatic oil degumming process was based on the addition of a phospholipase to an oil which was already water degummed.

In WO2006/008508 lipid acyltransferases were taught for use in enzymatic degumming of edible oils. WO 2006/008508 teaches addition of a lipid acyltransferase to a water-degummed oil or the addition of a lipid acyltransferase to a crude oil without the need for the oil to undergo a water-degumming process.

"Water-degummed oil" may typically be obtained by a conventional "water degumming process" comprising mixing 1-2% w/w of hot soft water with warm (70-90°C) crude oil (AOCS Introduction to the Processing of Fats and Oils – Table 8 – Degumming Processes - http://www.aocs.org/meetings/education/mod3sample.pdf). A rule of thumb is that that amount of water added to crude oil is typically approximately equal to the amount of phospholipids in the crude oil. Usual treatment periods are 30-60 minutes. The water-degumming step removes the phosphatides and mucilaginous gums which become insoluble in the oil when hydrated. The hydrated phosphatides and gums can be separated from the oil by settling, filtration or centrifugation - centrifugation being the more prevalent practice. The essential object in said water-degumming process is to separate the hydrated phosphatides from the oil. The mixing of hot water into the oil, described above, should herein be understood broadly as mixing of an aqueous solution into the oil according to standard water-degumming procedures in the art.

In the conventional water degumming process the main part of the phosphatides are removed in a heavy gum phase. At the end of the water degumming process an oil phase is separated from a gum phase. Although the gum phase can be processed further into commercial products it is essentially viewed as a bi-product of oil refining. It is the oil phase which is commercially important. However, because the phosphatides can be good emulsifiers some oil is inevitably lost in the gum phase during water degumming. This leads to reduced yields of oil in the oil phase following water degumming.

With increases in oil prices and an increasing need for vegetable oil for biodiesel it is important to optimise the processing of edible oils for high oil yield.

SUMMARY ASPECTS OF THE PRESENT INVENTION

Aspects of the present invention are presented in the claims and in the following commentary.

It has surprisingly been found that by adding one or more lipid acyltransferases to a crude edible oil during or before carrying out a water degumming process the yield of oil in the oil phase can be significantly increased. In other words, losses of oil to the gum phase can be significantly reduced.

In addition, it has surprisingly been found that by adding one or more lipid acyltransferases to a crude edible oil during or before carrying out a water degumming process the gum phase obtained is much less viscous. This may allow for more favourable centrifugation parameters.

It has also surprisingly been found that by adding one or more lipid acyltransferases to a crude edible oil during or before carrying out a water degumming process, the gum phase obtained from this process can be incubated or stored and (due to residual active lipid acyltransferase) further hydrolysis of phospholipids in the gum phase can be observed. The inventors have then found that it is then possible to isolate an oily phase containing free fatty acids (the acid oil) and the remaining triglycerides in the gum phase. This acid oil can be sold with a higher value than the normal gum phase which is added to meal. In addition, it has surprisingly been found that the remaining solid phase (after separation of the acid oil) has higher a phosphor level than normal gum and thus can be used as a source of organic phosphor.

It has also been surprisingly found that the combination of one or more lipid acyltransferases and one or more phospholipase C (PLC) enzymes results in synergistic effects when used in the degumming of edible oils (e.g. vegetable oils).

DETAILED ASPECTS OF THE PRESENT INVENTION

According to a first aspect of the present invention there is provided a process of water degumming an edible oil (preferably a crude edible oil) comprising the steps of:

a) admixing approximately 0.1-5% w/w water with an edible oil (preferably a crude edible oil) and a lipid acyltransferase, b) agitating the admixture for between about 10 minutes and 180 minutes at about 45°C to about 90°C, and c) separating the oil phase and the gum phase.

According to a second aspect of the present invention there is provided a use of a lipid acyltransferase during water degumming of an edible oil (preferably during the water degumming of a crude edible oil) for increasing the yield of oil in the oil phase after completion of the water degumming process.

According to a third aspect of the present invention there is provided a use of a lipid acyltransferase during water degumming of an edible oil (preferably during the water degumming of a crude edible oil) for decreasing the viscosity of the gum phase after completion of the water degumming process.

The increase in yield and/or decrease in viscosity is when compared with the oil phase and/or gum phase of a comparable oil degummed (either water degummed or enzymatically water degummed) without the use of the lipid acyltransferase.

According to a fourth aspect the present invention provides a process of water degumming an edible oil (preferably a crude edible oil) comprising the steps of: a) admixing approximately 0.1-5% w/w water with an edible oil (preferably a crude edible oil) and a lipid acyltransferase, b) agitating the admixture for between about 10 minutes and 180 minutes at about 45°C to about 90°C, c) separating the oil phase and the gum phase, d) incubating the gum phase comprising active lipid acyltransferase enzyme for between a minimum of about 2 hours and a maximum of 7 days (suitably up to about 1-2 days) and e) separating (e.g. by centrifugation) the oil from the gum phase.

The present invention further provides a method of treating a gum phase (preferably obtainable or obtained from degumming – such as water degumming or enzymatic degumming or a combination thereof – an edible oil) wherein the gum phase is incubated with one or more (active) lipid acyltransferase enzymes (alone or in combination with one or more phospholipase C enzyme) for between a minimum of

about 2 hours and a maximum of 7 days (suitably up to about 1-2 days) and separating (e.g. by centrifugation) the oil from the gum phase.

The present invention yet further provides the use of a lipid acyltransferase (alone or in combination with a phospholipase C) in the incubation of a gum phase (obtainable or obtained from degumming – such as water degumming, enzymatic degumming or a combination thereof – an edible oil) for increasing the yield of oil and/or producing a solid phase (after separation of the acid oil) with an improved phosphor level than normal gum.

The use of the enzyme(s) increases the value of the acid oil compared with the gum because the acid oil can be used for fatty acid production. Fatty acid has a higher value than a gum which is otherwise added to meal.

The improvements and/or increases are when compared with a gum phase which has not been treated by a lipid acyltransferase (alone or in combination with a phospholipase C).

Suitably the one or more lipid acyltransferase enzymes in the gum phase may have residual active enzyme which may have been transferred to the gum phase after enzymatic degumming of the edible oil. Alternatively the lipid acyltransferase enzyme in the gum phase may be added lipid acyltransferase – which enzyme may be added at the beginning or during the incubation of the gum phase.

Notably the oil at the end of the process in the fourth aspect (and other treatments of the gum phase) is an “acid oil”. This acid oil can be sold with a higher value than the normal gum phase which is added to meal. The remaining gum phase (after separation of the acid oil) is sometimes referred to as a solid phase. It has surprisingly been found that the remaining solid phase (after separation of the acid oil) has higher a phosphor level than normal gum and thus can be used as a source of organic phosphor.

Suitably the gum phase may be incubated with the lipid acyltransferase (either alone or with one or more phospholipase C enzymes) at about 30 to about 70°C, preferably at about 40 to about 60°C, preferably at about 40 to about 50°C, preferably at about 40 to about 45°C.

Preferably, the gum phase obtained from enzymatic water degumming of crude oil with lipid acyltransferase may be incubated at about about 30 to about 70°C, preferably at about 40 to about 60°C, preferably at about 40 to about 50°C, preferably at about 40 to about 45°C.

Suitably the lipid acyltransferase is one classified under the Enzyme Nomenclature classification (E.C. 2.3.1.43).

In one embodiment preferably the lipid acyl transferase is used in combination with a phospholipase C (E.C. 3.1.4.3).

In one preferable embodiment a lipid acyltransferase (E.C. 2.3.1.43) is used in combination with a phospholipase C (E.C. 3.1.4.3).

Therefore according to one aspect of the present invention there is provided a process of water degumming an edible oil (preferably a crude edible oil) comprising the steps of: a) admixing approximately 0.1-5% w/w water with an edible oil (preferably a crude edible oil) and a combination of a lipid acyltransferase and a phospholipase C, b) agitating the admixture for between about 10 minutes and 180 minutes at about 45°C to about 90°C, and c) separating the oil phase and the gum phase.

Without wishing to be bound by theory it has surprisingly been found that the lipid acyltransferase can use the diglyceride (produced by the reaction of the phospholipase C) as an acceptor molecule to produce triglyceride. Thus when a lipid acyltransferase is used in combination with a phospholipase C the interaction between these enzymes results in a synergistic increase in the amount of triglyceride in an oil comprising both enzymes compared with a comparable oil comprising either enzyme alone or a comparable oil comprising no enzyme. Advantageously when a

lipid acyltransferase is used in combination with a phospholipase C the interaction between these enzymes results in a synergistic decrease in the amount of diglyceride in an oil comprising both enzymes compared with a comparable oil comprising either enzyme alone or a comparable oil comprising no enzyme. When a lipid acyltransferase is used in combination with a phospholipase C the interaction between these enzymes results in a synergistic increase oil yield in an oil comprising both enzymes compared with a comparable oil comprising either enzyme alone or a comparable oil comprising no enzyme.

The use of a combination of these enzymes has significant advantages over the use of a phospholipase C alone as the accumulation of diglycerides in an oil (which can occur when a phospholipase C is used alone) can be detrimental to the oil because it can have a negative impact on the "smoke point" of the oil and/or can have a negative impact on the crystallisation properties of more saturated fat sources.

Hence in the present invention another advantage of the use of lipid acyltransferases (particularly when in combination with a phospholipase C) is that the amount of diglyceride in the oil can be reduced compared with a comparable oil without the lipid acyltransferase and/or particularly compared with a comparable oil treated with phospholipase C alone.

In another aspect of the present invention there is provided a use of a lipid acyltransferase in combination with a phospholipase C during water degumming of an edible oil (preferably during the water degumming of a crude edible oil) for increasing the yield of oil and/or for increasing triglyceride levels in the oil phase after completion of the water degumming process and/or for reducing the diglyceride level in the oil phase after completion of the water degumming process.

According to yet another aspect of the present invention there is provided a use of a lipid acyltransferase in combination with a phospholipase C during water degumming of an edible oil (preferably during the water degumming of a crude edible oil) for decreasing the viscosity of the gum phase after completion of the water degumming process.

These increases and/or reductions are when compared with a comparable degummed edible oil which has not been treated with a lipid acyltransferase in combination with a phospholipase C.

Generally the increases and/or reductions discussed herein are when compared with a comparable process or a comparable oil which has not been treated with a lipid acyltransferase (either alone or in combination with a phospholipase C).

According to another aspect the present invention provides a process of water degumming an edible oil (preferably a crude edible oil) comprising the steps of: a) admixing approximately 0.1-5% w/w water with an edible oil (preferably a crude edible oil) and a lipid acyltransferase in combination with a phospholipase C, b) agitating the admixture for between about 10 minutes and 180 minutes at about 45°C to about 90°C, c) separating the oil phase and the gum phase, d) incubating the gum phase comprising active lipid acyltransferase for between a minimum of about 2 hours and a maximum of 7 days (suitably for up to about 1-2 days) and e) separating (e.g. by centrifugation) oil from the gum phase.

When a phospholipid degrading enzyme (preferably a lipid acyltransferase) is used in combination with a phospholipase C the phospholipase C may be added before, at the same time or after the addition of the lipid acyltransferase enzyme.

In one embodiment preferably the phospholipase C is added before the lipid acyltransferase.

It has been surprisingly found that using a combination of a lipid acyltransferase and a phospholipase C significantly increases the yield of oil in the oil phase after completion of the water degumming process.

Without wishing to be bound by theory, it is envisaged that the phospholipase C hydrolyses the phospholipid (e.g. phosphatidylcholine) to a diglyceride (e.g. 1,2-diacylglycerol) and a phosphate moiety (e.g. choline phosphate) and the lipid acyltransferase then transfers a fatty acid onto the diglyceride formed by the

phospholipase C – thus forming more triglyceride and increasing the oil yield. This effect leads to a synergistic (i.e. preferably more than additive) increase on oil yield.

In one embodiment, suitably the method of degumming an edible oil and/or use according to the present invention may be carried out at between about 45-90°C, preferably between about 45 to about 70°C.

In another embodiment, suitably the method of degumming an edible oil process and/or use according to the present invention may be carried out at above about 44°C, more preferably above about 45°C, more preferably above about 50°C.

In another embodiment, suitably the process and/or use according to the present invention may be carried out at below about 60°C, preferably below about 65°C, preferably below about 70°C.

In one embodiment, suitably the process and/or use according to the present invention may be carried out at between about 45-70°C, preferably between about 45-68°C, more preferably between about 50-65°C degrees Celsius.

Suitably the temperature of the oil and/or water may be at the desired reaction temperature when the enzyme is admixed therewith.

The oil and/or water may be heated and/or cooled to the desired temperature before and/or during enzyme addition. Therefore in one embodiment it is envisaged that a further step of the process according to the present invention may be the cooling and/or heating of the oil and/or water.

Preferably the water content for the process according to the present invention may be between about 0.1-4% w/w, more preferably between about 0.1-3% w/w, more preferably between about 0.5-3% w/w.

In one embodiment the water content for the process according to the present invention may be between about 1-3% w/w.

In one embodiment the water content for the process according to the present invention may be less than about 3% w/w, suitably less than about 2%.

In one embodiment the water content for the process may be less than 1%. Reducing the amount of water to less than about 1% can result in a significant financial advantage in a water degumming process. Therefore being able to reduce the amount of water to less than about 1% can lead to significant cost reductions.

Suitably the reaction time (i.e. the time period in which the admixture is agitated) may be between about 10 minutes and about 180 minutes, preferably between about 15 minutes and about 180 minutes, more preferably between about 15 minutes and 60 minutes, even more preferably between about 15 minutes and about 35 minutes.

In one embodiment suitably the reaction time may be between about 30 minutes and about 180 minutes, preferably between about 30 minutes and about 60 minutes.

In one embodiment the process is preferably carried out at above about pH 4.5, above about pH 5 or above about pH 6.

Preferably the process is carried out between about pH 4.6 and about pH 10.0, more preferably between about pH 5.0 and about pH 10.0, more preferably between about pH 6.0 and about pH 10.0, more preferably between about pH 5.0 and about pH 7.0, more preferably between about pH 5.0 and about pH 6.5, and even more preferably between about pH 5.5 and pH 6.0.

In one embodiment the process may be carried out at a pH between about 5.3 to 8.3.

In one embodiment the process may be carried out at a pH between about 6-6.5, preferably about 6.3.

Suitably the pH may be neutral (about pH 5.0-about pH 7.0) in the methods and/or uses of the present invention.

Preferably the enzyme treatment occurs in the degumming process without pH adjustment of the oil and/or water. Therefore typically, the pH will be about 5.5-7.5.

This results in a significant advantage over prior art processes using phospholipase A enzymes which are typically only highly active in acid pH conditions, i.e. pH4-5. Therefore typically in prior art processes (for example using phospholipase A enzymes) the pH of the oil must be adjusted to more acidic conditions.

In addition, the use of a lipid acyltransferase with a phospholipase C enzyme has a significant advantage compared with the use of say a phospholipase A with a phospholipase C enzyme because the pH optima for lipid acyltransferases typically coincide much better with the pH optima for phospholipase C enzymes. Therefore, generally there is no "pH-conflict" when lipid acyltransferases are used in combination with phospholipase C enzymes. This contrasts sharply with the use of phospholipase A enzymes in combination with phospholipase C enzymes. Therefore, the use of lipid acyltransferases in combination with phospholipase C enzymes provides a significant improvement as both enzymes can work in their optimal pH range or simultaneously.

The separation of the oil phase and the gum phase may be carried out by any conventional separation method. Preferably the separation is carried out by centrifugation.

One significant advantage of the use of lipid acyltransferases (either alone or preferably in combination with a phospholipase C enzyme) is that the enzyme treatment makes it possible to adjust the centrifuge to control the amount of phosphor in the final oil. Without wishing to be bound by theory this is achievable because the viscosity of the oil is significantly reduced compared with an oil not treated with the lipid acyltransferase (either alone or preferably in combination with a phospholipase C enzyme). This is a significant advance over prior art processes. Typically, in conventional degumming processes the centrifugation results in a phosphor level in the oil of about 50ppm. In fact the specification guide for the level of phosphor in an edible oil is that it should be less than 200ppm. It is actually optimal to have oils with a phosphor level as close as possible to the 200ppm level. The use of the lipid acyltransferase (either alone or preferably in combination with a phospholipase C enzyme) results in an oil which can be centrifuged to a phosphor level of between about 100-200 ppm, preferably about 170-190ppm, more preferably about 180ppm. Adjustment of the centrifuge to give these levels of phosphor had prior to the present

invention been very difficult and provides a significant improvement in respect of the present invention.

Suitably the water may be admixed with the edible oil, prior to or at the same time as admixing with the enzyme. Alternatively, the edible oil and enzyme may be admixed before admixing with the water.

In one embodiment the oil, water and enzyme may be pumped in a stream simultaneously or substantially simultaneously through a mixer and into a holding tank.

Suitably the enzyme may be inactivated at during and/or at the end of the process.

The enzyme may be inactivated before or after separation of the oil phase and the gum phase.

Suitably the enzyme may be heat deactivated by heating for 10mins at 75-85°C or at above 92°C.

In one embodiment suitably the enzyme may be not deactivated in the gum phase. Thus when the gum phase is collected and incubated the enzyme may further degrade the phospholipids in the gum phase. After an extended incubation of the gum phase a further separation may be carried out (e.g. by centrifugation) in order to recover yet more oil from the gum phase. This may increase yet further the oil yield.

Without wishing to be bound by theory, the enzyme is thought to degrade the phospholipids to free fatty acids in the gum phase thus releasing triacylglyceride which had been previously emulsified with the phospholipids. This lowers the viscosity of the gum phase and allows the triacylglycerides and free fatty acids to be separated, for example by centrifugation.

In one embodiment suitably the process of the present invention may be carried out without the addition of an alkaline, such as NaOH for example.

In another embodiment suitably the process of the present invention may be carried out in the presence of an alkali, such as NaOH for example. When NaOH is added, preferably it is not added in an amount which exceeds about 0.2ml (4% solution) NaOH per 100 g oil.

Enzymes suitable for use in the methods and/or uses of the invention may have lipid acyltransferase activity as determined using the "Transferase Assay (Cholesterol:Phospholipid) (TrU)" below.

Determination of Transferase activity "TRANSFERASE ASSAY (CHOLESTEROL:PHOSPHOLIPID)" (TrU)

Substrate: 50 mg Cholesterol (Sigma C8503) and 450 mg Soya phosphatidylcholine(PC), Avanti #441601 is dissolved in chloroform, and chloroform is evaporated at 40°C under vacuum.

300 mg PC:cholesterol 9:1 is dispersed at 40°C in 10 ml 50mM HEPES buffer pH 7.

Enzymation:

250 µl substrate is added in a glass with lid at 40°C.

25 µl enzyme solution is added and incubated during agitation for 10 minutes at 40°C.

The enzyme added should esterify 2-5% of the cholesterol in the assay.

Also a blank with 25µl water instead of enzyme solution is analysed.

After 10 minutes 5 ml Hexan:Isopropanol 3:2 is added.

The amount of cholesterol ester is analysed by HPTLC using Cholesteryl stearate (Sigma C3549) standard for calibration.

Transferase activity is calculated as the amount of cholesterol ester formation per minute under assay conditions.

One Transferase Unit (TrU) is defined as µmol cholesterol ester produced per minute at 40°C and pH 7 in accordance with the transferase assay given above.

Preferably, the lipid acyltransferase used in the method and uses of the present invention will have a specific transferase unit (TrU) per mg enzyme of at least 25 TrU/mg enzyme protein.

Suitably the lipid acyltransferase for use in the present invention may be dosed in amount of 0.05 to 50 TrU per g oil, suitably in an amount of 0.5 to 5 TrU per g oil.

More preferably the enzymes suitable for use in the methods and/or uses of the present invention have lipid acyl-transferase activity as defined by the protocol below:

Protocol for the determination of % acyltransferase activity:

An edible oil to which a 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 and HPLC according to the procedure detailed hereinbelow. From the GLC and HPLC analyses the amount of free fatty acids and one or more of sterol/stanol esters; 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 and HPLC analyses the increase in free fatty acids and sterol/stanol esters can be calculated:

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

Mv fatty acid = average molecular weight of the fatty acids;

A = Δ % sterol ester/Mv sterol ester (where Δ % sterol ester = % sterol/stanol ester(enzyme) - % sterol/stanol ester(control) and Mv sterol ester = average molecular weight of the sterol/stanol esters);

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

$$\% \text{ transferase activity} = \frac{A \times 100}{A + \Delta \% \text{ fatty acid}/(\text{Mv fatty acid})}$$

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.

The edible oil used for the acyltransferase activity assay is preferably the soya bean oil supplemented with plant sterol (1%) and phosphatidylcholine (2%) oil using the method:

Plant sterol and phosphatidylcholine were dissolved in soya bean oil by heating to 95°C during agitation. The oil was then cooled to 40 °C and the enzymes were added. Water was added to a total concentration of 5% of the oil phase. The sample was maintained at 40 °C with magnetic stirring and samples were taken out after 4 and 20 hours and analysed by TLC.

For the assay the enzyme dosage used is preferably 0.2 TIPU-K/g oil, more preferably 0.08 TIPU-K/g oil, preferably 0.01 TIPU-K/g oil. The level of phospholipid present in the oil and/or the % conversion of sterol is preferably determined after 0.5, 1, 2, 4 and 20 hours, more preferably after 20 hours.

When the enzyme used is a lipid acyltransferase enzyme preferably the incubation time is effective to ensure that there is at least 5% transferase activity, preferably at least 10% transferase activity, preferably at least 15%, 20%, 25% 26%, 28%, 30%, 40% 50%, 60% or 75% transferase activity.

The % transferase activity (i.e. the transferase activity as a percentage of the total enzymatic activity) may be determined by the protocol taught above.

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 a edible oil treated with an lipid acyltransferase according to the present invention is less than the amount of free fatty acid produced in the edible oil 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 phospholipase enzyme, e.g. Lecitase Ultra™ (Novozymes A/S, Denmark), had been used.

In addition to, or instead of, assessing the % transferase activity in an oil (above), to identify the lipid acyl transferase enzymes most preferable for use in the methods of the invention the following assay entitled "Protocol for identifying lipid acyltransferases for use in the present invention" can be employed.

Protocol for identifying lipid acyltransferases

A lipid acyltransferase in accordance with the present invention is one which results in:

i) the removal of phospholipid present in a soya bean oil supplemented with plant sterol (1%) and phosphatidylcholine (2%) oil (using the method: Plant sterol and phosphatidylcholine were dissolved in soya bean oil by heating to 95°C during agitation. The oil was then cooled to 40 °C and the enzymes were added. The sample was maintained at 40 °C with magnetic stirring and samples were taken out after 0.5, 1, 2, 4 and 20 hours and analysed by TLC);
and/or
ii) the conversion (% conversion) of the added sterol to sterol-ester (using the method taught in i) above). The GLC method for determining the level of sterol and sterol esters as taught in Example 2 may be used.

For the assay the enzyme dosage used may be 0.2 TIPU-K/g oil, preferably 0.08 TIPU-K/g oil, preferably 0.01 TIPU-K/g oil. The level of phospholipid present in the oil and/or the conversion (% conversion) of sterol is preferably determined after 0.5, 1, 2, 4 and 20 hours, more preferably after 20 hours.

In the protocol for identifying lipid acyl transferases, after enzymatic treatment, 5% water is preferably added and thoroughly mixed with the oil. The oil is then separated into an oil and water phase using centrifugation (see "Enzyme-catalyzed degumming of vegetable oils" by Buchold, H. and Laurgi A.-G., Fett Wissenschaft Technologie (1993), 95(8), 300-4, ISSN: 0931-5985), and the oil phase can then be analysed for phosphorus content using the following protocol ("Assay for Phosphorus Content"):

Assay for Phosphorus Content

The level of phospholipid present in an oil after water degumming is determined by first preparing the oil sample according to the sample preparation taught in the AOAC Official Method 999.10 (>Lead, Cadmium, Zinc, Copper, and Iron in Foods Atomic Absorption Spectrophotometry after Microwave Digestion, First Action 1999 NMKL-AOAC Method). The amount of phospholipids in the oil is then measured by analysing the phosphorus content in the oil sample after degumming according to the AOAC Official Method Ca 20-99: Analysis of Phosphorus in oil by inductively Coupled Plasma Optical Emission Spectroscopy.

The amount of phosphorus present in the oil phase after using the present invention is typically not significantly different from the phosphorus content in the oil phase after conventional water degumming (i.e. without enzyme).

The oil yield using the present invention in the oil phase using the present invention is substantially increased compared with oil phase after using a conventional water degumming process (i.e. without enzyme). Suitably the process and/or use according to the present invention improves yield by about 0.25 to 7%, such as by about 0.25 to 3%, or about 0.5 to 2%, or about 1 to 2% compared with the same oil which has undergone the same water degumming process without addition of the enzyme.

Surprisingly it was found that the addition of enzyme in the process according to the present invention provides significantly higher oil yield in the oil phase without necessarily significantly reducing the phosphorus content of the oil phase compared with a comparable oil phase obtained using a comparative water degumming process but without addition of enzyme.

Suitably the amount of phosphorus in the oil phase when the oil has been treated in accordance with a process or use of the present invention may be 0-80%, suitably 0-50%, suitably 0-10%, suitably 0-1% less than the phosphorus content of an oil phase obtained using a comparative water degumming process but without addition of enzyme.

Notably the oil phase obtained in the process according to the present invention may be further degummed to remove phosphatides and/or phospholipids. For example the oil phase may undergo either enzymatic degumming and/or acid degumming.

The % conversion of the sterol present in the oil is at least 1%, preferably at least 5%, preferably at least 10%, preferably at least 20%, preferably at least 30%, preferably at least 40%, preferably at least 50%, preferably at least 60%, preferably at least 70%, preferably at least 80%, preferably at least 90%, preferably at least 95%.

In one embodiment the % conversion of the sterol present in the oil is at least 5%, preferably at least 20%.

In some aspects, the lipid acyltransferase for use in any one of the methods and/or uses of the present invention may comprise a GDSx motif and/ or a GANDY motif.

Preferably, the lipid acyltransferase enzyme is characterised as an enzyme which possesses acyltransferase 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.

Suitably, the nucleotide sequence encoding a lipid acyltransferase or lipid acyltransferase for use in any one of the methods and/or uses of the present invention may be obtainable, preferably obtained, 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*. Preferably, the lipid acyltransferase is obtainable, preferably obtained, from an organism from the genus *Aeromonas*.

In some aspects of the present invention, the nucleotide sequence encoding a lipid acyltransferase for use in any one of the methods and/or uses of the present invention encodes a lipid acyltransferase that comprises an aspartic acid residue at a position corresponding to N-80 in the amino acid sequence of the *Aeromonas salmonicida* lipid acyltransferase shown as SEQ ID No. 35.

In some aspects of the present invention, the lipid acyltransferase for use in any one of the methods and/or uses of the present invention is a lipid acyltransferase that comprises an aspartic acid residue at a position corresponding to N-80 in the amino acid sequence of the *Aeromonas salmonicida* lipid acyltransferase shown as SEQ ID No. 35.

In addition or in the alternative, the nucleotide sequence encoding a lipid acyltransferase for use in any one of the methods and/or uses of the present invention encodes a lipid acyltransferase that may comprise the amino acid sequence shown as SEQ ID No. 16, or an amino acid sequence which has 75% or more homology thereto. Suitably, the nucleotide sequence encoding a lipid acyltransferase encodes a lipid acyltransferase that may comprise the amino acid sequence shown as SEQ ID No. 16.

In addition or in the alternative, the nucleotide sequence encoding a lipid acyltransferase for use in any one of the methods and/or uses of the present invention encodes a lipid acyltransferase that may comprise the amino acid sequence shown as SEQ ID No. 68, or an amino acid sequence which has 75% or more homology thereto. Suitably, the nucleotide sequence encoding a lipid acyltransferase encodes a lipid acyltransferase that may comprise the amino acid sequence shown as SEQ ID No. 68.

In one embodiment the lipid acyltransferase for use in any one of the methods and/or uses of the present invention has an amino acid sequence shown in SEQ ID No. 16 or SEQ ID No. 68, or has an amino acid sequence which has at least 75% identity therewith, preferably at least 80%, preferably at least 85%, preferably at least 95%, preferably at least 98% identity therewith.

In one embodiment the lipid acyltransferase for use in any one of the methods and/or uses of the present invention is encoded by a nucleotide sequence shown in SEQ ID No. 49, or is encoded by a nucleotide sequence which has at least 75% identity therewith, preferably at least 80%, preferably at least 85%, preferably at least 95%, preferably at least 98% identity therewith.

In one embodiment preferably the lipid acyltransferase for use in any one of the methods and/or uses of the present invention is a lipid acyltransferase that is expressed in *Bacillus licheniformis* by transforming said *B. licheniformis* with a nucleotide sequence shown in SEQ ID No. 1 or a nucleotide sequence having at least 75% therewith (more preferably at least 80%, more preferably at least 85%, more preferably at least 95%, more preferably at least 98% identity therewith); culturing said *B. licheniformis* and isolating the lipid acyltransferase(s) produced therein.

The term "edible oil" as uses herein may encompass vegetable oils.

Preferably, the edible oil prior to treatment in accordance with the present invention is a crude edible oil comprising a non-hydratable phosphorus content of about 50-3000 ppm, more preferably in the range of about 50-1400 ppm, more preferably in the range of about 200-1400 ppm, and even more preferably in the range of about 400-1200 ppm.

In one aspect, the crude edible oil has, prior to carrying out the method of the invention, a phosphorous content above 350 ppm, more preferably above 400 ppm, even more preferably above 500 ppm, and most preferably above 600 ppm.

Preferably the edible oil is a vegetable oil.

Oils encompassed by the method according to the present invention may include, but are not limited to, one or more of soya bean oil, canola oil, corn oil, cottonseed oil, palm oil, coconut oil, rice bran oil, peanut oil, olive oil, safflower oil, palm kernel oil, rape seed oil and sunflower oil.

Preferably, the oil is one or more of soya bean oil, corn oil, sunflower oil and rape seed oil (sometimes referred to as canola oil).

More preferably, the oil is one or more of soya bean oil, sunflower oil or rape seed oil.

Most preferably, the oil is soya bean oil.

As used herein, "crude oil" (also referred to herein as a non-degummed oil) may be a pressed or extracted oil or a mixture thereof.

The phosphatide content in a crude oil may vary from 0.5-3% w/w corresponding to a phosphorus content in the range of 200-1200 ppm, more preferably in the range of 250-1200 ppm.

Apart from the phosphatides the crude oil may also contain small concentrations of carbohydrates, sugar compounds and metal/phosphatide acid complexes of Ca, Mg and Fe.

Advantageously, the method and uses of the present invention enable degumming of edible oils in a low water (<5%, preferably less than 2%, more preferably less than 1%) environments. Therefore water degumming can be performed with adding less water than when using a conventional water degumming process.

A further advantage of the present invention is the production of sterol esters in the oil phase.

Suitably the enzyme may be dosed in a range of about 0.01-10 TIPU-K/g oil, suitably the enzyme may be dosed in the range of about 0.05 to 1.5 TIPU-K/g oil, more preferably at 0.2-1 TIPU-K/g oil.

When the enzyme is a lipid acyltransferase suitably it may be dosed in the range of about 0.01 TIPU-K units/g oil to 5 TIPU-K units/g oil. In one embodiment the lipid acyltransferase may be dosed in the range of about 0.1 to about 1 TIPU-K units/g oil, more preferably the lipid acyltransferase may be dosed in the range of about 0.1 to about 0.5 TIPU-K units/g oil, more preferably the lipid acyltransferase may be dosed in the range of about 0.1 to about 0.3 TIPU-K units/g oil.

When the enzyme is a phospholipase suitably it may be dosed in the range of about 0.5-10 TIPU-K units/g oil. In one embodiment the phospholipase may be dosed in the range of about 0.5-5 TIPU-K units/g oil, preferably the phospholipase may be dosed in the range of about 0.5-1.5 TIPU-K units/g oil. Suitably the phospholipase may be dosed in the range of about 1.0-3 TIPU-K units/g oil.

Phospholipase activity, TIPU-K:

Substrate: 1.75% L- Plant Phosphatidylcholin 95% (441601, Avanti Polar Lipids), 6.3 % Triton X-100 (#T9284, Sigma) and 5 mM $CaCl_2$ dissolved in 50 mm Hepes pH 7.0. *Assay procedure*: Samples, calibration, and control were diluted in 10 mM HEPES pH 7.0, 0.1% Triton X-100 (#T9284, Sigma). Analysis was carried out using a Konelab Autoanalyzer (Thermo, Finland). The assay was run at 30C. 34 µL substrate was thermostatted for 180 seconds, before 4 µL sample was added. Enzymation lasted 600 sec. The amount of free fatty acid liberated during enzymation was measured using the NEFA C kit (999-75406, WAKO, Germany). 56 µL NEFA A was added and the mixture was incubated for 300 sec. Afterwards, 113 µL NEFA B was added and the mixture was incubated for 300 sec. OD 520 nm was then measured. Enzyme activity (µmol FFA/minmL) was calculated based on a standard enzyme preparation.

Enzyme activity TIPU-K was calculated as micromole free fatty acid (FFA) produced per minute under assay conditions.

In the present invention the process is preferably not a caustic neutralisation process (i.e. is not an acid-water degumming process and/or is not a acid-caustic degumming process). In other words, the process preferably does not comprise the addition of acids (such as phosphoric, citric, ascorbic, sulphuric, fumaric, maleic, hydrochloric and/or acetic acids) or caustics (such as KOH and NaOH), or does not comprise the addition of substantial amounts of acids or caustics. In other words if acids and/or caustics are added in the process of the present invention they are added at less than 0.004%.

For the ease of reference, these and further aspects of the present invention are now discussed under appropriate section headings. However, the teachings under each section are not necessarily limited to each particular section.

Phospholipase C

As mentioned above, the phospholipid degrading enzyme (preferably a lipid acyltransferase) may be used in combination with a phospholipase C (E.C. 3.1.4.3).

The phospholipase C may be any available phospholipase C enzyme and may be selected from one or more of the following phospholipase C enzymes: Purifine® (available from Verenium, US); a phospholipase C from *Clostridium perfringens* (such as the phospholipase C available from Sigma, Ref P7633); a phospholipase C from *Bacillus cereus* (such as the phospholipase C available from Sigma, Ref P6621); a phospholipase C enzyme taught in WO2008/036863.

ADVANTAGES

One advantage of the present invention is that an increased oil yield is obtained at the end of the water degumming process. The increase in oil yield is compared with a comparable water degumming process but without the addition of an enzyme in accordance with the present invention.

Without wishing to be bound by theory, the increased yield may be due to a decreased emulsifying effect caused by the removal of the phospholipids to the gum phase. Phospholipids are good emulsifiers and may be emulsified with triacylglyceride thus when the phospholipids are removed to the gum phase some oil in the form of triacylglyceride (oil) is also removed. A reduction in the viscosity of the gum phase due to the degradation of the phospholipids helps prevent the loss of oil to the gum phase (as separation is of the gum phase and the oil is much easier).

In addition or alternatively (without wishing to be bound by theory) when a lipid acyltransferase is used in accordance with the present invention sterol esters are formed by transferring a fatty acid moiety from a phospholipids to a sterol. This fatty acid moiety esterified to sterol by the lipid acyltransferase enzyme reaction is found in the oil phase and not in the gum phase. In conventional water degumming processes (without addition of lipid acyltransferase) these fatty acid moieties are lost to the gum phase.

A further advantage of the present invention is that when a lipid acyltransferase is used the pH in the water degumming process (about pH 5.0 or 5.5 to about pH 6.5 or 7) does not need to be adjusted. This pH results in a high reactivity of the lipid acyltransferase.

Another advantage of the present invention when using a lipid acyltransferase is the fatty acid from the phospholipids is transferred onto a sterol to form sterol esters. This on its own may contribute from between 0.1 to 0.15% increase in yield in the oil phase.

A further advantage of the present invention (particularly when using a lipid acyltransferase) is that the gum phase is less viscous compared with the gum phase from a comparable water degumming process but without the addition of an enzyme in accordance with the present invention. Lower viscosity in the gum phase results in it being easier to separate from the oil phase, i.e. by centrifugation.

In addition the gum phase may have a lower water content hence it may be easier to dry out.

A yet further advantage of the present invention is that there is a reduced triglyceride concentration in the gum phase.

The process of the present invention may result in a decreased fouling in the processing plant. This means that cleaning of the plant may be easier.

Without wishing to be bound by theory it has surprisingly been found that the lipid acyltransferase can use the diglyceride (produced by the reaction of the phospholipase C) as an acceptor molecule to produce triglyceride. Thus when a lipid acyltransferase is used in combination with a phospholipase C the interaction between these enzymes results in a synergistic increase in the amount of triglyceride in an oil comprising both enzymes compared with a comparable oil comprising either enzyme alone or a comparable oil comprising no enzyme. When a lipid acyltransferase is used in combination with a phospholipase C the interaction between these enzymes results in a synergistic increase oil yield in an oil comprising both enzymes compared with a comparable oil comprising either enzyme alone or a comparable oil comprising no enzyme.

The use of a combination of these enzymes has significant advantages over the use of a phospholipase C alone as the accumulation of diglycerides in an oil (which can

occur when a phospholipase C is used alone) can be detrimental to the oil because it can have a negative impact on the "smoke point" of the oil and/or can have a negative impact on the crystallisation properties of more saturated fat sources.

Hence in the present invention another advantage of the use of lipid acyltransferases (particularly when in combination with a phospholipase C) is that the amount of diglyceride in the oil can be reduced compared with a comparable oil without the lipid acyltransferase and/or particularly compared with a comparable oil treated with phospholipase C alone.

Use of the enzyme(s) in accordance with the present invention can reducing the amount of water needed in the process to less than about 1%. This can result in a significant financial advantage in a water degumming process. Therefore being able to reduce the amount of water to less than about 1% can lead to significant cost reductions.

Preferably the enzyme treatment occurs in the degumming process without pH adjustment of the oil and/or water. This results in a significant advantage over prior art processes using phospholipase A enzymes which are typically only highly active in acid pH conditions. Typically in prior art processes (for example using phospholipase A enzymes) the pH of the oil must be adjusted before and/or during the degumming process. This is not necessary with the present invention.

In addition, the use of a lipid acyltransferase in combination with a phospholipase C enzyme has a significant advantage compared with the use of say a phospholipase A with a phospholipase C enzyme because the pH optima for lipid acyltransferases typically coincide much better with the pH optima for phospholipase C enzymes. Therefore, generally there is no "pH-conflict" when lipid acyltransferases are used in combination with phospholipase C enzymes. This contrasts sharply with the use of phospholipase A enzymes in combination with phospholipase C enzymes. Therefore, the use of lipid acyltransferases in combination with phospholipase C enzymes provides a significant improvement as both enzymes can work in their optimal pH range or simultaneously.

Notably in the method which comprises treatment of the gum phase with a lipid acyltransferase (either alone or in combination with a phospholipase C) the "acid oil" produced at the end of this process can be sold with a higher value than the normal gum phase which is added to meal. In addition the remaining gum phase (after separation of the acid oil) has surprisingly been found to have a higher phosphor level than normal gum and thus can be used as a source of organic phosphor.

HOST CELL

The host organism can be a prokaryotic or a eukaryotic organism.

In one embodiment of the present invention the lipid acyl transferase according to the present invention in expressed in a host cell, for example a bacterial cells, such as a *Bacillus* spp, for example a *Bacillus licheniformis* host cell.

Alternative host cells may be fungi, yeasts or plants for example.

It has been found that the use of a *Bacillus licheniformis* host cell results in increased expression of a lipid acyltransferase when compared with other organisms, such as *Bacillus subtilis.*

A lipid acyltransferase from *Aeromonas salmonicida* has been inserted into a number of conventional expression vectors, designed to be optimal for the expression in *Bacillus subtilis*, *Hansenula polymorpha*, *Schizosaccharomyces pombe* and *Aspergillus tubigensis*, respectively. Only very low levels were, however, detected in *Hansenula polymorpha*, *Schizosaccharomyces pombe* and *Aspergillus tubigensis.* The expression levels were below 1 µg/ml, and it was not possible to select cells which yielded enough protein to initiate a commercial production (results not shown). In contrast, *Bacillus licheniformis* was able to produce protein levels, which are attractive for an economically feasible production.

In particular, it has been found that expression in *B. licheniformis* is approximately 100-times greater than expression in *B. subtilis* under the control of aprE promoter or is approximately 100-times greater than expression in *S. lividans* under the control of an A4 promoter and fused to cellulose (results not shown herein).

The host cell may be any *Bacillus* cell other than *B.subtilis.* Preferably, said *Bacillus* host cell being from one of the following species: *Bacillus licheniformis; B. alkalophilus; B. amyloliquefaciens; B. circulans; B. clausii; B. coagulans; B. firmus; B. lautus; B. lentus; B. megaterium; B. pumilus* or *B. stearothermophilus.*

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

Suitably, the host cell may be a protease deficient or protease minus strain and/or an α-amylase deficient or α-amylase minus strain.

The term "heterologous" as used herein means a sequence derived from a separate genetic source or species. A heterologous sequence is a non-host sequence, a modified sequence, a sequence from a different host cell strain, or a homologous sequence from a different chromosomal location of the host cell.

A "homologous" sequence is a sequence that is found in the same genetic source or species i.e. it is naturally occurring in the relevant species of host cell.

The term "recombinant lipid acyltransferase" as used herein means that the lipid acyltransferase has been produced by means of genetic recombination. For instance, the nucleotide sequence encoding the lipid acyltansferase has been inserted into a cloning vector, resulting in a *B. licheniformis* cell characterised by the presence of the heterologous lipid acyltransferase.

REGULATORY SEQUENCES

In some applications, a lipid acyltransferase sequence for use in the methods and/or uses of the present invention may be obtained by operably linking a nucleotide sequence encoding same to a regulatory sequence which is capable of providing for

the expression of the nucleotide sequence, such as by the chosen host cell (such as a *B. licheniformis* cell).

By way of example, 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, may be used.

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 regulatory regions, e.g. promoter, secretion leader and terminator regions that are not regulatory regions for the nucleotide sequence encoding the enzyme in nature.

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

Suitably, the nucleotide sequence encoding a lipid acyltransferase may be operably linked to at a nucleotide sequence encoding a terminator sequence. Examples of suitable terminator sequences for use in any one of the vectors, host cells, methods and/or uses of the present invention include: an α-amylase terminator sequence (for instance, CGGGACTTACCGAAAGAAACCATCAATGATGGTTTCTTTTTTGTTCATAAA – SEQ ID No. 64), an alkaline protease terminator sequence (for instance, CAAGACTAAAGACCGTTCGCCCGTTTTTTGCAATAAGCGGGCGAATCTTACATAAAA ATA – SEQ ID No. 65), a glutamic-acid specific terminator sequence (for instance,

ACGGCCGTTAGATGTGACAGCCCGTTCCAAAAGGAAGCGGGCTGTCTTCGTGTAT TATTGT – SEQ ID No. 66), a levanase terminator sequence (for instance, TCTTTTAAAGGAAAGGCTGGAATGCCCGGCATTCCAGCCACATGATCATCGTTT – SEQ ID No. 67) and a subtilisin E terminator sequence (for instance, GCTGACAAATAAAAAGAAGCAGGTATGGAGGAACCTGCTTCTTTTTACTATTATTG). Suitably, the nucleotide sequence encoding a lipid acyltransferase may be operably linked to an α-amylase terminator, such as a *B. licheniformis* α-amylase terminator.

PROMOTER

The promoter sequence to be used in accordance with the present invention may be heterologous or homologous to the sequence encoding a lipid acyltransferase.

The promoter sequence may be any promoter sequence capable of directing expression of a lipid acyltransferase in the host cell of choice.

Suitably, the promoter sequence may be homologous to a *Bacillus* species, for example *B. licheniformis*. Preferably, the promoter sequence is homologous to the host cell of choice.

Suitably the promoter sequence may be homologous to the host cell. "Homologous to the host cell" means originating within the host organism; i.e. a promoter sequence which is found naturally in the host organism.

Suitably, the promoter sequence may be selected from the group consisting of a nucleotide sequence encoding: an α-amylase promoter, a protease promoter, a subtilisin promoter, a glutamic acid-specific protease promoter and a levansucrase promoter.

Suitably the promoter sequence may be a nucleotide sequence encoding: the LAT (e.g. the alpha-amylase promoter from *B. licheniformis,* also known as AmyL), AprL (e.g. subtilisin Carlsberg promoter), EndoGluC (e.g. the glutamic-acid specific promoter from *B. licheniformis*), AmyQ (e.g. the alpha amylase promoter from *B. amyloliquefaciens* alpha-amylase promoter) and SacB (e.g. the *B. subtilis* levansucrase promoter).

Other examples of promoters suitable for directing the transcription of a nucleic acid sequence in the methods of the present invention include: the promoter of the *Bacillus lentus* alkaline protease gene (aprH), ; the promoter of the *Bacillus subtilis* alpha-amylase gene (amyE); the promoter of the *Bacillus stearothermophilus* maltogenic amylase gene (amyM); the promoter of the *Bacillus licheniformis* penicillinase gene (penP); the promoters of the *Bacillus subtilis* xylA and xylB genes; and/or the promoter of the *Bacillus thuringiensis* subsp. *tenebrionis* CryIIIA gene.

In a preferred embodiment, the promoter sequence is an α-amylase promoter (such as a *Bacillus licheniformis* α-amylase promoter). Preferably, the promoter sequence comprises the -35 to -10 sequence of the B. *licheniformis* α-amylase promoter – see Figures 53 and 55.

The "-35 to -10 sequence" describes the position relative to the transcription start site. Both the "-35" and the "-10" are boxes, i.e. a number of nucleotides, each comprising 6 nucleotides and these boxes are separated by 17 nucleotides. These 17 nucleotides are often referred to as a "spacer". This is illustrated in Figure 55, where the -35 and the -10 boxes are underlined. For the avoidance of doubt, where "-35 to -10 sequence" is used herein it refers to a sequence from the start of the -35 box to the end of the -10 box i.e. including both the -35 box, the 17 nucleotide long spacer and the -10 box.

SIGNAL PEPTIDE

The lipid acyltransferase produced by a host cell by expression of the nucleotide sequence encoding the lipid acyltransferase may be secreted or may be contained intracellularly depending on the sequence and/or the vector used.

A signal sequence may be used to direct secretion of the coding sequences through a particular cell membrane. The signal sequences may be natural or foreign to the lipid acyltransferase coding sequence. For instance, the signal peptide coding sequence may be obtained form an amylase or protease gene from a *Bacillus* species, preferably from *Bacillus licheniformis.*

Suitable signal peptide coding sequences may be obtained from one or more of the following genes: maltogenic α-amylase gene, subtilisin gene, beta-lactamase gene, neutral protease gene, prsA gene, and/or acyltransferase gene.

Preferably, the signal peptide is a signal peptide of *B. licheniformis* α-amylase, Aeromonas acyltransferase (for instance, mkkwfvcllglialtvqa - SEQ ID No. 21), *B. subtilis* subtilisin (for instance, mrskklwisllfaltliftmafsnmsaqa - SEQ ID No. 22) or *B. licheniformis* subtilisin (for instance, mmrkksfwfgmltafmlvftmefsdsasa - SEQ ID No. 23). Suitably, the signal peptide may be the signal peptide of *B. licheniformis* α-amylase.

However, any signal peptide coding sequence capable of directing the expressed lipid acyltransferase into the secretory pathway of a *Bacillus host* cell (preferably a *B. licheniformis* host cell) of choice may be used.

In some embodiments of the present invention, a nucleotide sequence encoding a signal peptide may be operably linked to a nucleotide sequence encoding a lipid acyltransferase of choice.

The lipid acyltransferase of choice may be expressed in a host cell as defined herein as a fusion protein.

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, such as a *B. licheniformis* host. The term "incorporated" preferably covers stable incorporation into the genome.

The nucleotide sequence encoding a lipid acyltransferase 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 (such as *B. licheniformis*), i.e. the vector is an expression vector.

The vectors of the present invention may be transformed into a suitable host cell as described above to provide for expression of a polypeptide having lipid acyltransferase activity as defined herein.

The choice of vector, e.g. plasmid, cosmid, virus or phage vector, genomic insert, will often depend on the host cell into which it is to be introduced. The present invention may cover other forms of expression vectors which serve equivalent functions and which are, or become, known in the art.

Once transformed into the host cell of choice, the vector may replicate and function independently of the host cell's genome, or may integrate into the genome itself.

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.

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.

LIPID ACYL TRANSFERASE

The nucleotide sequence encoding a lipid acyl transferase for use in any one of the methods and/or uses of the present invention may encode a natural lipid acyl transferase or a variant lipid acyl transferase.

The lipid acyl transferase for use in any one of the methods and/or uses of the present invention may be a natural lipid acyl transferase or a variant lipid acyl transferase.

For instance, the nucleotide sequence encoding a lipid acyl transferase for use in the present invention may be one as described in WO2004/064537, WO2004/064987, WO2005/066347, or WO2006/008508.

The term "lipid acyl transferase" as used herein preferably means an enzyme that has acyltransferase activity (generally classified as E.C. 2.3.1.x, for example 2.3.1.43), whereby the enzyme is capable of transferring an acyl group from a lipid to one or more acceptor substrates, such as one or more of the following: a sterol; a stanol; a carbohydrate; a protein; a protein subunit; a sugar alcohol, such as ascorbic acid and/or glycerol – preferably glycerol and/or a sterol, such as cholesterol.

Preferably, the lipid acyl transferase for use in any one of the methods and/or uses of the present invention is a lipid acyltransferase that is capable of transferring an acyl group from a phospholipid (as defined herein) to a sugar alcohol, such as ascorbic acid and/or glycerol and/or a sterol, preferably glycerol or a sterol, most preferably a sterol (e.g. cholesterol).

For some aspects the "acyl acceptor" according to the present invention may be any compound comprising a hydroxy group (-OH), such as for example, polyvalent alcohols, including glycerol; sterols; stanols; carbohydrates; hydroxy acids including fruit acids, citric acid, tartaric acid, lactic acid and ascorbic acid; proteins or a sub-unit thereof, such as amino acids, protein hydrolysates and peptides (partly hydrolysed protein) for example; and mixtures and derivatives thereof. Preferably, the "acyl acceptor" according to the present invention is not water.

The acyl acceptor is preferably not a monoglyceride.

In one embodiment the acyl acceptor may be a diglyceride.

In one aspect, the lipid acyltransferase for use in the methods and/or uses of the present invention preferably is able to transfer an acyl group from a lipid to a sterol and/or a stanol.

In another aspect, the lipid acyltransferase for use in the methods and/or uses of the present invention may, as well as being able to transfer an acyl group from a lipid to a sterol and/or a stanol, additionally be able to transfer the acyl group from a lipid to one or more of the following: a carbohydrate, a protein, a protein subunit, glycerol, fatty alcohol.

Suitably, the acyl acceptor may be naturally found in the oil. Alternatively the acyl acceptor may be added to the oil (e.g. the acyl acceptor may be extraneous to the oil). For instance, in some embodiments a sterol and/or stanol may be added to the oil prior to or during the degumming process. This is particularly important if the amount of acyl acceptor is rate limiting on the acyltransferase reaction. Addition of an acyl acceptor may lead to reductions in free fatty acids and/or higher acyl acceptor ester formation compared to an oil where no additional acyl acceptor is added.

Preferably, the lipid substrate upon which the lipid acyl acts is one or more of the following lipids: a phospholipid, such as a lecithin, e.g. phosphatidylcholine and/or phophatidylethanolamine.

This lipid substrate may be referred to herein as the "lipid acyl donor". The term lecithin as used herein encompasses phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and phosphatidylglycerol.

Preferred lipid acyltransferases for use in the present invention are identified as those which have a high activity such as high phospholipid hydrolytic activity or high phospholipid transferase activity on phospholipids in an oil environment, most preferably lipid acyl transferases for use in the present invention have a high phospholipid to sterol transferase activity.

As detailed above, other acyl-transferases suitable for use in the methods of the invention may be identified by identifying the presence of the GDSx, GANDY and HPT blocks either by alignment of the pFam00657 consensus sequence (SEQ ID No 1),

and/or alignment to a GDSx acyltransferase, for example SEQ ID No 28. In order to assess their suitability for degumming, i.e. identify those enzymes which have a transferase activity of at least 5%, more preferably at least 10%, more preferably at least 20%, more preferably at least 30%, more preferably at least 40%, more preferably 50%, more preferably at least 60%, 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, such acyltransferases are tested using the "Protocol for the determination of % acyltransferase activity" assay detailed hereinabove.

For some aspects, preferably the lipid acyl transferase for use in any one of the methods and/or uses of the present invention is a lipid acyltransferase that is incapable, or substantially incapable, of acting on a triglyceride and/or a 1-monoglyceride and/or 2-monoglyceride.

For some aspects, preferably the lipid acyl transferase for use in any one of the methods and/or uses of the present invention is a lipid acyltransferase that does not exhibit triacylglycerol lipase activity (E.C. 3.1.1.3) or does not exhibit significant triacylglycerol lipase activity (E.C. 3.1.1.3).

The ability to hydrolyse triglyeride (E.C. 3.1.1.3 activity) may be determined by lipase activity is determined according to Food Chemical Codex (3rd Ed., 1981, pp 492-493) modified to sunflower oil and pH 5.5 instead of olive oil and pH 6.5. The lipase activity is measured as LUS (lipase units sunflower) where 1 LUS is defined as the quantity of enzyme which can release 1 [mu]mol of fatty acids per minute from sunflower oil under the above assay conditions. Alternatively the LUT assay as defined in WO9845453 may be used.

The lipid acyl transferase for use in any one of the methods and/or uses of the present invention may be a lipid acyltransferase which is substantially incapable of acting on a triglyceride may have a LUS/mg of less than 1000, for example less than 500, such as less than 300, preferably less than 200, more preferably less than 100, more preferably less than 50, more preferably less than 20, more preferably less than 10, such as less than 5, less than 2, more preferably less than 1 LUS/mg. Alternatively LUT/mg activity is less than 500, such as less than 300, preferably less than 200, more preferably less than 100, more preferably less than 50, more

preferably less than 20, more preferably less than 10, such as less than 5, less than 2, more preferably less than 1 LUT/mg.

The lipid acyl transferase for use in any one of the methods and/or uses of the present invention may be a lipid acyltransferase which is substantially incapable of acting on a monoglyceride. This may be determined by using mono-oleate (M7765 1-Oleoyl-*rac*-glycerol 99%) in place of the sunflower oil in the LUS assay. 1 MGHU is defined as the quantity of enzyme which can release 1 [mu]mol of fatty acids per minute from monoglyceride under the assay conditions.

The lipid acyl transferase for use in any one of the methods and/or uses of the present invention is a lipid acyltransferase which is preferably substantially incapable of acting on a triglyceride may have a MGHU/mg of less than 5000, for example less than 1000, for example less than 500, such as less than 300, preferably less than 200, more preferably less than 100, more preferably less than 50, more preferably less than 20, more preferably less than 10, such as less than 5, less than 2, more preferably less than 1 MGHU/mg.

Suitably, the lipid acyltransferase for use in any one of the methods and/or uses of the present invention is a lipid acyltransferase which in addition to its lipid acyltransferase activity may also exhibit one or more of the following phospholipase activities: phospholipase A2 activity (E.C. 3.1.1.4) and/or phospholipase A1 activity (E.C. 3.1.1.32). The lipid acyl transferase may also have phospholipase B activity (E.C 3.1.1.5).

Suitably, for some aspects the lipid acyltransferase may be capable of transferring an acyl group from a phospholipid to a stanol and/or sterol, preferably cholesterol.

For some aspects, preferably the lipid acyltransferase for use any one of the methods and/or uses of the present invention encodes a lipid acyltransferase that is capable of transferring an acyl group from a phospholipid to a sterol and/or a stanol to form at least a sterol ester and/or a stanol ester.

Thus, in one embodiment the "acyl acceptor" according to the present invention may be a plant sterol/stanol.

Preferably, the lipid acyltransferase enzyme may be characterised using the following criteria:

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 an acyl acceptor to form a new ester; and

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.

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

The GDSX motif is comprised of four conserved amino acids. Preferably, the serine within the motif is a catalytic serine of the lipid acyl transferase enzyme. Suitably, the serine of the GDSX motif may be in a position corresponding to Ser-16 in *Aeromonas hydrophila* lipid acyltransferase 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 in accordance with the procedures taught in WO2004/064537 or WO2004/064987.

Preferably the lipid acyl transferase enzyme can be aligned using the Pfam00657 consensus sequence (for a full explanation see WO2004/064537 or WO2004/064987).

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 methods or uses of the invention may 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. See WO2004/064537 or WO2004/064987 for further details.

Preferably, residues of the GANDY motif are selected from GANDY, GGNDA, GGNDL, most preferably GANDY.

Preferably, when aligned with the Pfam00657 consensus sequence the enzyme for use in the methods or uses 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. 1: 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 3 as SEQ ID No. 2. 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 see WO2004/064537 or WO2004/064987).

In one embodiment, the lipid acyl transferase enzyme for use in any one of the methods and/or uses of the present invention is a lipid acyltransferase that 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* lipid acyltransferase enzyme shown in Figures 2 and 4 (SEQ ID No. 1 or SEQ ID No. 3).

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

In SEQ ID No. 3 or SEQ ID No. 1 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.

In one embodiment, the lipid acyl transferase enzyme for use any one of the methods and uses of the present invention is a lipid acyltransferase that comprises the following catalytic triad: Ser-34, Asp-306 and His-309 or comprises a serine residue, an aspartic acid residue and a histidine residue, respectively, at positions corresponding to Ser-34, Asp-306 and His-309 in the *Aeromonas hydrophila* lipid acyl transferase enzyme shown in Figure 4 (SEQ ID No. 3) or Figure 2 (SEQ ID No. 1). As stated above, in the sequence shown in SEQ ID No. 3 or SEQ ID No. 1 the first 18 amino acid residues form a signal sequence. Ser-34, Asp-306 and His-309 of the full length sequence, that is the protein including the signal sequence, equate to Ser-16, Asp-288 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 3 (SEQ ID No. 2) the active site residues correspond to Ser-7, Asp-345 and His-348.

In one embodiment, the lipid acyl transferase enzyme for use any one of the methods and/or uses of the present invention is a lipid acyltransferase that may be characterised using the following criteria:

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

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-306 and His-309, respectively, in the *Aeromonas hydrophila* lipid acyltransferase enzyme shown in SEQ ID No. 3 or SEQ ID No. 1.

Suitably, the lipid acyltransferase enzyme for use in any one of the methods and/or uses of the present invention may be encoded by one of the following nucleotide sequences:

(a) the nucleotide sequence shown as SEQ ID No. 36 (see Figure 29);
(b) the nucleotide sequence shown as SEQ ID No. 38 (see Figure 31);
(c) the nucleotide sequence shown as SEQ ID No. 39 (see Figure 32);
(d) the nucleotide sequence shown as SEQ ID No. 42 (see Figure 35);
(e) the nucleotide sequence shown as SEQ ID No. 44 (see Figure 37);
(f) the nucleotide sequence shown as SEQ ID No. 46 (see Figure 39);
(g) the nucleotide sequence shown as SEQ ID No. 48 (see Figure 41);
(h) the nucleotide sequence shown as SEQ ID No. 49 (see Figure 57);
(i) the nucleotide sequence shown as SEQ ID No. 50 (see Figure 58);
(j) the nucleotide sequence shown as SEQ ID No. 51 (see Figure 59);
(k) the nucleotide sequence shown as SEQ ID No. 52 (see Figure 60);
(l) the nucleotide sequence shown as SEQ ID No. 53 (see Figure 61);
(m) the nucleotide sequence shown as SEQ ID No. 54 (see Figure 62);
(n) the nucleotide sequence shown as SEQ ID No. 55 (see Figure 63);
(o) the nucleotide sequence shown as SEQ ID No. 56 (see Figure 64);
(p) the nucleotide sequence shown as SEQ ID No. 57 (see Figure 65);
(q) the nucleotide sequence shown as SEQ ID No. 58 (see Figure 66);
(r) the nucleotide sequence shown as SEQ ID No. 59 (see Figure 67);
(s) the nucleotide sequence shown as SEQ ID No. 60 (see Figure 68);
(t) the nucleotide sequence shown as SEQ ID No. 61 (see Figure 69);
(u) the nucleotide sequence shown as SEQ ID No. 62 (see Figure 70);
(v) the nucleotide sequence shown as SEQ ID No. 63 (see Figure 71);
(w) or

a nucleotide sequence which has 70% or more, preferably 75% or more, identity with any one of the sequences shown as SEQ ID No. 36, SEQ ID No. 38, SEQ ID No. 39, SEQ ID No. 42, SEQ ID No. 44, SEQ ID No. 46, SEQ ID No. 48, SEQ ID No. 49, SEQ ID No. 50, SEQ ID No. 51, SEQ ID No. 52, SEQ ID No. 53, SEQ ID No. 54, SEQ ID No. 55, SEQ ID No. 56, SEQ ID No. 57, SEQ ID No. 58, SEQ ID No. 59, SEQ ID No. 60, SEQ ID No. 61, SEQ ID No. 62 or SEQ ID No. 63.

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. 36, SEQ ID No. 38, SEQ ID No. 39, SEQ ID No. 42, SEQ ID No. 44, SEQ ID No. 46, SEQ ID No. 48, SEQ ID No. 49, SEQ ID No. 50, SEQ ID No. 51, SEQ ID No. 52, SEQ ID No. 53, SEQ ID No. 54, SEQ ID No. 55, SEQ ID No. 56, SEQ ID No. 57, SEQ ID No. 58, SEQ ID No. 59, SEQ ID No. 60, SEQ ID No. 61, SEQ ID No. 62 or SEQ ID No. 63.

In one embodiment, the nucleotide sequence encoding a lipid acyltransferase enzyme for use any one of the methods and uses of the present invention is a nucleotide sequence which has 70% or more, preferably 75% or more, identity with any one of the sequences shown as: SEQ ID No. 49, SEQ ID No. 50, SEQ ID No. 51, SEQ ID No. 62, and SEQ ID No. 63. 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. 49, SEQ ID No. 50, SEQ ID No. 51, SEQ ID No. 62, and SEQ ID No. 63.

In one embodiment, the nucleotide sequence encoding a lipid acyltransferase enzyme for use in any one of the methods and uses of the present invention is a nucleotide sequence which has 70% or more, 75% or more, 80% or more, preferably 85% or more, more preferably 90% or more and even more preferably 95% or more identity the sequence shown as SEQ ID No. 49.

Suitably, the lipid acyl transferase enzyme for use any one of the methods and/or uses of the present invention may be a lipid acyltransferase that comprises one or more of the following amino acid sequences:

(i) the amino acid sequence shown as SEQ ID No. 68

(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 SEQ ID No. 5
(v) the amino acid sequence shown as SEQ ID No. 6
(vi) the amino acid sequence shown as SEQ ID No. 7
(vii) the amino acid sequence shown as SEQ ID No. 8
(viii) the amino acid sequence shown as SEQ ID No. 9
(ix) the amino acid sequence shown as SEQ ID No. 10
(x) the amino acid sequence shown as SEQ ID No. 11
(xi) the amino acid sequence shown as SEQ ID No. 12
(xii) the amino acid sequence shown as SEQ ID No. 13
(xiii) the amino acid sequence shown as SEQ ID No. 14
(xiv) the amino acid sequence shown as SEQ ID No. 1
(xv) the amino acid sequence shown as SEQ ID No. 15
(xvi) the amino acid sequence shown as SEQ ID No. 16
(xvii) the amino acid sequence shown as SEQ ID No. 17
(xviii) the amino acid sequence shown as SEQ ID No. 18
(xix) the amino acid sequence shown as SEQ ID No. 34
(xx) the amino acid sequence shown as SEQ ID No. 35 or

an amino acid sequence which has 75%, 80%, 85%, 90%, 95%, 98% or more identity with any one of the sequences shown as SEQ ID No. 68, SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14 or SEQ ID No. 15, SEQ ID No. 16, SEQ ID No. 17, SEQ ID No. 18, SEQ ID No. 34 or SEQ ID No. 35.

Suitably, the lipid acyl transferase enzyme for use any one of the methods and uses of the present invention may be a lipid acyltransferase that comprises either the amino acid sequence shown as SEQ ID No. 68, or as SEQ ID No. 3 or as SEQ ID No. 4 or SEQ ID No. 1 or SEQ ID No. 15 or SEQ ID No. 16, or SEQ ID No. 34 or SEQ ID No. 35 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. 68 or the amino acid sequence shown as SEQ ID No. 3 or the amino acid sequence shown as SEQ ID No. 4 or the amino acid sequence shown as SEQ ID No. 1 or the amino acid sequence shown as SEQ ID No. 15 or the amino acid sequence shown as SEQ ID No. 16 or the

amino acid sequence shown as SEQ ID No. 34 or the amino acid sequence shown as SEQ ID No. 35.

Suitably the lipid acyl transferase enzyme for use any one of the methods and/or uses of the present invention may be a lipid acyltransferase that 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. 68, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 1, SEQ ID No. 15, SEQ ID No. 16, SEQ ID No. 17, SEQ ID No. 18, SEQ ID No. 34 or SEQ ID No. 35.

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Suitably, the lipid acyltransferase enzyme for use any one of the methods and/or uses of the present invention may be a lipid acyltransferase that 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. 3 or SEQ ID No. 1;

(b) an amino acid sequence shown as amino acids residues 101-200 of SEQ ID No. 3 or SEQ ID No. 1;

(c) an amino acid sequence shown as amino acid residues 201-300 of SEQ ID No. 3 or SEQ ID No. 1; 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 lipid acyl transferase enzyme for use in methods and uses of the present invention may comprise 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. 3 or SEQ ID No. 1;

(b) an amino acid sequence shown as amino acids residues 77-88 of SEQ ID No. 3 or SEQ ID No. 1;

(c) an amino acid sequence shown as amino acid residues 126-136 of SEQ ID No. 3 or SEQ ID No. 1;

(d) an amino acid sequence shown as amino acid residues 163-175 of SEQ ID No. 3 or SEQ ID No. 1;

(e) an amino acid sequence shown as amino acid residues 304-311 of SEQ ID No. 3 or SEQ ID No. 1; 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.

In one aspect, the lipid acyl transferase enzyme for use any one of the methods and/or uses of the present invention is a lipid acyltransferase that may be the lipid acyl transferase from *Candida parapsilosis* as taught in EP 1 275 711. Thus in one aspect the lipid acyl transferase for use in the method and uses of the present invention may be a lipid acyl transferase comprising one of the amino acid sequences taught in SEQ ID No. 17 or SEQ ID No. 18.

Much by preference, the lipid acyl transferase enzyme for use in any one of the methods and uses of the present invention is a lipid acyltransferase that may be a lipid acyl transferase comprising the amino acid sequence shown as SEQ ID No. 16, or an amino acid sequence which has 75% or more, preferably 85% or more, more preferably 90% or more, even more preferably 95% or more, even more preferably 98% or more, or even more preferably 99% or more identity to SEQ ID No. 16. This enzyme could be considered a variant enzyme.

In one aspect, the lipid acyltransferase enzyme for use any one of the methods and/or uses of the present invention is a lipid acyltransferase that may be a lecithin:cholesterol acyltransferase (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 lipid acyltransferase enzyme for use any one of the methods and/or uses of the present invention is a lipid acyltransferase that 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 NCIMB 41204 and NCIMB 41205, respectively.

A lipid acyltransferase enzyme for use in any one of the methods and/or uses of the present invention may be a phospholipid glycerol acyl transferase. Phospholipid glycerol acyl transferases include those isolated from *Aeromonas spp.*, preferably *Aeromonas hydrophila* or *A. salmonicida*, most preferably *A. salmonicida* or variants thereof.

Most preferred lipid acyl transferases for use in the present invention are encoded by SEQ ID No.s 1, 3, 4, 15, 16, 34 and 35. It will be recognised by the skilled person that it is preferable that the signal peptides of the acyl transferase has been cleaved during expression of the transferase. The signal peptide of SEQ ID No.s 1, 3, 4, 15 and 16 are amino acids 1-18. Therefore the most preferred regions are amino acids 19-335 for SEQ ID No. 1 and SEQ ID No. 3 (*A. hydrophilia*) and amino acids 19-336 for SEQ ID No. 4, SEQ ID No. 15 and SEQ ID No. 16. (*A. salmonicida*). When used to determine the homology of identity of the amino acid sequences, it is preferred that the alignments as herein described use the mature sequence.

In one embodiment, suitably the lipid acyl transferase for use in the present invention comprises (or consists of) the amino acid sequence shown in SEQ ID No. 16 or comprises (or consists of) an amino acid sequence which has at least 70%, at least 75%, at least 85%, at least 90%, at least 95%, at least 98% identity to SEQ ID No. 16.

In one embodiment, suitably the lipid acyl transferase for use in the present invention is encoded by a nucleotide sequence encoding the amino acid sequence comprising (or consisting of) the amino acid sequence shown in SEQ ID No. 68 or comprises (or consists of) an amino acid sequence which has at least 70%, at least 75%, at least 85%, at least 90%, at least 95%, at least 98% identity to SEQ ID No. 68.

Therefore the most preferred regions for determining homology (identity) are amino acids 19-335 for SEQ ID No. 1 and 3 (*A. hydrophilia*) and amino acids 19-336 for SEQ

ID No.s 4, 15 and 16 (*A. salmonicida*). SEQ ID No.s 34 and 35 are mature protein sequences of a lipid acyl transferase from *A. hydrophilia* and *A. salmonicida* respectively which may or may not undergo further post-translational modification.

A lipid acyltransferase enzyme for use any one of the methods and uses of the present invention may be a lipid acyltransferase that may also be isolated from *Thermobifida*, preferably *T. fusca*, most preferably that encoded by SEQ ID No. 28.

Suitable lipid acyltransferases 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:

a) a nucleic acid which encodes a polypeptide exhibiting lipid acyltransferase activity and is at least 70% identical (preferably at least 80%, more preferably at least 90% identical) with the polypeptide sequence shown in SEQ ID No. 16 or with the polypeptide shown in SEQ ID no. 68;

b) a (isolated) polypeptide comprising (or consisting of) an amino acid sequence as shown in SEQ ID No. 16 or SEQ ID No. 68 or an amino acid sequence which is at least 70% identical (preferably at least 80% identical, more preferably at least 90% identical) with SEQ ID No. 16 or SEQ ID No. 68;

c) a nucleic acid encoding a lipid acyltransferase, which nucleic acid comprises (or consists of) a nucleotide sequence shown as SEQ ID No. 49 or a nucleotide sequence which is at least 70% identical (preferably at least 80%, more preferably at least 90% identical) with the nucleotide sequence shown as SEQ ID No. 49;

d) a nucleic acid which hybridises under medium or high stringency conditions to a nucleic acid probe comprising the nucleotide sequence shown as SEQ ID No. 49 and encodes for a polypeptide exhibiting lipid acyltransferase activity;

e) a nucleic acid which is a fragment of the nucleic acid sequences specified in a), c) or d); or

f) a polypeptide which is a fragment of the polypeptide specified in b).

A lipid acyltransferase enzyme for use any one of the methods and uses of the present invention may be a lipid acyltransferase that may also be isolated from *Streptomyces*, preferable *S. avermitis*, most preferably that encoded by SEQ ID No. 32. Other possible enzymes for use in the present invention from *Streptomyces* include those encoded by SEQ ID No.s 5, 6, 9, 10, 11, 12, 13, 14, 31, and 33.

An enzyme for use in the invention may also be isolated from *Corynebacterium*, preferably *C. efficiens*, most preferably that encoded by SEQ ID No. 29.

Suitably, the lipid acyltransferase enzyme for use any one of the methods and/or uses of the present invention may be a lipid acyltransferase that comprises any one of the amino acid sequences shown as SEQ ID No.s 37, 38, 40, 41, 43, 45, or 47 or an amino acid sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith, or may be encoded by any one of the nucleotide sequences shown as SEQ ID No.s 36, 39, 42, 44, 46, or 48 or a nucleotide sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith.

In one embodiment, the nucleotide sequence encoding a lipid acyltransferase enzyme for use any one of the methods and/or uses of the present invention is selected from the group consisting of:

a) a nucleic acid comprising a nucleotide sequence shown in SEQ ID No. 36;
b) a nucleic acid which is related to the nucleotide sequence of SEQ ID No. by the degeneration of the genetic code; and
c) a nucleic acid comprising a nucleotide sequence which has at least 70% identity with the nucleotide sequence shown in SEQ ID No. 36.

In one embodiment, the lipid acyltransferase enzyme for use any one of the methods and/or uses of the present invention is a lipid acyltransferase that comprises an amino acid sequence as shown in SEQ ID No. 37 or an amino acid sequence which has at least 60% identity thereto.

In a further embodiment the lipid acyltransferase enzyme for use any one of the methods and/or uses of the present invention may be a lipid acyltransferase comprising any one of the amino acid sequences shown as SEQ ID No. 37, 38, 40, 41, 43, 45 or 47 or an amino acid sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith, or may be encoded by any one of the nucleotide sequences shown as SEQ ID No. 39, 42, 44, 46 or 48 or a nucleotide sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith.

In a further embodiment the lipid acyltransferase enzyme for use any one of the methods and/or uses of the present invention may be a lipid acyltransferase comprising any one of amino sequences shown as SEQ ID No. 38, 40, 41, 45 or 47 or an amino acid sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith for the uses described herein.

In a further embodiment the lipid acyltransferase for use in any one of the methods and/or uses of the present invention may be a lipid acyltransferase comprising any one of amino sequences shown as SEQ ID No. 38, 40, or 47 or an amino acid sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith for the uses described herein.

More preferably in one embodiment the lipid acyltransferase for use in any one of the methods and/or uses of the present invention may be a lipid acyltransferase comprising the amino acid sequence shown as SEQ ID No. 47 or an amino acid sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith.

In another embodiment the lipid acyltransferase for use in any one of the methods and uses of the present invention may be a lipid acyltransferase comprising the amino acid sequence shown as SEQ ID No. 43 or 44 or an amino acid sequence which has at least 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith.

In another embodiment the lipid acyltransferase for use in any one of the methods and uses of the present invention may be a lipid acyltransferase comprising the amino acid sequence shown as SEQ ID No. 41 or an amino acid sequence which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97% or 98% identity therewith.

In one embodiment the lipid acyltransferase for use in any one of the methods and uses of the present invention may be encoded by a nucleic acid selected from the group consisting of:

a) a nucleic acid comprising a nucleotide sequence shown in SEQ ID No. 36;
b) a nucleic acid which is related to the nucleotide sequence of SEQ ID No. 36 by the degeneration of the genetic code; and

c) a nucleic acid comprising a nucleotide sequence which has at least 70% identity with the nucleotide sequence shown in SEQ ID No. 36.

In one embodiment the lipid acyltransferase according to the present invention may be a lipid acyltransferase obtainable, preferably obtained, from the *Streptomyces* strains L130 or L131 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 25 June 2004 under accession numbers NCIMB 41226 and NCIMB 41227, respectively.

Suitable nucleotide sequences encoding a lipid acyltransferase for use in any one of the methods and/or uses of the present invention may encode a polynucleotide encoding a lipid acyltransferase (SEQ ID No. 16 or SEQ ID No. 68); or may encode an amino acid sequence of a lipid acyltransferase (SEQ ID No. 16 or SEQ ID No. 68).

A suitable lipid acyltransferases for use in any one of the methods and/or uses of the present invention may be an amino acid sequence which may be identified by alignment to the L131 (SEQ ID No. 37) sequence using Align X, the Clustal W pairwise alignment algorithm of VectorNTI using default settings.

An alignment of the L131 and homologues from *S. avermitilis* and *T. fusca* illustrates that the conservation of the GDSx motif (GDSY in L131 and *S. avermitilis* and *T. fusca*), the GANDY box, which is either GGNDA or GGNDL, and the HPT block (considered to be the conserved catalytic histidine). These three conserved blocks are highlighted in Figure 42.

When aligned to either the pfam Pfam00657 consensus sequence (as described in WO04/064987) and/ or the L131 sequence herein disclosed (SEQ ID No 37) it is possible to identify three conserved regions, the GDSx block, the GANDY block and the HTP block (see WO04/064987 for further details).

When aligned to either the pfam Pfam00657 consensus sequence (as described in WO04/064987) and/ or the L131 sequence herein disclosed (SEQ ID No 37)

i) The lipid acyltransferase for use in any one of the methods and uses of the present invention may be a lipid acyltransferase that has a GDSx motif, more preferably a GDSx motif selected from GDSL or GDSY motif.

and/or

ii) The lipid acyltransferase for use in any one of the methods and uses of the present invention may be a lipid acyltransferase that, has a GANDY block, more preferably a GANDY block comprising amino GGNDx, more preferably GGNDA or GGNDL.

and/or

iii) The lipid acyltransferase for use in any one of the methods and uses of the present invention may be a lipid acyltransferase that has preferably an HTP block.

and preferably

iv) the lipid acyltransferase for use in any one of the methods and uses of the present invention may be a lipid acyltransferase that has preferably a GDSx or GDSY motif, and a GANDY block comprising amino GGNDx, preferably GGNDA or GGNDL, and a HTP block (conserved histidine).

In one embodiment the enzyme according to the present invention may be preferably not a phospholipase enzyme, such as a phospholipase A1 classified as E.C. 3.1.1.32 or a phospholipase A2 classified as E.C. 3.1.1.4.

Variant lipid acyl transferase

In a preferred embodiment the nucleotide sequence encoding a lipid acyltransferase for use in any one of the methods and/or uses of the present invention may encode a lipid acyltransferase that is a variant lipid acyl transferase.

Variants which have an increased activity on phospholipids, such as increased hydrolytic activity and/ or increased transferase activity, preferably increased transferase activity on phospholipids may be used.

Preferably the variant lipid acyltransferase is prepared by one or more amino acid modifications of the lipid acyl transferases as defined hereinabove.

Suitably, the lipid acyltransferase for use in any one of the methods and uses of the present invention may be a lipid acyltransferase that may be a variant lipid acyltransferase, in which case the enzyme may be characterised in that 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, and wherein the variant enzyme comprises one or more amino acid modifications compared with a parent sequence at any one or more of the amino acid residues defined in set 2 or set 4 or set 6 or set 7 (as defined WO2005/066347 and hereinbelow).

For instance the variant lipid acyltransferase may be characterised in that 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, and wherein the variant enzyme comprises one or more amino acid modifications compared with a parent sequence at any one or more of the amino acid residues detailed in set 2 or set 4 or set 6 or set 7 (as defined in WO2005/066347 and hereinbelow) identified by said parent sequence being structurally aligned with the structural model of P10480 defined herein, which is preferably obtained by structural alignment of P10480 crystal structure coordinates with 1IVN.PDB and/or 1DEO.PDB as defined WO2005/066347 and hereinbelow.

In a further embodiment a lipid acyltransferase for use in any one of the methods and/or uses of the present invention may be a variant lipid acyltransferase that may be characterised in that 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, and wherein the variant enzyme comprises one or more amino acid modifications compared with a parent sequence at any one or more of the amino acid residues taught in set 2 identified when said parent sequence is aligned to the pfam consensus sequence (SEQ ID No. 2 –Figure 3) and modified according to a structural model of P10480 to ensure best fit overlap as defined WO2005/066347 and hereinbelow.

Suitably a lipid acyltransferase for use in any one of the methods and uses of the present invention may be a variant lipid acyltransferase enzyme that may comprise an amino acid sequence, which amino acid sequence is shown as SEQ ID No. 68, SEQ ID No. 16, SEQ ID No. 34, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No.

6, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 1, SEQ ID No. 15, SEQ ID No. 25, SEQ ID No. 26, SEQ ID No. 27, SEQ ID No. 28, SEQ ID No. 29, SEQ ID No. 30, , SEQ ID No. 32, SEQ ID No. 33 or SEQ ID No. 35 except for one or more amino acid modifications at any one or more of the amino acid residues defined in set 2 or set 4 or set 6 or set 7 (as defined WO2005/066347 and hereinbelow) identified by sequence alignment with SEQ ID No. 34.

Alternatively the lipid acyltransferase may be a variant lipid acyltransferase enzyme comprising an amino acid sequence, which amino acid sequence is shown as SEQ ID No. 34, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 1, SEQ ID No. 15, SEQ ID No. 25, SEQ ID No. 26, SEQ ID No. 27, SEQ ID No. 28, SEQ ID No. 29, SEQ ID No. 30, SEQ ID No. 16, SEQ ID No. 68, SEQ ID No. 32, SEQ ID No. 33 or SEQ ID No. 35 except for one or more amino acid modifications at any one or more of the amino acid residues defined in set 2 or set 4 or set 6 or set 7 as defined WO2005/066347 and hereinbelow, identified by said parent sequence being structurally aligned with the structural model of P10480 defined herein, which is preferably obtained by structural alignment of P10480 crystal structure coordinates with 1IVN.PDB and/or 1DEO.PDB as taught within WO2005/066347 and hereinbelow.

Alternatively, the lipid acyltransferase may be a variant lipid acyltransferase enzyme comprising an amino acid sequence, which amino acid sequence is shown as SEQ ID No. 34, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 1, SEQ ID No. 15, SEQ ID No. 25, SEQ ID No. 26, SEQ ID No. 27, SEQ ID No. 28, SEQ ID No. 29, SEQ ID No. 30, SEQ ID No. 32, SEQ ID No. 33, SEQ ID No. 16, SEQ ID No. 68 or SEQ ID No. 35 except for one or more amino acid modifications at any one or more of the amino acid residues taught in set 2 identified when said parent sequence is aligned to the pfam consensus sequence (SEQ ID No. 2) and modified according to a structural model of P10480 to ensure best fit overlap as taught within WO2005/066347 and hereinbelow.

Preferably, the parent enzyme is an enzyme which comprises, or is homologous to, the amino acid sequence shown as SEQ ID No. 34 and/or SEQ ID No. 15 and/or SEQ ID No. 35.

Preferably, the lipid acyltransferase may be a variant enzyme which comprises an amino acid sequence, which amino acid sequence is shown as SEQ ID No. 34 or SEQ ID No. 35 except for one or more amino acid modifications at any one or more of the amino acid residues defined in set 2 or set 4 or set 6 or set 7 as defined in WO2005/066347 and hereinbelow.

DEFINITION OF SETS

Amino acid set 1:

Amino acid set 1 (note that these are amino acids in 1IVN – Figure 53 and Figure 54) Gly8, Asp9, Ser10, Leu11, Ser12, Tyr15, Gly44, Asp45, Thr46, Glu69, Leu70, Gly71, Gly72, Asn73, Asp74, Gly75, Leu76, Gln106, Ile107, Arg108, Leu109, Pro110, Tyr113, Phe121, Phe139, Phe140, Met141, Tyr145, Met151, Asp154, His157, Gly155, Ile156, Pro158

The highly conserved motifs, such as GDSx and catalytic residues, were deselected from set 1 (residues underlined). For the avoidance of doubt, set 1 defines the amino acid residues within 10Å of the central carbon atom of a glycerol in the active site of the 1IVN model.

Amino acid set 2:

Amino acid set 2 (note that the numbering of the amino acids refers to the amino acids in the P10480 mature sequence)
Leu17, Lys22, Met23, Gly40, Asn80, Pro81, Lys82, Asn87, Asn88, Trp111, Val112, Ala114, Tyr117, Leu118, Pro156, Gly159, Gln160, Asn161, Pro162, Ser163, Ala164, Arg165, Ser166, Gln167, Lys168, Val169, Val170, Glu171, Ala172, Tyr179, His180, Asn181, Met209, Leu210, Arg211, Asn215, Lys284, Met285, Gln289 and Val290.

Table of selected residues in Set 1 compared with Set 2:

IVN model			P10480 Mature sequence Residue Number
IVN	A.hyd homologue		
	PFAM	Structure	
Gly8	Gly32		
Asp9	Asp33		
Ser10	Ser34		
Leu11	Leu35		Leu17
Ser12	Ser36		Ser18
			Lys22
			Met23
Tyr15	Gly58		Gly40
Gly44	Asn98		Asn80
Asp45	Pro99		Pro81
Thr46	Lys100		Lys82
			Asn87
			Asn88
Glu69	Trp129		Trp111
Leu70	Val130		Val112
Gly71	Gly131		
Gly72	Ala132		Ala114
Asn73	Asn133		
Asp74	Asp134		
Gly75	Tyr135		Tyr117
Leu76	Leu136		Leu118
Gln106		Pro174	Pro156
Ile107		Gly177	Gly159
Arg108		Gln178	Gln160
Leu109		Asn179	Asn161
Pro110		180 to 190	Pro162
Tyr113			Ser163
			Ala164
			Arg165

			Ser166
			Gln167
			Lys168
			Val169
			Val170
			Glu171
			Ala172
Phe121	His198	Tyr197	Tyr179
		His198	His180
		Asn199	Asn181
Phe139	Met227		Met209
Phe140	Leu228		Leu210
Met141	Arg229		Arg211
Tyr145	Asn233		Asn215
			Lys284
Met151	Met303		Met285
Asp154	Asp306		
Gly155	Gln307		Gln289
Ile156	Val308		Val290
His157	His309		
Pro158	Pro310		

Amino acid set 3:

Amino acid set 3 is identical to set 2 but refers to the *Aeromonas salmonicida* (SEQ ID No. 4) coding sequence, i.e. the amino acid residue numbers are 18 higher in set 3 as this reflects the difference between the amino acid numbering in the mature protein (SEQ ID No. 34) compared with the protein including a signal sequence (SEQ ID No. 25).

The mature proteins of *Aeromonas salmonicida* GDSX (SEQ ID No. 4) and *Aeromonas hydrophila* GDSX (SEQ ID No. 34) differ in five amino acids. These are Thr3Ser, Gln182Lys, Glu309Ala, Ser310Asn, and Gly318-, where the *salmonicida* residue is listed first and the *hydrophila* residue is listed last. The *hydrophila* protein is only 317 amino acids long and lacks a residue in position 318. The *Aeromonas salmonicida* GDSX has considerably high activity on polar lipids such as galactolipid substrates than the *Aeromonas hydrophila* protein. Site scanning was performed on all five amino acid positions.

Amino acid set 4:

Amino acid set 4 is S3, Q182, E309, S310, and -318.

Amino acid set 5:

F13S, D15N, S18G, S18V, Y30F, D116N, D116E, D157 N, Y226F, D228N Y230F.

Amino acid set 6:

Amino acid set 6 is Ser3, Leu17, Lys22, Met23, Gly40, Asn80, Pro81, Lys82, Asn 87, Asn88, Trp111, Val112, Ala114, Tyr117, Leu118, Pro156, Gly159, Gln160, Asn161, Pro162, Ser163, Ala164, Arg165, Ser166, Gln167, Lys168, Val169, Val170, Glu171, Ala172, Tyr179, His180, Asn181, Gln182, Met209, Leu210, Arg211, Asn215, Lys284, Met285, Gln289, Val290, Glu309, Ser310, -318.

The numbering of the amino acids in set 6 refers to the amino acids residues in P10480 (SEQ ID No. 25) – corresponding amino acids in other sequence backbones can be determined by homology alignment and/or structural alignment to P10480 and/or 1IVN.

Amino acid set 7:

Amino acid set 7 is Ser3, Leu17, Lys22, Met23, Gly40, Asn80, Pro81, Lys82, Asn 87, Asn88, Trp111, Val112, Ala114, Tyr117, Leu118, Pro156, Gly159, Gln160, Asn161, Pro162, Ser163, Ala164, Arg165, Ser166, Gln167, Lys168, Val169, Val170, Glu171,

Ala172, Tyr179, His180, Asn181, Gln182, Met209, Leu210, Arg211, Asn215, Lys284, Met285, Gln289, Val290, Glu309, Ser310, -318, Y30X (where X is selected from A, C, D, E, G, H, I, K, L, M, N, P, Q, R, S, T, V, or W), Y226X (where X is selected from A, C, D, E, G, H, I, K, L, M, N, P, Q, R, S, T, V, or W), Y230X (where X is selected from A, C, D, E, G, H, I, K, L, M, N, P, Q, R, S, T, V, or W), S18X (where X is selected from A, C, D, E, F, H, I, K, L, M, N, P, Q, R, T, W or Y), D157X (where X is selected from A, C, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W or Y).

The numbering of the amino acids in set 7 refers to the amino acids residues in P10480 (SEQ ID No. 25) – corresponding amino acids in other sequence backbones can be determined by homology alignment and/or structural alignment to P10480 and/or 1IVN).

Suitably, the variant enzyme comprises one or more of the following amino acid modifications compared with the parent enzyme:
S3E, A, G, K, M, Y, R, P, N, T or G
E309Q, R or A, preferably Q or R
-318Y, H, S or Y, preferably Y.

Preferably, X of the GDSX motif is L. Thus, preferably the parent enzyme comprises the amino acid motif GDSL.

Suitably, said first parent lipid acyltransferase may comprise any one of the following amino acid sequences: SEQ ID No. 34, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 1, SEQ ID No. 15, SEQ ID No. 25, SEQ ID No. 26, SEQ ID No. 27, SEQ ID No. 28, SEQ ID No. 29, SEQ ID No. 30, SEQ ID No. 32, SEQ ID No. 33 or SEQ ID No. 35.

Suitably, said second related lipid acyltransferase may comprise any one of the following amino acid sequences: SEQ ID No. 3, SEQ ID No. 34, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 1, SEQ ID No. 15, SEQ ID No. 25, SEQ ID No. 26, SEQ ID No. 27, SEQ ID No. 28, SEQ ID No. 29, SEQ ID No. 30, SEQ ID No. 32, SEQ ID No. 33 or SEQ ID No. 35.

The variant enzyme must comprise at least one amino acid modification compared with the parent enzyme. In some embodiments, the variant enzyme may comprise at least 2, preferably at least 3, preferably at least 4, preferably at least 5, preferably at least 6, preferably at least 7, preferably at least 8, preferably at least 9, preferably at least 10 amino acid modifications compared with the parent enzyme.

When referring to specific amino acid residues herein the numbering is that obtained from alignment of the variant sequence with the reference sequence shown as SEQ ID No. 34 or SEQ ID No. 35.

In one aspect preferably the variant enzyme comprises one or more of the following amino acid substitutions:

S3A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, T, V, W, or Y; and/or
L17A, C, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y; and/or
S18A, C, D, E, F, H, I, K, L, M, N, P, Q, R, T, W, or Y; and/or
K22A, C, D, E, F, G, H, I, L, M, N, P, Q, R, S, T, V, W, or Y; and/or
M23A, C, D, E, F, G, H, I, K, L, N, P, Q, R, S, T, V, W, or Y; and/or
Y30A, C, D, E, G, H, I, K, L, M, N, P, Q, R, S, T, V, or W; and/or
G40A, C, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; and/or
N80A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y; and/or
P81A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y; and/or
K82A, C, D, E, F, G, H, I, L, M, N, P, Q, R, S, T, V, W, or Y; and/or
N87A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y; and/or
N88A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y; and/or
W111A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W or Y; and/or
V112A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, W, or Y; and/or
A114C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; and/or
Y117A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, or W; and/or
L118A, C, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y; and/or
P156A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y; and/or
D157A, C, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y; and/or
G159A, C, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; and/or
Q160A, C, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y; and/or

N161A, C, D, E, F, G, H, I, K, L, M P, Q, R, S, T, V, W, or Y; and/or
P162A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y; and/or
S163A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, T, V, W, or Y; and/or
A164C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; and/or
R165A, C, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W, or Y; and/or
S166A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, T, V, W, or Y; and/or
Q167A, C, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y; and/or
K168A, C, D, E, F, G, H, I, L, M, N, P, Q, R, S, T, V, W, or Y; and/or
V169A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, W, or Y; and/or
V170A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, W, or Y; and/or
E171A, C, D, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; and/or
A172C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; and/or
Y179A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, or W; and/or
H180A, C, D, E, F, G, I, K, L, M, P, Q, R, S, T, V, W, or Y; and/or
N181A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y; and/or
Q182A, C, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y, preferably K; and/or
M209A, C, D, E, F, G, H, I, K, L, N, P, Q, R, S, T, V, W, or Y; and/or
L210 A, C, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W, or Y; and/or
R211 A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; and/or
N215 A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; and/or
Y226A, C, D, E, G, H, I, K, L, M, N, P, Q, R, S, T, V, or W; and/or
Y230A, C, D, E, G, H, I, K, L, M, N, P, Q, R, S, T, V or W; and/or
K284A, C, D, E, F, G, H, I, L, M, N, P, Q, R, S, T, V, W, or Y; and/or
M285A, C, D, E, F, G, H, I, K, L, N, P, Q, R, S, T, V, W, or Y; and/or
Q289A, C, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W, or Y; and/or
V290A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, W, or Y; and/or
E309A, C, D, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y; and/or
S310A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, T, V, W, or Y.

In addition or alternatively thereto there may be one or more C-terminal extensions. Preferably the additional C-terminal extension is comprised of one or more aliphatic amino acids, preferably a non-polar amino acid, more preferably of I, L, V or G. Thus, the present invention further provides for a variant enzyme comprising one or more of the following C-terminal extensions: 318I, 318L, 318V, 318G.

Preferred variant enzymes may have a decreased hydrolytic activity against a phospholipid, such as phosphatidylcholine (PC), may also have an increased transferase activity from a phospholipid.

Preferred variant enzymes may have an increased transferase activity from a phospholipid, such as phosphatidylcholine (PC), these may also have an increased hydrolytic activity against a phospholipid.

Modification of one or more of the following residues may result in a variant enzyme having an increased absolute transferase activity against phospholipid:

S3, D157, S310, E309, Y179, N215, K22, Q289, M23, H180, M209, L210, R211, P81, V112, N80, L82, N88; N87

Specific preferred modifications which may provide a variant enzyme having an improved transferase activity from a phospholipid may be selected from one or more of the following:

S3A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, T, V, W or Y; preferably N, E, K, R, A, P or M, most preferably S3A

D157A, C, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W or Y; preferably D157S, R, E, N, G, T, V, Q, K or C

S310A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, T, V, W or Y; preferably S310T -318 E

E309A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, T, V, W or Y; preferably E309 R, E, L, R or A

Y179A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V or W; preferably Y179 D, T, E, R, N, V, K, Q or S, more preferably E, R, N, V, K or Q

N215A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W or Y; preferably N215 S, L, R or Y

K22A, C, D, E, F, G, H, I, L, M, N, P, Q, R, S, T, V, W or Y; preferably K22 E, R, C or A

Q289A, C, D, E, F, G, H, I, K, L, M, N, P, R, S, T, V, W or Y; preferably Q289 R, E, G, P or N

M23A, C, D, E, F, G, H, I, K, L N, P, Q, R, S, T, V, W or Y; preferably M23 K, Q, L, G, T or S

H180A, C, D, E, F, G, I, K, L, M, P, Q, R, S, T, V, W or Y; preferably H180 Q, R or K

M209 A, C, D, E, F, G, H, I, K, L, N, P, Q, R, S, T, V, W or Y; preferably M209 Q, S, R, A, N, Y, E, V or L

L210A, C, D, E, F, G, H, I, K, M, N, P, Q, R, S, T, V, W or Y; preferably L210 R, A, V, S, T, I, W or M

R211A, C, D, E, F, G, H, I, K, L, M, N, P, Q, S, T, V, W or Y; preferably R211T

P81A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W or Y; preferably P81G

V112A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, W or Y; preferably V112C

N80A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W or Y; preferably N80 R, G, N, D, P, T, E, V, A or G

L82A, C, D, E, F, G, H, I, M, N, P, Q, R, S, T, V, W or Y; preferably L82N, S or E

N88A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W or Y; preferably N88C

N87A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W or Y; preferably N87M or G

Preferred modification of one or more of the following residues results in a variant enzyme having an increased absolute transferase activity against phospholipid:

S3 N, R, A, G

M23 K, Q, L, G, T, S

H180 R

L82 G

Y179 E, R, N, V, K or Q

E309 R, S, L or A

One preferred modification is N80D. This is particularly the case when using the reference sequence SEQ ID No. 35 as the backbone. Thus, the reference sequence may be SEQ ID No. 16. This modification may be in combination with one or more further modifications. Therefore in a preferred embodiment of the present invention the nucleotide sequence encoding a lipid acyltransferase for use in any one of the methods and uses of the present invention may encode a lipid acyltransferase that comprises SEQ ID No. 35 or an amino acid sequence which has 75% or more, preferably 85% or more, more preferably 90% or more, even more preferably 95% or more, even more preferably 98% or more, or even more preferably 99% or more identity to SEQ ID No. 35.

As noted above, when referring to specific amino acid residues herein the numbering is that obtained from alignment of the variant sequence with the reference sequence shown as SEQ ID No. 34 or SEQ ID No. 35

Much by preference, the nucleotide sequence encoding a lipid acyltransferase for use in any one of the methods and uses of the present invention may encode a lipid comprising the amino acid sequence shown as SEQ ID No. 16 or the amino acid sequence shown as SEQ ID No. 68, or an amino acid sequence which has 70% or more, preferably 75% or more, preferably 85% or more, more preferably 90% or more, even more preferably 95% or more, even more preferably 98% or more, or even more preferably 99% or more identity to SEQ ID No. 16 or SEQ ID No. 68. This enzyme may be considered a variant enzyme.

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 for amino acid sequences may be suitably determined by means of computer programs known in the art, such as Vector NTI 10 (Invitrogen Corp.). For pairwise alignment the score used is preferably BLOSUM62 with Gap opening penalty of 10.0 and Gap extension penalty of 0.1.

Suitably, the degree of identity with regard to an amino acid sequence is determined over at least 20 contiguous amino acids, preferably over at least 30 contiguous amino acids, preferably over at least 40 contiguous amino acids, preferably over at least 50 contiguous amino acids, preferably over at least 60 contiguous amino acids.

Suitably, the degree of identity with regard to an amino acid sequence may be determined over the whole sequence.

Suitably, the nucleotide sequence encoding a lipid acyltransferase or the lipid acyl transferase enzyme for use in 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, Candida, Thermobifida* and *Corynebacterium.*

Suitably, the nucleotide sequence encoding a lipid acyltransferase or the lipid acyl transferase enzyme for use in 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, Streptomyces thermosacchari, Streptomyces avermitilis 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 , Candida parapsilosis, Thermobifida fusca* and *Corynebacterium efficiens.*

In one aspect, preferably the nucleotide sequence encoding a lipid acyltransferase for use in any one of the methods and/or uses of the present invention encodes a lipid acyl transferase enzyme according to the present invention is obtainable, preferably obtained or derived, from one or more of *Aeromonas spp., Aeromonas hydrophila* or *Aeromonas salmonicida*.

In one aspect, preferably the lipid acyltransferase for use in any one of the methods and/or uses of the present invention is a lipid acyl transferase enzyme obtainable, preferably obtained or derived, from one or more of *Aeromonas spp., Aeromonas hydrophila* or *Aeromonas salmonicida.*

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

Suitably, the 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.

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); 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 5%, more preferably at least 10%, more preferably at least 20%, more preferably at least 30%, more preferably at least 40%, more preferably 50%, more preferably at least 60%, 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 the "Assay for Transferase Activity" given above.

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 a edible oil treated with an lipid acyltransferase according to the present invention is less than the amount of free fatty acid produced in the edible oil 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 phospholipase enzyme, e.g. Lecitase Ultra™ (Novozymes A/S, Denmark), had been used.

The term 'essentially consists' as used herein, when referring to a product or composition, preferably means that the product or composition, may consist of other products or compositions but only to a maximum concentration of, preferably 10%, such as 5%, such as 3%, such as 2% or 1%, or 0.5% or 0.1%.

In one preferred embodiment the lipid acyltransferase is used in combination with a lipase having one or more of the following enzyme activities: glycolipase activity (E.C. 3.1.1.26, phospholipase A2 activity (E.C. 3.1.1.4) or phospholipase A1 activity (E.C. 3.1.1.32). Suitably, lipase enzymes are well known within the art and include by way of example the following lipases: a phospholipase A1 LECITASE® ULTRA (Novozymes A/S, Denmark), phospholipase A2 (e.g. phospholipase A2 from LIPOMOD™ 22L from Biocatalysts, LIPOMAX™ and LysoMax PLA2™ from Genecor), LIPOLASE® (Novozymes A/S, Denmark).

In some embodiments it may be beneficial to combine the use of lipid acyltransferase with a phospholipase, such as phospholipase A1, phospholipase A2, phospholipase B, Phospholipase C and/or phospholipase D.

The combined use may be performed sequentially or concurrently, e.g. the lipid acyl transferase treatment may occur prior to or during the further enzyme treatment. Alternatively, the further enzyme treatment may occur prior to or during the lipid acyl transferase treatment.

In the case of sequential enzyme treatments, in some embodiments it may be advantageous to remove the first enzyme used, e.g. by heat deactivation or by use of an immobilised enzyme, prior to treatment with the second (and/or third etc.) enzyme.

POST-TRANSCRIPTION AND POST-TRANSLATIONAL MODIFICATIONS

Suitably the lipid acyltransferase in accordance with the present invention may be encoded by any one of the nucleotide sequences taught herein.

Depending upon the host cell used post-transcriptional and/or post-translational modifications may be made. It is envisaged that the lipid acyltransferase for use in the present methods and/or uses encompasses lipid acyltransferases which have undergone post-transcriptional and/or post-translational modification.

By way of example only, the expression of the nucleotide sequence shown herein as SEQ ID No. 49 (see Figure 57) in a host cell (such as *Bacillus licheniformis* for example) results in post-transcriptional and/or post-translational modifications which leads to the amino acid sequence shown herein as SEQ ID No. 68 (see Figure 73).

SEQ ID No. 68 is the same as SEQ ID No. 16 (shown herein in Figure 1) except that SEQ ID No. 68 has undergone post-translational and/or post-transcriptional modification to remove 38 amino acids.

ISOLATED

In one aspect, the lipid acyltransferase is a recovered/isolated lipid acyltransferase. Thus, the lipid acyltransferase produced may be in an isolated form.

In another aspect, the nucleotide sequence encoding a lipid acyltransferase for use in the present invention may be in an isolated form.

The term "isolated" means that the sequence or protein is at least substantially free from at least one other component with which the sequence or protein is naturally associated in nature and as found in nature.

PURIFIED

In one aspect, the lipid acyltransferase may be in a purified form.

In another aspect, the nucleotide sequence encoding a lipid acyltransferase for use in the present invention may be 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, labeled 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 nucleotide sequence encoding a lipid acyltransferase used in the invention may encode a variant lipid acyltransferase, i.e. the lipid acyltransferase may contain at least one amino acid substitution, deletion or addition, when compared to a parental enzyme. Variant enzymes retain at least 1%, 2%, 3%, 5%, 10%, 15%, 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 nucleotide sequence encoding a lipid acyltransferase for use in any one of the methods and/or uses of the present invention may encode a lipid acyltransferase that 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 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 may have increased thermostability.

The variant enzyme may 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 the use of amino acid sequences encoded by a nucleotide sequence which encodes a lipid acyltransferase for use in any one of the methods and/or uses of the present invention.

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

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.

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 Vector NTI (Invitrogen Corp.). 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), and FASTA (Altschul *et al* 1990 J. Mol. Biol. 403-410). 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 Vector NTI 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. Vector NTI 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 default values for the Vector NTI package.

Alternatively, percentage homologies may be calculated using the multiple alignment feature in Vector NTI (Invitrogen Corp.), 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.

Should Gap Penalties be used when determining sequence identity, then preferably the following parameters are used for pairwise alignment:

FOR BLAST	
GAP OPEN	0
GAP EXTENSION	0

FOR CLUSTAL	DNA	PROTEIN	
WORD SIZE	2	1	K triple
GAP PENALTY	15	10	
GAP EXTENSION	6.66	0.1	

In one embodiment, preferably the sequence identity for the nucleotide sequences is determined using CLUSTAL with the gap penalty and gap extension set as defined above.

Suitably, the degree of identity with regard to a nucleotide sequence is determined over at least 20 contiguous nucleotides, preferably over at least 30 contiguous nucleotides, preferably over at least 40 contiguous nucleotides, preferably over at least 50 contiguous nucleotides, preferably over at least 60 contiguous nucleotides, preferably over at least 100 contiguous nucleotides.

Suitably, the degree of identity with regard to a nucleotide sequence may be determined over the whole sequence.

In one embodiment the degree of amino acid sequence identity in accordance with the present invention may be suitably determined by means of computer programs known in the art, such as Vector NTI 10 (Invitrogen Corp.). For pairwise alignment the matrix used is preferably BLOSUM62 with Gap opening penalty of 10.0 and Gap extension penalty of 0.1.

Suitably, the degree of identity with regard to an amino acid sequence is determined over at least 20 contiguous amino acids, preferably over at least 30 contiguous amino acids, preferably over at least 40 contiguous amino acids, preferably over at least 50 contiguous amino acids, preferably over at least 60 contiguous amino acids.

Suitably, the degree of identity with regard to an amino acid sequence may be determined over the whole sequence.

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 the use of 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 the use of 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 the use of sequences that are complementary to sequences that are capable of hybridising under high stringency 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 the use of nucleotide sequences that can hybridise to the nucleotide sequences discussed herein (including complementary sequences of those discussed herein).

The present invention also relates to the use of 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 the use of 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 the use of 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 the use of nucleotide sequences that can hybridise to the nucleotide sequences discussed herein, or the complement thereof, under high stringency 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.

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.

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.

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 promoter not associated with a sequence encoding a lipid acyltransferase in nature.

TRANSFORMATION OF HOST CELLS/ORGANISM

The host organism can be a prokaryotic or a eukaryotic organism.

Examples of suitable prokaryotic hosts include bacteria such as *E. coli* and *Bacillus licheniformis*, preferably *B. licheniformis*.

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 secretion leader sequences not associated with a nucleotide sequence encoding a lipid acyltransferase in nature 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

The lipid acyltransferase for use in the present invention 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.

The amino acid sequence of a polypeptide having the specific properties as defined herein may be ligated to a non-native 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 non-native epitope that is recognised 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 the amino acid sequence of a mutant *Aeromonas salmonicida* mature lipid acyltransferase (GCAT) with a mutation of Asn80Asp (notably, amino acid 80 is in the mature sequence) (SEQ ID 16);

Figure 2 shows an amino acid sequence (SEQ ID No. 1) a lipid acyl transferase from *Aeromonas hydrophila* (ATCC #7965);

Figure 3 shows a pfam00657 consensus sequence from database version 6 (SEQ ID No. 2);

Figure 4 shows an amino acid sequence (SEQ ID No. 3) obtained from the organism *Aeromonas hydrophila* (P10480; GI:121051);

Figure 5 shows an amino acid sequence (SEQ ID No. 4) obtained from the organism *Aeromonas salmonicida* (AAG098404; GI:9964017);

Figure 6 shows an amino acid sequence (SEQ ID No. 5) obtained from the organism *Streptomyces coelicolor* A3(2) (Genbank accession number NP_631558);

Figure 7 shows an amino acid sequence (SEQ ID No. 6) obtained from the organism *Streptomyces coelicolor* A3(2) (Genbank accession number: CAC42140);

Figure 8 shows an amino acid sequence (SEQ ID No. 7) obtained from the organism *Saccharomyces cerevisiae* (Genbank accession number P41734);

Figure 9 shows an amino acid sequence (SEQ ID No. 8) obtained from the organism *Ralstonia* (Genbank accession number: AL646052);

Figure 10 shows SEQ ID No. 9. Scoe1 NCBI protein accession code CAB39707.1 GI:4539178 conserved hypothetical protein [Streptomyces coelicolor A3(2)];

Figure 11 shows an amino acid shown as SEQ ID No. 10. Scoe2 NCBI protein accession code CAC01477.1 GI:9716139 conserved hypothetical protein [Streptomyces coelicolor A3(2)];

Figure 12 shows an amino acid sequence (SEQ ID No. 11) Scoe3 NCBI protein accession code CAB88833.1 GI:7635996 putative secreted protein. [Streptomyces coelicolor A3(2)];

Figure 13 shows an amino acid sequence (SEQ ID No. 12) Scoe4 NCBI protein accession code CAB89450.1 GI:7672261 putative secreted protein. [Streptomyces coelicolor A3(2)];

Figure 14 shows an amino acid sequence (SEQ ID No. 13) Scoe5 NCBI protein accession code CAB62724.1 GI:6562793 putative lipoprotein [Streptomyces coelicolor A3(2)];

Figure 15 shows an amino acid sequence (SEQ ID No. 14) Srim1 NCBI protein accession code AAK84028.1 GI:15082088 GDSL-lipase [Streptomyces rimosus];

Figure 16 shows an amino acid sequence (SEQ ID No. 15) of a lipid acyltransferase from *Aeromonas salmonicida* subsp. *Salmonicida* (ATCC#14174);

Figure 17 shows SEQ ID No. 19. Scoe1 NCBI protein accession code CAB39707.1 GI:4539178 conserved hypothetical protein [Streptomyces coelicolor A3(2)];

Figure 18 shows an amino acid sequence (SEQ ID No. 25) of the fusion construct used for mutagenesis of the *Aeromonas hydrophila* lipid acyltransferase gene. The underlined amino acids is a xylanase signal peptide;

Figure 19 shows a polypeptide sequence of a lipid acyltransferase enzyme from *Streptomyces* (SEQ ID No. 26);

Figure 20 shows a polypeptide sequence of a lipid acyltransferase enzyme from *Thermobifida* (SEQ ID No. 27);

Figure 21 shows a polypeptide sequence of a lipid acyltransferase enzyme from *Thermobifida* (SEQ ID No. 28);

Figure 22 shows a polypeptide of a lipid acyltransferase enzyme from *Corynebacterium efficiens* GDSx 300 amino acid (SEQ ID No. 29);

Figure 23 shows a polypeptide of a lipid acyltransferase enzyme from *Novosphingobium aromaticivorans* GDSx 284 amino acid (SEQ ID No. 30);

Figure 24 shows a polypeptide of a lipid acyltransferase enzyme from *Streptomyces coelicolor* GDSx 269 aa (SEQ ID No. 31);

Figure 25 shows a polypeptide of a lipid acyltransferase enzyme from *Streptomyces avermitilis* \ GDSx 269 amino acid (SEQ ID No. 32);

Figure 26 shows a polypeptide of a lipid acyltransferase enzyme from *Streptomyces* (SEQ ID No. 33);

Figure 27 shows an amino acid sequence (SEQ ID No. 34) obtained from the organism *Aeromonas hydrophila* (P10480; GI:121051) (notably, this is the mature sequence);

Figure 28 shows the amino acid sequence (SEQ ID No. 35) of a mutant *Aeromonas salmonicida* mature lipid acyltransferase (GCAT) (notably, this is the mature sequence);

Figure 29 shows a nucleotide sequence (SEQ ID No. 36) from *Streptomyces thermosacchari;*

Figure 30 shows an amino acid sequence (SEQ ID No. 37) from *Streptomyces thermosacchari;*

Figure 31 shows an amino acid sequence (SEQ ID No. 38) from *Thermobifida fusca*/GDSx 548 amino acid;

Figure 32 shows a nucleotide sequence (SEQ ID No. 39) from *Thermobifida fusca;*

Figure 33 shows an amino acid sequence (SEQ ID No. 40) from *Thermobifida fusca*/GDSx;

Figure 34 shows an amino acid sequence (SEQ ID No. 41) from *Corynebacterium efficiens*/GDSx 300 amino acid;

Figure 35 shows a nucleotide sequence (SEQ ID No. 42) from *Corynebacterium efficiens;*

Figure 36 shows an amino acid sequence (SEQ ID No. 43) from *S. coelicolorl* GDSx 268 amino acid;

Figure 37 shows a nucleotide sequence (SEQ ID No. 44) from *S. coelicolor;*

Figure 38 shows an amino acid sequence (SEQ ID No. 45) from *S. avermitilis;*

Figure 39 shows a nucleotide sequence (SEQ ID No. 46) from *S. avermitilis;*

Figure 40 shows an amino acid sequence (SEQ ID No. 47) from *Thermobifida fusca/GDSx;*

Figure 41 shows a nucleotide sequence (SEQ ID No. 48) from *Thermobifida fusca/GDSx;*

Figure 42 shows an alignment of the L131 and homologues from *S. avermitilis* and *T. fusca* illustrates that the conservation of the GDSx motif (GDSY in L131 and *S. avermitilis* and *T. fusca*), the GANDY box, which is either GGNDA or GGNDL, and the HPT block (considered to be the conserved catalytic histidine). These three conserved blocks are highlighted;

Figure 43 shows SEQ ID No 17 which is the amino acid sequence of a lipid acyltransferase from *Candida parapsilosis*;

Figure 44 shows SEQ ID No 18 which is the amino acid sequence of a lipid acyltransferase from *Candida parapsilosis*;

Figure 45 shows a ribbon representation of the 1IVN.PDB crystal structure which has glycerol in the active site. The Figure was made using the Deep View Swiss-PDB viewer;

Figure 46 shows 1IVN.PDB Crystal Structure – Side View using Deep View Swiss-PDB viewer, with glycerol in active site - residues within 10Å of active site glycerol are coloured black;

Figure 47 shows 1IVN.PDB Crystal Structure – Top View using Deep View Swiss-PDB viewer, with glycerol in active site – residues within 10Å of active site glycerol are coloured black;

Figure 48 shows alignment 1;

Figure 49 shows alignment 2;

Figures 50 and 51 show an alignment of 1IVN to P10480 (P10480 is the database sequence for *A. hydrophila* enzyme), this alignment was obtained from the PFAM database and used in the model building process;

Figure 52 shows an alignment where P10480 is the database sequence for *Aeromonas hydrophila*. This sequence is used for the model construction and the site selection. Note that the full protein (SEQ ID No. 25) is depicted, the mature protein (equivalent to SEQ ID No. 34) starts at residue 19. A. sal is *Aeromonas salmonicida* (SEQ ID No. 4) GDSX lipase, A. hyd is *Aeromonas hydrophila* (SEQ ID No. 34) GDSX lipase. The consensus sequence contains a * at the position of a difference between the listed sequences;

Figure 53 shows a gene construct used in Example 1;

Figure 54 shows a codon optimised gene construct (no. 052907) used in Example 1; and

Figure 55 shows the sequence of the XhoI insert containing the LAT-KLM3' precursor gene, the -35 and -10 boxes are underlined;

Figure 56 shows BML780-KLM3'CAP50 (comprising SEQ ID No. 16 – upper colony) and BML780 (the empty host strain – lower colony) after 48h growth at 37°C on 1% tributyrin agar;

Figure 57 shows a nucleotide sequence from *Aeromonas salmonicida* (SEQ ID No. 49) including the signal sequence (preLAT - positions 1 to 87);

Figure 58 shows a nucleotide sequence (SEQ ID No. 50) encoding a lipid acyl transferase according to the present invention obtained from the organism *Aeromonas hydrophila*;

Figure 59 shows a nucleotide sequence (SEQ ID No. 51) encoding a lipid acyl transferase according to the present invention obtained from the organism *Aeromonas salmonicida*;

Figure 60 shows a nucleotide sequence (SEQ ID No. 52) 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 61 shows a nucleotide sequence (SEQ ID No. 53) 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 62 shows a nucleotide sequence (SEQ ID No. 54) encoding a lipid acyl transferase according to the present invention obtained from the organism *Saccharomyces cerevisiae* (Genbank accession number Z75034);

Figure 63 shows a nucleotide sequence (SEQ ID No. 55) encoding a lipid acyl transferase according to the present invention obtained from the organism *Ralstonia*;

Figure 64 shows a nucleotide sequence shown as SEQ ID No. 56 encoding NCBI protein accession code CAB39707.1 GI:4539178 conserved hypothetical protein [*Streptomyces coelicolor* A3(2)];

Figure 65 shows a nucleotide sequence shown as SEQ ID No. 57 encoding Scoe2 NCBI protein accession code CAC01477.1 GI:9716139 conserved hypothetical protein [*Streptomyces coelicolor* A3(2)];

Figure 66 shows a nucleotide sequence shown as SEQ ID No. 58 encoding Scoe3 NCBI protein accession code CAB88833.1 GI:7635996 putative secreted protein. [*Streptomyces coelicolor* A3(2)];

Figure 67 shows a nucleotide sequence shown as SEQ ID No. 59 encoding Scoe4 NCBI protein accession code CAB89450.1 GI:7672261 putative secreted protein. [*Streptomyces coelicolor* A3(2)];

Figure 68 shows a nucleotide sequence shown as SEQ ID No. 60, encoding Scoe5 NCBI protein accession code CAB62724.1 GI:6562793 putative lipoprotein [*Streptomyces coelicolor* A3(2)];

Figure 69 shows a nucleotide sequence shown as SEQ ID No. 61 encoding Srim1 NCBI protein accession code AAK84028.1 GI:15082088 GDSL-lipase [*Streptomyces rimosus*];

Figure 70 shows a nucleotide sequence (SEQ ID No. 62) encoding a lipid acyltransferase from *Aeromonas hydrophila* (ATCC #7965);

Figure 71 shows a nucleotide sequence (SEQ ID No 63) encoding a lipid acyltransferase from *Aeromonas salmonicida* subsp. *Salmonicida* (ATCC#14174);

Figure 72 shows a nucleotide sequence (SEQ ID No. 24) encoding an enzyme from *Aeromonas hydrophila* including a xylanase signal peptide;

Figure 73 shows the amino acid sequence of a mutant *Aeromonas salmonicida* mature lipid acyltransferase (GCAT) with a mutation of Asn80Asp (notably, amino acid 80 is in the mature sequence) – shown herein as SEQ ID No. 16 - and after undergoing post-translational modification as SEQ ID No. 68 – amino acid residues 235 and 236 of SEQ ID No. 68 are not covalently linked following post-translational modification. The two peptides formed are held together by one or more S-S bridges. Amino acid 236 in SEQ ID No. 68 corresponds with the amino acid residue number 274 in SEQ ID No. 16 shown herein;

Figure 74a shows a conventional process for water degumming/refining crude edible oil. At the end of the water degumming the oil phase and the gum phase are separated. After this the oil phase and gum phase may be further processed by conventional/known methods;

Figure 74b shows the process according to the present invention for water degumming/refining crude edible oil with an enzyme. The oil phase obtained when the oil and gum phase are separated has a much higher yield compared with the oil phase of a comparative process (i.e. one shown in Figure 74a – i.e. water degumming without the addition of an enzyme). The oil phase and/or gum phase may optionally undergo further processing, such as further conventional processing

Figure 75 shows a flow diagram of a lab scale water degumming process according to the present invention;

Figure 76 shows a diagram for analysis of the gum phase and the oil phase following water degumming (i.e. Step 1 of figure 74a or b);

Figure 77 shows the gum phase after 3hours following water degumming of crude soyabean oil in accordance with the present invention;

Figure 78 shows the %age gum after 30minutes water degumming with and without enzyme of crude soya oil;

Figure 79 shows the effect of the amount of water (1.5, 2 or 2.5%) on the amount of gum following water degumming of crude soya oil;

Figure 80 shows the effect with and without enzyme by degumming with different amounts of water (1.5, 2 or 2.5%) on the amount of gum following water degumming of crude soya oil with and without enzyme;

Figure 81 shows the ppm of phosphorus in the oil phase following water degumming of crude soya oil with different dosages of enzyme. Column 1 is the control without enzyme;

Figure 82 shows the % triglyceride in the gum phase following water degumming of crude soya oil at different enzyme dosages. Column 1 is the control without enzyme;

Figure 83 shows the relative % PA in the gum phase following water degumming of crude soya oil at different enzyme dosages. Column 1 is the control without enzyme;

Figure 84 shows the relative % PE in the gum phase following water degumming of crude soya oil at different enzyme dosages. Column 1 is the control without enzyme;

Figure 85 Increased oil yield (%) obtained in enzymatic degumming compared to control. Oils are centrifuged at different relative centrifuging force for 3 min;

Figure 86 shows the content (%) of gum and amount of triglyceride in gum, obtained from oils centrifuged at different times (minutes shown in bars) and different relative centrifuging forces are shown. Batch 3: control, 55°C, 4: with enzyme (KLM3'), 55°C;

Figure 87 shows viscosity as a function of shear rate. Measurements are based on gum from batch 1: control, 70°C and batch 2: with enzyme, 70°C;

Figure 88 shows oil yield (%) calculated from the amount of gum (control) subtracted amount of gum (enzymatic sample);

Figure 89 shows results from TLC analysis of the gum phase. Triglyceride content (%) in gums obtained from degumming with increasing amount (0, 0.1, 0.2, 0.5, 1, 1.5 and 1.9 ml 4%-solution) of NaOH;

Figure 90 shows GC-results. Contents (%) of FFA's, phytosterols and phytosterol esters in oils, degummed with increasing ml of NaOH- Sample 1: control (without enzyme and NaOH); Samples 2-8: enzymatic samples with KLM3' (0.1 TIPU-k/g) and increasing amounts (0, 0.1, 0.2, 0.5, 1, 1.5 and 1.9 ml 4%-solution) of NaOH;

Figure 91 shows results from TLC analysis of the gum phase. Relative degradation of phospholipids (PA, PE, PC and PI) in gums. Sample 1: control (without enzyme and NaOH), sample 2-7: enzymatic samples with KLM3' (0.1 TIPU-K/g) and increasing ml of NaOH;

Figure 92 shows microscopy analysis of gums from conventional water degumming and enzymatic water degumming in accordance with the present invention (pictures 200 and 400 magnifications at 25°C);

Figure 93 shows X-ray analysis on gum phases from conventional and enzymatic degumming;

Figure 94 shows sedimentation funnels (day 3). Left: control, right: enzyme treated oil;

Figure 95 shows microscopy analysis on gums from conventional and enzymatic water degumming;

Figure 96 shows increased oil yield obtained in enzymatic degumming compared to the control;

Figure 97 shows oil loss in the control and an enzymatic water degummed sample (in accordance with the present invention) carried out with 1, 1.5 and 2 % water. Calculation oil loss: (% gum / % triglyceride in gum) x 100%;

Figure 98 shows the relative degradation of phosphatidic acid and phosphatidylethanolamine in enzymatic (KLM3') gum samples compared to the control (no enzyme);

Figure 99 shows viscosity measurements of enzymatic gum phases, obtained from degumming with varying amount of water (1.25, 1.5, 1.75 and 2%);

Figure 100 shows Gum Phase from water degumming of crude soya with KLM3', and with addition of acceptor as shown in Table 1 of Example 9;

Figure 101 shows the relative amount of phospholipid in gum phase analysed by HPTLC;

Figure 102 shows ICP analysis of phosphor in oil from water degumming of crude soya oil (table 1 of Example 9);

Figure 103: Example 13 TLC (running buffer 1) of sample 1 to 9 after 30 minutes incubation;

Figure 104: Example 13 TLC (running buffer 1) of sample 1 to 9 after 240 minutes incubation;

Figure 105: Example 13 TLC (running buffer 6) of sample 1 to 9 after 30 minutes incubation. PE = phosphatidylethanolamine, PA = phosphatidic acid, PI = phosphatidylinositol and PC = phosphatidylcholine;

Figure 106: Example 13 TLC (running buffer 6) of sample 1 to 9 after 240 minutes incubation. PE = phosphatidylethanolamine, PA = phosphatidic acid, PI = phosphatidylinositol and PC = phosphatidylcholine;

Figure 107: Example 13 Relative degradation of phospholipids by enzymatic treatment of crude oil with lipid acyltransferase (KLM3') and phospholipase C (PLC). 240 minutes reaction time;

Figure 108: Example 13 Phospholipid diglyceride acyltransferase reaction;

Figure 109: Example 13 Interaction of Phospholipase C and KLM3' on diglyceride(DAG) level in degumming of crude soya oil;

Figure 110: Example 13 TLC analysis;

Figure 111 shows the effect of enzyme addition on triglyceride;

Figure 112 shows the effect of reaction time on triglyceride;

Figure 113 shows TLC analysis of diglyceride/PC substrate incubated with acyltransferase for 30 and 90 minutes as detailed in Example 13;

Figure 114 shows TLC analysis of diglyceride/PC substrate incubated with acyltransferase for 30 and 90 minutes as detailed in Example 13;

Figure 115 shows the effect of acyltransferase enzyme on triglyceride formation in a substrate of diglyceride/PC 80/20;

Figure 116 shows the effect of incubation time on triglyceride formation in a substrate of diglyceride/PC 80/20;

Figure 117 shows a flow diagram for enzymatic water degumming;

Figure 118 shows TLC analysis of the gum phase samples following water degumming at 55°C and incubation for 0d, 1d or 7d as detailed in Example 15; and

Figure 119 shows TLC analysis of the gum phase samples following water degumming at 45°C and incubation for 0d, 1d or 7d as detailed in Example 15.

EXAMPLE 1

Expression of KLM3' in *Bacillus licheniformis*

A nucleotide sequence (SEQ ID No. 49) encoding a lipid acyltransferase (SEQ. ID No. 16, hereinafter KLM3') was expressed in *Bacillus licheniformis* as a fusion protein with the signal peptide of *B. licheniformis* [alpha]-amylase (LAT) (see FIGS. 53 and 54). For optimal expression in Bacillus, a codon optimized gene construct (no. 052907) was ordered at Geneart (Geneart AG, Regensburg, Germany).

Construct no. 052907 contains an incomplete LAT promoter (only the –10 sequence) in front of the LAT-KLM3' precursor gene and the LAT transcription (Tlat) downstream of the LAT-KLM3' precursor gene (see FIGS 53 and 55). To create a *Xho*I fragment that contains the LAT-KLM3' precursor gene flanked by the complete LAT promoter at the 5' end and the LAT terminator at the 3' end, a PCR (polymerase chain reaction) amplification was performed with the primers Plat5XhoI_FW and EBS2XhoI_RV and gene construct 052907 as template.

Plat5XhoI_FW:

ccccgctcgaggcttttcttttggaagaaaatatagggaaaatggtacttgttaaaaattc

ggaatatttatacaatatcatatgtttcacattgaaagggg

EBS2XhoI_RV: tggaatctcgaggttttatcctttaccttgtctcc

PCR was performed on a thermocycler with Phusion High Fidelity DNA polymerase (Finnzymes OY, Espoo, Finland) according to the instructions of the manufacturer (annealing temperature of 55[deg.] C.).

The resulting PCR fragment was digested with restriction enzyme *Xho*I and ligated with T4 DNA ligase into *Xho*I digested pICatH according to the instructions of the supplier (Invitrogen, Carlsbad, Calif. USA).

The ligation mixture was transformed into *B. subtilis* strain SC6.1 as described in U.S. Patent Application US20020182734 (International Publication WO 02/14490). The sequence of the *Xho*I insert containing the LAT-KLM3' precursor gene was confirmed by DNA sequencing (BaseClear, Leiden, The Netherlands) and one of the correct plasmid clones was designated pICatH-KLM3'(ori1) (Figure 53). pICatH-KLM3'(ori1) was transformed into *B. licheniformis* strain BML780 (a derivative of BRA7 and BML612, see WO2005111203) at the permissive temperature (37[deg.] C.).

One neomycin resistant (neoR) and chloramphenicol resistant (CmR) transformant was selected and designated BML780(pICatH-KLM3'(ori1)). The plasmid in BML780(pICatH-KLM3'(ori1)) was integrated into the catH region on the *B. licheniformis* genome by growing the strain at a non-permissive temperature (50[deg.] C) in medium with 5 [mu]g/ml chloramphenicol. One CmR resistant clone was selected and designated BML780-pICatH-KLM3'(ori1). BML780-pICatH- KLM3'(ori1) was grown again at the permissive temperature for several generations without antibiotics to loop-out vector sequences and then one neomycin sensitive (neoS), CmR clone was selected. In this clone, vector sequences of pICatH on the chromosome are excised (including the neomycin resistance gene) and only the catH - LATKLM3' cassette is left. Next, the catH - LATKLM3' cassette on the chromosome was amplified by growing the strain in/on media with increasing concentrations of chloramphenicol. After various rounds of amplification, one clone (resistant against 50

[mu]g/ml chloramphenicol) was selected and designated BML780-KLM3'CAP50. To verify KLM3'expression, BML780-KLM3'CAP50 and BML780 (the empty host strain) were grown for 48h at 37 [deg.] C on a Heart Infusion (Bacto) agar plate with 1% tributyrin. A clearing zone, indicative for lipid acyltransferase activity, was clearly visible around the colony of BML780-KLM3'CAP50 but not around the host strain BML780 (see Figure 56). This result shows that a substantial amount of KLM3' is expressed in *B. licheniformis* strain BML780-KLM3'CAP50 and that these KLM3' molecules are functional.

COMPARATIVE EXAMPLE 1

Vector construct

The plasmid construct is pCS32new N80D, which is a pCCmini derivative carrying the sequence encoding the mature form of the native *Aeromonas salmonicida* Glycerophospholipid-cholesterol acyltransferase with a Asn to Asp substitution at position 80 (KLM3'), under control of the p32 promoter and with a CGTase signal sequence.

The host strain used for the expression, is in the *bacillus subtilis* OS21ΔAprE strain

The expression level is measured as transferase activity, expressed as % cholesterol esterified, calculated from the difference in free cholesterol in the reference sample and free cholesterol in the enzyme sample in reactions with PC (T_{PC}) as donor and cholesterol as acceptor molecule.

Culture conditions

5 ml of LB broth (Casein enzymatic digest, 10 g/l; low-sodium Yeast extract, 5 g/l; Sodium Chloride, 5 g/l; Inert tableting aids, 2 g/l) supplemented with 50 mg/l kanamycin, was inoculated with a single colony and incubated at 30 °C for 6 hours at 205 rpm. 0.7 ml of this culture was used to inoculate 50 ml of SAS media (K_2HPO_4, 10 g/l; MOPS (3-morpholinopropane sulfonic acid), 40 g/l; Sodium Chloride, 5 g/l; Antifoam (Sin 260), 5 drops/l; Soy flour degreased, 20 g/l; Biospringer 106 (100 % dw YE), 20 g/l) supplemented with 50 mg/l kanamycin and a solution of high maltose

starch hydrolysates (60 g/l). Incubation was continued for 40 hours at 30 °C and 180 rpm before the culture supernatant was separated by centrifugation at 19000 rpm for 30 min. The supernatant was transferred into a clean tube and directly used for transferase activity measurement.

Preparation of substrates and enzymatic reaction

PC (Avanti Polar Lipids #441601) and cholesterol (Sigma C8503) was scaled in the ratio 9:1, dissolved in chloroform, and evaporated to dryness.

The substrate was prepared by dispersion of 3% PC:Cholesterol 9:1 in 50 mM Hepes buffer pH 7.

0.250 ml substrate solution was transferred into a 3 ml glass tube with screw lid. 0.025 ml culture supernatant was added and the mixture was incubated at 40 °C for 2 hours. A reference sample with water instead of enzyme was also prepared. Heating the reaction mixture in a boiling water bath for 10 minutes stopped the enzyme reaction. 2 ml of 99% ethanol was added to the reaction mixture before submitted to cholesterol assay analysis.

Cholesterol assay

100 µl substrate containing 1.4 U/ml Cholesterol oxidase(SERVA Electrophoresis GmbH cat. No 17109), 0.4 mg/ml ABTS (Sigma A-1888), 6 U/ml Peroxidase (Sigma 6782) in 0.1 M Tris-HCl, pH 6.6 and 0.5 % Triton X-100 (Sigma X-100) was incubated at 37°C for 5 minutes before 5 µl enzyme reaction sample was added and mixed. The reaction mixture was incubated for further 5 minutes and OD_{405} was measured. The content of cholesterol was calculated from the analyses of standard solutions of cholesterol containing 0.4 mg/ml, 0.3 mg/ml, 0.20 mg/ml, 0.1 mg/ml, 0.05 mg/ml, and 0 mg/ml cholesterol in 99 % EtOH.

Results

The table shows the average of 8 separate expression cultures

Strain	T_{PC}[a]
OS21ΔAprE[pCS32new]	74.2 ±10.1[b]
[a] T_{PC} is the transferase activity, expressed as % cholesterol esterified, calculated from the difference in free cholesterol in the reference sample and free cholesterol in the enzyme sample in reactions with PC as donor molecule and cholesterol as acceptor molecule.	
[b] Average of 8 separate expression cultures	

EXAMPLE 2

Use of a lipid acyltransferase in water degumming

MATERIALS AND METHODS

Enzyme:

KLM3': a lipid acyltransferase taught in Example 1 having SEQ ID No. 68 (Also referred to herein as "K932") - 1128 TIPU/ml

Oil:

SBO 1:Crude soya bean oil from Solae, Aarhus, DK. 27.09.2007 Delite (Based on beans from Canada)

SBO 2: Crude Soya Oil from Brazil

RSO 3: Crude extracted Rapeseed Oil from Aarhus Karlshamn

RSO 4: Crude pressed Rapeseed Oil from Scanola, Aarhus, DK

Soy Lecithin Mix Standard (ST16) from Spectra Lipid, Germany

Methods:

HPTLC:

Applicator: Automatic TLC Sampler 4, CAMAG

HPTLC plate: 20 x 10 cm, Merck no. 1.05641. Activated 10 minutes at 160°C before use.

Application:

Oil phase: 5 µl of a 8% solution of oil in Chloroform:Methanol 2:1 was applied to the HPTLC plate using Automatic TLC Sampler.

Gum phase: Gum phase from 10 gram oil was dissolved in 7.5 ml chloroform:methanol 2:1.

1 µl of the sample was applied to the HPTLC plate.

TLC applicator.

Running buffer 6: Chloroform:1-propanol:Methylacetate:Methanol: 0.25% KCl in water 25:25:25:10:9

Running buffer 5: P-ether: MTBE 30:70

Elution: The plate was eluted 7 cm using an Automatic Developing Chamber ADC2 from Camag.

Development:

The plate was dried in an oven for 10 minutes at 160°C, cooled, and dipped into 6% cupri acetate in 16% H_3PO_4. Dried additionally 10 minutes at 160°C and evaluated directly.

After development the plates were scanned on a Camag Scanner and the area of each component (spot) on the TLC plate was calculated.

Calculation

Oil phase:

The amount of phospholipid in the oil phase was calculated by analysing a Standard lecithin with known concentrations of phospholipids (PE, PA, PI, PC, PS) at different concentrations on the same TLC plate as the oil samples. Based on the standard mixture a calibration curve for each phospholipid was produced and used for calculation of the phospholipid concentration of each phospholipid in the oil sample.

Based on the mol weight of the concentration of phospholipids were converted to ppm P (phosphorus).

Gum Phase:

The content of triglyceride in the gum phase was calculated based on analysing a standard refined vegetable oil on the same plate as the gum phase. Based on the analysis of the vegetable oil a calibration curve was produced and used for calculation of the triglyceride in the gum phase.

The analysis of the phospholipids in the gum phase was based on applying different volumes of the gum phase from the control (without enzyme added) on the same plate as the other gum phases. Based on the analysis of phospholipids (PE and PA) in the control gum phase a calibration curve was produced and used for calculation of the amount of phospholipids in the enzyme treated samples relative to the amount of phospholipid in the control which was defined as 100%.

pH measurement:

The pH of samples from oil degumming was analysed by a fluorescence method described in http://www.3i-usa.com/downloads/hydrop_man.pdf, i.e. The pH

measurement was conducted by using a HydroPlate® HP96C from Presens, Josef Engert Str. 11, D-93053 Regensburg, Germany.

The HydroPlate® is a sterile, polystyrene microtiter plate in the common 96-well format with 96 integrated sensors. A sensor is immobilised on the bottom of each well. The sensor can be read out from the bottom side. This can be done by almost any commercially available fluorescence plate reader. The assay is bases on 2 different, fluorescent dyes: A pH-sensitive indicator and an inert reference dye. This combination ensures a precise, internally referenced signal for achieving the most exact results of the experiments.

pH can alternatively be measured by using a pH electrode according Bo Yang *et al* JAOCS, Vol. 83, No. 7 (2006) pp653-658.

Determination of water in oil

Residual water in the oil is determined by AOCS method Ca 2c-25 or equivalent.

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 (used since 30.10.2003):	1	2	3
Oven temperature, °C.	90	280	350
Isothermal, time, min.	1	0	10
Temperature rate, °C/min.	15	4	

Sample preparation: 50 mg sample was dissolved in 12 ml Pyridin, containing internal standard heptadecane, 0.5 mg/ml. 500µl sample solution was then transferred to a crimp vial, 100 µl MSTFA:TMCS – 99:1 (N-Methyl-N-trimethylsilyl-trifluoraceamid) was added and reacted for 20 minutes at 60°C.

Calculation: Response factors for sterol, sterol palmitate and sterol stearate were determined from pure reference material (weighing pure material 8-10mg in 12 ml Pyridin, containing internal standard heptadecane, 0.5 mg/ml.).

Enzyme assay, TIPU

Substrate:

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

Assay procedure:

34 µl substrate was added to a cuvette, using a KoneLab automatic analyzer. At time T= 0 min, 4µl enzyme solution was added. Also a blank with water instead of enzyme was analyzed. The sample was mixed and incubated at 30°C for 10 minutes.

The free fatty acid content of sample was analyzed by using the NEFA C kit from WAKO GmbH.

Enzyme activity TIPU pH 7 was calculated as micromole fatty acid produced per minute under assay conditions.

Degumming procedure lab scale.

100 g crude soya oil was scaled into a 250 ml Blue Cap flask with lid and heated to 50°C or 55°C or 60°C or 65°C or 70°C.

Water was then added to the oil followed by enzyme addition. The oil was homogenised with an Ultra Turrax mixer for 30 seconds, and then agitated for 30 minutes with magnetic stirring at 450 rpm.

After 30, 120 or 180 minutes, 10 ml oil was transferred to a 12 ml centrifuge tube (previously scaled). The oil was heated to 97°C in a boiling water bath for 10 minutes, and then immediately centrifuged at 5000 g for 5 minutes.

Oil was decanted from the gum phase and the tubes were drained for 30 minutes and the weight of both phases measured. (See Figure 75).

The oil phase was analysed for free sterols, sterol esters and free fatty acids by GLC, and the oil phase was also analysed by TLC. (See Figure 76).

Results

EXAMPLE 2a:

In this experiment KLM3' was tested in the water degumming process of crude SBO 1.

Different dosages of KLM3' from 0.1 to 0.5 TIPU/g oil were tested and also the impact of Ultra Turrax mixing was tested.

The Table below together with Figure 77 show a clear reduction of the gum phase and improved oil yield (in the oil phase) in the samples treated with KLM3'.

An increase of about 2% oil was seen and there was a tendency that an increased yield was obtained by increasing the enzyme dosage.

The mixing also had an impact on the gum phase. It was seen that Ultra Turrax treatment of the oil for 30 sec just after enzyme addition gave a smaller gum phase, but the effect of the enzyme addition was almost the same with or without Ultra Turrax mixing. In the industry it is normal to pump the oil through a static mixer or a dynamic mixer after water addition, and in order to imitate this at laboratory scale it was decided to use Ultra Turrax mixing.

2460-150 (Example 2a)		**1***	2	3	4	**5***	6	7	8
Crude Soya oil Solae d.27-9-07	g	**100**	100	100	100	**100**	100	100	100
KLM3' 100 TIPU/ml	ml	**0**	0,1	0,25	0,5	**0**	0,1	0,25	0,5
Extra Water	ml	**2,00**	1,90	1,75	1,50	**2,00**	1,90	1,75	1,50
TIPU/g oil		**0,00**	0,10	0,25	0,50	**0,00**	0,10	0,25	0,50
% water		**2**	2	2	2	**2**	2	2	2
Ultra Turrax		**-**	-	-	-	**+**	+	+	+
pH		**5,39**	5,7	5,91	5,72	**5,55**	5,99	5,72	5,49
Gum Phase, %		**8,48**	6,36	5,73	4,76	**6,19**	4,63	4,44	4,19
Oil Phase %		**91,5**	93,6	94,3	95,2	**93,8**	95,4	95,6	95,8

*control without enzyme addition

EXAMPLE 2b:

Two different crude SBOs were tested in water degumming according to standard procedure with or without the addition of the KLM3' enzyme. The enzyme dosage was 0.25 TIPU/g.

Recipe 2460-151 (Example 2b)		1	2	3	4
SBO 1	g	100	100		
SBO 2	g			100	100
KLM3' 100 TIPU/ml	ml	0	0,25	0	0,25
Extra Water	ml	2,00	1,75	2,00	1,75
TIPU/g oil		0,00	0,25	0,00	0,25
% water		2	2	2	2
pH		5,78	5,75	5,73	5,68

The results shown in the table below indicate a clear reduction of the gum phase both after 30 minutes and 120 minutes reaction time, which corresponds to a higher oil yield. Analysis of sterol and sterol ester in the oil phase showed a high conversion of sterol to sterol ester in the enzyme treated samples. It is also observed that the amount of free fatty acid (FFA) increased, because a hydrolytic activity also had taken place.

Results 2460-151	SBO 1	SBO 1	SBO 2	SBO 2
KLM3', U/g oil	0	0,25	0	0,25
% Gum, 30 min	6,20	5,21	5,66	4,80
% Gum, 120 min	5,59	4,86	5,24	3,90
% Oil, 30 min	93.8	94.79	94,34	95.2
% Oil, 120 min	94.41	95.14	94.76	96.1
Oil Phase				
FFA total	0,37	0,53	0,64	0,85
Sterols	0,31	0,09	0,27	0,07
Sterol ester	0,14	0,47	0,12	0,50

EXAMPLE 2c:

In this experiment different dosages of KLM3' were tested in water degumming of SBO 2 at 50 °C. Different levels of water, namely 1.5%, 2% and 2.5%, were also tested in the process with and without addition of enzyme.

Recipe 2460-152 (Example 2c)		1	2	3	4	5	6	7	8
SBO 2	g	100	100	100	100	100	100	100	100
KLM3' 100 TIPU/ml	ml	0	0,1	0,25	0,4	0	0,25	0	0,25
Extra Water	ml	2,00	1,90	1,75	1,60	1,50	1,25	2,50	2,25
TIPU/g oil		0,00	0,10	0,25	0,40	0,00	0,25	0,00	0,25
% water		2	2	2	2	1,5	1,5	2,5	2,5
pH		5,32	5,92	5,72	5,59	5,58	5,73	5,30	5,81

The results shown in the tables and also in Figure 78, Figure 79, Figure 80, Figure 81, Figure 82, Figure 83 and Figure 84 below clearly indicate a reduced amount of gum phase and because the sum of gum phase and oil phase is 100% it is concluded that the acyltransferase (KLM3') contributes to improvement in oil yield in the oil phase.

It was also observed that the content of phospholipid in the gum phase was reduced in the enzyme treated samples. Both the phosphatidylethanolamine (PE) and phosphatidic acid (PA) were reduced in the gum phase relative to the amount of these phospholipids in the gum phase without enzyme treatment. The amount of triglyceride in the gum phase was also smaller in the enzyme treated gum phases, which also confirms that the increase in oil yield (in the oil phase) in the enzyme treated samples.

The amount of water added to the crude soya oil also showed as expected an impact on the amount of gum phase, but the results also confirmed the effect of acyltransferese on yield at different water addition relative to the control without enzyme addition (see Figure 80).

In the water degumming experiments the pH was in the range of 5.5 to 6 which explains high enzyme activity at low dosage and a high conversion of sterol to sterol esters.

Results

2460-152		1	2	3	4	5	6	7	8
Gum phase									
Gum, 30 min	%	6,48	5,14	5,68	5,19	5,73	4,85	7,06	6,03
Gum, 120 min	%	5,79	5,88	4,86	4,94	5,65	5,07	6,12	5,96
TLC analysis									
Phosphor	ppm	66	73	64	58	76	62	65	62
PA,	% rel.	100	61	45	35	86	47	105	50
PE	% rel.	100	45	24	18	88	26	102	34
Triglyceride	%	65	26	37	29	62	41	62	38
GLC analysis									
FFA,	%	0,63	0,71	0,78	0,87	0,57	0,79	0,57	0,73
Free Sterols		0,27	0,12	0,06	0,05	0,27	0,06	0,26	0,11
Sterol Esters		0,18	0,41	0,47	0,51	0,12	0,53	0,13	0,40

The analyses were made in duplicate and the results were used for Statistical evaluation of results using StatGraphic S Plus software.

EXAMPLE 2d:

In order to investigate the effect of KLM3' on oil yield at different temperature the enzyme was tested in water degumming of SBO2 at 55, 60, 65 and 70 °C.

Recipe

2460-154, 155, 156 and 157		1	2	3	4
SBO 2	g	100	100	100	100
KLM3' 100 TIPU/ml	ml	0	0,10	0,20	0,30
Extra Water	ml	2,00	1,90	1,80	1,70
TIPU/g oil		0,00	0,10	0,20	0,30
% water		2	2	2	2

The results shown in the Table below clearly illustrate the effect of KLM3' on the amount of gum phase. A dosage of 0.1 TIPU/g oil at all temperatures gave a significant reduction in the amount of gum. Increasing the amount of enzyme to 0.2 and 0.3 further decreased the gum phase a little.

Results

% Gum phase by water degumming of SBO 2 at different temperature, reaction times and enzyme dosages.

Temperature °C	Reaction time minutes	Enzyme 0 TIPU/g	Enzyme 0.1 TIPU/g	Enzyme 0.2 TIPU/g	Enzyme 0.3 TIPU/g
55	30	6,53	4,77	5,12	5,54
60	30	6,64	4,83	4,73	4,55
65	30	6,79	5,63	5,05	4,94
70	30	6,49	4,58	4,36	4,23
55	120	6,29	4,94	4,72	4,80
60	120	5,79	4,76	4,47	4,05
65	120	6,70	5,37	4,84	5,39
70	120	5,05	4,41	3,39	3,00

EXAMPLE 3

Enzymatic water degumming in pilot plant

Recipe

Ingredients applied in pilot water degumming trials.

Batch 1: control, 70°C,

Batch 2: with enzyme (namely the lipid acyltransferase K932 – sometimes referred to herein as KLM3' – which has the amino acid sequence shown herein as SEQ ID No. 68), 70°C,

Batch 3: control, 55°C and

Batch 4: with enzyme (namely the lipid acyltransferase K932 – sometimes referred to herein as KLM3' – which has the amino acid sequence shown herein as SEQ ID No. 68), 55°C.

	Amount	Batch			
		1	2	3	4
Journal no.		2460-158		2460-160	
Crude Soya Oil	kg	20	20	20	20
K932, 1128 TIPU/ml	Ml	0	3.55	0	3.55
Extra Water	Ml	400.30	396.10	400.1	396.47
TIPU-K/g oil		0.00	0.2	0.00	0.2
Water	%	2	2	2	2

Water degumming pilot plant procedure

The oil was initially heated under N_2 coverage and agitation in a 50-liter tank. Afterwards, water (and enzyme) was added to the oil. In the initial experiments (batches 1 and 2), the oil was re-circulated after addition of the water and enzyme, using a homogenizer (Silverson, Chesham Sweden). In batches 3 and 4 only a re-circulation pump was used to lower the agitation in the tank.

Oil samples were collected (batches 1-4) for laboratory analysis after 30 minutes of enzyme activity and placed in a boiling water bath (10 minutes) in order to inactivate the enzyme. Inactivation of the remaining oil in the tank was done by heating the oil to 75 °C (under agitation). Subsequently, centrifuging was carried out in a preheated (hot water) centrifuge (Alfa Laval) and the oil phase was tapped in buckets and weighed. Different centrifuge capacity adjustments were tested, it was not possible to monitor the separated gum phase, as the volume of the centrifuge was too large compared to the amount of oil. The gum phase was, thus, collected from the lid of the centrifuge, where it had accumulated.

Laboratory water degumming and centrifuging

100g crude soya oil was scaled into a 250ml blue cap flask with lid and heated to 55°C. Water was added to the oil followed by enzyme addition. The oil was homogenised using an Ultra Turrax mixer for 30 seconds, and then agitated for 30 minutes with magnetic stirring at 450rpm. After 30 minutes, 10ml oil was transferred to a 12ml centrifuge tube (previously scaled). The oil was heated to 97°C in a boiling water bath for 10 minutes, and then immediately centrifuged at different relative centrifuging forces (500, 1000, 2500 and 5000) for varying times (3, 6 and 10 minutes).

Oil was decanted from the gum phase, and the tubes were drained for 15 minutes, and the weights of both phases were measured. The oil phase was analysed for free phytosterols, sterol esters and free fatty acids by GLC, and the oil phase was analysed by HPTLC.

Results and discussion

Oil yield

Figure 85 shows the increased oil yield obtained from enzymatic degumming of crude soybean oil in accordance with the present invention compared to the control. The oil is centrifuged at increasing relative centrifuging force (rcf) (500, 1000, 2500 and 5000) for 3 minutes and oil yield is calculated from amount (%) of gum in the control subtracted amount of gum in enzymatic samples.

Clearly it is seen that the oil yield increases in enzymatic degumming compared to the control and that the oil yield increases with decreasing rcf.

Effect of centrifugation

The amount of triglycerides in gums and amount of gum, obtained from oil samples centrifuged at different times (minutes in bars) are shown for batches 3 and 4 in Figure 86.

The results illustrate that rcf affects the amount (%) of gum obtained from conventional degumming (blue bars). Initially, at low rcf (500-1000), the amount of gum is high (high triglyceride content) compared to the amount obtained at relative centrifuging forces of 2500 to 5000. Centrifuging time (3, 6 and 10 minutes) does not seem to affect the amount of gum, at least not when centrifuged at 5000 rcf.

Inspecting the gum obtained from enzymatic degumming according to the present invention, the amount does not seem to be affected by rcf and time. Without wishing to be bound by theory this may be explained by differences in viscosity between gums obtained from conventional and enzymatic degumming according to the present

invention. In Figure 87, measurements of the viscosity, based on gum phases, are shown. The viscosity decreases with increasing shear rate for both types of gum, however, the viscosity decreases to a higher extent in gums obtained from enzymatic degumming in accordance with the present invention.

Besides, increased oil yield, the decreased viscosity achieved with the present invention may have other benefits for an industrial water degumming processing. It is likely that production capacity may be increased.

EXAMPLE 4

Evaluation of NaOH in water degumming of crude soy bean oil

Recipe

Table 1: Samples for water degumming trials

Journal 2460-181		1	2	3	4	5	6	7	8
Crude soya bean oil	g	100	100	100	100	100	100	100	100
K932 100 TIPU/ml	ml	0	0.1	0.1	0.1	0.1	0.1	0.1	0.1
4% NaOH-solution	ml	0	0	0.1	0.2	0.5	1	1.5	1.9
Extra Water	ml	2.00	1.90	1.80	1.70	1.40	0.90	0.40	0.00
TIPU-K/g oil		0.00	0.10	0.10	0.10	0.10	0.10	0.10	0.10
% water		2	2	2	2	2	2	2	2

Water degumming lab procedure

100g crude soya oil was scaled into a 250ml blue cap flask with lid and heated to 55°C. Water and NaOH was added to the oil followed by enzyme addition. The oil was homogenised using an Ultra Turrax mixer for 30 seconds and agitated for 30 minutes with magnetic stirring at 450rpm. After 30 minutes, approximately 10ml oil was transferred to a 12ml centrifuge tube (previously scaled). The oil was heated to 97°C in a boiling water bath for 10 minutes.

Results and discussion

Analysis of oil yield

Figure 88 shows the increased oil yield, obtained from enzymatic degumming with KLM3' (namely the lipid acyltransferase K932 – sometimes referred to herein as KLM3' – which has the amino acid sequence shown herein as SEQ ID No. 68) (0.1 TIPU-K/g) and increasing amount of NaOH (0, 0.1, 0.2, 0.5, 1, 1.5 and 1.9 ml 4%-solution). Calculations are based on the amount of gum in the control subtracted the amount of gum in enzymatic samples.

Highest oil yield increase is achieved by enzymatic degumming without NaOH and generally increased oil yield (%) decreases with increasing amount of NaOH. This most likely may be explained from the increased saponification of triglycerides with increasing amount of NaOH. However, inspecting the triglycerides in the control and enzymatic gum samples (Figure 89), the content is not markedly higher in NaOH-treated gums than usually observed without NaOH. The level of triglyceride in enzymatic samples without NaOH likewise is comparable to previous observations.

Analysis of fatty acids, phytosterols and phytosterol ester in oil

The content of phytosterols, phytosterol esters and free fatty acids in the control and enzymatic degummed oils is depicted in Figure 90. The content of phytosterol esters increases from 0.19% (control) to 0.42% (0.2 ml NaOH), where it reaches a maximum. After this point the phytosterol esters decrease to 0.15%. Accordingly, an initial decrease of phytosterols from 0.3-0.12%, followed by an increase from 0.12-0.28%, is observed.

The FFA's similarly increase to the point of pH 6.3 (0.2 ml NaOH), most likely because of increased saponification.

The results clearly illustrate that running the water degumming at higher pH increases the transferase activity of the lipid acyltransferase KLM3'. Even a slight increase in pH (e.g. 0.1 ml NaOH) increases the formation of phytosterol esters with approximately 50%, almost without affecting the formation of FFA's in the oil (increases 0.02%). The

increase in FFA's is important to consider, as the FFA's evaporate during the deodorization step and thus are regarded as oil loss.

Analysis of phospholipid content in oil

Table 2 shows the content (ppm) of phospholipids (phosphatidyl-ethanolamine and phosphatidic acid) in oils (control and enzymatic samples) degummed with increasing amount (0, 0.1, 0.2, 0.5, 1 and 1.9 ml) of NaOH.

Table 2: Content (ppm) phosphor from PA, PE, PC and total phosphor in oils, degummed with increasing amount (0, 0.1, 0.2, 0.5, 1, 1.5 and 1.9 ml) of 4%-NaOH-solution.

	Sample							
pH	5.3	5.9	6.3	6.6	7.4	7.8	8.2	8.3
KLM3' (TIPU-K/g)	0	0.1	0.1	0.1	0.1	0.1	0.1	0.1
NaOH (ml)	0	0	0.1	0.2	0.5	1	1.5	1.9
PA	34.0	33.8	**35.3**	38.4	36.8	36.7	34.8	38.8
PE	6.8	5.9	**5.0**	5.6	4.9	4.0	5.0	4.6
PC	1.9	0.8	**0**	0	0	0.7	2.8	0.9
Total phosphor content	42.8	40.6	**40.2**	44.1	41.8	41.5	42.6	44.3

Highest reduction (40.2 ppm) of phosphor is observed in oils, degummed with 0.1 ml NaOH (pH 6.3), however, a comparable content is obtained under normal degumming conditions (0 ml NaOH). Hence, it appears that increasing the pH 1.0 unit does affect the hydrolytic activity of KLM3'. At pH higher than 6.3 (> 0.2 ml NaOH), a reduced phospholipid degradation is observed compared to "normal" enzymatic conditions.

Analysis of phospholipid content in gum

Figure 91 shows the relative degradation of phosphatidic acid (PA), phosphatidyl-ethanolamine (PE), phosphatidylcholine and phosphatidylinositol (PI) in enzymatic gum samples compared to the control. The degradation of phospholipids in the control is set to 100% and the content in enzymatic samples is calculated relatively to the control.

The degradation of phospholipid in enzymatic samples with 0, 0.1 and 0.2 ml NaOH is analogous. Hence, applying NaOH in amounts less than 0.2 ml does not impair the degradation of phospholipids compared to enzymatic degumming with KLM3' only. On the contrary, reduced degradation is observed in oils with NaOH applied in higher amounts (0.5, 1 and 1.9 ml).

CONCLUSION

Increasing the pH with NaOH in water degumming of crude soy bean oil turned out, as expected, to increase the activity of KLM3'. Formation of phytosterol esters increased concurrent with increasing amount of NaOH. Maximum phytosterol ester level (0.42%) was obtained at pH 6.3 (0.2 ml NaOH), where after a continuous decrease followed. A similar pattern was observed for the FFA's in the oil, which increased from 0.46% in the control to 0.60% in oils, degummed with 0.2 ml NaOH, where after it decreased.

Small amounts of NaOH did not affect the hydrolytic activity of KLM3', as observed from comparable levels of phospholipids in oils, degummed with 0 and 0.1 ml NaOH. Degradation of phospholipids in the gum phase was reduced compared to normal enzymatic degumming (KLM3' only) at pH above 7.5 (>0.5 ml NaOH).

Highest oil yield increase was achieved by enzymatic degumming without NaOH and generally the % increased oil yield decreased with increasing amount of NaOH.

The conclusion of the present experiment is that small amounts of NaOH may be advantageous for the formation of phytosterol esters in water degumming, however, NaOH does not add positively to the oil yield and phospholipid degradation.

EXAMPLE 5

Analysis of gum phase from enzymatic water degumming - Microscopy and x-ray analysis

Recipe

		1	2
Crude Soya oil Solae	g	100	100
K932 100 TIPU-K/ml	ml	0	0,20
Extra Water	ml	2,00	1,80
TIPU-K/g oil		0,00	0,20
% water		2	2

Water degumming laboratory procedure

100g crude soya oil was scaled into a 250ml blue cap flask with lid and heated to 55°C. Water was added to the oil followed by enzyme addition. The oil was homogenised using an Ultra Turrax mixer for 30 seconds and agitated for 30 minutes with magnetic stirring at 450rpm. After 30 minutes, the oil was centrifuged (2000 rcf for 3 minutes). The gum phase was taken for microscopy- and x-ray analysis.

Results and discussion

Microscopy/x-ray analysis

Gums from control and enzymatic water degumming trials (the latter in accordance with the present invention) were collected for microscopy and x-ray analysis. The gum phases were studied in the microscope (plane polarised light) at different temperatures (25, 35, 45, 55 and 65°C). At all temperatures the gum was in a lamellar phase (lipid bi-layers separated by water layers), as seen for the control and enzymatic sample (25°C) in Figure 92.

Some differences appear between the control and enzymatic sample. The control gum appears coarser than the enzymatically gummed sample in accordance with the

present invention. Differences between the control and enzymatic sample also can be observed from x-ray analysis, as seen in Figure 93.

The larger spacing of approximately 20 Å in the control compared to the enzyme treated sample corresponds to the length of a fatty acid chain (C18). The spacing expresses the water and phospholipid layer, hence, the larger spacing in the control could explain that the control contains an extra monolayer of fatty acids or that more water is absorbed in the gum phase.

EXAMPLE 6

Sedimentation study

Recipe

		1	2
Crude Soya oil Solae	g	200	200
K932 100 TIPU-K/ml	ml	0	0,4
Extra Water	ml	4,00	3,60
TIPU-K/g oil		0,00	0,20
% water		2	2

Procedure

200g crude soya oil was scaled into a 250ml blue cap flask with lid and heated to 55°C. Water was added to the oil followed by enzyme addition. The oil was homogenised using an Ultra Turrax mixer for 30 seconds and agitated for 30 minutes with magnetic stirring at 450rpm. After 30 minutes, the samples were placed in separation funnels. Pictures of the gum phase were taken after 1, 3 and 6 days. After day six, the gums were taken for microscopy analysis.

Results

Pictures of gum phases/microscopy

In Figure 94 the oil and gum phase can be seen for the control and enzymatic sample. Sedimentation by gravity has been carried out for 3 days. Clear differences exist between the control and enzymatic sample, as seen from both the oil and gum phase.

The oil phase of enzymatic treated oil (i.e. treated in accordance with the present invention) is clearer than the control and a decreased amount of gum is observed compared to the control. The results may be explained from microscopy analysis (Figure 95). The enzymatic treated gum is observed as an emulsion, while the control gum is lamellar phase.

EXAMPLE 7

Evaluation of varying amount of water in enzymatic degumming of crude soybean oil

Recipes

Journal 2460-165		1	2	3	4	5	6	7	8
Crude Soya oil Solae	G	100	100	100	100	100	100	100	100
K932 100 TIPU-K/ml	Ml	0	0.2	0	0.2	0	0.2	0	0.2
Extra Water	Ml	1.00	0.800	1.50	1.30	2.00	1.80	2.50	2.30
KLM3' activity (TIPU-K/g oil)		0.00	0.20	0.00	0.20	0.00	0.20	0.00	0.20
% water		1	1	1.5	1.5	2	2	2.5	2.5

Journal 2460-169		1	2	3	4	5	6
Crude Soya oil Solae	g	100	100	100	100	100	100
K932 100 TIPU-K/ml	ml				0.2	0.2	0.2
Extra Water	ml	1.00	1.50	2.00	0.80	1.30	1.80
KLM3' activity (TIPU-K/g oil)					0.20	0.20	0.20
% water		1	1.5	2	1	1.5	2

Journal 2460-170		1	2	3	4	5	6	7	8	9	10
Crude Soya oil Solae	g	100	100	100	100	100	100	100	100	100	100
K932 100 TIPU-K/ml	ml	0	0	0	0	0	0.2	0.2	0.2	0.2	0.2
Extra Water	ml	1.00	1.25	1.50	1.75	2.00	0.80	1.05	1.30	1.55	1.80
KLM3' activity (TIPU-K/g oil)		0.00	0.00	0.00	0.00	0	0.20	0.20	0.20	0.20	0.20
% water		1.00	1.25	1.50	1.75	2.00	1.00	1.25	1.50	1.75	2.00

Water degumming laboratory procedure

100g crude soya oil was scaled into a 250ml blue cap flask with lid and heated to 55°C. Water was added to the oil followed by enzyme addition. The oil was homogenised using an Ultra Turrax mixer for 30 seconds and agitated for 30 minutes with magnetic stirring at 450rpm. After 30 minutes, approximately 10ml oil was transferred to a 12ml centrifuge tube (previously scaled). The oil was heated to 97°C in a boiling water bath for 10 minutes. The tubes were centrifuges at 300 rcf for 3 minutes. Oil was decanted from the gum phase and drained for 15 minutes by turning the tube upside down. Based on the weight of the gum phase the oil yield was calculated.

Results and discussion

Oil yield

Figure 96 shows the increased oil yield obtained from enzymatic water degumming of crude soybean oil with varying amounts of water. Increased oil yield is calculated from the amount of gum in the control subtracted amount of gum in enzymatic samples.

Enzymatic degumming attributes to an increased oil yield compared to the control and it appears that the oil yield increases with decreasing amount of water. The oil yield approximately increases 50% in enzymatic degumming compared to the control, when water is reduced from 2 to 1%.

These calculations are based on amount of gum and hence also include the triglyceride content in the gum phase. Inspecting the actual oil loss (based on amount of gum and triglyceride content in gum) (Figure 97), the oil loss decreases in the control with increasing water content. However, in enzymatic degumming, the oil loss is somewhat unaffected by amount of water. Approximately 2% oil is lost in enzymatic degumming compared to 3.5-6.5% in the control.

The decreased amount of water in enzymatic water degumming may be a financial advantage for the industry (less process water) and most likely also with regard to energy savings during the drying of the gum phase.

Phospholipid degradation in gum phase

The relative degradation (%) of phosphatidic acid (PA) and phosphatidylethanolamine (PE) in the enzymatic gum phases relative to the control is shown in Figure 98.

Phospholipid degradation with KLM3' appears to be more pronounced at lower water concentrations. In overall enzymatic degumming with KLM3' and 1% water appears to be an advantage in respect to phospholipid degradation compared to degumming with 2% water.

Viscosity measurements of the gum phase

The viscosity of enzymatic (KLM3' 0.2 TIPU-K/g) gum phases, from degumming with different amounts of water is shown in Figure 99. The viscosity is not markedly affected by the different water content. At lower shear rate (up to approximately 10) the viscosity is somewhat similar for all samples, however, after this point the viscosity of samples with lowest amount (1.25%) of water increases, while gum samples highest amount (2%) of water increases.

EXAMPLE 8

Water degumming of crude Corn Oil.

ABSTRACT

Lipid acyltransferase, KLM3' (sometimes referred to as K932 and having the amino acid sequence shown herein as SEQ ID No. 68 was tested in a crude corn oil with the aim to study effects on oil yield in water degumming of this oil.

MATERIALS AND METHODS

Enzyme:

KLM3' K932. 1128 TIPU/g

Oil:

Crude corn oil from Cargill, May 2008

Degumming procedure:

100 g crude corn oil was scaled into a 250 ml Blue Cap flask with lid and heated to 55°C.

Water and enzyme was added and the oil was homogenised with an Ultra Turrax mixer for 30 seconds, and then agitated for 30 minutes with magnetic stirring at 450 rpm.

After 30 minutes, 10 ml oil was transferred to a 12 ml tarred centrifuge tube and the oil weight noticed. The oil was heated to 97°C in a boiling water bath for 10 minutes, and then immediately centrifuged at 3000 rcf for 3 minutes.

Oil was decanted from the gum phase and drained for 15 minutes by turning the tube upside down. Based on the weight of the gum phase the oil yield was calculated relative to an oil not treated with enzyme.

The gum phase was then analysed by HPTLC, and the degradation of the phospholipids in the gum phase was calculated.

Results

The oil degumming process was conducted with different concentrations of KLM3'

Table 1. Recipe for degumming of Crude Corn Oil

2460-182		1	2	3	4	5
Crude Corn oil	g	100	100	100	100	100
K932 100 TIPU-K/ml	ml	0	0,050	0,10	0,20	0,50
Extra Water	ml	2,00	1,95	1,90	1,80	1,50
TIPU/g oil		0,00	0,05	0,10	0,20	0,50
% water		2	2	2	2	2

The samples were treated as described in 'degumming procedure' and the amount of wet gum was determined in duplicate with results shown below.

Table 2 Gum Phase, % from water degumming of crude corn oil

Sample	Enzyme, Units/g	Gum Phase	Yield increase
1	0	6,0	0,00
2	0,05	5,7	0,28
3	0,1	5,5	0,44
4	0,2	5,6	0,36
5	0,5	5,6	0,38

From the result in table 2 it is seen that KLM3' contribute to a decrease in the amount of gum phase by water degumming of crude corn oil. The reduced amount of gum phase corresponds to an increase in the oil phase of 0,28 to 0,44%.

The gum phase isolated from water degumming of crude corn oil was analysed by TLC and the reduction of phosphatidylethanolamine and phosphatidic acid was calculated relative to the amount in the gum without enzyme treatment. (Table 3)

Table 3: TLC analysis of Gum phase. PE= phosphatidylethanolamine

PA=Phosphatidic acid

Enzyme dosage TIPU/g oil	PA Relative %	PE Relative %
0	100	100
0,05	88	85
0,1	73	68
0,2	75	72
0,5	72	64

The results from table 3 indicate the activity of KLM3' on phospholipids in crude corn oil. An increased enzyme activity is seen up to a dosage of 0,1 TIPU/g oil. At higher enzyme dosage the activity on the phospholipids levels off.

EXAMPLE 9

Water degumming of crude Soya Oil , and addition of acceptors.

Lipid acyltransferase, KLM3', was tested in an crude soya bean oil from Solae with the aim to study effects of adding acceptor substrate for the enzyme KLM3'.

In this study a phytosterol product Generol 122 from Henkel, Germany, and a fatty alcohol, laurylalcohol was tested.

Addition of phytosterol to the oil produced more sterol ester concomitant with a reduction of free fatty acid formation. It is concluded that a higher degree of phospholipid conversion can be achieved without increased fatty acid production when more acceptor substrate is available.

MATERIALS AND METHODS

Enzyme:

KLM3' K932 (having amino acid sequence shown as SEQ ID No. 68 - 1128 TIPU/g

Phytosterol from soya : Generol 122 N, from Grünau, Illertissen, Germany.

Laurylalcohol: Sigma L-5375

Oil:

Crude Soya Bean oil from Solae, January 2008

Soy Lecithin Mix Standard (ST16) from Spectra Lipid, Germany.

Degumming procedure:

100 g crude soya oil, phytosterol and layrylalcohol was scaled into a 250 ml Blue Cap flask with lid and heated to 55°C. The phytosterol was completely dissolved in the oil before further processing.

Water and enzyme was added and the oil was homogenised with an Ultra Turrax mixer for 30 seconds, and then agitated for 30 minutes with magnetic stirring at 450 rpm.

After 30 minutes, 10 ml oil was transferred to a 12 ml tarred centrifuge tube and the oil weight noticed. The oil was heated to 97°C in a boiling water bath for 10 minutes, and then immediately centrifuged at 3000 rcf for 3 minutes.

Oil was decanted from the gum phase and drained for 15 minutes by turning the tube upside down. Based on the weight of the gum phase the oil yield was calculated.

The oil phase and the gum phase was then analysed by HPTLC, and the amount of triglyceride in the gum phase and the degradation of the phospholipids in the oil phase was calculated

Results

The oil degumming process was conducted with different concentrations of KLM3, phytosterol and fatty alcohol as shown table 1.

Table 1. Recipe for degumming of Crude Soya Oil

2460-182		1	2	3	4	5	6	7	8	9
Crude Soya oil, Solae d.16-01-2008	g	100	100	100	100	100	100	100	100	100
K932 100 TIPU-K/ml	ml	0	0,20	0,20	0,20	0,2	0,2	1	1	0,2
Generol 122 N	g		0	0,25	0,50	0,75	0,75		0,75	
4% NaOH	ml						0,2			
Lauryl alcohol	g									0,5
Extra Water	ml	2,00	1,80	1,80	1,80	1,80	1,80	1,00	1,00	1,80
pH		4,90	5,65	5,55	5,48	5,41	6,18	5,29	5,27	5,57
TIPU/g oil		0,00	0,20	0,20	0,20	0,20	0,20	1,00	1,00	0,20
% water		2	2	2	2	2	2	2	2	2

The samples were treated as described in 'degumming procedure' and the amount of wet gum was determined in duplicate with results shown in Figure 100.

Addition of increasing amount of phytosterol did not contribute to any decrease in % gum, and pH adjustment (trial 6) did not have any significant effect on the amount of gum although there is a tendency to more gum in this trial. Addition of 0.2 TIPU/g of KLM3' had a significant effect on the gum content, and it was shown that an increase to 1 TIPU/g further decreased the amount of gum. Lauryl alcohol did not have any effect on the amount of gum.

The oil phase separated from the gum was analysed for free fatty acids, sterols and sterol esters by GLC.

The results in table 2 indicate an increase of 0,09 % free fatty acid by enzymatic treatment with 0.2 TIPU/g (sample 2), but it is observed that sample 3 to 5 with increased level of phytosterols contains less free fatty acids. Also in sample 7 and 8 treated with 1 TIPU/g a reduction in free fatty acids is observed when more sterol is added to the oil. These results indicate that the hydrolytic reaction decreases with increased mount of sterols in the oil.

It should then be expected that the amount of sterol ester increase with increase sterol in the oil. This is also seen for sample 3, but with increased amount of sterols (sample 4 and 5), the amount of sterol esters does not change. Even a tendency to decreased amount of sterol ester in sample 5 is observed, but this is within the experimental error. Adjusting the pH by addition of NaOH however has a strong effect on sterol ester formation as seen before. Increased amount of enzyme (sample 7 and 8) also contribute to increase in sterol ester formation.

Table 2. GLC analyses of oil phase form water degumming of samples (see table 1)

Sample	Free fatty acids, %	Sterols %	Sterol ester, %
1	0,46	0,30	0,20
2	0,55	0,15	0,40
3	0,54	0,36	0,45
4	0,52	0,60	0,40
5	0,50	0,83	0,38
6	0,55	0,69	0,63
7	0,86	0,12	0,47
8	0,80	0,65	0,64
9	0,53	0,20	0,39

The gum phase isolated by water degumming of samples (table1) were analysed by HPTLC and the degradation of certain phospholipids phosphatidylethanolamine(PE) and phosphatic acid (PA) were quantified relative to the control sample no 1. (Figure 101)

The results in Figure 101 indicate an increased degradation of PA and PE when 0,25% sterol is added,

But increased dosage (0.5 and 0.75% sterol) does not contribute to further phospholipid degradation. This is in agreement with the observation about the effect on sterol ester formation (see table 2). pH adjustment with NaOH also has a strong effect on phospholipid degradation, but this is related to more enzyme activity with increased pH.

It is also seen that increase in enzyme dosage to 1 TIPU/g further degrades the phospholipids.

The oil phase isolated form the water degumming was analysed by ICP with the aim to analyse the amount of residual phosphor in the oil.

The results in Figure 102 indicate that the level of phosphor in the oil is not very much dependent of the amount of sterol in the oil, but the results indicate that increased enzyme dosage (1 TIPU/g) has an effect on the phosphor level. Addition of laurylalcohol (C12-alcohol) has a negative effect on the level of phosphor in the oil phase.

Conclusion.

Addition of lipid acyltransferase KLM3' to crude oil catalyses the transfer of fatty acid moiety from phospholipid to sterol, during formation of sterol esters. On a molecular level the amount of sterol is less than 1/3 of the amount of phospholipids in crude soya oil. Because the acyl acceptor sterol is the limiting factor for KLM3' in crude soya oil, the hydrolysis reaction might occur depending on enzyme dosage and reaction time.

In this study it was found that the addition of more sterol to the crude oil will produces more sterol ester, when the oil is treated with lipid acyltransferase KLM3', and the amount of free fatty acids formed is reduced compared with an oil where no sterol was added.

Addition of extra sterol does not have much impact in the level of phosphor in the oil phase after water degumming, but it is observed that increased dosage of KLM3' reduces the level of phosphor in the oil phase. Addition of 0.5% laurylalcohol did not have much effect on the level of free fatty acid and no laurylalcohol ester was seen by GLC analysis.

Example 10

Combination of a lipid acyltransferase and a phospholipase C

MATERIALS AND METHODS

Enzyme:

Lipid Acyltransferase KLM3' K932. 1128 LATU/g (having the amino acid sequence shown herein as SEQ ID No. 68)

Phospholipase C, Sigma P7633 15 Units/mg

Oil:

Crude Soya Bean oil from Solae, Aarhus, DK

Degumming procedure

100 g crude soya oil is scaled into a 250 ml Blue Cap flask with lid and heated to 55°C. 0,14 ml 50% citric acid monohydrate is added. The oil is homogenised with an Ultra Turrax mixer for 30 seconds, and then agitated for 15 minutes with magnetic stirring at 450 rpm. 0,367 ml 1N NaOH is added followed by 2,5% water and 5 Units/g oil of Phospholipase C. The oil is again homogenised with an Ultra Turrax mixer for 30 seconds and agitated at 450 rpm with magnetic stirrer. After 2 hours reaction time 0,2 LATU/g oil of enzyme Lipid acyltransferase KLM3' is added and the reaction is continued for one hour more with stirring.

The oil is heated to 97°C in a boiling water bath for 10 minutes, and then immediately centrifuged at 3000 rcf for 3 minutes.

Oil phase is decanted from the gum phase. The weight of the gum phase the oil phase is measured.

The oil phase is analysed for residual phospholipids by TLC, and ppm phosphor is analysed by ICP. Free sterol, sterol ester, free fatty acid and diglyceride are analysed by GLC.

The gum phase is analysed for triglyceride, diglyceride, residual phospholipids and free fatty acid.

The degradation of phospholipids in the gum phase is analysed by TLC

Results

The degumming process with a combination of lipid acyltransferase and phospholipase C is expected to increase the oil yield by more than 2 % compared with an oil without enzyme treatment. Initial studies suggest that diglyceride has been produced in the oil phase in the enzyme treated sample.

In the oil phase after centrifugation a main part of the sterols will be esterified.

Preliminary investigations show that the phosphor level is below 5 ppm in the oil phase and a strong degradation of phospholipids in the gum phase. (i.e. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) almost completely disappearing and a strong degradation of phosphatidylinositol (PI) and phosphatidic acid (PA)).

Example 11

Lipid acyltransferase in combination with Phospholipase C

MATERIALS AND METHODS

Enzyme:
Lipid Acyltransferase KLM3' K932. 1128 LATU/g
Phospholipase C Sigma P7633 15 Units/mg

Oil:
Crude Soya Bean oil from Solae, Aarhus, DK

Degumming procedure

100 g crude soya oil is scaled into a 250 ml Blue Cap flask with lid and heated to 55°C.

3 % water is added followed by 0,1 Units/g oil of Acyltransferase KLM3' and 5 Units Phospholipase C. The oil is homogenised with an Ultra Turrax mixer for 30 seconds, and then agitated for 30 minutes with magnetic stirring at 450 rpm.

After 30 minutes, 10 ml oil is transferred to a 12 ml tarred centrifuge tube and the oil weight noticed. The oil is heated to 97°C in a boiling water bath for 10 minutes, and then immediately centrifuged at 3000 rcf for 3 minutes.

Oil phase is decanted from the gum phase and drained for 15 minutes by turning the tube upside down. Based on the weight of the gum phase the oil yield is calculated. The oil phase is analysed for residual phospholipids by TLC and ICP. Free sterol, sterol ester, free fatty acid and diglyceride are analysed by GLC.

The gum phase is analysed for triglyceride residual phospholipids and free fatty acid.

Results

Preliminary investigations suggest that the water degumming process with a combination of Lipid acyltransferase and phospholipase C results in a significant increase in the oil yield with more than 2% compared with an oil without enzyme treatment. Initial studies show that diglyceride is produced in the oil phase and a main part of the sterols in the oil phase is esterified.

Example 12

Enzymatic degumming with lipid acytransferase KLM3 and Phospholipase C (PLC)

MATERIALS AND METHODS

Enzyme:

Lipid Acyltransferase KLM3' K932. 1128 LATU/g

Phospholipase C Sigma P7633 15 Units/mg

Oil:

Crude Soya Bean oil from Solae, Aarhus, DK

Degumming procedure

100 g crude soya oil is scaled into a 250 ml Blue Cap flask with lid and heated to 55°C.

3 % water is added followed by 5 Units/g oil of Phospholipase C. pH is adjusted to 5.5 with NaOH. The oil is homogenised with an Ultra Turrax mixer for 30 seconds, and then agitated for 15 minutes with magnetic stirring at 450 rpm. After 15 minutes a sample is taken out and 0,1 Units/g oil of Acyltransferase is added. The oil is agitated for a further 15 minutes at 55°C.

After 2x15 minutes reaction time, 10 ml oil is transferred to a 12 ml tarred centrifuge tube and the oil weight noticed. The oil is heated to 97°C in a boiling water bath for 10 minutes, and then immediately centrifuged at 3000 rcf for 3 minutes.

Oil is decanted from the gum phase and drained for 15 minutes by turning the tube upside down. Based on the weight of the gum phase the oil yield is calculated.

The oil phase is analysed for residual phospholipids by TLC and ICP. Free sterol, sterol ester, free fatty acid and diglyceride are analysed by GLC.

The gum phase is analysed for triglyceride residual phospholipids and free fatty acid.

<u>Results</u>

Initial studies suggest that the water degumming process using a combination of Lipid acyltransferase and phospholipase C increases the oil yield by more than 2.5% compared with an oil without enzyme treatment. Preliminary investigations suggest that diglyceride has been produced after 15 minutes in the oil phase.

A main part of the sterols in the oil phase will be esterified.

Preliminary investigations show that after 15 minutes a main part of the phosphatidylethanolamine (PE) and phosphatidylcholine (PC) has disappeared but less activity may be seen on phosphatidylinositol (PI) and phosphatidic acid (PA). In the sample after 30 minutes and centrifugation also a main part of the PI and PA will have disappeared.

Example 13

Enzymatic degumming with lipid acytransferase KLM3 and Phospholipase C (PLC)

Lipid Acyltransferase KLM3' and Phospholipase C (PLC) from Sigma were tested alone and in combinations in water degumming of crude soya oil. Phospholipase C in oil degumming produced diglyceride from phospholipids in the oil. It was surprisingly shown that KLM3' can use the diglyceride as an acceptor molecule during production of triglyceride. Model experiments with substrate containing diglyceride and phosphatidylcholine confirmed that lipid acyltransferase (KLM3') catalyzes a transfer reaction of fatty acid moiety from phospholipid to diglyceride during production of triglyceride.

COMMERCIAL RELEVANCE OF THE RESULTS

This study was initiated with the aim to show that the combination of KLM3' and Phospholipase C (PLC) is highly advantageous when degumming of crude vegetable oils.

A phospholipase C from Verenium, U.S. (namely Purifine®) has been introduced for use in oil degumming (WO 2008/036863).

This enzyme is active on phospholipids (such as phosphatidylcholine and phosphatidylethanolamine) in crude oil forming diglyceride (diacylglycerol) and phosphor-choline, -ethanolamine, -inositol or –acid. Diglyceride produced during this process will form part of the oil during the oil degumming process and thus contribute to improved oil yield.

The inventors have shown that lipid acyltransferases (such as KLM3') can contribute to improved yield in oil degumming by modification of the phospholipids concomitant with sterol ester formation.

Lipid acyltransferases (such as KLM3') can use sterols as an acyl acceptor as well as other acceptors like alcohols including fatty alcohols.

The aim of the current study was to investigate any synergistic effect when a lipid acyltransferase (e.g. KLM3') was used in combination a phospholipase C.

Material and Methods:

- KLM3' :Glycerophospholipid cholesterol acyltransferase (FoodPro LysoMax Oil) (K932) (SEQ ID No. 68)
  Lot no 102629600. Activity 1128 LATU/g
- Phospholipase C P7633 Sigma, from *Clostridium perfringens*, 135.3mg solid:3.8 unit/mg solid, 13.2 unit/mg protein
- Phospholipase C P6621 Sigma, from *Bacillus cereus*, 250 Units

Diglyceride. Destilled diglyceride from sunflower oil, Jour 2641/064

Phosphatidylcholine, Avanti #441601

Mono-di-triglyceride: GRINDSTED® MONO-DI R 50/D

Crude soya oil no 18: from, Argentina

HPTLC analysis

The degradation of phospholipids in the gum phase from enzyme treated samples was analysed by HPTLC.

Applicator: Automatic TLC Sampler 4, CAMAG

HPTLC plate: 20 x 10 cm, Merck no. 1.05641. Activated 10 minutes at 160°C before use.

Application:

Gum phase from 10 gram oil was dissolved in 7.5 ml Hexan:Isopropanol 3:2.

1 µl of the sample was applied to the HPTLC plate.

A phospholipid standard (0.5% phospholipid (Spectra Lipid, Germany) was applied (0.1, 0.3, 0.5, 0.8 and 1.5 µl) and used for the calculation of the individual phospholipids in the gum.

In some applications the phospholipid content was calculated relative to a control gum not treated with enzyme. This control sample was applied 0.1 – 0.3 – 0.5 – 0.8 – 1 µl and used for making calibrations curves.

Oil phase. Approximately 90 mg was scaled and dissolved in 1 ml Hexan:Isopropanol 3:2.

5 µl of the sample was applied to the HPTLC plate. Mono-diglyceride 5 mg/ml of known concentration was applied at 0.1 - 0.3 - 0.5 - 0.8 - 1.5 µl and used for calculation of individual glyceride components

TLC applicator.

Running buffer no. 1: P-ether : Methyl Tert Butyl Ketone : Acetic acid 50:50:1

Running buffer no 6: Chloroform : 1-propanol:Methylacetate:Methanol : 0.25% KCl in water 25:25:25:10:9

Elution: The plate was eluted 7 cm using an Automatic Developing Chamber ADC2 from Camag.

Development:

The plate was dried on a Camag TLC Plate Heater III for 6 minutes at 160°C, cooled, and dipped into 6% cupri acetate in 16% H_3PO_4. Additionally dried 10 minutes at 160°C and evaluated directly.

The density of the components on the TLC plate was analysed by a Camag TLC Scanner 3.

Gas chromatography

Free fatty acid in the gum phase was analysed by GLC.

Mono-di-triglyceride, sterol and sterol ester of the oil phase was also analysed by GLC

Apparatus:

- Perkin Elmer Autosystem 9000 Capillary Gas Chromatograph equipped with WCOT fused silica column 12.5 m x 0.25mm ID x 0.1µ 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 (used since 30.10.2003):

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

Sample preparation:

The sample was dissolved in 12ml Heptane:Pyridin, 2:1 containing internal standard heptadecane, 0.5mg/ml. 500µl sample solution was transferred to a crimp vial, 100µl MSTFA (N-Methyl-N-trimethylsilyl-trifluoraceamid) was added and reacted for 15 minutes at 60°C.

Calculation:

Response factors for sterol, sterol ester, free fatty acids, mono- di- and tri-glyceride were determined based on pure reference material.

Experimental:

Acyltransferase KLM3' and PLC was tested in a water degumming process using crude soya oil with the recipes shown in Table 1

Table 1

		1	2	3	4	5	6	7	8	9
Crude soya oil from Argentina n	g	10	10	10	10	10	10	10	10	10
Phospholipase C P7633	ml		0,2	0,2	0,2					
Phospholipase C P6621								0,2	0,2	0,2
K932 10 U/ml	ml			0,01	0,05	0,01	0,05		0,01	0,05
Water	ml	0,250	0,050	0,040	0,000	0,240	0,200	0,050	0,040	0,000
% water		2,50	2,50	2,50	2,50	2,50	2,50	2,50	2,50	2,50

Phospholipase C P7633 Sigma, From *C. perfringens*, 135.3mg solid:3.8 unit/mg solid, 32.9 mg enzyme in 0.5 ml water

Phospholipase C P6621 Sigma, From *Bacillus cereus*, 250 Units dissolved in 1 ml water

Acyltransferase KLM3' (K932) diluted to 10 LATU/ml

The crude soya was heated to 45°C in a 20 ml Wheaton glass. Water and enzyme was added.

The sample was homogenized by high shear mixing for 30 seconds.

The samples were placed on a heating block at 45 °C with magnetic agitation.

Samples of 1 ml were taken out after 30 and 240 minutes in an Eppendorf tube and the enzymes inactivated for 10 minutes at 97 °C. Notably although deactivation of the enzyme is carried out in the experiments – this is not generally done in practice in industry. The deactivation is only carried out in the experiments herein so that an accurate analysis of the enzyme degradation.

The samples were centrifuged at 3000 rcf for 3 minutes. The oil phase was separated from the gum phase, and both phases were analysed by TLC and GLC.

<u>Results</u>

TLC analysis

Samples taken out after 30 minutes and 240 minutes were analysed by TLC with results shown in Figures 103 to 106.

The TLC plates (Figure 103 and Figure 104) were scanned and used for quantitative determination of 1,2 diglyceride (DAG sn1,2) with results shown in Table 2 and 3 below.

The relative degradation of the phospholipids are shown in Figure 107.

Table 2: TLC analysis of oil phase after 30 minutes reaction time.

Test no.	Phospholipase C P7633 U/g	Phospholipase C P6621 U/g	K932 U/ml LATU/g	DAG sn_1,2 %
1	0	0	0	0.33
2	5	0	0	0.72
3	5	0	0.01	0.67
4	5	0	0.05	0.60
5	0	0	0.01	0.37
6	0	0	0.05	0.29
7	0	5	0	1.28
8	0	5	0.01	1.22
9	0	5	0.05	1.19

Table 3: TLC analysis of oil phase after 240 minutes reaction time.

Test no.	Phospholipase C P7633 U/g	Phospholipase C P6621 U/g	K932 LATU/g	DAG sn_1,2 %
1	0	0	0	0.27
2	5	0	0	0.64
3	5	0	0.01	0.60
4	5	0	0.05	0.50
5	0	0	0.01	0.34
6	0	0	0.05	0.27
7	0	5	0	1.06
8	0	5	0.01	1.04
9	0	5	0.05	1.01

The results from Tables 2 and 3 above clearly indicate the formation of diglyceride caused by the PLC degradation of phospholipids. It is observed that with the dosage of PLC used the formation of sn 1,2 diglyceride has already reached its maximum after 30 minutes reaction time. It is also observed that the amount of sn 1,2 diglyceride decreases with increased dosage of KLM3' when used in combination with PLC.

This effect was observed for both phospholipase C enzymes but the effect was most pronounced when KLM3' was combined with Phospholipase C P7633 Sigma, from *C. perfringens*. This is most probably explained by the fact that PLC from C. *perfringens* only degraded a small part of the phospholipids, so more substrate was available for KLM3'.

The results in Figure 107 also clearly show that Phospholipase C P7633 Sigma, from *C. perfringens* is mainly active on phosphatidylcholine (PC), and Phospholipase C P6621 Sigma, from *Bacillus cereus* has main activity on phosphatidylcholine (PC) and phosphatidylethanolamine (PE) and less activity on phosphatidic acid (PA) and phosphatidylinositol (PI). The results also proof that KLM3' can use all four types of phospholipids.

It is therefore concluded that acyltransferase KLM3' can use sn1,2 diglyceride as an acceptor molecule and catalyses the reaction in Figure 108.

GLC analysis

The samples no 1 to 6 of oil phase from the experiment in Table 1 were also analysed by GLC.

The GLC analysis of total diglyceride (DAG), sterol sterol ester and FFA are listed in Table 4 below.

Table 4 GLC analysis of oil phase after 30minutes and 240 minutes incubation.

sample no	PLC U/g	KLM3 U/g	Reaction Time minutes	DAG %	Sterol %	Sterol ester %	FFA %
1	0	0	30	1.34	0.25	0.12	0.22
2	5	0	30	2.58	0.26	0.13	0.21
3	5	0.05	30	2.39	0.18	0.26	0.22
4	5	0.1	30	2.10	0.09	0.42	0.28
5	0	0.05	30	1.43	0.15	0.33	0.22
6	0	0.1	30	1.24	0.06	0.49	0.33
1	0	0	240	1.63	0.22	0.13	0.20
2	5	0	240	2.33	0.25	0.13	0.20
3	5	0.05	240	2.13	0.08	0.45	0.29
4	5	0.1	240	2.08	0.04	0.48	0.43
5	0	0.05	240	1.69	0.04	0.49	0.32
6	0	0.1	240	1.68	0.04	0.50	0.56

The GLC analysis of samples taken out after 30 and 240 minutes reaction time confirmed what was already observed by TLC analysis, that Phospholipase C P7633 Sigma, from *C. perfringens* produced diglyceride from the phospholipids in the oil. The results also confirm the synergistic effect by reduced amount of diglyceride when Phospholipase C is combined with KLM3'. A statistical evaluation by ANOVA using

Statgraphic software of the effect of PLC and KLM3' on the amount of diglyceride clearly indicates the interaction effect between these two enzymes, see Figure 109.

PLC had no significant effect on the sterols in the oil but KLM3' converts free sterols to sterols esters. Sterols are a better acceptor molecule than DAG for KLM3' and therefore only 10-15% of the DAG in the reaction mixture were converted to triglyceride.

PLC does not have much impact on the level of free fatty acids (FFA) but it is observed that KLM3' in the high dosage and at extended reaction time contribute to increased level of FFA.

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Without wishing to be bound by theory the decrease in diglyceride by combining acyltransferase (KLM3') and phospholipase C (PLC) may be caused by substrate (phospholipid) competition when the two enzymes are used together.

In order to prove that KLM3' is able to use diglyceride as acceptor and catalyse the reaction mentioned in Figure 108 a model experiment with the recipe shown in Table 5 below was conducted.

Table 5 Recipe for investigation of acyltransferase effect of KLM3' on diglyceride/phosphatidylcholine substrate.

		1	2	3	4	5	6
Diglyceride/PC 80/20	g	3	3	3	3	3	3
Acyltransferase KLM3': 300 LATU/g	ml	0	0.01		0.01		0.01
Buffer	ml	0.03	0.03	0.03	0.03	0.03	0.03
Water 3% salt		0.01		0.01		0.01	
Buffer :1 100 mM Acetate pH 5.5		X	X				
Buffer 2: 100 mM HEPES pH 7				X	X		
Buffer 3. 100 mM MES pH 6						X	X

Distilled diglyceride based on sunflower oil and phosphatidylcholine (PC) was mixed during heating and agitation to 80°C until PC dissolved in the diglyceride.

The substrate was scaled in a 7 ml Dram Glass with screw lid and heated to 55°C. Enzyme, buffer and water was added, and the sample was agitated with magnetic stirring at 450 rpm.

After 30 and 180 minutes a sample was taken and analysed by TLC (Figure 110).

The TLC plate was scanned and the triglyceride content in the samples was quantified from a standard curve made form the analysis of Canola oil with results shown in Table 6 below.

Table 6

Buffer pH	Enzyme U/g	Reaction time minutes	Triglyceride %
5.5	0	30	1.42
5.5	1	30	1.74
6	0	30	1.63
6	1	30	1.79
7	0	30	1.49
7	1	30	1.55
5.5	0	180	1.75
5.5	1	180	1.79
6	0	180	1.76
6	1	180	1.80
7	0	180	1.67
7	1	180	2.01

The results shown in Table 6 were analysed statistically by ANOVA using Statgraphic software with results shown in Figures 111 and 112.

The statistical evaluation of the triglyceride results from Table 6 confirm a significant increase in amount of triglyceride by addition of acyltransferase KLM3' to a substrate containing diglyceride and phosphatidylcholine.

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The experiment mentioned above in Table 5 was studied in further detail to investigate the effect of higher level of water on the transfer reaction of fatty acid moiety from phospholipid to diglyceride during formation of triglyceride. The experimental set up is listed in Table 7 below.

Table 7: Recipe for investigation of acyltransferase effect of KLM3' on diglyceride/phosphatidylcholine substrate.

		1	2	3	4	5
Diglyceride/Phosphatidylcholine 80/20	g	3	3	3	3	3
Acyltransferase KLM3': 1128 LATU/ml	ml	0	0.01	0.01	0.01	0.01
Buffer :1 100 mM Acetate pH 5.5	ml	0.05	0.05	0.05	0.05	
Water	ml	0.01		0.09	0.165	0.14
% water		2.00	2.00	5.00	7.50	5.00
LATU/g substrate		0.0	3.8	3.8	3.8	3.8

Distilled diglyceride based on sunflower oil and phosphatidylcholine (PC) was mixed during heating and agitation to 80°C until PC dissolved in the diglyceride.

The substrate was scaled in a 7 ml Dram Glass with screw lid and heated to 55°C. Enzyme, buffer and water was added, and the sample was agitated with magnetic stirring at 450 rpm

After 30, 90 and 240 minutes a sample was taken and analysed by TLC

TLC chromatograms are shown in Figure 113 and Figure 114.

The TLC plates were scanned and the content of triglyceride in the samples calculated based on a calibration curve made from triglyceride (Canola Oil). The results of triglyceride determination is shown in Table 8.

Table 8: Triglyceride analysis in diglyceride/PC substrate incubated with acyltransferase KLM3'

Test no	Triglyceride, % 30 minutes	Triglyceride, % 90 minutes	Triglyceride, % 240 minutes
1	1.33	1.36	1.58
2	1.55	1.91	2.56
3	1.59	2.02	2.65
4	1.57	1.81	2.29
5	1.56	1.91	2.46

The results in Table 8 were analysed statistically by ANOVA using Statgraphic software with results shown in Figure 115 and Figure 116.

The results from Table 8 and Figure 115 and Figure 116 clearly demonstrate the ability of acyltransferase KLM3' to produce triglyceride from a substrate of diglyceride and phosphatidylcholine.

Conclusion

Lipid acyltransferase KLM3' as well as phospholipase C (PLC) are known to contribute to increased oil yield in degumming of vegetable oil.

The effect of lipid acyltransferase KLM3' in oil degumming is based on a transfer reaction of fatty acid moiety from phospholipids to sterol during production lysophospholipids and sterol esters.

The effect of phospholipase C (PLC) relies on the conversion of phospholipids into diglyceride and water soluble phosphor-derivatives. The diglyceride produced in this reaction will accumulate in the oil phase by the degumming process, but it is not always preferable to have high diglyceride in the oil because it will have an impact on the smoke point of the oil and will also have an impact in the crystallisation properties of more saturated fat sources.

In the current study lipid acyltransferase KLM3' and Phospholipase C (PLC) were tested alone and in combination in a water degumming process. The experiments showed that PLC in the water degumming of soya oil produces diglyceride which

forms part of the oil phase. When PLC was used in combination with KLM3' it was surprisingly shown that the amount of diglyceride produced by PLC was reduced and the sterol was converted to sterol esters indicating a synergistic effect between these two enzymes because KLM3' catalyses the transfer reaction of fatty acid moiety from phospholipid to diglyceride during formation of triglyceride.

The transfer reaction catalyzed by KLM3' of fatty acid moiety from phospholipid to diglyceride during formation of triglyceride was confirmed in a model system composed of diglyceride and phospholipid.

The results also showed that the two phospholipids tested do not have the same activity on all types of phospholipids, but KLM3 has almost the same activity on all four types of phospholipids found in crude soya oil. This also opens the possibility to use Phospholipase C in combination with KLM3' in order to get a further conversion of phospholipids.

Example 14

Use of KLM3' in water degumming of crude soya oil.

Vegetable oil including soya bean oil contains 1 to 3 % phospholipids, which are removed by an oil degumming process. The oil degumming process is normally divided into a water degumming process and a neutralisation process. Crude Soya bean oil with 1-3% phosopholipids can not be shipped for export without water degumming aimed at reducing the phosphor level down to 200 ppm Phosphor or below to meet the specification for water degummed crude oil.

If the phosphor level is much lower than 200 ppm then this can be disadvantageous. Typically conventional degumming results in a phosphor level post-centrifugation of about 50ppm. This is because it is not possible to control the centrifuge to give levels of phosphor which are less than 200 ppm but as close as possible to this level.

In contrast in the present case the use of the lipid acyltransferase the water degummed oil might preferably be adjusted to about 180 ppm phosphor.

Adjustment of the phosphor level in the enzymatic water degumming process of the present invention can preferably be done by adjusting the interphase between gum and oil in the centrifuge to get a little more phospholipid into the oil phase. In a conventional water degumming process the gum phase is however very thick and viscous, and it is therefore not easy to adjust the interphase in the centrifuge.

The present inventors have surprisingly found that when lipid acyltransferase (e.g. KLM3') is used in the water degumming process the interphase could be adjusted without problems in the centrifuge and could produce a degummed oil which was closer to the specification of a maximum of 200 ppm phosphor.

Experimental

The lipid acyltransferase KLM3' (SEQ ID No. 68) was used in water degumming of crude soya oil in the process outlined in Figure 117.

The crude soya oil containing 1100 ppm phosphor was exposed to the water degumming process shown in Figure 117. In the first experiment the degumming process was run without addition of the enzyme. In the second experiment the enzyme KLM3' was added, and after analysing the phosphor content of the water degummed oil the interphase between gum and oil in the centrifuge was adjusted towards the centre of the centrifuge. When the process was in balance again the phosphor was analysed again.

The result from the trials are shown in Table 1 below:

Table 1

Water degumming	1	2	3
Enzyme KLM3', LATU/kg	0	200	200
Centrifuge fine Tuner setting	185	185	195
Phosphor in oil after centrifuge, ppm	44*	35*	185

* not significant

Conclusion

In the experiment with enzymatic water degumming using KLM3' it was shown that the interphase between oil and gum in the centrifuge could easily be adjusted or controlled to produce water degummed oil with a phosphor level closer to specification (i.e. closer to but less than 200ppm).

Under conventional water degumming conditions it is not always easy to adjust the interphase because of the consistency (high viscosity) of the gum phase does not allow such adjustment.

Example 15

Enzymatic reaction in the "gum phase" after enzymatic water degumming of vegetable oils.

Lipid acyltransferase, LysoMax Oil (KLM3') was tested in water degumming of crude soya oil. Notably, the enzyme was not inactivated at the end of the enzymatic water degumming process – as would be routine in practice in industry. Therefore the enzymatic water degumming process was carried out in accordance with the Experimental protocol shown below. Notably enzyme was not inactivated after degumming.

The isolated gum phase from this process was incubated at 40°C, and the further degradation of phospholipid in the gum phase was analysed. The results surprisingly showed that the enzyme further hydrolysed phospholipid into lysophospholipids and free fatty acid. This is explained by the fact that the enzyme associates with the gum phase when the gum phase is separated from the oil phase by centrifugation.

Also the lyso-phospholipids were hydrolysed during storage, and after 7 days storage almost all phospholipids had disappeared from the gum phase.

COMMERCIAL RELEVANCE OF THE RESULTS

Enzymatic oil degumming of crude soya oil with KLM3' has shown that it is possible to improve the oil yield from 0.5 to 1.5%. The gum phase isolated from this process typically still contains some oil and phospholipids (EP1 624 047). It is known that by hydrolysis of the gum phase an oil phase can separate form the gum, which can be isolated by centrifugation or other means of separation. This oil phase containing high levels of free fatty acid can be sold as acid oil with higher value than the normal gum phase which is added to the meal.

A further aspect is that the remaining solid phase after separation of acid oil has higher phosphor level then normal gum and can be used as a source or organic phosphor.

Introduction

The inventors have surprisingly shown that the lipid acyltransferase LysoMax Oil (KLM3') is active in the gum phase isolated from enzymatic water degumming of crude soya oil. It was therefore speculated whether the enzyme could further degrade the phospholipids into free fatty acids which, by centrifugation, could be isolated as an acid oil together with the remaining triglyceride in the gum phase.

In this study the effect of different enzyme dosages and water degumming temperatures on the phospholipid degradation in the gum phase was examined.

Material and Methods:

- KLM3':Glycerophospholipid cholesterol acyltransferase (FoodPro LysoMax Oil) (K932)
  Lot no 102629600. 1 Activity 1128 LATU/g

Crude soya oil no 18: from, Argentina

HPTLC analysis

The degradation of phospholipids in the gum phase from enzyme treated samples was analysed HPTLC.

Applicator: Automatic TLC Sampler 4, CAMAG

HPTLC plate: 20 x 10 cm, Merck no. 1.05641. Activated 10 minutes at 160°C before use.

Application:

Gum phase from 10 gram oil was dissolved in 7.5 ml Hexan:Isopropanol 3:2.

1 µl of the sample was applied to the HPTLC plate.

A phospholipid standard (0.5% phospholipid (Spectra Lipid, Germany) was applied (0.1, 0.3, 0.5, 0.8 and 1.5 µl) and used for the calculation of the individual phospholipids in the gum.

In some applications the phospholipid content was calculated relative to a control gum n0ot treated with enzyme. This control sample was applied 0.1 – 0.3 – 0.5 – 0.8 – 1 µl and used for making calibrations curves.

Oil phase. Approximate 90 mg was scaled and dissiloved in 1 ml Hexan:Isopropanol 3:2.

5 µl of the sample was applied to the HPTLC plate. Mono-diglyceride 5 mg/ml of known concentration was applied at 0.1 - 0.3 - 0.5 - 0.8 - 1.5 µl and used for calculation of individual glyceride components

TLC applicator.

Running buffer no. 1: P-ether: Methyl Tert Butyl Ketone : Acetic acid 50:50:1

Running buffer 6: Chloroform:1-propanol:Methylacetate:Methanol: 0.25% KCl in water 25:25:25:10:9

Elution: The plate was eluted 7 cm using an Automatic Developing Chamber ADC2 from Camag.

Development:

The plate was dried on a Camag TLC Plate Heater III for 10 minutes at 160°C, cooled, and dipped into 6% cupri acetate in 16% H_3PO_4. Additionally dried 10 minutes at 160°C and evaluated directly.

The density of the components on the TLC plate was analysed by a Camag TLC Scanner 3.

Gas chromatography

Free fatty acid in the gum phase was analysed by GLC.

Sterol, sterol ester and Mono-di-triglyceride of the oil phase was also analysed by GLC

Apparatus

- Perkin Elmer Autosystem 9000 Capillary Gas Chromatograph equipped with WCOT fused silica column 12.5 m x 0.25mm ID x 0.1µ 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 (used since 30.10.2003):

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

Sample preparation

The sample was dissolved in 12ml Heptane:Pyridin, 2:1 containing internal standard heptadecane, 0.5mg/ml. 500µl sample solution was transferred to a crimp vial, 100µl MSTFA (N-Methyl-N-trimethylsilyl-trifluoraceamid) was added and reacted for 15 minutes at 60°C.

Calculation

Response factors for free fatty acids, mono- di- and tri-glyceride were determined based on pure reference material.

Experimental:

Lipid acyltransferase KLM3' was tested in crude soya oil in the recipes shown in table 1 below.

The degumming experiments in Table 1 were conducted at both 45 and 55°C

Jour. 2460-220		1	2	3	4	5	6	7	8	9	10
Crude soya oil	g	10	10	10	10	10	10	10	10	10	10
K932: 100 LATU-K/ml	ml	0	0.01	0.02	0.05	0.01	0.02	0.05	0.01	0.02	0.05
Extra Water	ml	0.10	0.09	0.08	0.05	0.09	0.08	0.05	0.09	0.08	0.05
LATU-K/g oil		0.00	0.10	0.20	0.50	0.10	0.20	0.50	0.10	0.20	0.50
% water		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

The crude soya was heated to 55°C (or 45°C) in a 20 ml Wheaton glass. Water and enzyme was added. The sample was homogenized by high shear mixing for 30 seconds. The samples were placed on a heating block at 55°C (or 45°C) with magnetic agitation (450 rpm). After 30 minutes incubation the samples were centrifuged at 3000 rcf for 3 minutes.

The oil phase was separated form the gum phase by turning the tubes up side down for 15 minutes, which left the gum in the tubes.

The gum phase from each of samples 1 to 4 was then immediately frozen.

The gum phase from each of samples 5 to 8 were incubated at 40°C for 1 day and then frozen.

The gum phase from each of samples 9-12 were incubated 7 days at 40°C.

All samples were analysed at the same time by TLC and GLC.

Results:

TLC analysis of gum phase samples from degumming at 55°C are shown in Figure 118 and the samples from degumming at 45°C are shown in Figure 119

Based on the scanning of the TLC chromatogram the relative content of phospholipid in the enzyme treated gum phase compared with the gum phase without enzyme treatment, was calculated (see Tables 2 and 3 below).

Table 2: Relative phospholipid in gum phase from water degumming at 55°C

sample no	Enzyme LATU/g	Time days	LPC Rel. %	PC Rel. %	PA Rel. %	PE Rel. %	PI Rel. %
1	0	0	100.0	100	100	100	100
2	0.1	0	571.2	31.2	35.8	26.1	55.0
3	0.2	0	144.5	18.0	24.1	13.1	39.6
4	0.5	0	45.6	3.3	17.1	3.0	16.3
5	0.1	1	452.5	4.6	17.6	3.0	24.6
6	0.2	1	26.7	1.0	15.5	0.4	9.5
7	0.5	1	2.0	0.0	6.2	0.0	2.5
8	0.1	7	3.0	0.0	8.0	0.0	3.2
9	0.2	7	1.0	0.0	4.0	0.0	2.1
10	0.5	7	0.2	0.0	0.0	0.0	2.6

Table 2: Relative phospholipid in gum phase from water degumming at 45°C

sample no	Enzyme LATU/g	Time days	PC Rel. %	PA Rel. %	PE Rel. %	PI Rel. %
1	0	0	100.0	100.0	100.0	100.0
2	0.1	0	40.5	48.6	38.5	43.0
3	0.2	0	21.5	33.7	22.4	26.9
4	0.5	0	7.4	23.1	9.0	15.6
5	0.1	1	6.4	41.9	6.0	17.2
6	0.2	1	2.3	25.7	1.9	12.5
7	0.5	1	1.3	10.7	0.0	4.2
8	0.1	7	0.0	17.1	0.0	8.1
9	0.2	7	2.5	9.4	0.0	4.8
10	0.5	7	0.0	0.0	0.0	3.7

The gum phase samples from 0 days were taken out just after the degumming reaction and centrifugation. At this point already a main part of the phospholipid is degraded and it is seen that the amount of lyso-phospholipid increases (Table 2). During incubation of the gum phase further hydrolysis of the phospholipids occurs, but also the lyso-phospholipids are hydrolysed.

The gum phases were analysed by GLC for free fatty acids (FFA) and triglyceride (see Table 3 below).

A fraction of the gum phase was extracted twice with Hexan Isopropanol 2:1 and the insoluble part was dried and quantified gravimetrically.

Table 3: GLC analysis of FFA and triglyceride in the gum phase and insoluble material

Sample No	Incubation Days	Enzyme LATU/g	Dry basis % FFA	Dry basis % Triglyceride	Dry basis % FFA+Triglyceride	Hexan:IPA insoluble, %.
1	0	0	1.9	64.0	66.0	2.7
2	0	0.1	7.0	41.5	48.6	3.6
3	0	0.2	8.2	42.5	50.7	6.0
4	0	0.5	7.4	43.1	50.5	26.9
5	1	0.1	16.3	36.4	52.7	15.7
6	1	0.2	16.6	39.8	56.4	nd.
7	1	0.5	12.6	40.3	53.0	41.1
8	7	0.1	21.2	37.3	58.5	35.6
9	7	0.2	19.2	37.1	56.4	33.3
10	7	0.5	14.6	42.1	56.7	38.7

The results shown in Table 3 clearly confirm that the enzymatic hydrolysis continues during storage of the gum phase at 40°C up to 7 days.

The content of the gum phase which is not extractable with organic solvent (Hexan Isopropanol 2:1) is a measure for the amount of solid in the gum phase. When the phospholipids in the gum phase are hydrolyzed into FFA and phosphatidylglycerol the amount of material which is not soluble in Hexan:isopropanol increases. After 7 days incubation, more then 90% of the gum phase is composed of FFA, triglyceride and phosphatidylglycerol and no phospholipids are left in the gum phase. The composition of the gum phase after incubation makes it more easy to separate into an oily phase and a solid/water soluble phase, because no emulsifiers (phospholipids and lysophospholipids) are left in the gum.

Conclusion

During enzymatic degumming with a lipid acyltransferase (e.g. KLM3') a gum phase is isolated which contains active enzyme. Incubation of the gum phase at 40°C further hydrolyses the phospholipids in the gum phase. Depending on the enzyme dosage all the phospholipids as well as the lyso-phospholipids are hydrolysed into fatty acids and

phosphatidylglycerol. The elimination of the phospholipids in the gum phase makes it possible to isolate an oily phase containing free fatty acids and the remaining triglyceride in the gum phase.

In the degumming experiment conducted at 55°C, higher levels of phospholipid degradation were observed than running the experiment at 45°C. In both experiments the enzyme was active in the gum phase after separation and there was a tendency to an overall higher degree of hydrolysis during storage at 40°C when the water degumming was conduced at 55°c

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

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

NAME AND ADDRESS OF DEPOSITOR

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Escherichia coli TOP10pPet12aAhydro	NCIMB 41204

II. SCIENTIFIC 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.,	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorised official(s): Terence Dando
Address: 23 St Machar Drive Aberdeen AB24 3RY Scotland, UK.	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 sheet)

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

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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 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.

Form BP/9 (first page)

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):

Date: 9 January 2004

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

Form BP/9 (second and last page)

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Denmark

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RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
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INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

NAME AND ADDRESS OF DEPOSITOR

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Escherichia coli TOP10pPet12aAsalmp	NCIMB 41205

II. SCIENTIFIC 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.

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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.,	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorised official(s):
Address: 23 St Machar Drive Aberdeen AB24 3RY Scotland	Date: 9 January 2004

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

Form BP/9 (second and last page)

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

Danisco Intellectual Assets
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DK-1001 Copenhagen
Denmark

NAME AND ADDRESS OF DEPOSITOR

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
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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:
Streptomyces sp. L130	NCIMB 41226

II. SCIENTIFIC 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 23 June 2004 (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: 28 June 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)

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VIABILITY STATEMENT
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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 41226 Date of the deposit or of the transfer[1]: 23 June 2004

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 25 June 2004 [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.

Form BP/9 (first page)

'. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4].

". 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): Date: 28 June 2004

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

Form BP/9 (second and last page)

BUDAPEST TREATY ON THE INTERNATIONAL
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NAME AND ADDRESS OF DEPOSITOR

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I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:

Streptomyces sp.
L131

Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:

NCIMB 41227

II. SCIENTIFIC 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 23 June 2004 (date of the original deposit)

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: 28 June 2004

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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 41227 Date of the deposit or of the transfer[1]: 23 June 2003
III. VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on 25 June 2004 [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.

Form BP/9 (first page)

CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

INTERNATIONAL DEPOSITARY AUTHORITY	
ame: NCIMB Ltd., ddress: 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): Date: 28 June 2004

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

Form BP/9 (second and last page)

CLAIMS:

1. A method of water degumming an edible oil comprising the steps of: a) admixing approximately 0.1-5% w/w water with an edible oil and a lipid acyltransferase, wherein a phospholipase C is additionally admixed with the oil or water or lipid acyltransferase or a combination thereof, b) agitating the admixture for between about 10 minutes and 180 minutes at about 45 to about 90°C, and c) separating the oil phase and the gum phase, wherein the lipid acyltransferase used has a transferase activity (TrU) per mg enzyme of at least 25 TrU/mg enzyme protein as determined using the following assay:

a) 50mg cholesterol and 450 mg Soya phosphatidylcholine is dissolved in chloroform and chloroform is evaporated at 40 °C under vacuum; 300 mg PC: cholesterol 9:1 is dispersed at 40 °C in 10 ml 50mM HEPES buffer pH 7 to form the substrate;

b) 250 µl substrate is added in a glass with lid at 40 °C, 25 µl enzyme solution is added and incubated during agitation for 10 minutes at 40 °C;

c) after 10 minutes 5ml Hexan:Isopropanol 3:2 is added;

d) the amount of cholesterol ester is analyzed by High Performance Thin Layer Chromatography (HPTLC) using Cholesteryl stearate standard for calibration; and

e) transferase activity is calculated as the amount of cholesterol ester formation per minute.

2. A method according to claim 1 where the method further comprises d) incubating the gum phase comprising active lipid acyltransferase enzyme for between a minimum of about 2 hours and a maximum of 7 days and e) separating the oil from the gum phase.

3. A method of treating a gum phase wherein the gum phase is incubated with one or more lipid acyltransferase enzymes in combination with one or more phospholipase C enzyme for between a minimum of about 2 hours and a maximum of 7 days and separating the oil from the gum phase.

4. A method according to any one of claims 1-3 wherein the pH of the process is between about pH 5.0 to about pH 10.0.

5. A method according to any one of claims 1 to 4 wherein the lipid acyltransferase that comprises a GDSx motif and/ or a GANDY motif.

6. A method according to any one of claims 1 to 5 wherein the lipid acyltransferase enzyme is characterised as an enzyme which possesses acyltransferase 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.

7. A method according to any one of claims 1 to 6 wherein the lipid acyltransferase is obtainable, or obtained, from an organism from one or more of *Aeromonas*, *Streptomyces*, *Saccharomyces*, *Lactococcus*, *Mycobacterium*, *Streptococcus*, *Lactobacillus*, *Desulfitobacterium*, *Bacillus*, *Campylobacter*, *Vibrionaceae*, *Xylella*, *Sulfolobus*, *Aspergillus*, *Schizosaccharomyces*, *Listeria*, *Neisseria*, *Mesorhizobium*, *Ralstonia*, *Xanthomonas* or *Candida* genera.

8. A method according to claim 7 wherein lipid acyltransferase is obtainable, or obtained, from an organism from the genus *Aeromonas*.

9. A method according to any one of claims 1 to 8 wherein the lipid acyltransferase is a polypeptide having lipid acyltransferase activity which polypeptide is obtained by expression of any one of the nucleotide sequences shown as SEQ ID No. 49, SEQ ID No. 36, SEQ ID No. 38, SEQ ID No. 39, SEQ ID No. 42, SEQ ID No. 44, SEQ ID No. 46, SEQ ID No. 48, SEQ ID No. 50, SEQ ID No. 51, SEQ ID No. 52, SEQ ID No. 53, SEQ ID No. 54, SEQ ID No. 55, SEQ ID No. 56, SEQ ID No. 57, SEQ ID No. 58, SEQ ID No. 59, SEQ ID No. 60, SEQ ID No. 61, SEQ ID No. 62 or SEQ ID No. 63 or a nucleotide sequence which as has 75% or more identity therewith.

10. A method according to any one of claims 1 to 9 wherein the lipid acyltransferase is a polypeptide having lipid acyltransferase activity which polypeptide is obtained by expression of:

a) the nucleotide sequence shown as SEQ ID No. 49 or a nucleotide sequence which as has 75% or more identity therewith;

b) a nucleic acid which encodes said polypeptide wherein said polypeptide is at least 70% identical with the polypeptide sequence shown in SEQ ID No. 16 or with the polypeptide sequence shown in SEQ ID No. 68;

c) or a nucleic acid which hybridises under medium stringency conditions to a nucleic probe comprising the nucleotide sequence shown as SEQ ID No. 49.

11. A method according to claim 10 wherein the lipid acyltransferase is a polypeptide obtained by expression of the nucleotide sequences in *Bacillus licheniformis*.

12. A method according to any one of claims 1 to 11 wherein the lipid acyltransferase is a polypeptide having lipid acyltransferase activity which polypeptide comprises any one of the amino acid sequences shown as SEQ ID No. 68, SEQ ID No. 16, SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 15, SEQ ID No. 17, SEQ ID No. 17, SEQ ID No. 18, SEQ ID No. 34, SEQ ID No. 35 or an amino acid sequence which as has 75% or more identity therewith.

13. A method according to any one of claims 1 to 12 wherein the lipid acyltransferase is a polypeptide having lipid acyltransferase activity which polypeptide comprises the amino acid sequence shown as SEQ ID No. 68 or an amino acid sequence which as has 75% or more identity therewith.

14. A method according to claim 1, wherein the edible oil is a crude edible oil.

15. A method according to claim 3, wherein the gum phase is obtainable or obtained from degumming an edible oil.

16. A method according to claim 15 wherein the degumming is water degumming, or enzymatic degumming, or a combination thereof, an edible oil.

17. Use of lipid acyltransferase in combination with a phospholipase C in water degumming of an edible oil for increasing the yield of oil in the oil phase after completion of the water degumming process, wherein the lipid acyltransferase used has a transferase activity (TrU) per mg enzyme of at least 25 TrU/mg enzyme protein as determined using the following assay:

a) 50mg cholesterol and 450 mg Soya phosphatidylcholine is dissolved in chloroform and chloroform is evaporated at 40 °C under vacuum; 300 mg PC: cholesterol 9:1 is dispersed at 40 °C in 10 ml 50mM HEPES buffer pH 7 to form the substrate;

b) 250 µl substrate is added in a glass with lid at 40 °C, 25 µl enzyme solution is added and incubated during agitation for 10 minutes at 40 °C;

c) after 10 minutes 5ml Hexan:Isopropanol 3:2 is added;

d) the amount of cholesterol ester is analyzed by High Performance Thin Layer Chromatography (HPTLC) using Cholesteryl stearate standard for calibration; and

e) transferase activity is calculated as the amount of cholesterol ester formation per minute.

18. Use of a lipid acyltransferase in combination with a phospholipase C in water degumming of an edible oil for decreasing the viscosity of the gum phase after completion of the water degumming process, wherein the lipid acyltransferase used has a transferase activity (TrU) per mg enzyme of at least 25 TrU/mg enzyme protein as determined using the following assay:

a) 50mg cholesterol and 450 mg Soya phosphatidylcholine is dissolved in chloroform and chloroform is evaporated at 40 °C under vacuum; 300 mg PC: cholesterol 9:1 is dispersed at 40 °C in 10 ml 50mM HEPES buffer pH 7 to form the substrate;

b) 250 µl substrate is added in a glass with lid at 40 °C, 25 µl enzyme solution is added and incubated during agitation for 10 minutes at 40 °C;

c) after 10 minutes 5ml Hexan:Isopropanol 3:2 is added;

d) the amount of cholesterol ester is analyzed by High Performance Thin Layer Chromatography (HPTLC) using Cholesteryl stearate standard for calibration; and

e) transferase activity is calculated as the amount of cholesterol ester formation per minute.

19. Use of a lipid acyltransferase in combination with a phospholipase C in water degumming of an edible oil for increasing the yield of oil and/or for increasing triglyceride levels in the oil phase after completion of the water degumming process and/or for reducing the diglyceride level in the oil phase after completion of the water degumming process, wherein the lipid acyltransferase

used has a transferase activity (TrU) per mg enzyme of at least 25 TrU/mg enzyme protein as determined using the following assay:

a) 50mg cholesterol and 450 mg Soya phosphatidylcholine is dissolved in chloroform and chloroform is evaporated at 40 °C under vacuum; 300 mg PC: cholesterol 9:1 is dispersed at 40 °C in 10 ml 50mM HEPES buffer pH 7 to form the substrate;

b) 250 µl substrate is added in a glass with lid at 40 °C, 25 µl enzyme solution is added and incubated during agitation for 10 minutes at 40 °C;

c) after 10 minutes 5ml Hexan:Isopropanol 3:2 is added;

d) the amount of cholesterol ester is analyzed by High Performance Thin Layer Chromatography (HPTLC) using Cholesteryl stearate standard for calibration; and

e) transferase activity is calculated as the amount of cholesterol ester formation per minute.

20. Use of a lipid acyltransferase in combination with a phospholipase C in the incubation of a gum phase for increasing the yield of oil and/or producing a solid phase with an improved phosphor level compared with an untreated gum, wherein the lipid acyltransferase has a transferase activity (TrU) per mg enzyme of at least 25 TrU/mg enzyme protein as determined using the following assay:

a) 50mg cholesterol and 450 mg Soya phosphatidylcholine is dissolved in chloroform and chloroform is evaporated at 40 °C under vacuum; 300 mg PC: cholesterol 9:1 is dispersed at 40 °C in 10 ml 50mM HEPES buffer pH 7 to form the substrate;

b) 250 µl substrate is added in a glass with lid at 40 °C, 25 µl enzyme solution is added and incubated during agitation for 10 minutes at 40 °C;

c) after 10 minutes 5ml Hexan:Isopropanol 3:2 is added;

d) the amount of cholesterol ester is analyzed by High Performance Thin Layer Chromatography (HPTLC) using Cholesteryl stearate standard for calibration; and

e) transferase activity is calculated as the amount of cholesterol ester formation per minute.

21. Use according to any one of claims 17-20 wherein 0.1-4% w/w water is admixed with the edible oil.

22. Use according to any one of claims 17 to 21 wherein the enzyme is added to an edible oil at a temperature in the range of about 45 to about 90°C.

23. Use according to any one of claims 17-22 wherein the pH of the degumming process is between about pH5.0 to about pH10.0.

24. Use according to any one of claims 17-23 wherein the lipid acyltransferase is reacted with the edible oil for between about 10 minutes to 180 minutes.

25. Use according to any one of claims 17 to 24 wherein the lipid acyltransferase comprising a GDSx motif and/ or a GANDY motif.

26. Use according to any one of claims 17-25 wherein the lipid acyltransferase enzyme is characterised as an enzyme which possesses acyltransferase 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.

27. Use according to any one of claims 17-26 wherein the lipid acyltransferase is obtainable, or obtained, from an organism from one or more of *Aeromonas*, *Streptomyces*, *Saccharomyces*, *Lactococcus*, *Mycobacterium*, *Streptococcus*, *Lactobacillus*, *Desulfitobacterium*, *Bacillus*, *Campylobacter*, *Vibrionaceae*, *Xylella*, *Sulfolobus*, *Aspergillus*, *Schizosaccharomyces*, *Listeria*, *Neisseria*, *Mesorhizobium*, *Ralstonia*, *Xanthomonas* or *Candida* genera.

28. Use according to claim 27 wherein the lipid acyltransferase is obtainable, or obtained, from an organism from the genus *Aeromonas*.

29. Use according to any one of claims 17-28 wherein the lipid acyltransferase is obtained by expression of any one of the nucleotide sequences shown as SEQ ID No. 49, SEQ ID No. 36, SEQ ID No. 38, SEQ ID No. 39, SEQ ID No. 42, SEQ ID No. 44, SEQ ID No. 46, SEQ ID No. 48, SEQ ID No. 50, SEQ ID No. 51, SEQ ID No. 52, SEQ ID No. 53, SEQ ID No. 54, SEQ ID No. 55, SEQ ID No. 56, SEQ ID No. 57, SEQ ID No. 58, SEQ ID No. 59, SEQ ID No. 60, SEQ ID No. 61, SEQ ID No. 62 or SEQ ID No. 63 or a nucleotide sequence which as has 75% or more identity therewith.

30. Use according to any one of claims 17-29 wherein the lipid acyltransferase is a polypeptide obtained by expression of:

a) the nucleotide sequence shown as SEQ ID No. 49 or a nucleotide sequence which as has 75% or more identity therewith;

b) a nucleic acid which encodes said polypeptide wherein said polypeptide is at least 70% identical with the polypeptide sequence shown in SEQ ID No. 16 or with the polypeptide sequence shown in SEQ ID No. 68; or

c) a nucleic acid which hybridises under medium stringency conditions to a nucleic probe comprising the nucleotide sequence shown as SEQ ID No. 49.

31. Use according to claim 30 wherein the lipid acyltransferase is a polypeptide obtained by expression of the nucleotide sequences in *Bacillus licheniformis*.

32. Use according to any one of claims 17-31 wherein the lipid acyltransferase is polypeptide comprises any one of the amino acid sequences shown as SEQ ID No. 68, SEQ ID No. 16, SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 15, SEQ ID No. 17, SEQ ID No. 18, SEQ ID No. 34, SEQ ID No. 35 or an amino acid sequence which has 75% or more identity therewith.

33. Use according to any one of claims 17-32 wherein the lipid acyltransferase is a polypeptide comprises the amino acid sequence shown as SEQ ID No. 68 or an amino acid sequence which as has 75% or more identity therewith.

34. Use according to claim 20 wherein the gum phase is obtainable or obtained from degumming an edible oil.

35. Use according to claim 20 wherein the gum phase is obtainable or obtained from the water degumming, enzymatic degumming, or a combination thereof, of an edible oil.

FIGURE 1

SEQ ID No. 16

```
  1  ADTRPAFSRI VMFGDSLSDT GKMYSKMRGY LPSSPPYYEG RFSNGPVWLE QLTKQFPGLT
 61  IANEAEGGAT AVAYNKISWD PKYQVINNLD YEVTQFLQKD SFKPDDLVIL WVGANDYLAY
121  GWNTEQDAKR VRDAISDAAN RMVLNGAKQI LLFNLPDLGQ NPSARSQKVV EAVSHVSAYH
181  NKLLLNLARQ LAPTGMVKLF EIDKQFAEML RDPQNFGLSD VENPCYDGGY VWKPFATRSV
241  STDRQLSAFS PQERLAIAGN PLLAQAVASP MARRSASPLN CEGKMFWDQV HPTTVVHAAL
301  SERAATFIET QYEFLAHG
```

FIGURE 2

(SEQ ID No. 1)

```
  1  MKKWFVCLLG LVALTVQAAD SRPAFSRIVM FGDSLSDTGK MYSKMRGYLP
 51  SSPPYYEGRF SNGPVWLEQL TKQFPGLTIA NEAEGGATAV AYNKISWNPK
101  YQVINNLDYE VTQFLQKDSF KPDDLVILWV GANDYLAYGW NTEQDAKRVR
151  DAISDAANRM VLNGAKQILL FNLPDLGQNP SARSQKVVEA VSHVSAYHNQ
201  LLLNLARQLA PTGMVKLFEI DKQFAEMLRD PQNFGLSDVE NPCYDGGYVW
251  KPFATRSVST DRQLSAFSPQ ERLAIAGNPL LAQAVASPMA RRSASPLNCE
301  GKMFWDQVHP TTVVHAALSE RAATFIANQY EFLAH*
```

FIGURE 3

(SEQ ID No. 2)

```
  1 ivafGDSlTd geayygdsdg ggwgagladr Ltallrlrar prgvdvfnrg isGrtsdGrl
 61 ivDalvallF laqslglpnL pPYLsgdflr GANFAsagAt Ilptsgpfli QvqFkdfksq
121 vlelrqalgl lqellrllpv ldakspdlvt imiGtNDlit saffgpkste sdrnvsvpef
181 kdnlrqlikr Lrsnngarii vlitlvilnl gplGClPlkl alalassknv dasgclerln
241 eavadfneal relaiskled qlrkdglpdv kgadvpyvDl ysifqdldgi qnpsayvyGF
301 ettkaCCGyG gryNynrvCG naglcnvtak aCnpssylls flfwDgfHps ekGykavAea
361 l
```

FIGURE 4

(SEQ ID No. 3)

```
  1 mkkwfvcllg lvaltvqaad srpafsrivm fgdslsdtgk myskmrgylp ssppyyegrf
 61 sngpvwleql tnefpgltia neaeggptav aynkiswnpk yqvinnldye vtqflqkdsf
121 kpddlvilwv gandylaygw nteqdakrvr daisdaanrm vlngakeill fnlpdlgqnp
181 sarsqkvvea ashvsayhnq lllnlarqla ptgmvklfei dkqfaemlrd pqnfglsdqr
241 nacyggsyvw kpfasrsast dsqlsafnpq erlaiagnpl laqavaspma arsastlnce
301 gkmfwdqvhp ttvvhaalse paatfiesqy eflah
```

FIGURE 5

SEQ ID No. 4

```
  1 mkkwfvcllg lialtvqaad trpafsrivm fgdslsdtgk myskmrgylp ssppyyegrf
 61 sngpvwleql tkqfpgltia neaeggatav aynkiswnpk yqvynnldye vtqflqkdsf
121 kpddlvilwv gandylaygw nteqdakrvr daisdaanrm vlngakqill fnlpdlgqnp
181 sarsqkvvea vshvsayhnk lllnlarqla ptgmvklfei dkqfaemlrd pqnfglsdve
241 npcydggyvw kpfatrsvst drqlsafspq erlaiagnpl laqavaspma rrsasplnce
301 gkmfwdqvhp ttvvhaalse raatfietqy eflahg
```

FIGURE 6

SEQ ID No. 5

```
  1 mpkpalrrvm tatvaavgtl algltdatah aapaqatptl dyvalgdsys agsgvlpvdp
 61 anllclrsta nyphviadtt garltdvtcg aaqtadftra qypgvapqld algtgtdlvt
121 ltiggndnst finaitacgt agvlsggkgs pckdrhgtsf ddeieantyp alkeallgvr
181 arapharvaa lgypwitpat adpscflklp laagdvpylr aiqahlndav rraaeetgat
241 yvdfsgvsdg hdaceapgtr wiepllfghs lvpvhpnalg errmaehtmd vlgld
```

FIGURE 7

SEQ ID No. 6

```
  1 mpkpalrrvm tatvaavgtl algltdatah aapaqatptl dyvalgdsys agsgvlpvdp
 61 anllclrsta nyphviadtt garltdvtcg aaqtadftra qypgvapqld algtgtdlvt
121 ltiggndnst finaitacgt agvlsggkgs pckdrhgtsf ddeieantyp alkeallgvr
181 arapharvaa lgypwitpat adpscflklp laagdvpylr aiqahlndav rraaeetgat
241 yvdfsgvsdg hdaceapgtr wiepllfghs lvpvhpnalg errmaehtmd vlgld
```

FIGURE 8

SEQ ID No. 7

```
  1 mdyekfllfg dsitefafnt rpiedgkdqy algaalvney trkmdilqrg fkgytsrwal
 61 kilpeilkhe snivmatifl gandacsagp qsvplpefid nirqmvslmk syhirpiiig
121 pglvdrekwe kekseeialg yfrtnenfai ysdalaklan eekvpfvaln kafqqeggda
181 wqqlltdglh fsgkgykifh dellkvietf ypqyhpknmq yklkdwrdvl ddgsnims
```

FIGURE 9

(SEQ ID No. 8)

```
          10         20         30         40         50         60
          |          |          |          |          |          |
MNLRQWMGAA TAALALGLAA CGGGGTDQSG NPNVAKVQRM VVFGDSLSDI GTYTPVAQAV

          70         80         90         100        110        120
          |          |          |          |          |          |
GGGKFTTNPG PIWAETVAAQ LGVTLTPAVM GYATSVQNCP KAGCFDYAQG GSRVTDPNGI

         130        140        150        160        170        180
          |          |          |          |          |          |
GHNGGAGALT YPVQQQLANF YAASNNTFNG NNDVVFVLAG SNDIFFWTTA AATSGSGVTP

         190        200        210        220        230        240
          |          |          |          |          |          |
AIATAQVQQA ATDLVGYVKD MIAKGATQVY VFNLPDSSLT PDGVASGTTG QALLHALVGT

         250        260        270        280        290        300
          |          |          |          |          |          |
FNTTLQSGLA GTSARIIDFN AQLTAAIQNG ASFGFANTSA RACDATKINA LVPSAGGSSL

         310        320        330        340
          |          |          |          |
FCSANTLVAS GADQSYLFAD GVHPTTAGHR LIASNVLARL LADNVAH
```

FIGURE 10 (SEQ ID No. 9)

```
  1 migsyvavgd sftegvgdpg pdgafvgwad rlavlladrr pegdftytnl avrgrlldqi
 61 vaeqvprvvg lapdlvsfaa ggndiirpgt dpdevaerfe lavaaltaaa gtvlvttgfd
121 trgvpvlkhl rgkiatyngh vraiadrygc pvldlwslrs vqdrrawdad rlhlspeght
181 rvalragqal glrvpadpdq pwpplpprgt ldvrrddvhw areylvpwig rrlrgessgd
241 hvtakgtlsp daiktriaav a
```

FIGURE 11

(SEQ ID No. 10)

```
  1 mqtnpaytsl vavgdsfteg msdllpdgsy rgwadllatr maarspgfry anlavrgkli
 61 gqivdeqvdv aaamgadvit lvgglndtlr pkcdmarvrd lltqaverla phceqlvlmr
121 spgrqgpvle rfrprmealf aviddlagrh gavvvdlyga qsladprmwd vdrlhltaeg
181 hrrvaeavwq slghepedpe whapipatpp pgwvtrrtad vrfarqhllp wigrrltgrs
241 sgdglpakrp dllpyedpar
```

FIGURE 12

(SEQ ID No. 11)

```
  1 mtrgrdggag apptkhrall aaivtlivai saaiyagasa ddgsrdhalq aggrlprgda
 61 apastgawvg awatapaaae pgtettglag rsvrnvvhts vggtgaritl snlygqsplt
121 vthasialaa gpdtaaaiad tmrrltfggs arviipaggq vmsdtarlai pyganvlvtt
181 yspipsgpvt yhpqarqtsy ladgdrtadv tavayttptp ywryltaldv lsheadgtvv
241 afgdsitdga rsqsdanhrw tdvlaarlhe aagdgrdtpr ysvvnegisg nrlltsrpgr
301 padnpsglsr fqrdvlertn vkavvvvlgv ndvlnspela drdailtglr tlvdraharg
361 lrvvgatitp fggyggytea retmrqevne eirsgrvfdt vvdfdkalrd pydprrmrsd
421 ydsgdhlhpg dkgyarmgav idlaalkgaa pvka
```

FIGURE 13 (SEQ ID No. 12)

```
  1 mtsmsrarva rriaagaayg gggiglagaa avglvvaevq larrrvgvgt ptrvpnaqgl
 61 yggtlptagd pplrlmmlgd staagqgvhr agqtpgalla sglaavaerp vrlgsvaqpg
121 acsddldrqv alvlaepdrv pdicvimvga ndvthrmpat rsvrhlssav rrlrtagaev
181 vvgtcpdlgt iervrqplrw larrasrqla aaqtigaveq ggrtvslgdl lgpefaqnpr
241 elfgpdnyhp saegyataam avlpsvcaal glwpadeehp dalrregflp varaaaeaas
301 eagtevaaam ptgprgpwal lkrrrrrrvs eaepsspsgv
```

FIGURE 14 (SEQ ID No. 13)

```
  1 mgrgtdqrtr ygrrrarval aaltaavlgv gvagcdsvgg dspapsgsps krtrtapawd
 61 tspasvaavg dsitrgfdac avlsdcpevs watgssakvd slavrllgka daaehswnya
121 vtgarmadlt aqvtraaqre pelvavmaga ndacrsttsa mtpvadfraq feeamatlrk
181 klpkaqvyvs sipdlkrlws qgrtnplgkq vwklglcpsm lgdadsldsa atlrrntvrd
241 rvadynevlr evcakdrrcr sddgavhefr fgtdqlshwd wfhpsvdgqa rlaeiayrav
301 taknp
```

FIGURE 15 (SEQ ID No. 14)

```
  1 mrlsrraata sallltpala lfgasaavsa priqatdyva lgdsyssgvg agsydsssgs
 61 ckrstksypa lwaashtgtr fnftacsgar tgdvlakqlt pvnsgtdlvs itiggndagf
121 adtmttcnlq gesaclaria karayiqqtl paqldqvyda idsrapaaqv vvlgyprfyk
181 lggscavgls eksraainaa addinavtak raadhgfafg dvnttfaghe lcsgapwlhs
241 vtlpvensyh ptangqskgy lpvlnsat
```

FIGURE 16 (SEQ ID No. 15)

```
  1 MKKWFVCLLG LIALTVQAAD TRPAFSRIVM FGDSLSDTGK MYSKMRGYLP
 51 SSPPYYEGRF SNGPVWLEQL TKQFPGLTIA NEAEGGATAV AYNKISWNPK
101 YQVINNLDYE VTQFLQKDSF KPDDLVILWV GANDYLAYGW NTEQDAKRVR
151 DAISDAANRM VLNGAKQILL FNLPDLGQNP SARSQKVVEA VSHVSAYHNK
201 LLLNLARQLA PTGMVKLFEI DKQFAEMLRD PQNFGLSDVE NPCYDGGYVW
251 KPFATRSVST DRQLSAFSPQ ERLAIAGNPL LAQAVASPMA RRSASPLNCE
301 GKMFWDQVHP TTVVHAALSE RAATFIETQY EFLAHG*
```

FIGURE 17 (SEQ ID No. 19)

```
  1 migsyvavgd sftegvgdpg pdgafvgwad rlavlladrr pegdftytnl avrgrlldqi
 61 vaeqvprvvg lapdlvsfaa ggndiirpgt dpdevaerfe lavaaltaaa gtvlvttgfd
121 trgvpvlkhl rgkiatyngh vraiadrygc pvldlwslrs vqdrrawdad rlhlspeght
181 rvalragqal glrvpadpdq pwpplpprgt ldvrrddvhw areylvpwig rrlrgessgd
241 hvtakgtlsp daiktriaav a
```

FIGURE 18 (SEQ ID No. 25)

```
  1 MFKFKKNFLV GLSAALMSIS LFSATASAAS ADSRPAFSRI VMFGDSLSDT
 51 GKMYSKMRGY LPSSPPYYEG RFSNGPVWLE QLTKQFPGLT IANEAEGGAT
101 AVAYNKISWN PKYQVINNLD YEVTQFLQKD SFKPDDLVIL WVGANDYLAY
151 GWNTEQDAKR VRDAISDAAN RMVLNGAKQI LLFNLPDLGQ NPSARSQKVV
201 EAVSHVSAYH NQLLLNLARQ LAPTGMVKLF EIDKQFAEML RDPQNFGLSD
251 VENPCYDGGY VWKPFATRSV STDRQLSAFS PQERLAIAGN PLLAQAVASP
301 MARRSASPLN CEGKMFWDQV HPTTVVHAAL SERAATFIAN QYEFLAH**
```

FIGURE 19

(SEQ ID NO. 26)

MRLTRSLSAASVIVFALLLALLGISPAQAAGPAYVALGDSYSSGNGAGSYIDSSGDCHRSN

NAYPARWAAANAPSSFTFAACSGAVTTDVINNQLGALNASTGLVSITIGGNDAGFADAMTT

CVTSSDSTCLNRLATATNYINTTLLARLDAVYSQIKARAPNARVVVLGYPRMYLASNPWYC

LGLSNTKRAAINTTADTLNSVISSRATAHGFRFGDVRPTFNNHELFFGNDWLHSLTLPVWE

SYHPTSTGHQSGYLPVLNANSST

Figure 20

SEQ ID No. 27

ZP 00058717

```
       1 mlphpagerg evgaffallv gtpqdrrlrl echetrplrg rcgcgerrvp pltlpgdgvl
      61 cttsstrdae tvwrkhlqpr pdggfrphlg vgcllagqgs pgvlwcgreg crfevcrrdt
     121 pglsrtrngd ssppfragws lppkcgeisq sarktpavpr ysllrtdrpd 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
```

FIGURE 21

(SEQ ID No. 28)

```
       1 mgsgpraatr 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
```

FIGURE 22

(SEQ ID No. 29)

```
  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
```

FIGURE 23

(SEQ ID No. 30)

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 //
```

FIGURE 24

SEQ ID No. 31

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
//
```

FIGURE 25

SEQ ID No. 32

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
//
```

FIGURE 26

SEQ ID No. 33

```
MRLTRSLSAASVIVFALLLALLGISPAQAAGPAYVALGDSYSSGNGAGSYIDSSGDCHRSN
NAYPARWAAANAPSSFTFAACSGAVTTDVINNQLGALNASTGLVSITIGGNDAGFADAMTT
CVTSSDSTCLNRLATATNYINTTLLARLDAVYSQIKARAPNARVVVLGYPRMYLASNPWYC
LGLSNTKRAAINTTADTLNSVISSRATAHGFRFGDVRPTFNNHELFFGNDWLHSLTLPVWE
SYHPTSTGHQSGYLPVLNANSST
```

FIGURE 27

(SEQ ID No. 34)

ADSRPAFSRIVMFGDSLSDTGKMYSKMRGYLPSSPPYYEGRFSNGPVWLEQLTNEFPGLTIANEAEGGPT
AVAYNKISWNPKYQVINNLDYEVTQFLQKDSFKPDDLVILWVGANDYLAYGWNTEQDAKRVRDAISDAAN
RMVLNGAKEILLFNLPDLGQNPSARSQKVVEAASHVSAYHNQLLLNLARQLAPTGMVKLFEIDKQFAEML
RDPQNFGLSDQRNACYGGSYVWKPFASRSASTDSQLSAFNPQERLAIAGNPLLAQAVASPMAARSASTLN
CE
GKMFWDQVHPTTVVHAALSEPAATFIESQYEFLAH

FIGURE 28

(SEQ ID No. 35)

```
  1  ADTRPAFSRI VMFGDSLSDT GKMYSKMRGY LPSSPPYYEG RFSNGPVWLE QLTKQFPGLT
 61  IANEAEGGAT AVAYNKISWN PKYQVINNLD YEVTQFLQKD SFKPDDLVIL WVGANDYLAY
121  GWNTEQDAKR VRDAISDAAN RMVLNGAKQI LLFNLPDLGQ NPSARSQKVV EAVSHVSAYH
181  NKLLLNLARQ LAPTGMVKLF EIDKQFAEML RDPQNFGLSD VENPCYDGGY VWKPFATRSV
241  STDRQLSAFS PQERLAIAGN PLLAQAVASP MARRSASPLN CEGKMFWDQV HPTTVVHAAL
301  SERAATFIET QYEFLAHG
```

FIGURE 29

(SEQ ID No. 36)

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

FIGURE 30

(SEQ ID NO. 37):

MRLTRSLSAASVIVFALLLALLGISPAQAAGPAYVALGDSYSSGNGAGSYIDSSGDCHRSN
NAYPARWAAANAPSSFTFAACSGAVTTDVINNQLGALNASTGLVSITIGGNDAGFADAMTT
CVTSSDSTCLNRLATATNYINTTLLARLDAVYSQIKARAPNARVVVLGYPRMYLASNPWYC
LGLSNTKRAAINTTADTLNSVISSRATAHGFRFGDVRPTFNNHELFFGNDWLHSLTLPVWE
SYHPTSTGHQSGYLPVLNANSST

FIGURE 31

SEQ ID No. 38

```
  1 mlphpagerg evgaffallv gtpqdrrlrl echetrplrg rcgcgerrvp pltlpgdgvl
 61 cttsstrdae tvwrkhlqpr pdggfrphlg vgcllagqgs pgvlwcgreg crfevcrrdt
121 pglsrtrngd ssppfragws lppkcgeisq sarktpavpr ysllrtdrpd 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
```

FIGURE 32

(SEQ ID No. 39)

```
   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 acccttcgt
 961 ggacggtgcg gatgcggtga gcgtcgggtg cctcccctaa cgctccccgg tgacggagtg
1021 ttgtgcacca catctagcac gcgggacgcg gaaaccgtat ggagaaaaca cctacaaccc
1081 cggccggacg gtgggtttcg gccacactta ggggtcgggt gcctgcttgc cgggcagggc
1141 agtcccgggg tgctgtggtg cgggcgggag ggctgtcgct tcgaggtgtg ccggcgggac
1201 actccgggcc tcagccgtac ccgcaacggg gacagttctc ctcccttccg ggctggatgg
1261 tcccttcccc cgaaatgcgg cgagatctcc cagtcagccc ggaaaacacc cgctgtgccc
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
```

FIGURE 33

(SEQ ID No. 40)

```
  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
```

FIGURE 34

(SEQ ID No. 41)

```
  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
```

FIGURE 35

(SEQ ID No. 42)

```
   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
1741 ctgccgctcg tgtctgccgg ggactgcccc gaactggggg atgtctccga ggcggatcgt
1801 cgttgggcgg ttgagctgac cgggcagatc aacgagaccg tgcgcgaggc ggccgaacga
1861 cacgatgccc tctttgtcct gcccgacgat gccgatgagc acaccagttg tgcaccccca
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
```

FIGURE 36

(SEQ ID No. 43)

```
  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
```

Figure 37

(SEQ ID No. 44)

```
   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
 301 ctccagcgcg agatcagcag cgtccagccg tcgccctccg ccagcgtcgc gctgcggtcg
 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
1081 gatcagcatc ggcggcaacg acgccggttt cgccgacacc atgacgacct gtgtgctcca
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
```

FIGURE 38

(SEQ ID No. 45)

```
  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
```

FIGURE 39

SEQ ID No. 46

```
   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
 301 tccggatctt cttgctacgc agctgtgcca tacgagggag tcctcctctg ggcagcggcg
 361 cgcctgggtg gggcgcacgg ctgtgggggg tgcgcgcgtc atcacgcaca cggccctgga
 421 gcgtcgtgtt ccgccctggg ttgagtaaag cctcggccat ctacgggggt ggctcaaggg
 481 agttgagacc ctgtcatgag tctgacatga gcacgcaatc aacggggccg tgagcacccc
 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
1141 ccgtcggccc atgtggccgt gctgggctac ccccgcttct acaaactggg cggctcctgc
1201 ctcgcgggcc tctcggagac caagcggtcc gccatcaacg acgcggccga ctatctgaac
1261 agcgccatcg ccaagcgcgc cgccgaccac ggcttcacct tcggcgacgt caagagcacc
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
1621 tcgttccgct cgtgtcgtac gtggtgacga acacctgctt ctgctgggtc tttccgccgc
1681 tcgccgggaa ggacagcgtc ttccagcccg gatccgggac ctcgcccttc ttggtcaccc
1741 agcggtactc cacctcgacc ggcacccggc ccaccgtgaa ggtcgccgtg aacgtgggcg
1801 cctgggcggt gggcggcggg caggcaccgg agtagtcggt gtgcacgccg gtgaccgtca
1861 ccttcacgga ctgggccggc ggggtcgtcg taccgccgcc gccaccgccg cctcccggag
1921 tggagcccga gctgtggtcg cccccgccgt cggcgttgtc gtcctcgggg gttttcgaac
```

FIGURE 40

SEQ ID No. 47

```
  1 mgsgpraatr 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
```

FIGURE 41

SEQ ID No. 48

```
1      ctgcagacac ccgccccgcc ttctcccgga tcgtcatgtt cggcgactcc ctcagcgaca
61     ccggcaagat gtactccaag atgcgcggct acctgccgtc ctccccgccg tactacgagg
121    gccgcttctc gaacggcccg gtctggctgg agcagctgac gaagcagttc cccggcctga
181    cgatcgccaa cgaggccgag gggggcgcga ccgcagtcgc ctacaacaag atctcctgga
241    acccgaagta ccaggtcatt aacaacctcg actacgaggt cacccagttc ttgcagaagg
301    actcgttcaa gcccgacgac ctggtcatcc tgtgggtggg cgccaacgac tacctggcct
361    acggttggaa cacggagcag gacgccaagc gggtgcgcga cgccatctcg gacgcggcaa
421    accgcatggt cctgaacggc gcgaagcaga tcctgctgtt caacctgccc gacctgggcc
481    agaacccgtc cgcccgctcc cagaaggtcg tcgaggccgt ctcgcacgtg tccgcctacc
541    acaacaagct gctcctcaac ctcgcccggc agctcgcccc gacgggcatg gtcaagctgt
601    tcgagatcga caagcagttc gcggagatgc tgcgcgaccc ccagaacttc ggcctgagcg
661    acgtggagaa cccgtgctac gacggcggct acgtgtggaa gccgttcgcc acccggtccg
721    tctcgaccga ccggcagctg tcggccttct cgccccagga gcgcctggcg atcgctggca
781    acccgctcct ggcacaggcg gtagcttcgc cgatggcccg ccgctcggcc tcgcccctca
841    actgcgaggg caagatgttc tgggaccagg tccaccccac caccgtggtc cacgccgccc
901    tctcggagcg cgccgccacc ttcatcgaga cccagtacga gttcctcgcc cactagtcta
961    gaggatcc
```

Figure 42

1. L131
2. S.avermitilis
3. T.fusca
4. Consensus

```
                1                                                    50
1   (1) --------MRLTRSLSAASVIVFALLLALLGISPAQAAG----------
2   (1) --------MRRSRITAYVTSLLLAVGCALTGAATAQASPA----------
3   (1) VGSGPRAATRRRLFLGIPALVLVTALTLVLAVPTGRETLWRMWCEATQDW
4   (1)         MRRSRFLA  ALILLTLA AL GAA ARAAP

               51                                                   100
1   (32) ------------------------------P-AYVALGDSYSSGNGAGSYID
2   (33) ---------------------------AAATGYVALGDSYSSGVGAGSYLS
3   (51) CLGVPVDSRGQPAEDGEFLLLSPVQAATWGNYYALGDSYSSGDGARDYYP
4   (51)                                A A  YVALGDSYSSG GAGSY

               101                                                  150
1    (53) SSGD---CHRSNNAYPARWAAANAP---SSFTFAACSGAVTTDVIN----
2    (57) SSGD---CKRSSKAYPYLWQAAHSP---SSFSFMACSGARTGDVLA----
3   (101) GTAVKGGCWRSANAYPELVAEAYDFA--GHLSFLACSGQRGYAMLDAIDE
4   (101) SSGD   C RSTKAYPALWAAAHA    SSFSF ACSGARTYDVLA

               151                                                  200
1    (93) --NQLGALNAST--GLVSITIGGNDAGFADAMTTCVTS------SDSTCL
2    (97) --NQLGTLNSST--GLVSLTIGGNDAGFSDVMTTCVLQ------SDSACL
3   (149) VGSQLDWNSPHT--SLVTIGIGGNDLGFSTVLKTCMVR------VPLLDS
4   (151)    QL  LNS T   LVSITIGGNDAGFAD MTTCVL        SDSACL

               201                                                  250
1   (133) NRLATATNYINTTLLA-------RLDAVYSQIKARAPNARVVVLGYPRMY
2   (137) SRINTAKAYVDSTLPG-------QLDSVYTAISTKAPSAHVAVLGYPRFY
3   (191) KACTDQEDAIRKRMAKF----ETTFEELISEVRTRAPDARILVVGYPRIF
4   (201)  RIA AK YI  TLPA        RLDSVYSAI TRAP ARVVVLGYPRIY

               251                                                  300
1   (176) LASNPWYCLGLSNTKRAAINTTADTLNSVISSRATAH----------GF
2   (180) KLGG-SCLAGLSETKRSAINDAADYLNSAIAKRAADH----------GF
3   (237) PEEPTGAYYTLTASNQRWLNETIQEFNQQLAEAVAVHDEEIAASGGVGSV
4   (251)   SG    LGLS TKRAAINDAAD LNSVIAKRAADH          GF

               301                                                  350
1   (215) RFGDVRPTFNNHELFFGNDWLHSLTLP----------------VWESYH
2   (218) TFGDVKSTFTGHEICSSSTWLHSLDLLN---------------IGQSYH
3   (287) EFVDVYHALDGHEIGSDEPWVNGVQLRDLATG----------VTVDRSTFH
4   (301) TFGDV  TF GHELCSA PWLHSLTLP                 V  SYH

               351                                                  395
1   (248) PTSTGHQSGYLPVLNANSST-------------------------
2   (252) PTAAGQSGGYLPVMNSVA--------------------------
3   (328) PNAAGHRAVGERVIEQIETGPGRPLYATFAVVAGATVDTLAGEVG
4   (351) PTA GHAAGYLPVLNSI T
```

FIGURE 43

SEQ ID No 17 which is the amino acid sequence of a lipid acyltransferase from *Candida parapsilosis*;

MRYFAIAFLL INTISAFVLA PKKPSQDDFY TPPQGYEAQP LGSILKTRNV PNPLTNVFTP VKVQNAWQLL
VRSEDTFGNP NAIVTTIIQP FNAKKDKLVS YQTFEDSGKL DCAPSYAIQY GSDISTLTTQ GEMYYISALL
DQGYYVVTPD YEGPKSTFTV GLQSGRATLN SLRATLKSGN LTGVSSDAET LLWGYSGGSL ASGWAAAIQK
EYAPELSKNL LGAALGGFVT NITATAEAVD SGPFAGIISN ALAGIGNEYP DFKNYLLKKV SPLLSITYRL
GNTHCLLDGG IAYFGKSFFS
RIIRYFPDGW DLVNQEPIKT ILQDNGLVYQ PKDLTPQIPL FIYHGTLDAI VPIVNSRKTF QQWCDWGLKS
GEYNEDLTNG HITESIVGAP AALTWIINRF NGQPPVDGCQ HNVRASNLEY PGTPQSIKNY FEAALHAILG
FDLGPDVKRD KVTLGGLLKL ERFAF

FIGURE 44

SEQ ID No 18 which is the amino acid sequence of a lipid acyltransferase from *Candida parapsilosis*;

MRYFAIAFLL INTISAFVLA PKKPSQDDFY TPPQGYEAQP LGSILKTRNV PNPLTNVFTP VKVQNAWQLL
VRSEDTFGNP NAIVTTIIQP FNAKKDKLVS YQTFEDSGKL DCAPSYAIQY GSDISTLTTQ GEMYYISALL
DQGYYVVTPD YEGPKSTFTV GLQSGRATLN SLRATLKSGN LTGVSSDAET LLWGYSGGSL ASGWAAAIQK
EYAPELSKNL LGAALGGFVT NITATAEAVD SGPFAGIISN ALAGIGNEYP DFKNYLLKKV SPLLSITYRL
GNTHCLLDGG IAYFGKSFFS RIIRYFPDGW DLVNQEPIKT ILQDNGLVYQ PKDLTPQIPL FIYHGTLDAI
VPIVNSRKTF QQWCDWGLKS GEYNEDLTNG HITESIVGAP AALTWIINRF NGQPPVDGCQ HNVRASNLEY
PGTPQSIKNY FEAALHAILG FDLGPDVKRD KVTLGGLLKL ERFAFHHHHH H

FIG. 45

FIG. 46

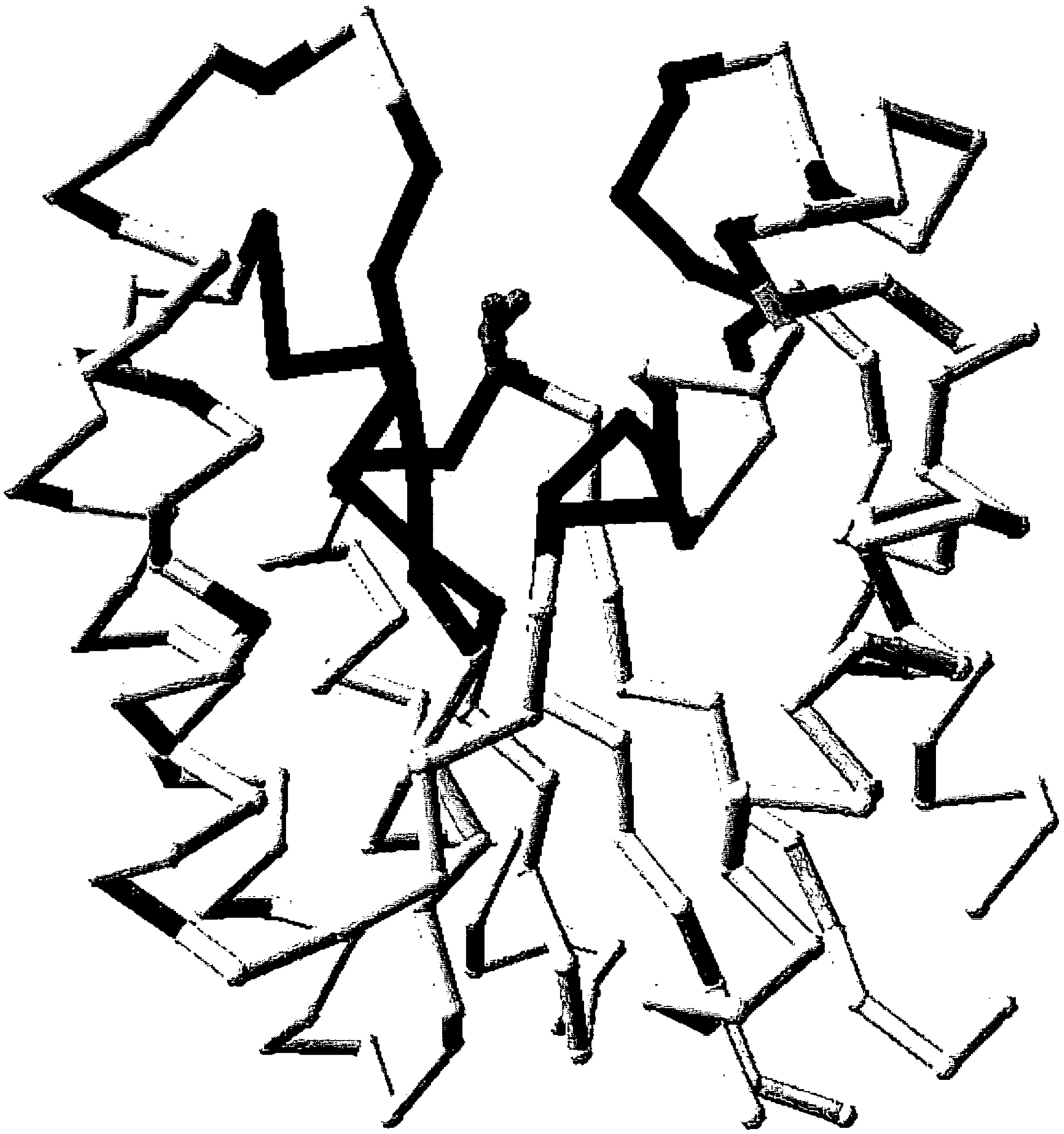
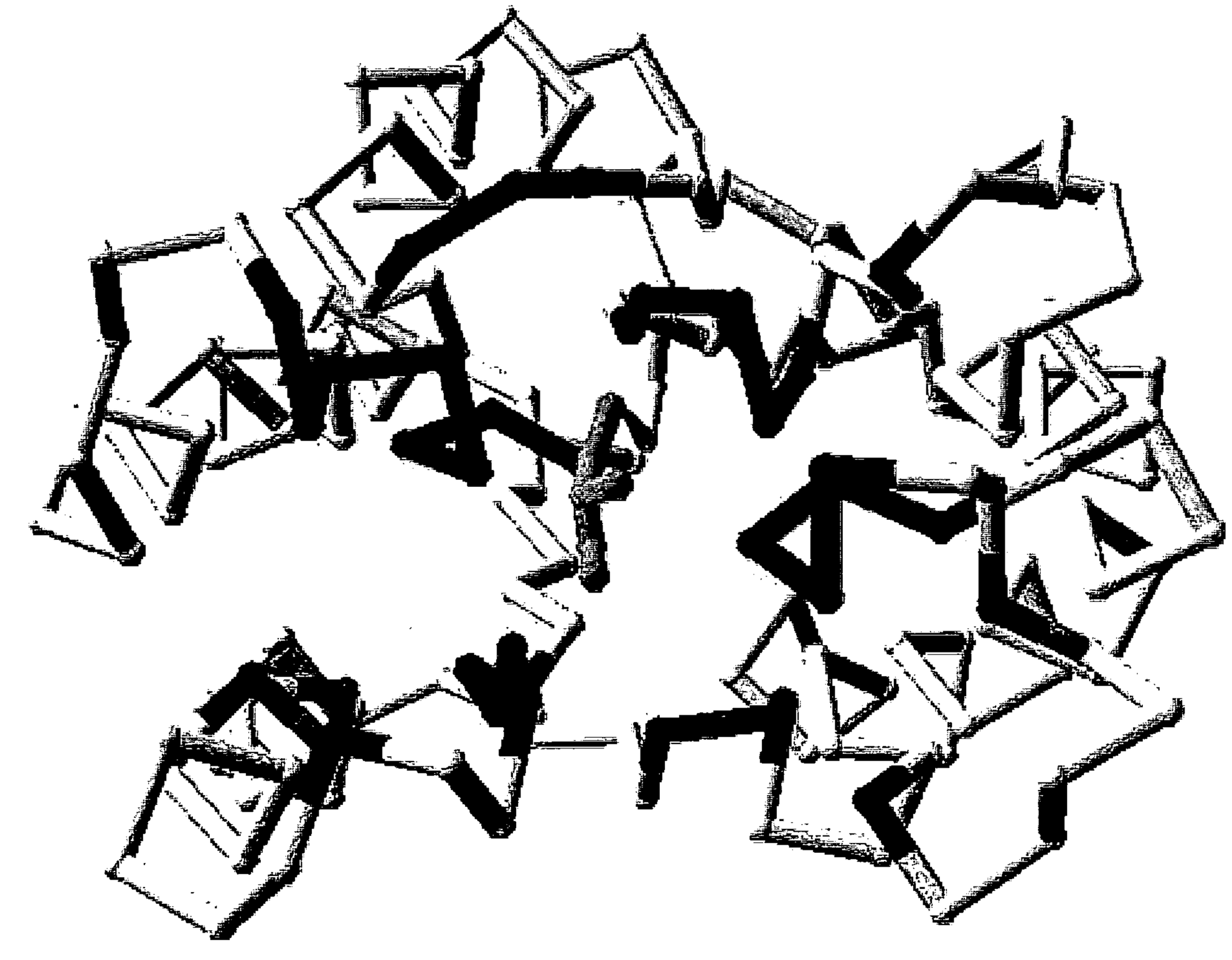


FIG. 47



FIGRURE 48

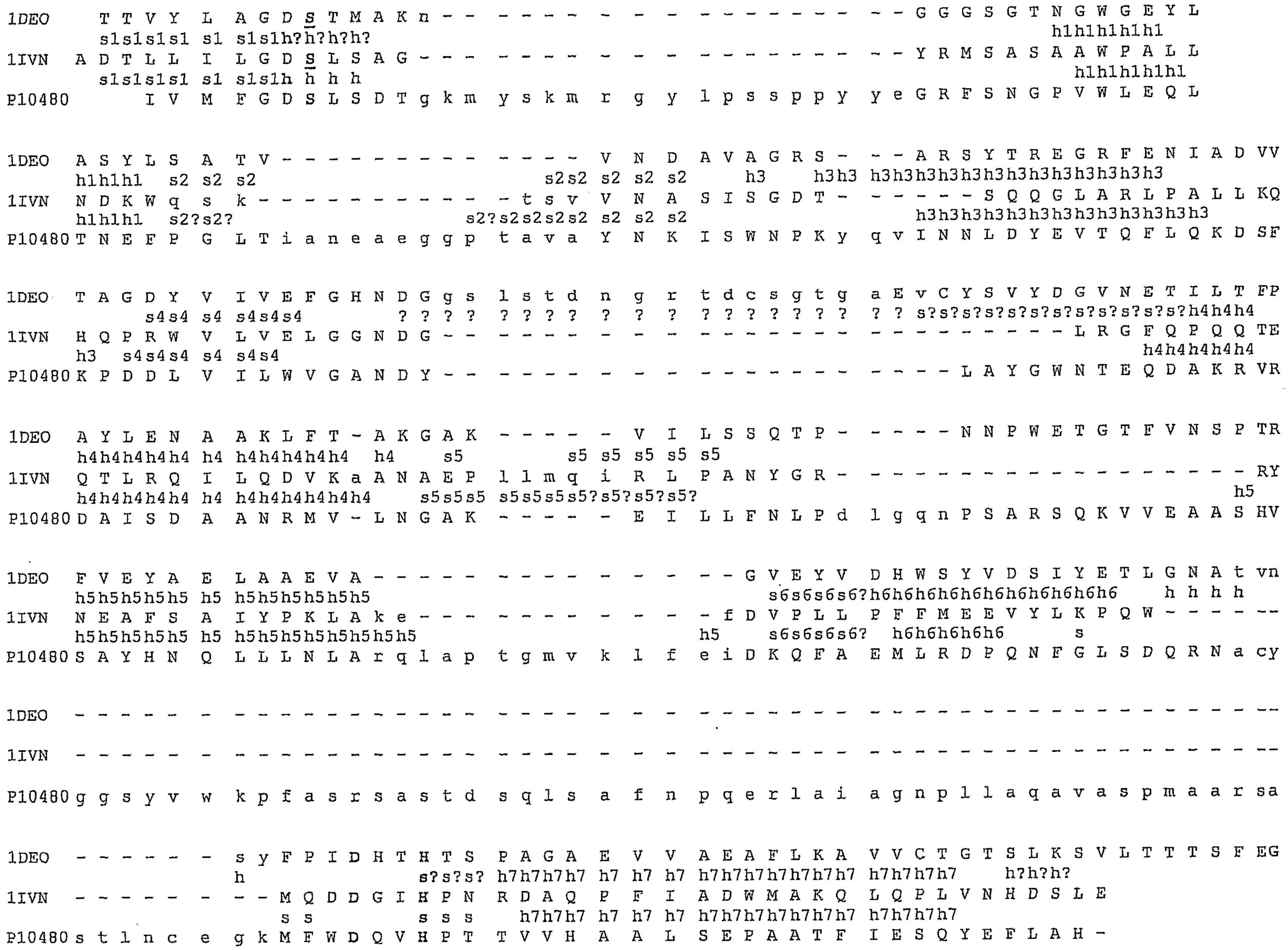


FIGURE 49

```
1DEOm     T T V Y  L  A G D S T M A K n - -  - - - - - - - - - -  - - - -  - - - - G G G S G T N G W G E Y L
          s1s1s1s1 s1 s1s1h?h?h?h?h?                                                  h1h1h1h1h1
1IVNm   A D T L L  I  L G D S L S A G - - -  - - - - - - - - - -  - - - -  - - - - Y R M S A S A A W P A L L
          s1s1s1s1 s1 s1s1h h h h                                                     h1h1h1h1h1
P10480m       I V  M  F G D S L S D T g k m  y s k m r g y l  p  s  s  p  p y y e G R F S N G P V W L E Q L

1DEOm   A S Y L S  A  T V - - - - - - - - - -  - - - - V N D A  V  A  G  R  S - - -        A R S Y T R E G R F E N IA
        h1h1h1  s2 s2 s2                        s2s2s2s2s2s2     h3                  h3h3h3h3h3h3h3h3h3h3h3h3h3h3
1IVNm   N D K W q  s  k - - - - - - - - - -  - t s v V N A S  I  S  G  D  T - - - - - - - S Q Q G L A     R L P A LL
        h1h1h1  s2?s2?                      s2?s2s2s2s2s2s2s2                    h3h3h3h3h3h3  h3h3h3h3h3h3h3
P10480mT N E F P  G  L T i a n e a e g g p  t a v a Y N K I  S  W  N  P  K         y q v I N N L D Y E V T Q F LQ

1DEOm   D   V V T  A  G D Y V I V E F G H N  D G g s l s t d  n  g  r  t  d c s g t g a E v C Y S V Y D G V N E TI
        h3h3       s4 s4s4s4s4s4       ? ?  ? ? ? ? ? ? ?  ?  ?  ?  ?  ? ? ? ? ? ? s?s?s?s?s?s?s?s?s?s?s?s?
1IVNm   K Q H Q P     R W V L V E L G G N  D G - - - - - - -  -  -  -  -  - - - - - - - - - - - - - - - - L R G F QP
        h3h3h3       s4s4s4s4s4s4                                                                          h4
P10480mK D S F K  P  D D L V I L W V G A N  D Y - - - - - - -  -  -  -  -  - - - - - - - - - - - - L A Y G W N T E Q DA

1DEOm   L T F P A  Y  L E N A A K L F T A K  G A K V I L S S  Q  T  P  N  N P W E T G T F V N S P T R
        h4h4h4h4 h4  h4h4h4h4h4h4h4h4h4h4h4  s5s5s5s5s5s5                                                 h5h5h5h5h5h5
1IVNm   Q Q T E Q  T  L R Q I L Q D V K a A  N A E P l l m q  i  R  L  P  A N Y G R - - - - - - - - - - R Y N E A
        h4h4h4h4h4 h4  h4h4h4h4h4h4h4h4h4h4  s5s5s5s5s5s5s5?s5?s5?s5?                                   h5h5h5h5h5h5
P10480mK R V R D  A  I S D A A N R M V L N  G A K E I L L F  N  L  P  d  l g q n P S A R S Q K V V E A A S H V SA

1DEOm     F V E Y  A  E L A A E V A - - - -  - - - - - - - - G  V  E  Y  V  D H W S Y V D S I Y E T L G N A t v n --
          h5h5h5h5 h5  h5h5h5h5h5h5h5                                s6 s6 s6 s6?h6h6h6h6h6h6h6h6h6h6h6    h h h h
1IVNm     F S A I  Y  P K L A k e - - - - -  - - - - - - - f D  V  P  L  L  P F F M E E V Y         L K P Q W - - --
        h5h5h5h5h5 h5  h5h5h5                    h5  s6s6s6s6?   h6 h6 h6 h6h6      s
P10480mY H N Q L  L  L N L A r q l a p t g  m v k l f e i D  K  Q  F  A  E M L R D P Q N F G L S D Q R N a c y gg

1DEOm   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - --

1IVNm   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - --

P10480ms y v w k  p  f a s r s a s t d s q  l s a f n p q e  r  l  a  i  a g n p l l a q a v a s p m a a r s a st

1DEOm   - - - - s  y  F P I D H T H T S P A  G A E V V A E A  F  L  K  A  V V C T G T S L K S V L T T T S F E G TC
              h                 s?s?s?h7h7 h7h7h7h7h7h7h7h7 h7 h7 h7 h7 h7h7h7h7    h?h?h?
1IVNm   - - - - - -  -  M Q D D G I H P N R D  A Q P F I A D W  M  A  K  Q  L Q P L V N H D S L E
                s  s  s  h7h7h7h7h7h7h7h7 h7h7h7h7h7h7h7h7 h7 h7 h7 h7 h7
P10480ml n c e g  k  M F W D Q V H P T T V  V H A A L S E P  A  A  T  F  I E S Q Y E F L A H -
```

FIGURE 50

```
1DEO      T T V Y  L  A G D S T M A K n - - - - - - - - - - - - - - - - - - G G G S G T N G W G E Y L
          s1s1s1s1 s1 s1s1h?h?h?h?h?                                                  h1h1h1h1h1h1
1IVN    A D T L L  I  L G D S L S A G - - - - - - - - - - - - - - - - - - Y R M S A S A A W P A L L
          s1s1s1s1 s1 s1s1h h h h h                                                    h1h1h1h1h1h1
P10480        I V  M  F G D S L S D T g k m y s k m r g y l p s s p p y y e G R F S N G P V W L E Q L

1DEOm     T T V Y  L  A G D S T M A K n - - - - - - - - - - - - - - - - - - G G G S G T N G W G E Y L
          s1s1s1s1 s1 s1s1h?h?h?h?h?                                                  h1h1h1h1h1h1
1IVNm   A D T L L  I  L G D S L S A G - - - - - - - - - - - - - - - - - - Y R M S A S A A W P A L L
          s1s1s1s1 s1 s1s1h h h h h                                                    h1h1h1h1h1h1
P10480m       I V  M  F G D S L S D T g k m y s k m r g y l p s s p p y y e G R F S N G P V W L E Q L


1DEO    A S Y L S  A  T V - - - - - - - - - - - - - - V N D A V A G R S - - - A R S Y T R E G R F E N I A D VV
        h1h1h1   s2 s2 s2                          s2s2 s2 s2 s2    h3      h3h3 h3h3h3h3h3h3h3h3h3h3h3h3h3h3
1IVN    N D K W q  s  k - - - - - - - - - - - t s v V N A S I S G D T - - - - - - S Q Q G L A R L P A L L KQ
        h1h1h1   s2?s2?                    s2?s2s2s2s2s2 s2 s2 s2                h3h3h3h3h3h3h3h3h3h3h3h3h3h3
P10480  T N E F P  G  L T i a n e a e g g p t a v a Y N K I S W N P K y q v I N N L D Y E V T Q F L Q K D SF

1DEOm   A S Y L S  A  T V - - - - - - - - - - - - - - V N D A V A G R S - - -       A R S Y T R E G R F E N IA
        h1h1h1   s2 s2 s2                          s2s2 s2 s2 s2    h3                h3h3h3h3h3h3h3h3h3h3h3h3h3h3
1IVNm   N D K W q  s  k - - - - - - - - - - - t s v V N A S I S G D T - - - - - - S Q Q G L A     R L P A LL
        h1h1h1   s2?s2?                    s2?s2s2s2s2s2 s2 s2 s2                h3h3h3h3h3h3  h3h3h3h3h3h3h3
P10480mT N E F P  G  L T i a n e a e g g p t a v a Y N K I S W N P K         y q v I N N L D Y E V T Q F LQ
```

```
1DEO    T A G D Y  V  I V E F G H N D G g s l s t d n g r t d c s g t g a E v C Y S V Y D G V N E T I L T FP
          s4s4 s4 s4s4s4      ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? s?s?s?s?s?s?s?s?s?s?s?s?h4h4h4
1IVN    H Q P R W  V  L V E L G G N D G - - - - - - - - - - - - - - - - - - - - - - - - - - - L R G F Q P Q Q TE
        h3  s4s4s4 s4 s4s4                                                                  h4h4h4h4h4h4
P10480  K P D D L  V  I L W V G A N D Y - - - - - - - - - - - - - - - - - - - - - - - L A Y G W N T E Q D A K R VR

1DEOm   D   V V T  A  G D Y V I V E F G H N D G g s l s t d n g r t d c s g t g a E v C Y S V Y D G V N E TI
        h3h3       s4 s4s4s4s4s4s4      ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? s?s?s?s?s?s?s?s?s?s?s?s?
1IVNm   K Q H Q P      R W V L V E L G G N D G - - - - - - - - - - - - - - - - - - - - - - - - - - - L R G F QP
        h3h3h3       s4s4s4s4s4s4s4                                                                 h4
P10480m K D S F K  P  D D L V I L W V G A N D Y - - - - - - - - - - - - - - - - - - - - - - - L A Y G W N T E Q DA


1DEO    A Y L E N  A  A K L F T - A K G A K - - - - - - V I L S S Q T P - - - - - N N P W E T G T F V N S P TR
        h4h4h4h4h4 h4 h4h4h4h4h4h4  h4    s5        s5 s5 s5 s5 s5
1IVN    Q T L R Q  I  L Q D V K a A N A E P l l m q i R L P A N Y G R - - - - - - - - - - - - - - - - - - - - RY
        h4h4h4h4h4 h4 h4h4h4h4h4h4h4    s5s5s5 s5s5s5s5?s5?s5?s5?s5?                                    h5
P10480  D A I S D  A  A N R M V - L N G A K - - - - - - E I L L F N L P d l g q n P S A R S Q K V V E A A S HV

1DEOm   L T F P A  Y  L E N A A K L F T A K G A K V I L S S Q T P N N P W E T G T F V N S P T R
          h4h4h4h4 h4 h4h4h4h4h4h4h4h4h4h4h4     s5s5s5 s5 s5 s5
```

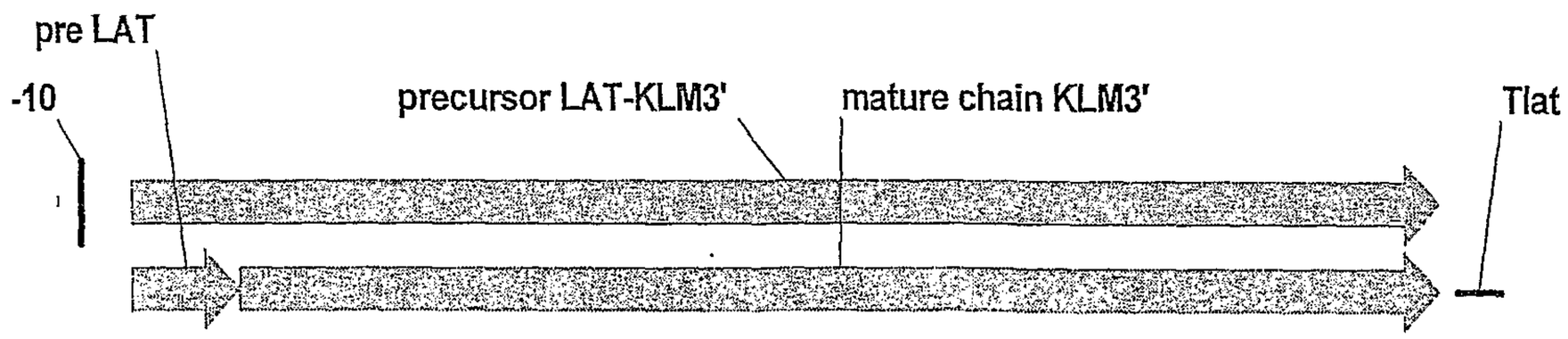
```
1IVNm   Q Q T E Q  T  L R Q I L Q D V K a A  N A E P  l  l  m  q  i  R  L  P  A N  Y G R - - - - - - - - - R Y N E A
        h4h4h4h4h4 h4 h4h4h4h4h4h4h4h4h4h4h4    s5s5s5 s5 s5 s5 s5?s5?s5?s5?                              h5h5h5h5h5h5
P10480m K R V R D  A  I S D A A N R M V L N  G A K E  I  L  L  F  N  L  P  d  l g  q n P S A R S Q K V V E A A S H V SA

1DEO    F V E Y A  E  L A A E V A - - - - -  - - - - -  -  -  -  -  -  G  V  E  Y V  D H W S Y V D S I Y E T L G N A t vn
        h5h5h5h5h5 h5 h5h5h5h5h5h5                                                  s6 s6 s6s6?h6h6h6h6h6h6h6h6h6h6h6    h h h h
1IVN    N E A F S  A  I Y P K L A k e - - -  - - - -  -  -  -  -  f  D  V  P  L L  P F F M E E V Y L K P Q W - - - - --
        h5h5h5h5h5 h5 h5h5h5h5h5h5h5h5h5                                  h5       s6 s6 s6s6?  h6h6h6h6h6      s
P10480  S A Y H N  Q  L L L N L A r q l a p  t g m v  k  l  f  e  i  D  K  Q  F A  E M L R D P Q N F G L S D Q R N a cy

1DEOm     F V E Y  A  E L A A E V A - - - -  - - - - -  -  -  -  G  V  E  Y  V  D H  W S Y V D S I Y E T L G N A t v n --
          h5h5h5h5 h5 h5h5h5h5h5h5h5h5                                        s6 s6 s6 s6?h6h6 h6h6h6h6h6h6h6h6h6    h h h h
1IVNm     F S A I  Y  P K L A k e - - - - -  - - - -  -  f  D  V  P  L  L  P F  F M E E V Y         L K P Q W - - --
        h5h5h5h5h5 h5 h5h5h5                  h5    s6 s6 s6 s6?   h6 h6 h6 h6h6       s                                
P10480m Y H N Q L  L  L N L A r q l a p t g  m v k l  f  e  i  D  K  Q  F A  E M  L R D P Q N F G L S D Q R N a c y gg

1DEO    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - --

1IVN    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - --

P10480  g g s y v  w  k p f a s r s a s t d  s q l s  a  f  n  p  q  e  r  l  a i  a g n p l l a q a v a s p m a a r sa

1DEOm   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - --

1IVNm   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - --

P10480m s y v w k  p  f a s r s a s t d  s q l s  a  f  n  p  q  e  r  l  a  i  a g  n p l l a q a v a s p m a a r s a st

1DEO    - - - - - -  s  y F P I D H T H  T S  P A G A  E  V  V  A  E  A  F  L  K A  V V C T G T S L K S V L T T T S F EG
                     h                    s?s?s? h7h7h7h7 h7 h7 h7 h7 h7 h7 h7 h7 h7h7 h7h7h7h7    h?h?h?
1IVN    - - - - - -  - -  M Q D D G I H P N  R D A Q  P  F  I  A  D  W  M  A  K Q  L Q P L V N H D S L E
                        s  s          s  s  s    h7h7h7 h7 h7 h7 h7 h7 h7 h7 h7 h7h7 h7h7h7h7
P10480  s t l n c  e  g k M F W D Q V H P T  T V V H  A  A  L  S  E  P  A  A  T F  I E S Q Y E F L A H -

1DEOm   - - - - s  y  F P I D H T H T S P A  G A E V  V  A  E  A  F  L  K  A  V V  C T G T S L K S V L T T T S F E G TC
                h                    s?s?s?h7h7 h7h7h7h7 h7 h7 h7 h7 h7 h7 h7 h7 h7h7 h7h7    h?h?h?
1IVNm   - - - - -  -  M Q D D G I H P N R D  A Q P F  I  A  D  W  M  A  K  Q  L Q  P L V N H D S L E
                s  s  s   h7h7h7h7h7h7h7h7 h7h7h7h7 h7 h7 h7 h7 h7 h7 h7 h7 h7
P10480m l n c e g  k  M F W D Q V H P T T V  V H A A  L  S  E  P  A  A  T  F  I E  S Q Y E F L A H -
```

FIGURE 51

```
                          10        20        30        40    .   50
60

....*....|....*....|....*....|....*....|....*....|....*....|
1IVN_A          4 LLILGDSLSAG--------------------YRMSASAAWPALLNDKWqsk---
------- 34
P10480         28
IVMFGDSLSDTgkmyskmrgylpssppyyeGRFSNGPVWLEQLTNEFPGLTianeaeggp 87

                          70        80        90       100       110
120
....*....|....*....|....*....|....*....|....*....|....*....|
1IVN_A          35 -tsvVNASISGDT-----------------------------
SQQGLARLPALLKQHQPRW 65
P10480          88 tavaYNKISWNPKyq----------------------
vINNLDYEVTQFLQKDSFKPDDL 125

                         130       140       150       160       170
180

....*....|....*....|....*....|....*....|....*....|....*....|
1IVN_A          66 VLVELGGNDG-----------------------------------
LRGFQPQQTEQT 87
P10480         126 VILWVGANDY---------------------------------LA--
YGWNTEQDAKRVRDA 152

                         190       200       210       220       230
240

....*....|....*....|....*....|....*....|....*....|....*....|
1IVN_A          88 LRQILQDVKaANAEPllmqiRLPANYGR-------------------------
------- 115
P10480         153 ISDAANRMV-LNGAK-----EILLFNLPdlg----------------------
----qnP 180

                         250       260       270       280       290
300

....*....|....*....|....*....|....*....|....*....|....*....|
1IVN_A         116 ------------RYNEAFSAIYPKLAke----------------------
fDVPLLPFFME 142
P10480         181 SARSQKVVEAASHVSAYHNQLLLNLArqlaptg----------
mvklfeiDKQFAEMLRD 230

                         310       320       330       340       350
360

....*....|....*....|....*....|....*....|....*....|....*....|
1IVN_A         143 EVYLKPQW-----------------------------------------
------- 150
P10480         231
PQNFGLSDQRNacyggsyvwkpfasrsastdsqlsafnpqerlaiagnpllaqavaspma 290

                              370       380       390       400
                         ....*....|....*....|....*....|....*....|
1IVN_A         151 ------------MQDDGI---------HPNRDAQPFIADWM 170
P10480         291 arsastlncegkMFWDQV---------HPTTVVHAALSEPA 322
```

```
                  1                                                  50
   P10480     (1) MKKWFVCLLGLVALTVQAADSRPAFSRIVMFGDSLSDTGKMYSKMRGYLP
   A. sal     (1) ------------------ADTRPAFSRIVMFGDSLSDTGKMYSKMRGYLP
   A. hyd     (1) ------------------ADSRPAFSRIVMFGDSLSDTGKMYSKMRGYLP
Consensus     (1)                   AD*RPAFSRIVMFGDSLSDTGKMYSKMRGYLP
                  51                                                 100
   P10480    (51) SSPPYYEGRFSNGPVWLEQLTNEFPGLTIANEAEGGPTAVAYNKISWNPK
   A. sal    (33) SSPPYYEGRFSNGPVWLEQLTKQFPGLTIANEAEGGATAVAYNKISWNPK
   A. hyd    (33) SSPPYYEGRFSNGPVWLEQLTKQFPGLTIANEAEGGATAVAYNKISWNPK
Consensus    (51) SSPPYYEGRFSNGPVWLEQLT**FPGLTIANEAEGG*TAVAYNKISWNPK
                 101                                                 150
   P10480   (101) YQVINNLDYEVTQFLQKDSFKPDDLVILWVGANDYLAYGWNTEQDAKRVR
   A. sal    (83) YQVINNLDYEVTQFLQKDSFKPDDLVILWVGANDYLAYGWNTEQDAKRVR
   A. hyd    (83) YQVINNLDYEVTQFLQKDSFKPDDLVILWVGANDYLAYGWNTEQDAKRVR
Consensus   (101) YQVINNLDYEVTQFLQKDSFKPDDLVILWVGANDYLAYGWNTEQDAKRVR
                 151                                                 200
   P10480   (151) DAISDAANRMVLNGAKEILLFNLPDLGQNPSARSQKVVEAASHVSAYHNQ
   A. sal   (133) DAISDAANRMVLNGAKQILLFNLPDLGQNPSARSQKVVEAVSHVSAYHNK
   A. hyd   (133) DAISDAANRMVLNGAKQILLFNLPDLGQNPSARSQKVVEAVSHVSAYHNQ
Consensus   (151) DAISDAANRMVLNGAK*ILLFNLPDLGQNPSARSQKVVEA*SHVSAYHN*
                 201                                                 250
   P10480   (201) LLLNLARQLAPTGMVKLFEIDKQFAEMLRDPQNFGLSDQRNACYGGSYVW
   A. sal   (183) LLLNLARQLAPTGMVKLFEIDKQFAEMLRDPQNFGLSDVENPCYDGGYVW
   A. hyd   (183) LLLNLARQLAPTGMVKLFEIDKQFAEMLRDPQNFGLSDVENPCYDGGYVW
Consensus   (201) LLLNLARQLAPTGMVKLFEIDKQFAEMLRDPQNFGLSD**N*CY*G*YVW
                 251                                                 300
   P10480   (251) KPFASRSASTDSQLSAFNPQERLAIAGNPLLAQAVASPMAARSASTLNCE
   A. sal   (233) KPFATRSVSTDRQLSAFSPQERLAIAGNPLLAQAVASPMARRSASPLNCE
   A. hyd   (233) KPFATRSVSTDRQLSAFSPQERLAIAGNPLLAQAVASPMARRSASPLNCE
Consensus   (251) KPFA*RS*STD*QLSAF*PQERLAIAGNPLLAQAVASPMA*RSAS*LNCE
                 301                                                 336
   P10480   (301) GKMFWDQVHPTTVVHAALSEPAATFIESQYEFLAH-
   A. sal   (283) GKMFWDQVHPTTVVHAALSERAATFIETQYEFLAHG
   A. hyd   (283) GKMFWDQVHPTTVVHAALSERAATFIANQYEFLAH-
Consensus   (301) GKMFWDQVHPTTVVHAALSE*AATFI**QYEFLAH*
```

FIGURE 53



Gene construct for KLM3' expression

1182 bp

FIGURE 54

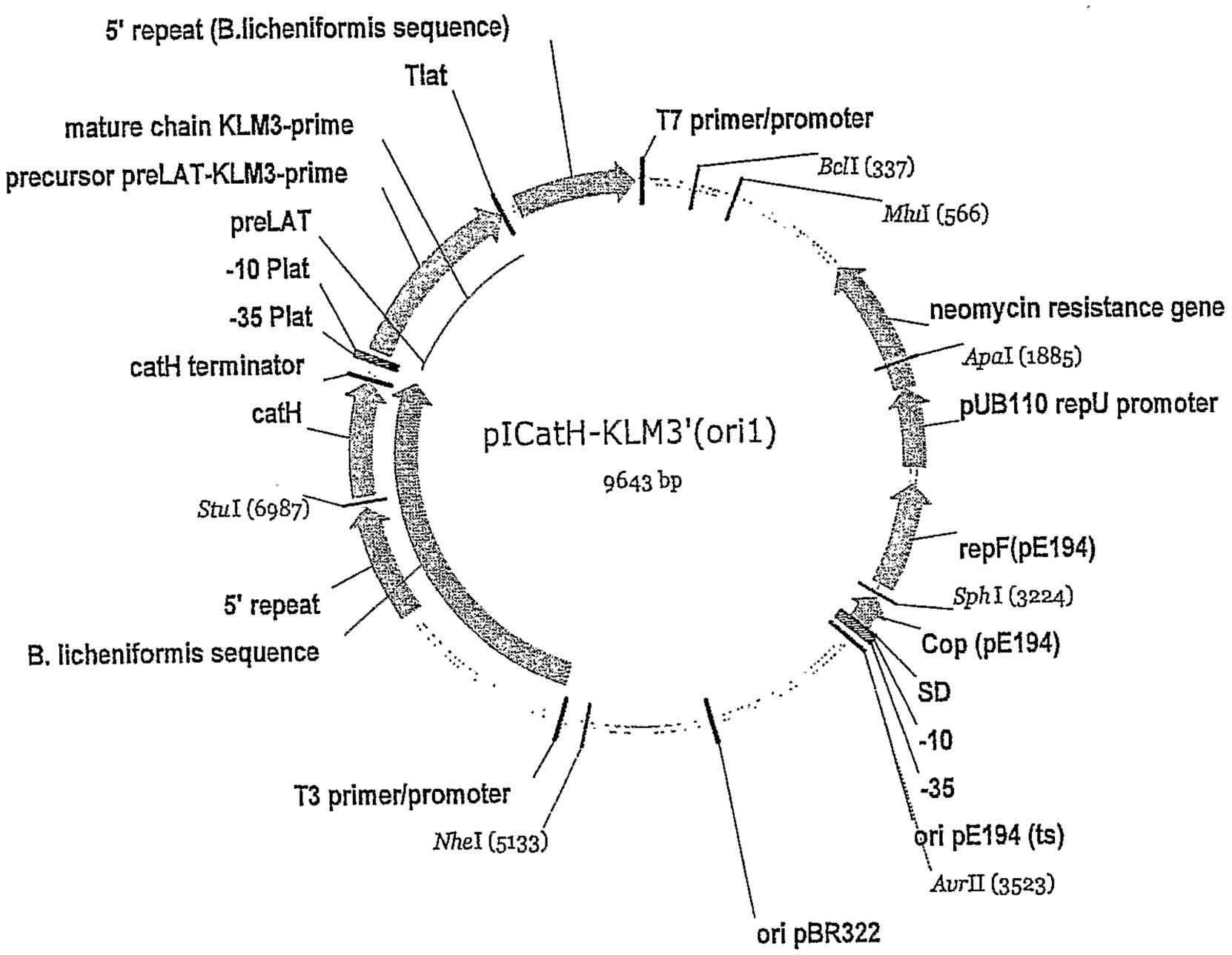

5' repeat (B.licheniformis sequence)
Tlat
mature chain KLM3-prime
precursor preLAT-KLM3-prime
preLAT
-10 Plat
-35 Plat
catH terminator
catH
StuI (6987)
5' repeat
B. licheniformis sequence
T7 primer/promoter
BclI (337)
MluI (566)
neomycin resistance gene
ApaI (1885)
pUB110 repU promoter
repF(pE194)
SphI (3224)
Cop (pE194)
SD
-10
-35
ori pE194 (ts)
AvrII (3523)
pICatH-KLM3'(ori1)
9643 bp
T3 primer/promoter
NheI (5133)
ori pBR322

FIGURE 55

```
                                                                -35
  1  GCTTTTCTTT TGGAAGAAAA TATAGGGAAA ATGGTACTTG TTAAAAATTC GGAATATTTA
     CGAAAAGAAA ACCTTCTTTT ATATCCCTTT TACCATGAAC AATTTTTAAG CCTTATAAAT
       -10                                                M  K  Q  Q  K  R  L  .
 61  TACAATATCA TATGTTTCAC ATTGAAAGGG GAGGAGAATC ATGAAACAAC AAAAACGGCT
     ATGTTATAGT ATACAAAGTG TAACTTTCCC CTCCTCTTAG TACTTTGTTG TTTTTGCCGA
     .  Y  A  R  L  L  T  L  L  F  A  L  I  F  L  L  P  H  S  A  A  .
121  TTACGCCCGA TTGCTGACGC TGTTATTTGC GCTCATCTTC TTGCTGCCTC ATTCTGCAGC
     AATGCGGGCT AACGACTGCG ACAATAAACG CGAGTAGAAG AACGACGGAG TAAGACGTCG
     .  S  A  A  D  T  R  P  A  F  S  R  I  V  M  F  G  D  S  L  S  .
181  TTCAGCAGCA GATACAAGAC CGGCGTTTAG CCGGATCGTC ATGTTTGGAG ATAGCCTGAG
     AAGTCGTCGT CTATGTTCTG GCCGCAAATC GGCCTAGCAG TACAAACCTC TATCGGACTC
     .  D  T  G  K  M  Y  S  K  M  R  G  Y  L  P  S  S  P  P  Y  Y  .
241  CGATACGGGC AAAATGTATA GCAAAATGAG AGGCTATCTT CCGTCAAGCC CGCCGTATTA
     GCTATGCCCG TTTTACATAT CGTTTTACTC TCCGATAGAA GGCAGTTCGG GCGGCATAAT
     .  E  G  R  F  S  N  G  P  V  W  L  E  Q  L  T  K  Q  F  P  G  .
301  TGAAGGCCGC TTTAGCAATG GACCGGTCTG GCTGGAACAA CTGACGAAAC AATTTCCGGG
     ACTTCCGGCG AAATCGTTAC CTGGCCAGAC CGACCTTGTT GACTGCTTTG TTAAAGGCCC
     .  L  T  I  A  N  E  A  E  G  G  A  T  A  V  A  Y  N  K  I  S  .
361  ACTGACGATC GCTAATGAAG CAGAAGGAGG AGCAACAGCG GTCGCCTATA ACAAAATCAG
     TGACTGCTAG CGATTACTTC GTCTTCCTCC TCGTTGTCGC CAGCGGATAT TGTTTTAGTC
     .  W  D  P  K  Y  Q  V  I  N  N  L  D  Y  E  V  T  Q  F  L  Q  .
421  CTGGGACCCG AAATATCAGG TCATCAACAA CCTGGACTAT GAAGTCACAC AGTTTCTTCA
     GACCCTGGGC TTTATAGTCC AGTAGTTGTT GGACCTGATA CTTCAGTGTG TCAAAGAAGT
     .  K  D  S  F  K  P  D  D  L  V  I  L  W  V  G  A  N  D  Y  L  .
481  GAAAGACAGC TTTAAACCGG ATGATCTGGT CATCCTTTGG GTCGGCGCCA ATGATTATCT
     CTTTCTGTCG AAATTTGGCC TACTAGACCA GTAGGAAACC CAGCCGCGGT TACTAATAGA
     .  A  Y  G  W  N  T  E  Q  D  A  K  R  V  R  D  A  I  S  D  A  .
541  GGCGTATGGC TGGAACACAG AACAAGATGC CAAAAGAGTC AGAGATGCCA TCAGCGATGC
     CCGCATACCG ACCTTGTGTC TTGTTCTACG GTTTTCTCAG TCTCTACGGT AGTCGCTACG
     .  A  N  R  M  V  L  N  G  A  K  Q  I  L  L  F  N  L  P  D  L  .
601  CGCTAATAGA ATGGTCCTGA ACGGCGCCAA ACAAATCCTG CTGTTTAACC TGCCGGATCT
     GCGATTATCT TACCAGGACT TGCCGCGGTT TGTTTAGGAC GACAAATTGG ACGGCCTAGA
     .  G  Q  N  P  S  A  R  S  Q  K  V  V  E  A  V  S  H  V  S  A  .
661  GGGACAAAAT CCGAGCGCCA GAAGCCAAAA AGTCGTCGAA GCAGTCAGCC ATGTCAGCGC
     CCCTGTTTTA GGCTCGCGGT CTTCGGTTTT TCAGCAGCTT CGTCAGTCGG TACAGTCGCG
     .  Y  H  N  K  L  L  L  N  L  A  R  Q  L  A  P  T  G  M  V  K  .
721  CTATCATAAC AAACTGCTGC TGAACCTGGC AAGACAATTG GCACCGACGG GAATGGTTAA
     GATAGTATTG TTTGACGACG ACTTGGACCG TTCTGTTAAC CGTGGCTGCC CTTACCAATT
     .  L  F  E  I  D  K  Q  F  A  E  M  L  R  D  P  Q  N  F  G  L  .
781  ATTGTTTGAA ATTGACAAAC AGTTTGCCGA AATGCTGAGA GATCCGCAAA ATTTTGGCCT
     TAACAAACTT TAACTGTTTG TCAAACGGCT TTACGACTCT CTAGGCGTTT TAAAACCGGA
     .  S  D  V  E  N  P  C  Y  D  G  G  Y  V  W  K  P  F  A  T  R  .
841  GAGCGATGTC GAAAACCCGT GCTATGATGG CGGATATGTC TGGAAACCGT TTGCCACAAG
     CTCGCTACAG CTTTTGGGCA CGATACTACC GCCTATACAG ACCTTTGGCA AACGGTGTTC
     .  S  V  S  T  D  R  Q  L  S  A  F  S  P  Q  E  R  L  A  I  A  .
901  AAGCGTCAGC ACGGATAGAC AACTGTCAGC GTTTAGCCCG CAAGAAAGAC TGGCAATCGC
     TTCGCAGTCG TGCCTATCTG TTGACAGTCG CAAATCGGGC GTTCTTTCTG ACCGTTAGCG
     .  G  N  P  L  L  A  Q  A  V  A  S  P  M  A  R  R  S  A  S  P  .
961  CGGAAATCCG CTTTTGGCAC AAGCAGTTGC TTCACCGATG GCAAGAAGAT CAGCAAGCCC
     GCCTTTAGGC GAAAACCGTG TTCGTCAACG AAGTGGCTAC CGTTCTTCTA GTCGTTCGGG
     .  L  N  C  E  G  K  M  F  W  D  Q  V  H  P  T  T  V  V  H  A  .
1021 GCTGAATTGC GAAGGCAAAA TGTTTTGGGA TCAGGTCCAT CCGACAACAG TTGTCCATGC
     CGACTTAACG CTTCCGTTTT ACAAAACCCT AGTCCAGGTA GGCTGTTGTC AACAGGTACG
     .  A  L  S  E  R  A  A  T  F  I  E  T  Q  Y  E  F  L  A  H  G  .
1081 TGCCCTTTCA GAAAGAGCGG CGACGTTTAT CGAAACACAG TATGAATTTC TGGCCCATGG
     ACGGGAAAGT CTTTCTCGCC GCTGCAAATA GCTTTGTGTC ATACTTAAAG ACCGGGTACC
     .stop
1141 CTGAGTTAAC AGAGGACGGA TTTCCTGAAG GAAATCCGTT TTTTTATTTT AAGCTTGGAG
     GACTCAATTG TCTCCTGCCT AAAGGACTTC CTTTAGGCAA AAAAATAAAA TTCGAACCTC
1201 ACAAGGTAAA GGATAAAACC TCGAG
     TGTTCCATTT CCTATTTTGG AGCTC
```

FIG. 56

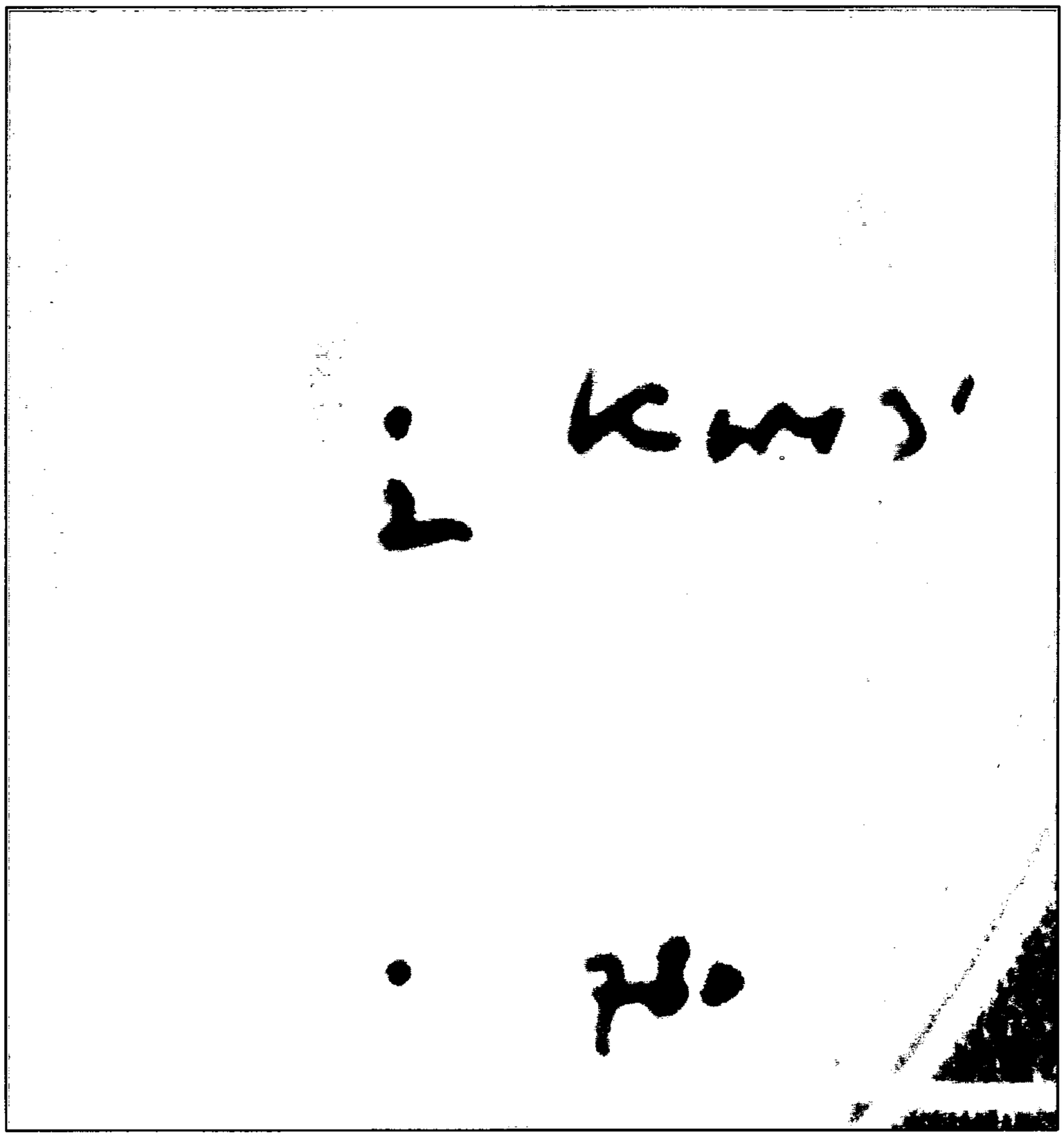


FIGURE 57 (SEQ ID No 49)

```
1     ATGAAACAAC AAAAACGGCT TTACGCCCGA TTGCTGACGC TGTTATTTGC
      TACTTTGTTG TTTTTGCCGA AATGCGGGCT AACGACTGCG ACAATAAACG

51    GCTCATCTTC TTGCTGCCTC ATTCTGCAGC TTCAGCAGCA GATACAAGAC
      CGAGTAGAAG AACGACGGAG TAAGACGTCG AAGTCGTCGT CTATGTTCTG

101   CGGCGTTTAG CCGGATCGTC ATGTTTGGAG ATAGCCTGAG CGATACGGGC
      GCCGCAAATC GGCCTAGCAG TACAAACCTC TATCGGACTC GCTATGCCCG

151   AAAATGTATA GCAAAATGAG AGGCTATCTT CCGTCAAGCC CGCCGTATTA
      TTTTACATAT CGTTTTACTC TCCGATAGAA GGCAGTTCGG GCGGCATAAT

201   TGAAGGCCGC TTTAGCAATG GACCGGTCTG GCTGGAACAA CTGACGAAAC
      ACTTCCGGCG AAATCGTTAC CTGGCCAGAC CGACCTTGTT GACTGCTTTG

251   AATTTCCGGG ACTGACGATC GCTAATGAAG CAGAAGGAGG AGCAACAGCG
      TTAAAGGCCC TGACTGCTAG CGATTACTTC GTCTTCCTCC TCGTTGTCGC

301   GTCGCCTATA ACAAAATCAG CTGGGACCCG AAATATCAGG TCATCAACAA
      CAGCGGATAT TGTTTTAGTC GACCCTGGGC TTTATAGTCC AGTAGTTGTT

351   CCTGGACTAT GAAGTCACAC AGTTTCTTCA GAAAGACAGC TTTAAACCGG
      GGACCTGATA CTTCAGTGTG TCAAAGAAGT CTTTCTGTCG AAATTTGGCC

401   ATGATCTGGT CATCCTTTGG GTCGGCGCCA ATGATTATCT GGCGTATGGC
      TACTAGACCA GTAGGAAACC CAGCCGCGGT TACTAATAGA CCGCATACCG

451   TGGAACACAG AACAAGATGC CAAAAGAGTC AGAGATGCCA TCAGCGATGC
      ACCTTGTGTC TTGTTCTACG GTTTTCTCAG TCTCTACGGT AGTCGCTACG

501   CGCTAATAGA ATGGTCCTGA ACGGCGCCAA ACAAATCCTG CTGTTTAACC
      GCGATTATCT TACCAGGACT TGCCGCGGTT TGTTTAGGAC GACAAATTGG

551   TGCCGGATCT GGGACAAAAT CCGAGCGCCA GAAGCCAAAA AGTCGTCGAA
      ACGGCCTAGA CCCTGTTTTA GGCTCGCGGT CTTCGGTTTT TCAGCAGCTT

601   GCAGTCAGCC ATGTCAGCGC CTATCATAAC AAACTGCTGC TGAACCTGGC
      CGTCAGTCGG TACAGTCGCG GATAGTATTG TTTGACGACG ACTTGGACCG

651   AAGACAATTG GCACCGACGG GAATGGTTAA ATTGTTTGAA ATTGACAAAC
      TTCTGTTAAC CGTGGCTGCC CTTACCAATT TAACAAACTT TAACTGTTTG

701   AGTTTGCCGA AATGCTGAGA GATCCGCAAA ATTTTGGCCT GAGCGATGTC
      TCAAACGGCT TTACGACTCT CTAGGCGTTT TAAAACCGGA CTCGCTACAG

751   GAAAACCCGT GCTATGATGG CGGATATGTC TGGAAACCGT TTGCCACAAG
      CTTTTGGGCA CGATACTACC GCCTATACAG ACCTTTGGCA AACGGTGTTC

801   AAGCGTCAGC ACGGATAGAC AACTGTCAGC GTTTAGCCCG CAAGAAAGAC
      TTCGCAGTCG TGCCTATCTG TTGACAGTCG CAAATCGGGC GTTCTTTCTG

851   TGGCAATCGC CGGAAATCCG CTTTTGGCAC AAGCAGTTGC TTCACCGATG
      ACCGTTAGCG GCCTTTAGGC GAAAACCGTG TTCGTCAACG AAGTGGCTAC

901   GCAAGAAGAT CAGCAAGCCC GCTGAATTGC GAAGGCAAAA TGTTTTGGGA
      CGTTCTTCTA GTCGTTCGGG CGACTTAACG CTTCCGTTTT ACAAAACCCT

951   TCAGGTCCAT CCGACAACAG TTGTCCATGC TGCCCTTTCA GAAAGAGCGG
      AGTCCAGGTA GGCTGTTGTC AACAGGTACG ACGGGAAAGT CTTTCTCGCC

1001  CGACGTTTAT CGAAACACAG TATGAATTTC TGGCCCATGG CTGA
      GCTGCAAATA GCTTTGTGTC ATACTTAAAG ACCGGGTACC GACT
```

FIGURE 58 (SEQ ID No. 50)

```
   1  ATGAAAAAAT GGTTTGTGTG TTTATTGGGA TTGGTCGCGC TGACAGTTCA GGCAGCCGAC
  61  AGCCGTCCCG CCTTCTCCCG GATCGTGATG TTTGGCGACA GCCTCTCCGA TACCGGCAAG
 121  ATGTACAGCA AGATGCGCGG TTACCTCCCC TCCAGCCCCC CCTACTATGA GGGCCGCTTC
 181  TCCAACGGGC CCGTCTGGCT GGAGCAGCTG ACCAACGAGT TCCCGGGCCT GACCATAGCC
 241  AACGAGGCGG AAGGCGGACC GACCGCCGTG GCTTACAACA AGATCTCCTG GAATCCCAAG
 301  TATCAGGTCA TCAACAACCT GGACTACGAG GTCACCCAGT TCCTGCAAAA AGACAGCTTC
 361  AAGCCGGACG ATCTGGTGAT CCTCTGGGTC GGCGCCAACG ACTATCTGGC CTATGGCTGG
 421  AACACAGAGC AGGATGCCAA GCGGGTGCGC GACGCCATCA GCGATGCGGC CAACCGCATG
 481  GTGCTGAACG GCGCCAAGGA GATACTGCTG TTCAACCTGC CGGATCTGGG CCAGAACCCC
 541  TCGGCCCGCA GCCAGAAGGT GGTCGAGGCG GCCAGCCATG TCTCCGCCTA CCACAACCAG
 601  CTGCTGCTGA ACCTGGCACG CCAGCTGGCT CCCACCGGCA TGGTGAAGCT GTTCGAGATC
 661  GACAAGCAGT TTGCCGAGAT GCTGCGTGAT CCGCAGAACT TCGGCCTGAG CGACCAGAGG
 721  AACGCCTGCT ACGGTGGCAG CTATGTATGG AAGCCGTTTG CCTCCCGCAG CGCCAGCACC
 781  GACAGCCAGC TCTCCGCCTT CAACCCGCAG GAGCGCCTCG CCATCGCCGG CAACCCGCTG
 841  CTGGCCCAGG CCGTCGCCAG CCCCATGGCT GCCCGCAGCG CCAGCACCCT CAACTGTGAG
 901  GGCAAGATGT TCTGGGATCA GGTCCACCCC ACCACTGTCG TGCACGCCGC CCTGAGCGAG
 961  CCCGCCGCCA CCTTCATCGA GAGCCAGTAC GAGTTCCTCG CCCAC
```

FIGURE 59 (SEQ ID No. 51)

```
   1  ATGAAAAAAT GGTTTGTTTG TTTATTGGGG TTGATCGCGC TGACAGTTCA GGCAGCCGAC
  61  ACTCGCCCCG CCTTCTCCCG GATCGTGATG TTCGGCGACA GCCTCTCCGA TACCGGCAAA
 121  ATGTACAGCA AGATGCGCGG TTACCTCCCC TCCAGCCCGC CCTACTATGA GGGCCGTTTC
 181  TCCAACGGAC CCGTCTGGCT GGAGCAGCTG ACCAAGCAGT TCCCGGGTCT GACCATCGCC
 241  AACGAAGCGG AAGGCGGTGC CACTGCCGTG GCTTACAACA AGATCTCCTG GAATCCCAAG
 301  TATCAGGTCT ACAACAACCT GGACTACGAG GTCACCCAGT TCTTGCAGAA AGACAGCTTC
 361  AAGCCGGACG ATCTGGTGAT CCTCTGGGTC GGTGCCAATG ACTATCTGGC ATATGGCTGG
 421  AATACGGAGC AGGATGCCAA GCGAGTTCGC GATGCCATCA GCGATGCGGC CAACCGCATG
 481  GTACTGAACG GTGCCAAGCA GATACTGCTG TTCAACCTGC CGGATCTGGG CCAGAACCCG
 541  TCAGCCCGCA GTCAGAAGGT GGTCGAGGCG GTCAGCCATG TCTCCGCCTA TCACAACAAG
 601  CTGCTGCTGA ACCTGGCACG CCAGCTGGCC CCCACCGGCA TGGTAAAGCT GTTCGAGATC
 661  GACAAGCAAT TTGCCGAGAT GCTGCGTGAT CCGCAGAACT TCGGCCTGAG CGACGTCGAG
 721  AACCCCTGCT ACGACGGCGG CTATGTGTGG AAGCCGTTTG CCACCCGCAG CGTCAGCACC
 781  GACCGCCAGC TCTCCGCCTT CAGTCCGCAG GAACGCCTCG CCATCGCCGG CAACCCGCTG
 841  CTGGCACAGG CCGTTGCCAG TCCTATGGCC CGCCGCAGCG CCAGCCCCCT CAACTGTGAG
 901  GGCAAGATGT TCTGGGATCA GGTACACCCG ACCACTGTCG TGCACGCAGC CCTGAGCGAG
 961  CGCGCCGCCA CCTTCATCGA GACCCAGTAC GAGTTCCTCG CCCACGGATG A
```

FIGURE 60 (SEQ ID No. 52)

```
   1  ATGCCGAAGC CTGCCCTTCG CCGTGTCATG ACCGCGACAG TCGCCGCCGT CGGCACGCTC
  61  GCCCTCGGCC TCACCGACGC CACCGCCCAC GCCGCGCCCG CCCAGGCCAC TCCGACCCTG
 121  GACTACGTCG CCCTCGGCGA CAGCTACAGC GCCGGCTCCG GCGTCCTGCC CGTCGACCCC
 181  GCCAACCTGC TCTGTCTGCG CTCGACGGCC AACTACCCCC ACGTCATCGC GGACACGACG
 241  GGCGCCCGCC TCACGGACGT CACCTGCGGC GCCGCGCAGA CCGCCGACTT CACGCGGGCC
 301  CAGTACCCGG GCGTCGCACC CCAGTTGGAC GCGCTCGGCA CCGGCACGGA CCTGGTCACG
 361  CTCACCATCG GCGGCAACGA CAACAGCACC TTCATCAACG CCATCACGGC CTGCGGCACG
 421  GCGGGTGTCC TCAGCGGCGG CAAGGGCAGC CCCTGCAAGG ACAGGCACGG CACCTCCTTC
 481  GACGACGAGA TCGAGGCCAA CACGTACCCC GCGCTCAAGG AGGCGCTGCT CGGCGTCCGC
 541  GCCAGGGCTC CCCACGCCAG GGTGGCGGCT CTCGGCTACC CGTGGATCAC CCCGGCCACC
 601  GCCGACCCGT CCTGCTTCCT GAAGCTCCCC CTCGCCGCCG GTGACGTGCC CTACCTGCGG
 661  GCCATCCAGG CACACCTCAA CGACGCGGTC CGGCGGGCCG CCGAGGAGAC CGGAGCCACC
 721  TACGTGGACT TCTCCGGGGT GTCCGACGGC CACGACGCCT GCGAGGCCCC CGGCACCCGC
 781  TGGATCGAAC CGCTGCTCTT CGGGCACAGC CTCGTTCCCG TCCACCCCAA CGCCCTGGGC
 841  GAGCGGCGCA TGGCCGAGCA CACGATGGAC GTCCTCGGCC TGGACTGA
```

FIGURE 61 (SEQ ID No. 53)

```
  1  TCAGTCCAGG CCGAGGACGT CCATCGTGTG CTCGGCCATG CGCCGCTCGC CCAGGGCGTT
 61  GGGGTGGACG GGAACGAGGC TGTGCCCGAA GAGCAGCGGT TCGATCCAGC GGGTGCCGGG
121  GGCCTCGCAG GCGTCGTGGC CGTCGGACAC CCCGGAGAAG TCCACGTAGG TGGCTCCGGT
181  CTCCTCGGCG GCCCGCCGGA CCGCGTCGTT GAGGTGTGCC TGGATGGCCC GCAGGTAGGG
241  CACGTCACCG GCGGCGAGGG GGAGCTTCAG GAAGCAGGAC GGGTCGGCGG TGGCCGGGGT
301  GATCCACGGG TAGCCGAGAG CCGCCACCCT GGCGTGGGGA GCCCTGGCGC GGACGCCGAG
361  CAGCGCCTCC TTGAGCGCGG GGTACGTGTT GGCCTCGATC TCGTCGTCGA AGGAGGTGCC
421  GTGCCTGTCC TTGCAGGGGC TGCCCTTGCC GCCGCTGAGG ACACCCGCCG TGCCGCAGGC
481  CGTGATGGCG TTGATGAAGG TGCTGTTGTC GTTGCCGCCG ATGGTGAGCG TGACCAGGTC
541  CGTGCCGGTG CCGAGCGCGT CCAACTGGGG TGCGACGCCC GGGTACTGGG CCCGCGTGAA
601  GTCGGCGGTC TGCGCGGCGC CGCAGGTGAC GTCCGTGAGG CGGGCGCCCG TCGTGTCCGC
661  GATGACGTGG GGGTAGTTGG CCGTCGAGCG CAGACAGAGC AGGTTGGCGG GGTCGACGGG
721  CAGGACGCCG GAGCCGGCGC TGTAGCTGTC GCCGAGGGCG ACGTAGTCCA GGGTCGGAGT
781  GGCCTGGGCG GGCGCGGCGT GGGCGGTGGC GTCGGTGAGG CCGAGGGCGA GCGTGCCGAC
841  GGCGGCGACT GTCGCGGTCA TGACACGGCG AAGGGCAGGC TTCGGCAT
```

FIGURE 62 (SEQ ID No. 54)

```
  1  ATGGATTACG AGAAGTTTCT GTTATTTGGG GATTCCATTA CTGAATTTGC TTTTAATACT
 61  AGGCCCATTG AAGATGGCAA AGATCAGTAT GCTCTTGGAG CCGCATTAGT CAACGAATAT
121  ACGAGAAAAA TGGATATTCT TCAAAGAGGG TTCAAAGGGT ACACTTCTAG ATGGGCGTTG
181  AAAATACTTC CTGAGATTTT AAAGCATGAA TCCAATATTG TCATGGCCAC AATATTTTTG
241  GGTGCCAACG ATGCATGCTC AGCAGGTCCC CAAAGTGTCC CCCTCCCCGA ATTTATCGAT
301  AATATTCGTC AAATGGTATC TTTGATGAAG TCTTACCATA TCCGTCCTAT TATAATAGGA
361  CCGGGGCTAG TAGATAGAGA GAAGTGGGAA AAAGAAAAAT CTGAAGAAAT AGCTCTCGGA
421  TACTTCCGTA CCAACGAGAA CTTTGCCATT TATTCCGATG CCTTAGCAAA ACTAGCCAAT
481  GAGGAAAAAG TTCCCTTCGT GGCTTTGAAT AAGGCGTTTC AACAGGAAGG TGGTGATGCT
541  TGGCAACAAC TGCTAACAGA TGGACTGCAC TTTTCCGGAA AAGGGTACAA AATTTTTCAT
601  GACGAATTAT TGAAGGTCAT TGAGACATTC TACCCCCAAT ATCATCCCAA AAACATGCAG
661  TACAAACTGA AAGATTGGAG AGATGTGCTA GATGATGGAT CTAACATAAT GTCTTGA
```

FIGURE 63 (SEQ ID No. 55)

```
atgaacctgc gtcaatggat gggcgccgcc acggctgccc ttgccttggg cttggccgcg        60
tgcgggggcg gtgggaccga ccagagcggc aatcccaatg tcgccaaggt gcagcgcatg       120
gtggtgttcg gcgacagcct gagcgatatc ggcacctaca cccccgtcgc gcaggcggtg       180
ggcggcggca agttcaccac caacccgggc ccgatctggg ccgagaccgt ggccgcgcaa       240
ctgggcgtga cgctcacgcc ggcggtgatg ggctacgcca cctccgtgca gaattgcccc       300
aaggccggct gcttcgacta tgcgcagggc ggctcgcgcg tgaccgatcc gaacggcatc       360
ggccacaacg gcggcgcggg ggcgctgacc tacccggttc agcagcagct cgccaacttc       420
tacgcggcca gcaacaacac attcaacggc aataacgatg tcgtcttcgt gctggccggc       480
agcaacgaca ttttcttctg gaccactgcg gcggccacca gcggctccgg cgtgacgccc       540
gccattgcca cggcccaggt gcagcaggcc gcgacggacc tggtcggcta tgtcaaggac       600
atgatcgcca agggtgcgac gcaggtctac gtgttcaacc tgcccgacag cagcctgacg       660
ccggacggcg tggcaagcgg cacgaccggc caggcgctgc tgcacgcgct ggtgggcacg       720
ttcaacacga cgctgcaaag cgggctggcc ggcacctcgg cgcgcatcat cgacttcaac       780
gcacaactga ccgcggcgat ccagaatggc gcctcgttcg gcttcgccaa caccagcgcc       840
cgggcctgcg acgccaccaa gatcaatgcc ctggtgccga gcgccggcgg cagctcgctg       900
ttctgctcgg ccaacacgct ggtggcttcc ggtgcggacc agagctacct gttcgccgac       960
ggcgtgcacc cgaccacggc cggccatcgc ctgatcgcca gcaacgtgct ggcgcgcctg      1020
ctggcggata acgtcgcgca ctga                                             1044
```

FIGURE 64 (SEQ ID No. 56)

```
  1 gtgatcgggt cgtacgtggc ggtgggggac agcttcaccg agggcgtcgg cgaccccggc
 61 cccgacgggg cgttcgtcgg ctgggccgac cggctcgccg tactgctcgc ggaccggcgc
121 cccgagggcg acttcacgta cacgaacctc gccgtgcgcg gcaggctcct cgaccagatc
181 gtggcggaac aggtcccgcg ggtcgtcgga ctcgcgcccg acctcgtctc gttcgcggcg
241 ggcggcaacg acatcatccg gcccggcacc gatcccgacg aggtcgccga gcggttcgag
301 ctggcggtgg ccgcgctgac cgccgcggcc ggaaccgtcc tggtgaccac cgggttcgac
361 acccgggggg tgcccgtcct caagcacctg cgcggcaaga tcgccacgta caacgggcac
421 gtccgcgcca tcgccgaccg ctacggctgc ccggtgctcg acctgtggtc gctgcggagc
481 gtccaggacc gcagggcgtg ggacgccgac cggctgcacc tgtcgccgga ggggcacacc
541 cgggtggcgc tgcgcgcggg gcaggccctg ggcctgcgcg tcccggccga ccctgaccag
601 ccctggccgc ccctgccgcc gcgcggcacg ctcgacgtcc ggcgcgacga cgtgcactgg
661 gcgcgcgagt acctggtgcc gtggatcggg cgccggctgc ggggcgagtc gtcgggcgac
721 cacgtgacgg ccaaggggac gctgtcgccg gacgccatca agacgcggat cgccgcggtg
781 gcctga
```

FIGURE 65 (SEQ ID No. 57)

```
  1 atgcagacga accccgcgta caccagtctc gtcgccgtcg gcgactcctt caccgagggc
 61 atgtcggacc tgctgcccga cggctcctac cgtggctggg ccgacctcct cgccacccgg
121 atggcggccc gctcccccgg cttccggtac gccaacctgg cggtgcgcgg gaagctgatc
181 ggacagatcg tcgacgagca ggtggacgtg gccgccgcca tgggagccga cgtgatcacg
241 ctggtcggcg ggctcaacga cacgctgcgg cccaagtgcg acatggcccg ggtgcgggac
301 ctgctgaccc aggccgtgga acggctcgcc ccgcactgcg agcagctggt gctgatgcgc
361 agtcccggtc gccagggtcc ggtgctggag cgcttccggc cccgcatgga ggccctgttc
421 gccgtgatcg acgacctggc cgggcggcac ggcgccgtgg tcgtcgacct gtacggggcc
481 cagtcgctgg ccgaccctcg gatgtgggac gtggaccggc tgcacctgac cgccgagggc
541 caccgccggg tcgcggaggc ggtgtggcag tcgctcggcc acgagcccga ggaccccgag
601 tggcacgcgc cgatcccggc gacgccgccg ccggggtggg tgacgcgcag gaccgcggac
661 gtccggttcg cccggcagca cctgctgccc tggataggcc gcaggctgac cgggcgctcg
721 tccggggacg gcctgccggc caagcgcccg gacctgctgc cctacgagga ccccgcacgg
781 tga
```

FIGURE 66 (SEQ ID No. 58)

```
   1 atgacccggg gtcgtgacgg gggtgcgggg gcgcccccca ccaagcaccg tgccctgctc
  61 gcggcgatcg tcaccctgat agtggcgatc tccgcggcca tatacgccgg agcgtccgcg
 121 gacgacggca gcagggacca cgcgctgcag gccggaggcc gtctcccacg aggagacgcc
 181 gcccccgcgt ccaccggtgc ctgggtgggc gcctgggcca ccgcaccggc cgcggccgag
 241 ccgggcaccg agacgaccgg cctggcgggc cgctccgtgc gcaacgtcgt gcacacctcg
 301 gtcggcggca ccggcgcgcg gatcaccctc tcgaacctgt acgggcagtc gccgctgacc
 361 gtcacacacg cctcgatcgc cctggccgcc gggcccgaca ccgccgccgc gatcgccgac
 421 accatgcgcc ggctcacctt cggcggcagc gcccgggtga tcatcccggc gggcggccag
 481 gtgatgagcg acaccgcccg cctcgccatc ccctacgggg cgaacgtcct ggtcaccacg
 541 tactccccca tcccgtccgg gccggtgacc taccatccgc aggcccggca gaccagctac
 601 ctggccgacg gcgaccgcac ggcggacgtc accgccgtcg cgtacaccac ccccacgccc
 661 tactggcgct acctgaccgc cctcgacgtg ctgagccacg aggccgacgg cacggtcgtg
 721 gcgttcggcg actccatcac cgacggcgcc cgctcgcaga gcgacgccaa ccaccgctgg
 781 accgacgtcc tcgccgcacg cctgcacgag gcggcgggcg acggccggga cacgccccgc
 841 tacagcgtcg tcaacgaggg catcagcggc aaccggctcc tgaccagcag gccggggcgg
 901 ccggccgaca acccgagcgg actgagccgg ttccagcggg acgtgctgga acgcaccaac
 961 gtcaaggccg tcgtcgtcgt cctcggcgtc aacgacgtcc tgaacagccc ggaactcgcc
1021 gaccgcgacg ccatcctgac cggcctgcgc accctcgtcg accgggcgca cgcccgggga
1081 ctgcgggtcg tcggcgccac gatcacgccg ttcggcggct acggcggcta caccgaggcc
1141 cgcgagacga tgcggcagga ggtcaacgag gagatccgct ccggccgggt cttcgacacg
1201 gtcgtcgact tcgacaaggc cctgcgcgac ccgtacgacc cgcgccggat gcgctccgac
1261 tacgacagcg gcgaccacct gcaccccggc gacaaggggt acgcgcgcat gggcgcggtc
1321 atcgacctgg ccgcgctgaa gggcgcggcg ccggtcaagg cgtag
```

FIGURE 67 (SEQ ID No. 59)

```
   1 atgacgagca tgtcgagggc gagggtggcg cggcggatcg cggccggcgc ggcgtacggc
  61 ggcggcggca tcggcctggc gggagcggcg gcggtcggtc tggtggtggc cgaggtgcag
 121 ctggccagac gcagggtggg ggtgggcacg ccgacccggg tgccgaacgc gcagggactg
 181 tacggcggca ccctgcccac ggccggcgac ccgccgctgc ggctgatgat gctgggcgac
 241 tccacggccg ccgggcaggg cgtgcaccgg gccgggcaga cgccgggcgc gctgctggcg
 301 tccgggctcg cggcggtggc ggagcggccg gtgcggctgg ggtcggtcgc ccagccgggg
 361 gcgtgctcgg acgacctgga ccggcaggtg gcgctggtgc tcgccgagcc ggaccgggtg
 421 cccgacatct gcgtgatcat ggtcggcgcc aacgacgtca cccaccggat gccggcgacc
 481 cgctcggtgc ggcacctgtc ctcggcggta cggcggctgc gcacggccgg tgcggaggtg
 541 gtggtcggca cctgtccgga cctgggcacg atcgagcggg tgcggcagcc gctgcgctgg
 601 ctggcccggc gggcctcacg gcagctcgcg gcggcacaga ccatcggcgc cgtcgagcag
 661 ggcgggcgca cggtgtcgct gggcgacctg ctgggtccgg agttcgcgca gaacccgcgg
 721 gagctcttcg gccccgacaa ctaccacccc tccgccgagg ggtacgccac ggccgcgatg
 781 gcggtactgc cctcggtgtg cgccgcgctc ggcctgtggc cggccgacga ggagcacccg
 841 gacgcgctgc gccgcgaggg cttcctgccg gtggcgcgcg cggcggcgga ggcggcgtcc
 901 gaggcgggta cggaggtcgc cgccgccatg cctacggggc ctcgggggcc ctgggcgctg
 961 ctgaagcgcc ggagacggcg tcgggtgtcg gaggcggaac cgtccagccc gtccggcgtt
1021 tga
```

FIGURE 68 (SEQ ID No. 60)

```
   1 atgggtcgag ggacggacca gcggacgcgg tacggccgtc gccgggcgcg tgtcgcgctc
  61 gccgccctga ccgccgccgt cctgggcgtg ggcgtggcgg gctgcgactc cgtgggcggc
 121 gactcacccg ctccttccgg cagcccgtcg aagcggacga ggacggcgcc cgcctgggac
 181 accagcccgg cgtccgtcgc cgccgtgggc gactccatca cgcgcggctt cgacgcctgt
 241 gcggtgctgt cggactgccc ggaggtgtcg tgggcgaccg gcagcagcgc gaaggtcgac
 301 tcgctggccg tacggctgct ggggaaggcg gacgcggccg agcacagctg gaactacgcg
 361 gtcaccgggg cccggatggc ggacctgacc gctcaggtga cgcgggcggc gcagcgcgag
 421 ccggagctgg tggcggtgat ggccgggggcg aacgacgcgt gccggtccac gacctcggcg
 481 atgacgccgg tggcggactt ccgggcgcag ttcgaggagg cgatggccac cctgcgcaag
 541 aagctcccca aggcgcaggt gtacgtgtcg agcatcccgg acctcaagcg gctctggtcc
 601 cagggccgca ccaacccgct gggcaagcag gtgtggaagc tcggcctgtg cccgtcgatg
 661 ctgggcgacg cggactccct ggactcggcg gcgaccctgc ggcgcaacac ggtgcgcgac
 721 cgggtggcgg actacaacga ggtgctgcgg gaggtctgcg cgaaggaccg gcggtgccgc
 781 agcgacgacg gcgcggtgca cgagttccgg ttcggcacgg accagttgag ccactgggac
 841 tggttccacc cgagtgtgga cggccaggcc cggctggcgg agatcgccta ccgcgcggtc
 901 accgcgaaga atccctga
```

FIGURE 69 (SEQ ID No. 61)

```
   1 ttcatcacaa cgatgtcaca acaccggcca tccgggtcat ccctgatcgt gggaatgggt
  61 gacaagcctt cccgtgacga aagggtcctg ctacatcaga aatgacagaa atcctgctca
 121 gggaggttcc atgagactgt cccgacgcgc ggccacggcg tccgcgctcc tcctcacccc
 181 ggcgctcgcg ctcttcggcg cgagcgccgc cgtgtccgcg ccgcgaatcc aggccaccga
 241 ctacgtggcc ctcggcgact cctactcctc gggggtcggc gcgggcagct acgacagcag
 301 cagtggctcc tgtaagcgca gcaccaagtc ctacccggcc ctgtgggccg cctcgcacac
 361 cggtacgcgg ttcaacttca ccgcctgttc gggcgcccgc acaggagacg tgctggccaa
 421 gcagctgacc ccggtcaact ccggcaccga cctggtcagc attaccatcg gcggcaacga
 481 cgcgggcttc gccgacacca tgaccacctg caacctccag ggcgagagcg cgtgcctggc
 541 gcggatcgcc aaggcgcgcg cctacatcca gcagacgctg cccgcccagc tggaccaggt
 601 ctacgacgcc atcgacagcc gggcccccgc agcccaggtc gtcgtcctgg gctacccgcg
 661 cttctacaag ctgggcggca gctgcgccgt cggtctctcg gagaagtccc gcgcggccat
 721 caacgccgcc gccgacgaca tcaacgccgt caccgccaag cgcgccgccg accacggctt
 781 cgccttcggg gacgtcaaca cgaccttcgc cgggcacgag ctgtgctccg gcgccccctg
 841 gctgcacagc gtcacccttc ccgtggagaa ctcctaccac cccacggcca acggacagtc
 901 caagggctac ctgcccgtcc tgaactccgc cacctgatct cgcggctact ccgcccctga
 961 cgaagtcccg ccccgggcg gggcttcgcc gtaggtgcgc gtaccgccgt cgcccgtcgc
1021 gccggtggcc ccgccgtacg tgccgccgcc cccggacgcg gtcggttc
```

FIGURE 70 (SEQ ID No. 62)

```
   1  ATGAAAAAAT GGTTTGTGTG TTTATTGGGA TTGGTCGCGC TGACAGTTCA
      TACTTTTTTA CCAAACACAC AAATAACCCT AACCAGCGCG ACTGTCAAGT

  51  GGCAGCCGAC AGTCGCCCCG CCTTTTCCCG GATCGTGATG TTCGGCGACA
      CCGTCGGCTG TCAGCGGGGC GGAAAAGGGC CTAGCACTAC AAGCCGCTGT

 101  GCCTCTCCGA TACCGGCAAA ATGTACAGCA AGATGCGCGG TTACCTCCCC
      CGGAGAGGCT ATGGCCGTTT TACATGTCGT TCTACGCGCC AATGGAGGGG

 151  TCCAGCCCGC CCTACTATGA GGGCCGTTTC TCCAACGGAC CCGTCTGGCT
      AGGTCGGGCG GGATGATACT CCCGGCAAAG AGGTTGCCTG GGCAGACCGA

 201  GGAGCAGCTG ACCAAACAGT TCCCGGGTCT GACCATCGCC AACGAAGCGG
      CCTCGTCGAC TGGTTTGTCA AGGGCCCAGA CTGGTAGCGG TTGCTTCGCC

 251  AAGGCGGTGC CACTGCCGTG GCTTACAACA AGATCTCCTG GAATCCCAAG
      TTCCGCCACG GTGACGGCAC CGAATGTTGT TCTAGAGGAC CTTAGGGTTC

 301  TATCAGGTCA TCAACAACCT GGACTACGAG GTCACCCAGT TCTTGCAGAA
      ATAGTCCAGT AGTTGTTGGA CCTGATGCTC CAGTGGGTCA AGAACGTCTT

 351  AGACAGCTTC AAGCCGGACG ATCTGGTGAT CCTCTGGGTC GGTGCCAATG
      TCTGTCGAAG TTCGGCCTGC TAGACCACTA GGAGACCCAG CCACGGTTAC

 401  ACTATCTGGC CTATGGCTGG AACACGGAGC AGGATGCCAA GCGGGTTCGC
      TGATAGACCG GATACCGACC TTGTGCCTCG TCCTACGGTT CGCCCAAGCG

 451  GATGCCATCA GCGATGCGGC CAACCGCATG GTACTGAACG GTGCCAAGCA
      CTACGGTAGT CGCTACGCCG GTTGGCGTAC CATGACTTGC CACGGTTCGT

 501  GATACTGCTG TTCAACCTGC CGGATCTGGG CCAGAACCCG TCAGCTCGCA
      CTATGACGAC AAGTTGGACG GCCTAGACCC GGTCTTGGGC AGTCGAGCGT

 551  GTCAGAAGGT GGTCGAGGCG GTCAGCCATG TCTCCGCCTA TCACAACCAG
      CAGTCTTCCA CCAGCTCCGC CAGTCGGTAC AGAGGCGGAT AGTGTTGGTC

 601  CTGCTGCTGA ACCTGGCACG CCAGCTGGCC CCCACCGGCA TGGTAAAGCT
      GACGACGACT TGGACCGTGC GGTCGACCGG GGGTGGCCGT ACCATTTCGA

 651  GTTCGAGATC GACAAGCAAT TTGCCGAGAT GCTGCGTGAT CCGCAGAACT
      CAAGCTCTAG CTGTTCGTTA AACGGCTCTA CGACGCACTA GGCGTCTTGA

 701  TCGGCCTGAG CGACGTCGAG AACCCCTGCT ACGACGGCGG CTATGTGTGG
      AGCCGGACTC GCTGCAGCTC TTGGGGACGA TGCTGCCGCC GATACACACC

 751  AAGCCGTTTG CCACCCGCAG CGTCAGCACC GACCGCCAGC TCTCCGCCTT
      TTCGGCAAAC GGTGGGCGTC GCAGTCGTGG CTGGCGGTCG AGAGGCGGAA

 801  CAGTCCGCAG GAACGCCTCG CCATCGCCGG CAACCCGCTG CTGGCACAGG
      GTCAGGCGTC CTTGCGGAGC GGTAGCGGCC GTTGGGCGAC GACCGTGTCC

 851  CCGTTGCCAG TCCTATGGCC CGCCGCAGCG CCAGCCCCCT CAACTGTGAG
      GGCAACGGTC AGGATACCGG GCGGCGTCGC GGTCGGGGGA GTTGACACTC

 901  GGCAAGATGT TCTGGGATCA GGTACACCCG ACCACTGTCG TGCACGCAGC
      CCGTTCTACA AGACCCTAGT CCATGTGGGC TGGTGACAGC ACGTGCGTCG

 951  CCTGAGCGAG CGCGCCGCCA CCTTCATCGC GAACCAGTAC GAGTTCCTCG
      GGACTCGCTC GCGCGGCGGT GGAAGTAGCG CTTGGTCATG CTCAAGGAGC

1001  CCCAC TGA
      GGGTG ACT
```

FIGURE 71 (SEQ ID No. 63)

```
   1  ATGAAAAAAT GGTTTGTTTG TTTATTGGGG TTGATCGCGC TGACAGTTCA
      TACTTTTTTA CCAAACAAAC AAATAACCCC AACTAGCGCG ACTGTCAAGT

  51  GGCAGCCGAC ACTCGCCCCG CCTTCTCCCG GATCGTGATG TTCGGCGACA
      CCGTCGGCTG TGAGCGGGGC GGAAGAGGGC CTAGCACTAC AAGCCGCTGT

 101  GCCTCTCCGA TACCGGCAAA ATGTACAGCA AGATGCGCGG TTACCTCCCC
      CGGAGAGGCT ATGGCCGTTT TACATGTCGT TCTACGCGCC AATGGAGGGG

 151  TCCAGCCCGC CCTACTATGA GGGCCGTTTC TCCAACGGAC CCGTCTGGCT
      AGGTCGGGCG GGATGATACT CCCGGCAAAG AGGTTGCCTG GGCAGACCGA

 201  GGAGCAGCTG ACCAAGCAGT TCCCGGGTCT GACCATCGCC AACGAAGCGG
      CCTCGTCGAC TGGTTCGTCA AGGGCCCAGA CTGGTAGCGG TTGCTTCGCC

 251  AAGGCGGTGC CACTGCCGTG GCTTACAACA AGATCTCCTG GAATCCCAAG
      TTCCGCCACG GTGACGGCAC CGAATGTTGT TCTAGAGGAC CTTAGGGTTC

 301  TATCAGGTCA TCAACAACCT GGACTACGAG GTCACCCAGT TCTTGCAGAA
      ATAGTCCAGT AGTTGTTGGA CCTGATGCTC CAGTGGGTCA AGAACGTCTT

 351  AGACAGCTTC AAGCCGGACG ATCTGGTGAT CCTCTGGGTC GGTGCCAATG
      TCTGTCGAAG TTCGGCCTGC TAGACCACTA GGAGACCCAG CCACGGTTAC

 401  ACTATCTGGC ATATGGCTGG AATACGGAGC AGGATGCCAA GCGAGTTCGC
      TGATAGACCG TATACCGACC TTATGCCTCG TCCTACGGTT CGCTCAAGCG

 451  GATGCCATCA GCGATGCGGC CAACCGCATG GTACTGAACG GTGCCAAGCA
      CTACGGTAGT CGCTACGCCG GTTGGCGTAC CATGACTTGC CACGGTTCGT

 501  GATACTGCTG TTCAACCTGC CGGATCTGGG CCAGAACCCG TCAGCCCGCA
      CTATGACGAC AAGTTGGACG GCCTAGACCC GGTCTTGGGC AGTCGGGCGT

 551  GTCAGAAGGT GGTCGAGGCG GTCAGCCATG TCTCCGCCTA TCACAACAAG
      CAGTCTTCCA CCAGCTCCGC CAGTCGGTAC AGAGGCGGAT AGTGTTGTTC

 601  CTGCTGCTGA ACCTGGCACG CCAGCTGGCC CCCACCGGCA TGGTAAAGCT
      GACGACGACT TGGACCGTGC GGTCGACCGG GGGTGGCCGT ACCATTTCGA

 651  GTTCGAGATC GACAAGCAAT TTGCCGAGAT GCTGCGTGAT CCGCAGAACT
      CAAGCTCTAG CTGTTCGTTA AACGGCTCTA CGACGCACTA GGCGTCTTGA

 701  TCGGCCTGAG CGACGTCGAG AACCCCTGCT ACGACGGCGG CTATGTGTGG
      AGCCGGACTC GCTGCAGCTC TTGGGGACGA TGCTGCCGCC GATACACACC

 751  AAGCCGTTTG CCACCCGCAG CGTCAGCACC GACCGCCAGC TCTCCGCCTT
      TTCGGCAAAC GGTGGGCGTC GCAGTCGTGG CTGGCGGTCG AGAGGCGGAA

 801  CAGTCCGCAG GAACGCCTCG CCATCGCCGG CAACCCGCTG CTGGCACAGG
      GTCAGGCGTC CTTGCGGAGC GGTAGCGGCC GTTGGGCGAC GACCGTGTCC

 851  CCGTTGCCAG TCCTATGGCC CGCCGCAGCG CCAGCCCCCT CAACTGTGAG
      GGCAACGGTC AGGATACCGG GCGGCGTCGC GGTCGGGGGA GTTGACACTC

 901  GGCAAGATGT TCTGGGATCA GGTACACCCG ACCACTGTCG TGCACGCAGC
      CCGTTCTACA AGACCCTAGT CCATGTGGGC TGGTGACAGC ACGTGCGTCG

 951  CCTGAGCGAG CGCGCCGCCA CCTTCATCGA GACCCAGTAC GAGTTCCTCG
      GGACTCGCTC GCGCGGCGGT GGAAGTAGCT CTGGGTCATG CTCAAGGAGC

1001  CCCACGGATG A
      GGGTGCCTAC T
```

FIGURE 72 (SEQ ID No. 24)

```
   1  ATGTTTAAGT TTAAAAAGAA TTTCTTAGTT GGATTATCGG CAGCTTTAAT
      TACAAATTCA AATTTTTCTT AAAGAATCAA CCTAATAGCC GTCGAAATTA

  51  GAGTATTAGC TTGTTTTCGG CAACCGCCTC TGCAGCTAGC GCCGACAGCC
      CTCATAATCG AACAAAAGCC GTTGGCGGAG ACGTCGATCG CGGCTGTCGG

 101  GTCCCGCCTT TTCCCGGATC GTGATGTTCG GCGACAGCCT CTCCGATACC
      CAGGGCGGAA AAGGGCCTAG CACTACAAGC CGCTGTCGGA GAGGCTATGG

 151  GGCAAAATGT ACAGCAAGAT GCGCGGTTAC CTCCCCTCCA GCCCGCCCTA
      CCGTTTTACA TGTCGTTCTA CGCGCCAATG GAGGGGAGGT CGGGCGGGAT

 201  CTATGAGGGC CGTTTCTCCA ACGGACCCGT CTGGCTGGAG CAGCTGACCA
      GATACTCCCG GCAAAGAGGT TGCCTGGGCA GACCGACCTC GTCGACTGGT

 251  AACAGTTCCC GGGTCTGACC ATCGCCAACG AAGCGGAAGG CGGTGCCACT
      TTGTCAAGGG CCCAGACTGG TAGCGGTTGC TTCGCCTTCC GCCACGGTGA

 301  GCCGTGGCTT ACAACAAGAT CTCCTGGAAT CCCAAGTATC AGGTCATCAA
      CGGCACCGAA TGTTGTTCTA GAGGACCTTA GGGTTCATAG TCCAGTAGTT

 351  CAACCTGGAC TACGAGGTCA CCCAGTTCTT GCAGAAAGAC AGCTTCAAGC
      GTTGGACCTG ATGCTCCAGT GGGTCAAGAA CGTCTTTCTG TCGAAGTTCG

 401  CGGACGATCT GGTGATCCTC TGGGTCGGTG CCAATGACTA TCTGGCCTAT
      GCCTGCTAGA CCACTAGGAG ACCCAGCCAC GGTTACTGAT AGACCGGATA

 451  GGCTGGAACA CGGAGCAGGA TGCCAAGCGG GTTCGCGATG CCATCAGCGA
      CCGACCTTGT GCCTCGTCCT ACGGTTCGCC CAAGCGCTAC GGTAGTCGCT

 501  TGCGGCCAAC CGCATGGTAC TGAACGGTGC CAAGCAGATA CTGCTGTTCA
      ACGCCGGTTG GCGTACCATG ACTTGCCACG GTTCGTCTAT GACGACAAGT

 551  ACCTGCCGGA TCTGGGCCAG AACCCGTCAG CTCGCAGTCA GAAGGTGGTC
      TGGACGGCCT AGACCCGGTC TTGGGCAGTC GAGCGTCAGT CTTCCACCAG

 601  GAGGCGGTCA GCCATGTCTC CGCCTATCAC AACCAGCTGC TGCTGAACCT
      CTCCGCCAGT CGGTACAGAG GCGGATAGTG TTGGTCGACG ACGACTTGGA

 651  GGCACGCCAG CTGGCCCCCA CCGGCATGGT AAAGCTGTTC GAGATCGACA
      CCGTGCGGTC GACCGGGGGT GGCCGTACCA TTTCGACAAG CTCTAGCTGT

 701  AGCAATTTGC CGAGATGCTG CGTGATCCGC AGAACTTCGG CCTGAGCGAC
      TCGTTAAACG GCTCTACGAC GCACTAGGCG TCTTGAAGCC GGACTCGCTG

 751  GTCGAGAACC CCTGCTACGA CGGCGGCTAT GTGTGGAAGC CGTTTGCCAC
      CAGCTCTTGG GGACGATGCT GCCGCCGATA CACACCTTCG GCAAACGGTG

 801  CCGCAGCGTC AGCACCGACC GCCAGCTCTC CGCCTTCAGT CCGCAGGAAC
      GGCGTCGCAG TCGTGGCTGG CGGTCGAGAG GCGGAAGTCA GGCGTCCTTG

 851  GCCTCGCCAT CGCCGGCAAC CCGCTGCTGG CACAGGCCGT TGCCAGTCCT
      CGGAGCGGTA GCGGCCGTTG GGCGACGACC GTGTCCGGCA ACGGTCAGGA

 901  ATGGCCCGCC GCAGCGCCAG CCCCCTCAAC TGTGAGGGCA AGATGTTCTG
      TACCGGGCGG CGTCGCGGTC GGGGGAGTTG ACACTCCCGT TCTACAAGAC

 951  GGATCAGGTA CACCCGACCA CTGTCGTGCA CGCAGCCCTG AGCGAGCGCG
      CCTAGTCCAT GTGGGCTGGT GACAGCACGT GCGTCGGGAC TCGCTCGCGC

1001  CCGCCACCTT CATCGCGAAC CAGTACGAGT TCCTCGCCCA CTGATGA
      GGCGGTGGAA GTAGCGCTTG GTCATGCTCA AGGAGCGGGT GACTACT
```

FIGURE 73

SEQ ID No. 68

```
  1  ADTRPAFSRI VMFGDSLSDT GKMYSKMRGY LPSSPPYYEG RFSNGPVWLE QLTKQFPGLT
 61  IANEAEGGAT AVAYNKISWD PKYQVINNLD YEVTQFLQKD SFKPDDLVIL WVGANDYLAY
121  GWNTEQDAKR VRDAISDAAN RMVLNGAKQI LLFNLPDLGQ NPSARSQKVV EAVSHVSAYH
181  NKLLLNLARQ LAPTGMVKLF EIDKQFAEML RDPQNFGLSD VENPCYDGGY VWKPF

236  RSASPLNCEG KMFWDQVHPT TVVHAALSER AATFIETQYE FLAHG
```

FIGURE 74a

CONVENTIONAL PROCESS (for comparison only)

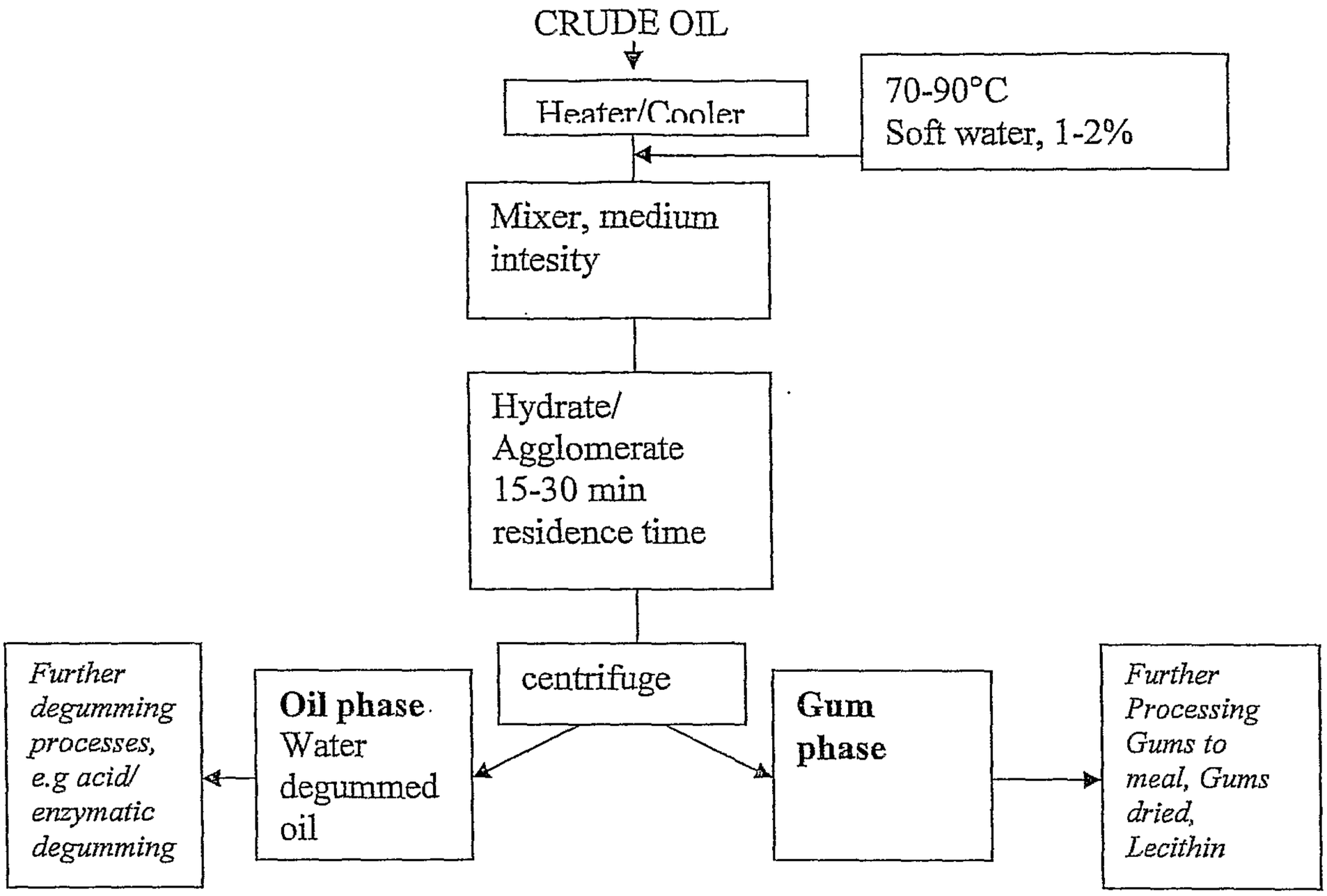


FIGURE 74b

PROCESS OF PRESENT INVENTION

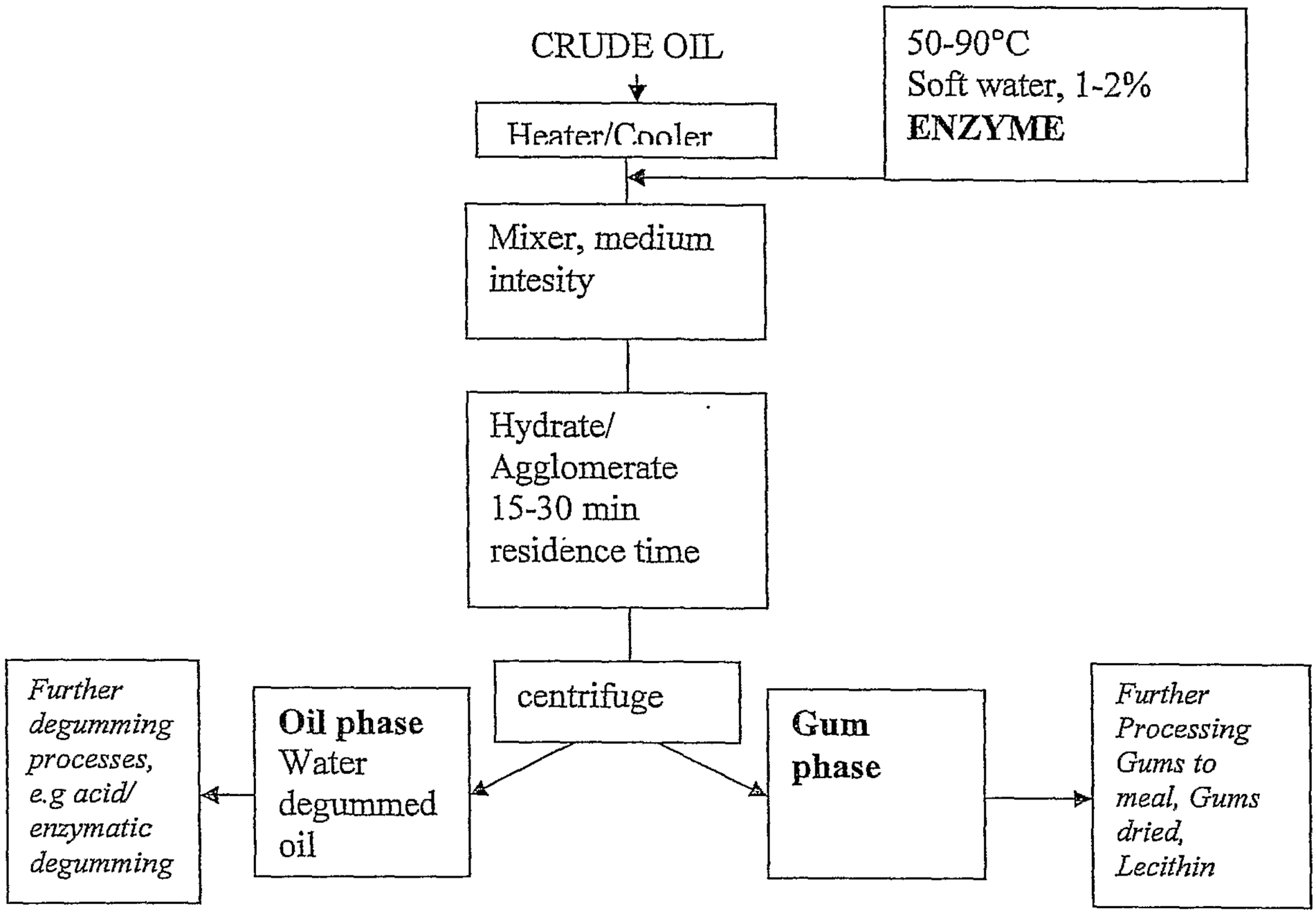


FIGURE 75

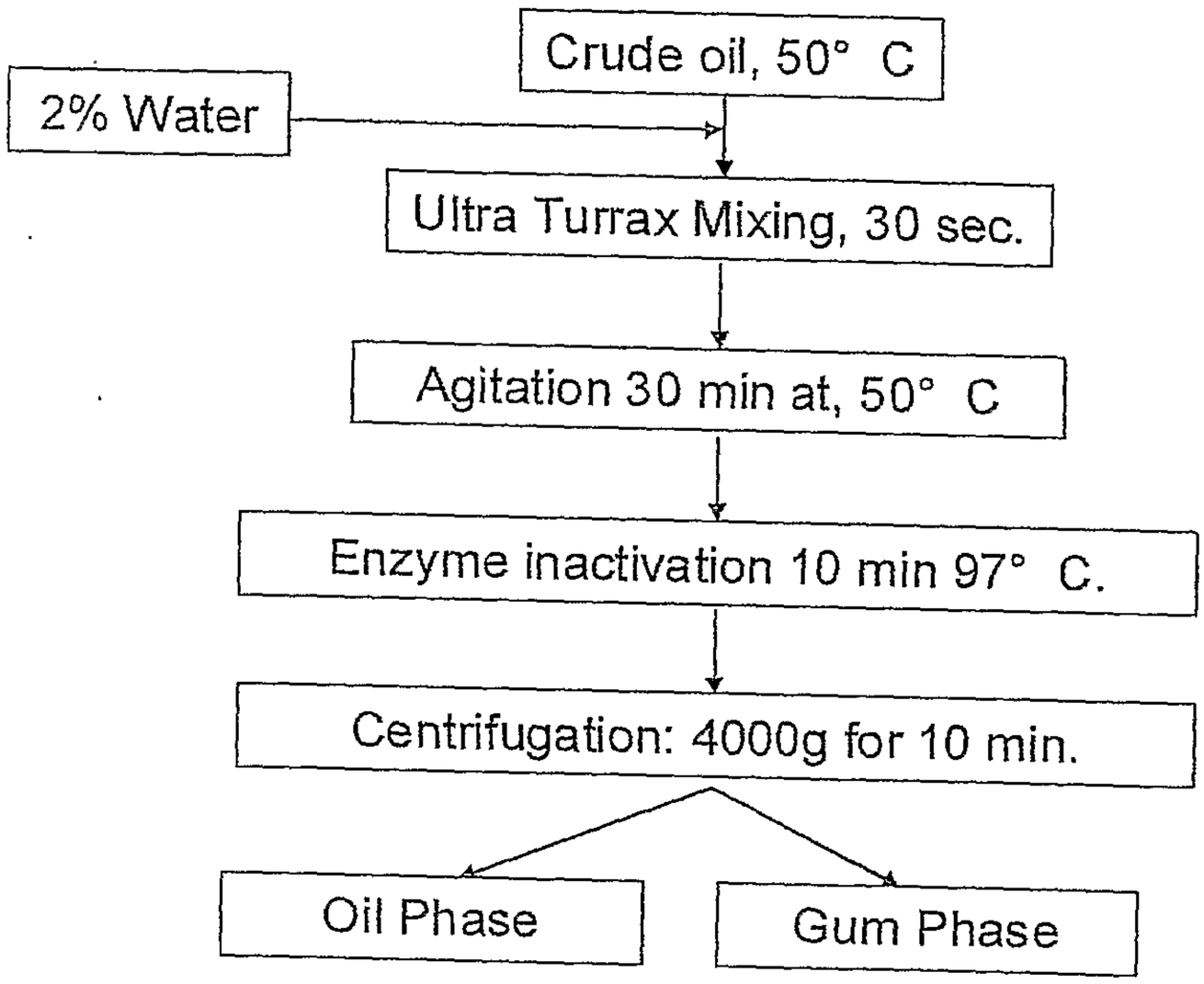

Crude oil, 50° C
2% Water
Ultra Turrax Mixing, 30 sec.
Agitation 30 min at, 50° C
Enzyme inactivation 10 min 97° C.
Centrifugation: 4000g for 10 min.
Oil Phase
Gum Phase

FIGURE 76

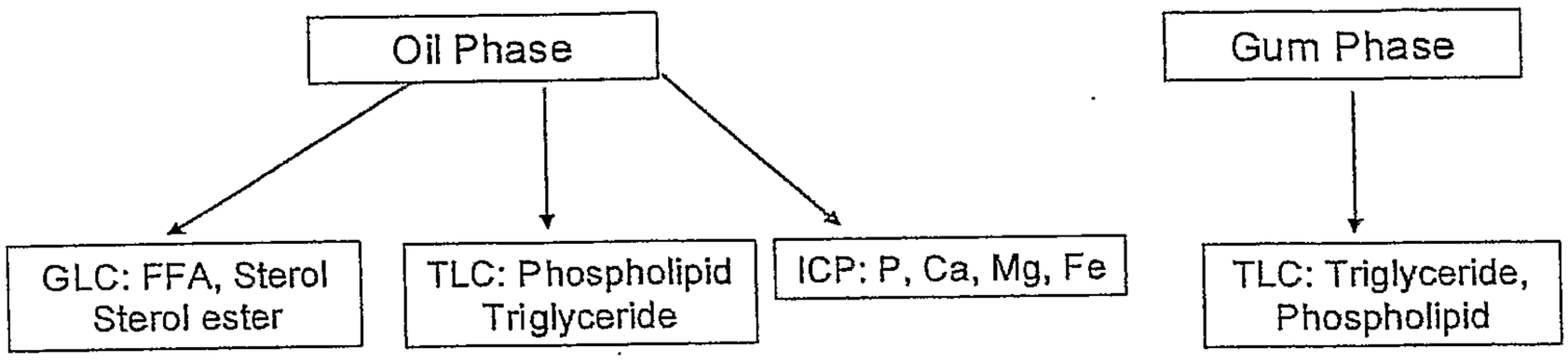

Oil Phase
Gum Phase
GLC: FFA, Sterol
Sterol ester
TLC: Phospholipid
Triglyceride
ICP: P, Ca, Mg, Fe
TLC: Triglyceride,
Phospholipid

FIGURE 77

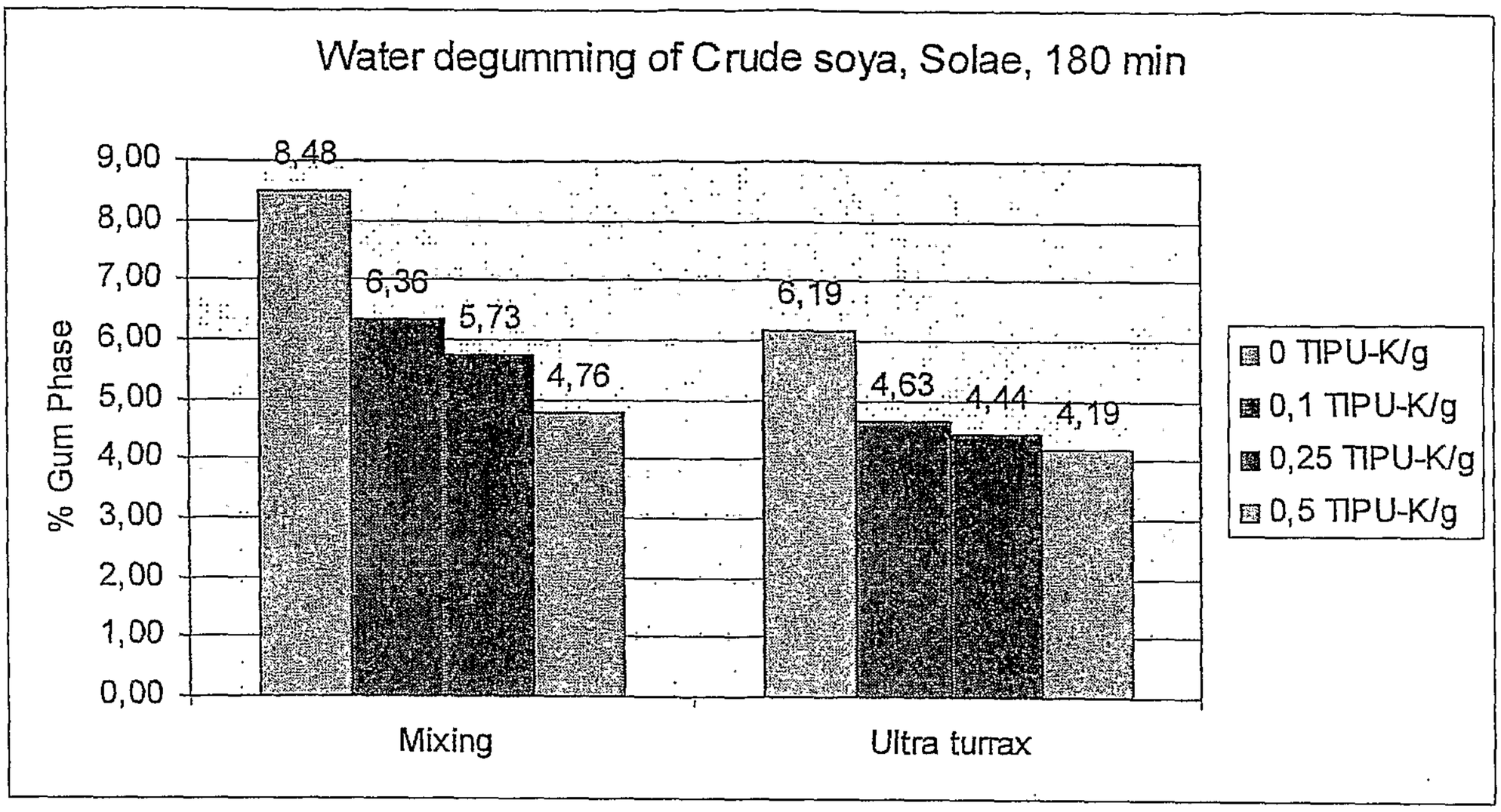

Water degumming of Crude soya, Solae, 180 min
% Gum Phase
9,00
8,00
7,00
6,00
5,00
4,00
3,00
2,00
1,00
0,00
8,48
6,36
5,73
4,76
6,19
4,63
4,44
4,19
Mixing
Ultra turrax
0 TIPU-K/g
0,1 TIPU-K/g
0,25 TIPU-K/g
0,5 TIPU-K/g

FIGURE 78

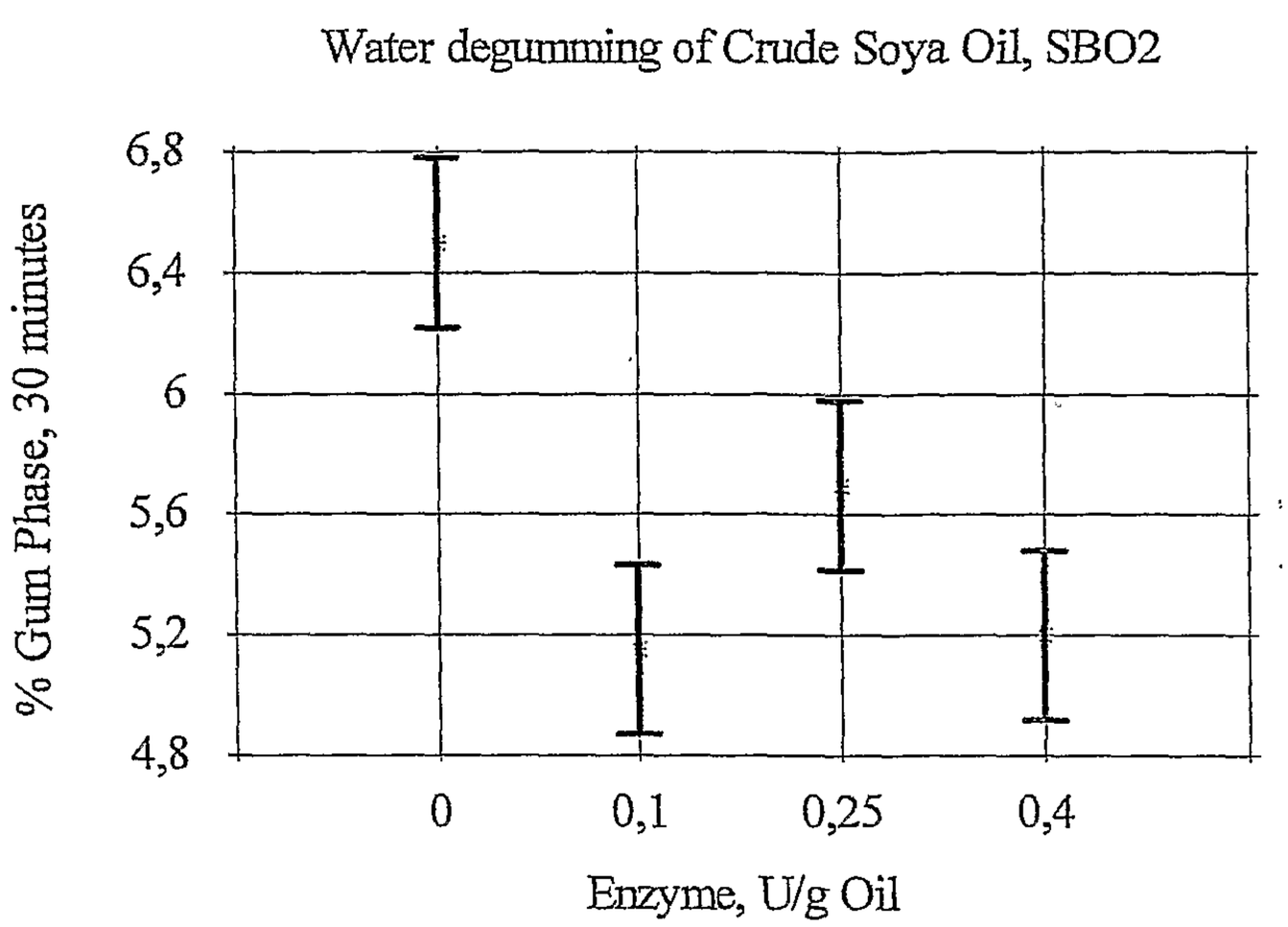

Water degumming of Crude Soya Oil, SBO2
% Gum Phase, 30 minutes
6,8
6,4
6
5,6
5,2
4,8
0
0,1
0,25
0,4
Enzyme, U/g Oil

FIGURE 79

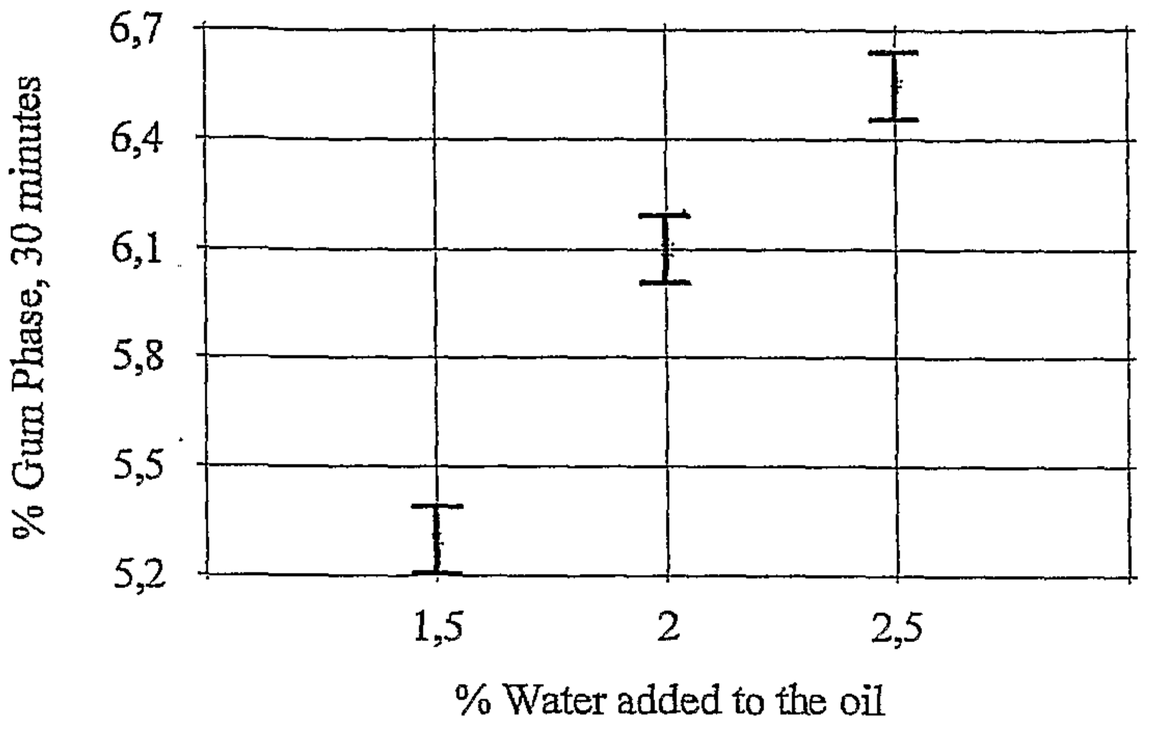

Water degumming of Crude Soya Oil, SBO2 with 1.5, 2.0 or 2.5% water
6,7
6,4
6,1
5,8
5,5
5,2
% Gum Phase, 30 minutes
1,5
2
2,5
% Water added to the oil

FIGURE 80

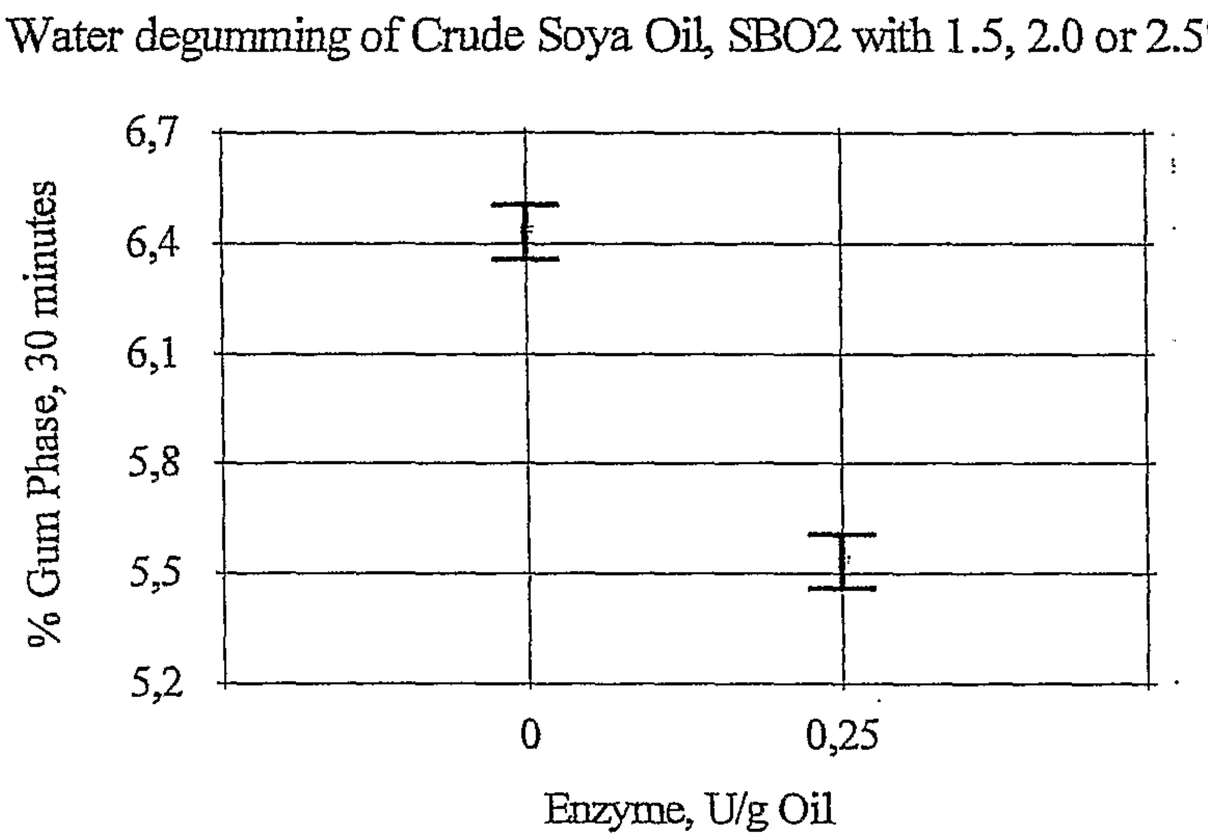

Water degumming of Crude Soya Oil, SBO2 with 1.5, 2.0 or 2.5% water
% Gum Phase, 30 minutes
6,7
6,4
6,1
5,8
5,5
5,2
0
0,25
Enzyme, U/g Oil

FIGURE 81

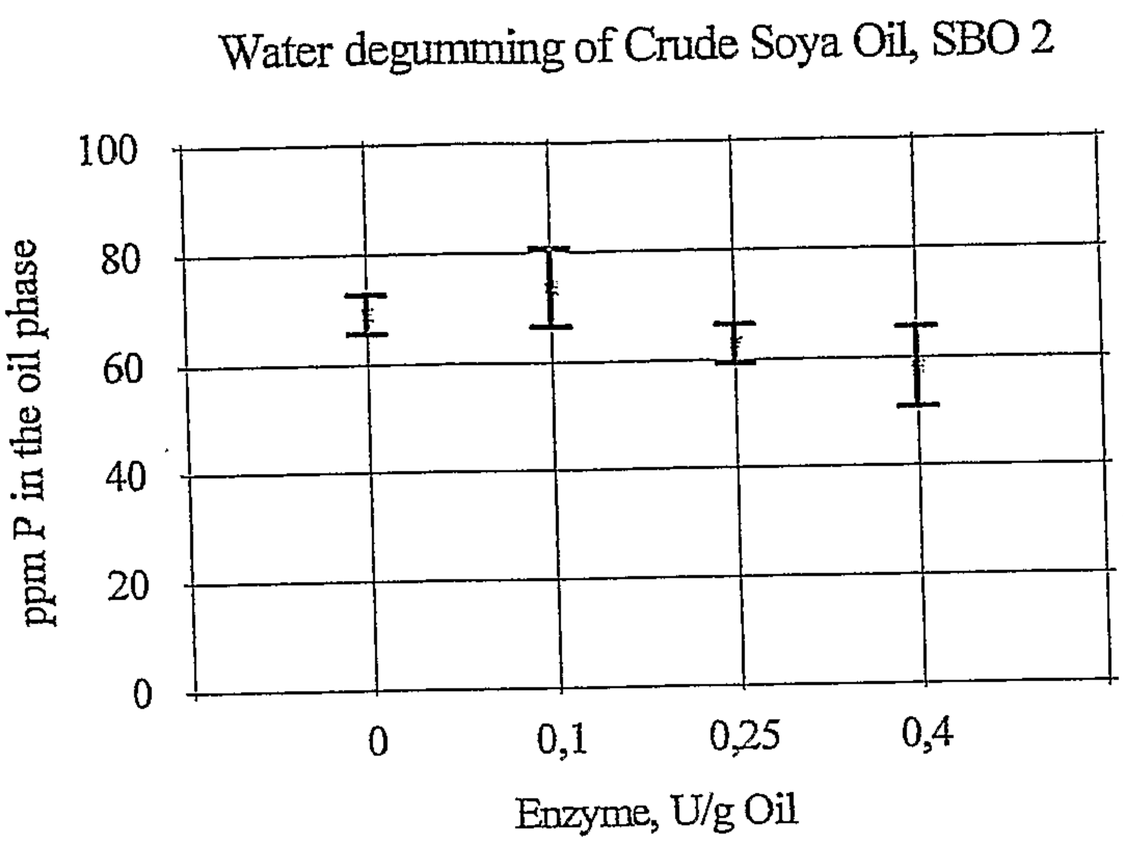

Water degumming of Crude Soya Oil, SBO 2
ppm P in the oil phase
100
80
60
40
20
0
0
0,1
0,25
0,4
Enzyme, U/g Oil

FIGURE 82

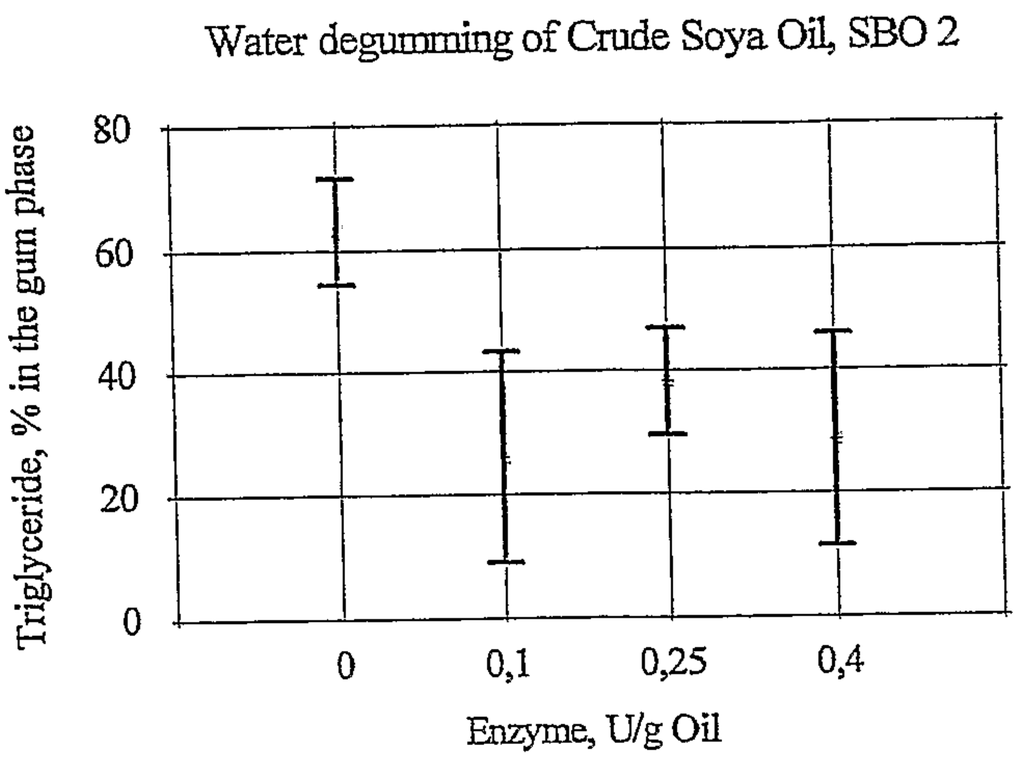

Water degumming of Crude Soya Oil, SBO 2
Triglyceride, % in the gum phase
80
60
40
20
0
0
0,1
0,25
0,4
Enzyme, U/g Oil

FIGURE 83

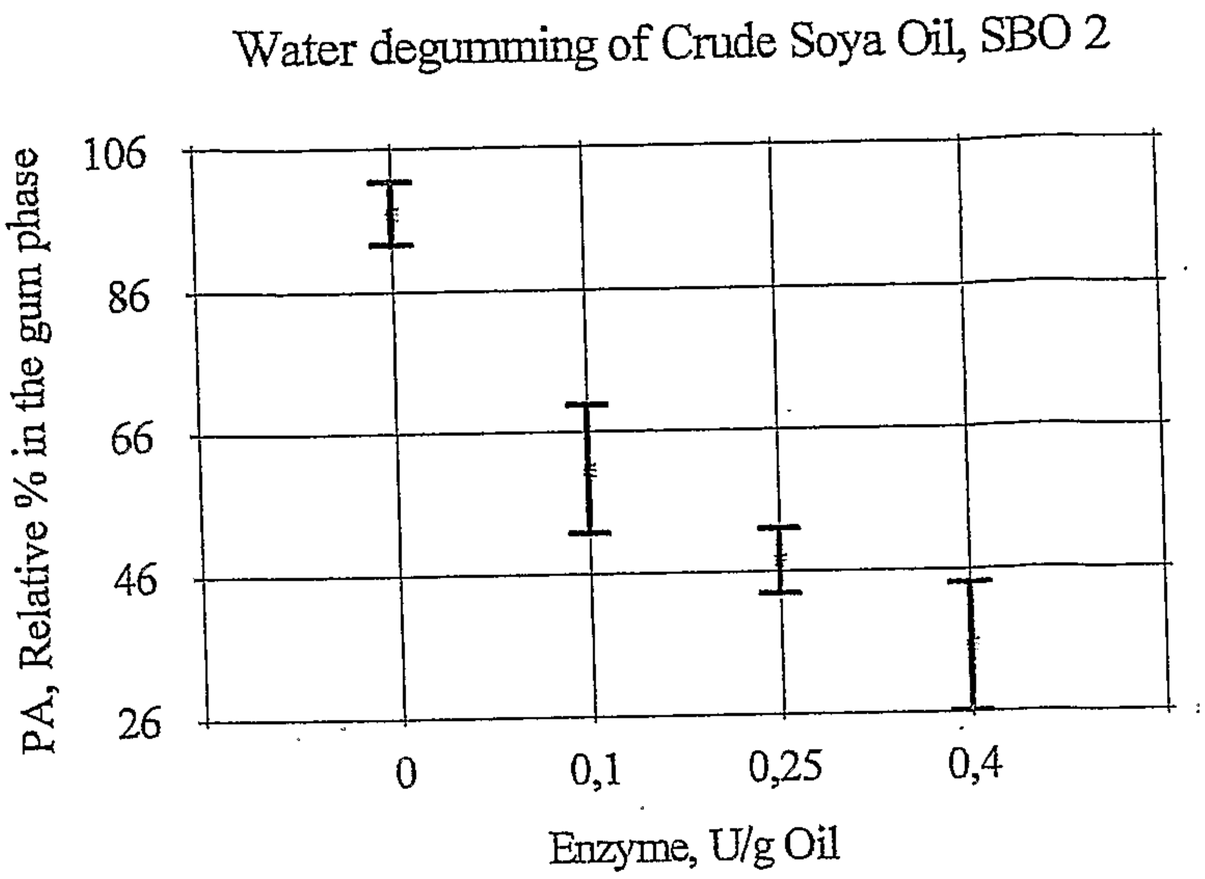

Water degumming of Crude Soya Oil, SBO 2
PA, Relative % in the gum phase
106
86
66
46
26
0
0,1
0,25
0,4
Enzyme, U/g Oil

FIGURE 84

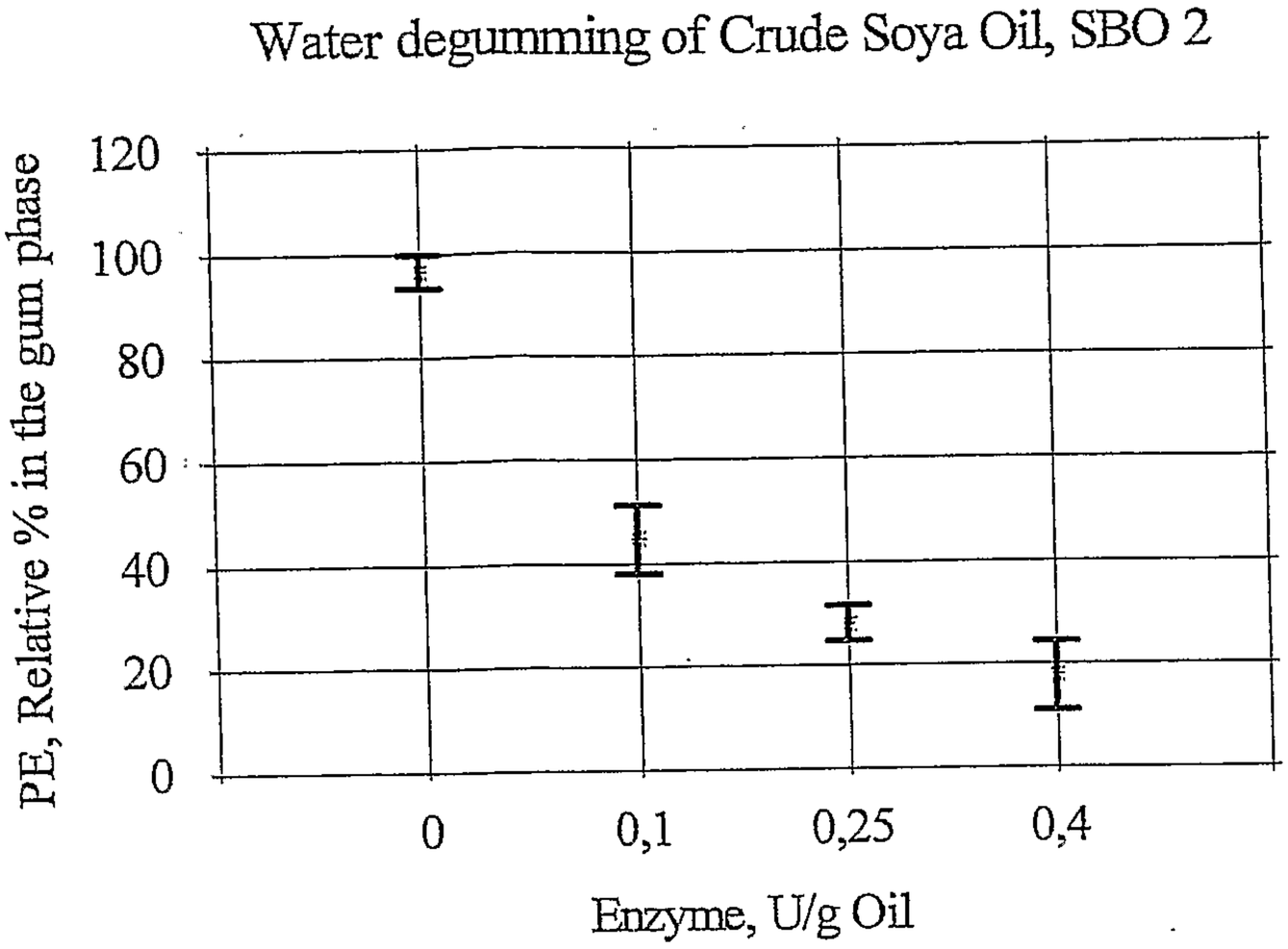

Water degumming of Crude Soya Oil, SBO 2
PE, Relative % in the gum phase
120
100
80
60
40
20
0
0
0,1
0,25
0,4
Enzyme, U/g Oil

FIGURE 85

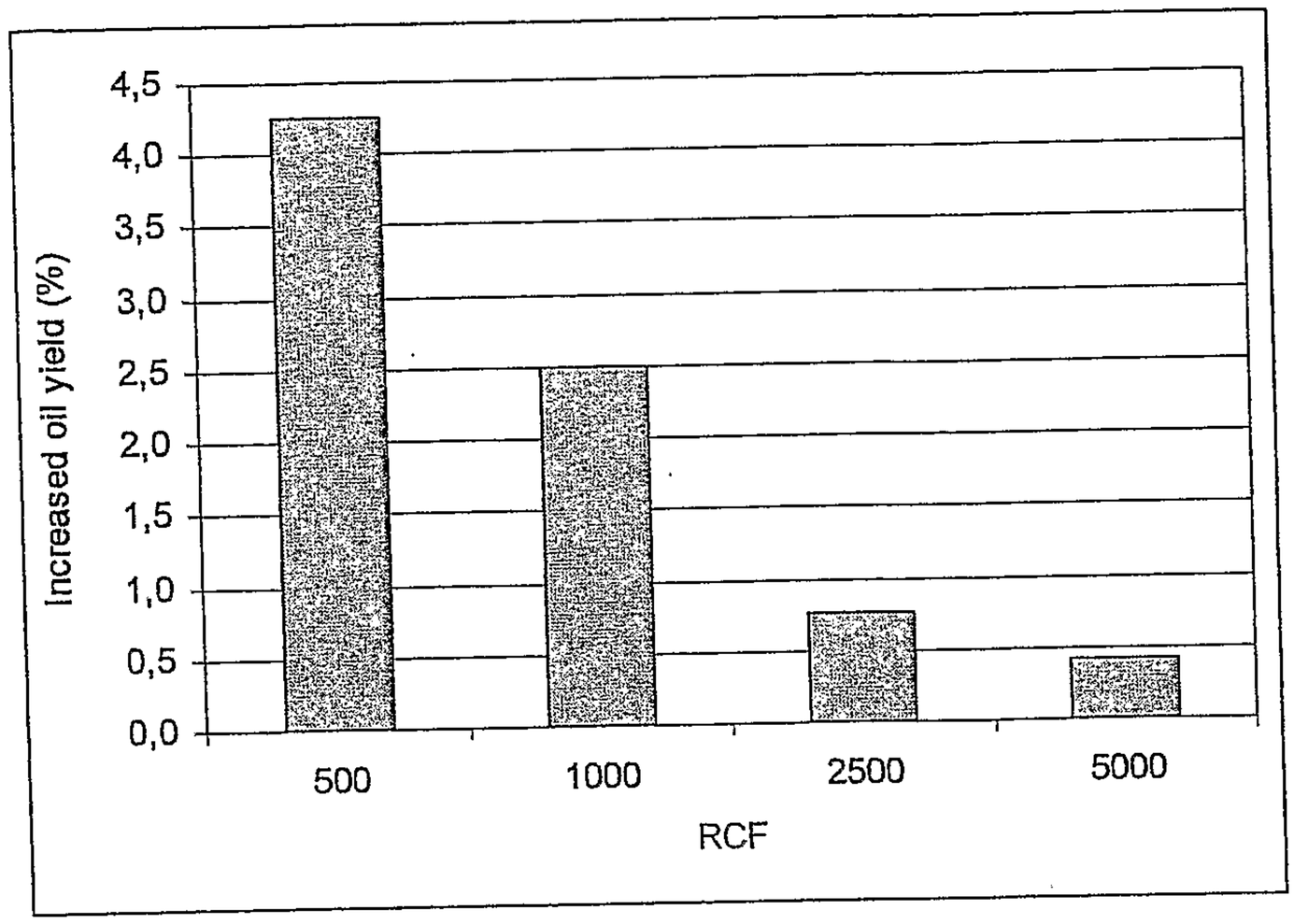

Increased oil yield (%)
4,5
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
500
1000
2500
5000
RCF

FIGURE 86

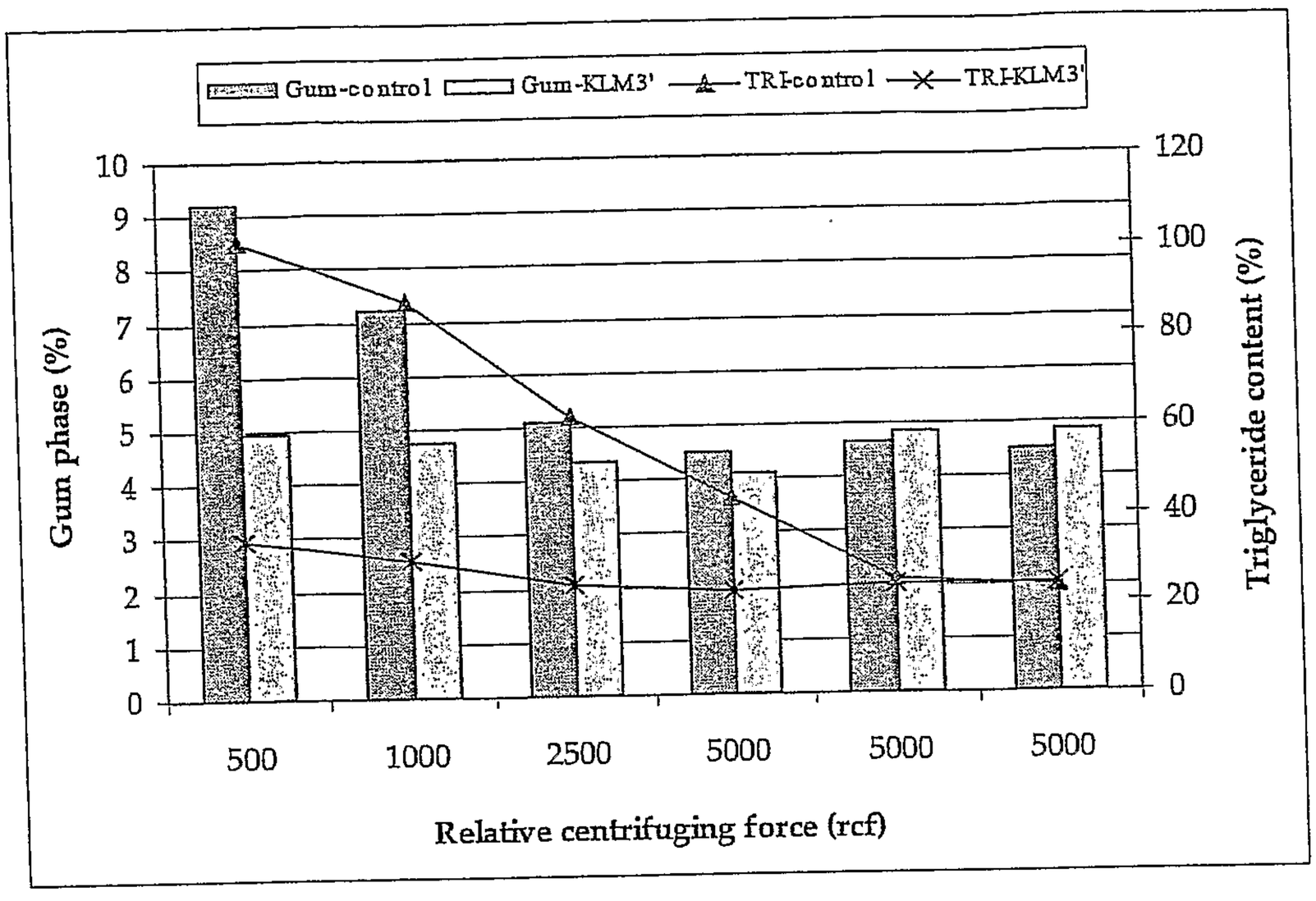

Gum-control
Gum-KLM3'
TRI-control
TRI-KLM3'
Gum phase (%)
Triglyceride content (%)
0
1
2
3
4
5
6
7
8
9
10
0
20
40
60
80
100
120
500
1000
2500
5000
5000
5000
Relative centrifuging force (rcf)

FIGURE 87

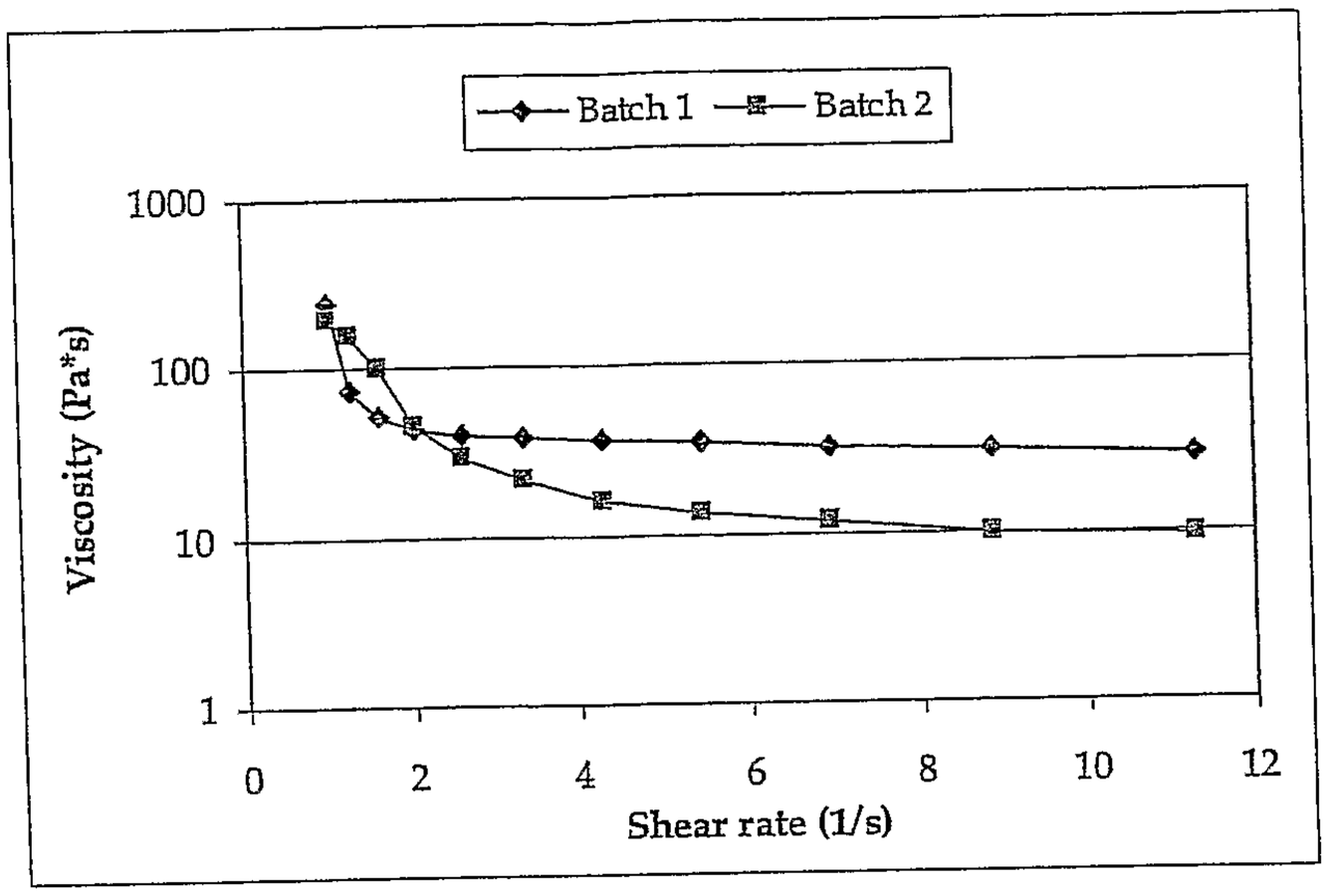

Batch 1
Batch 2
1000
100
10
1
Viscosity (Pa*s)
0
2
4
6
8
10
12
Shear rate (1/s)

FIGURE 88

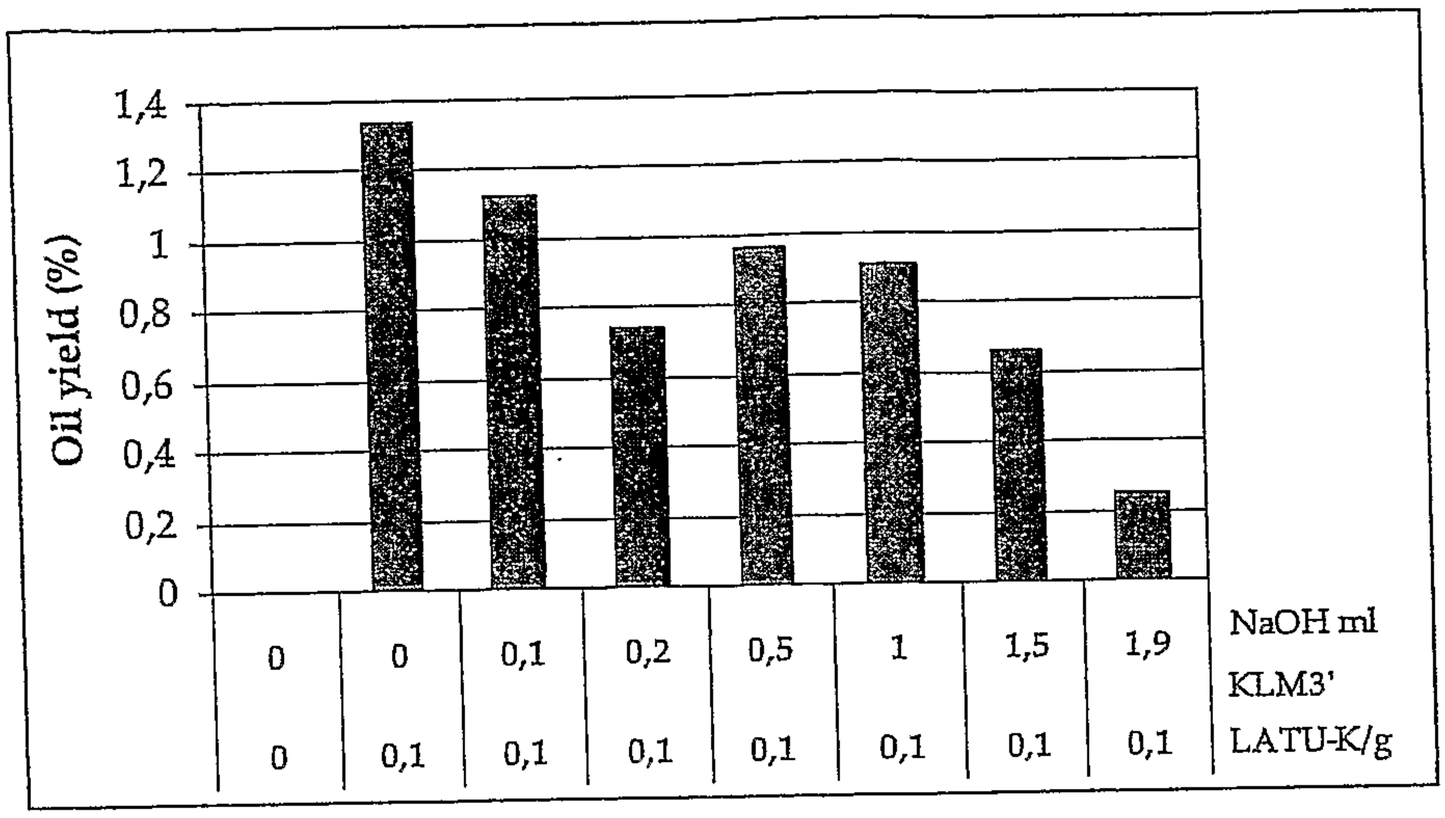

Oil yield (%)
1,4
1,2
1
0,8
0,6
0,4
0,2
0
NaOH ml
KLM3'
LATU-K/g
0 0 0,1 0,2 0,5 1 1,5 1,9
0 0,1 0,1 0,1 0,1 0,1 0,1 0,1

FIGURE 89

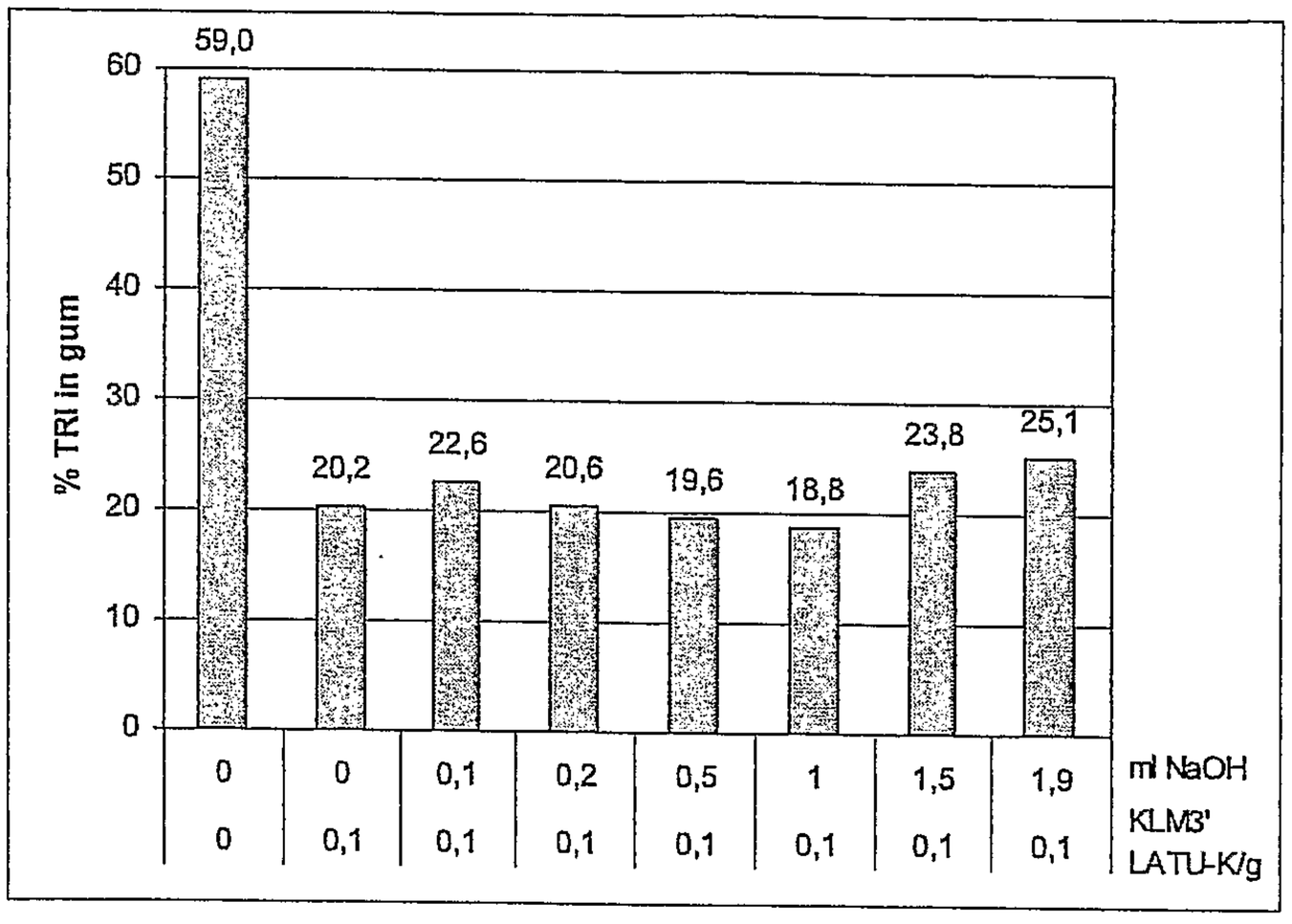
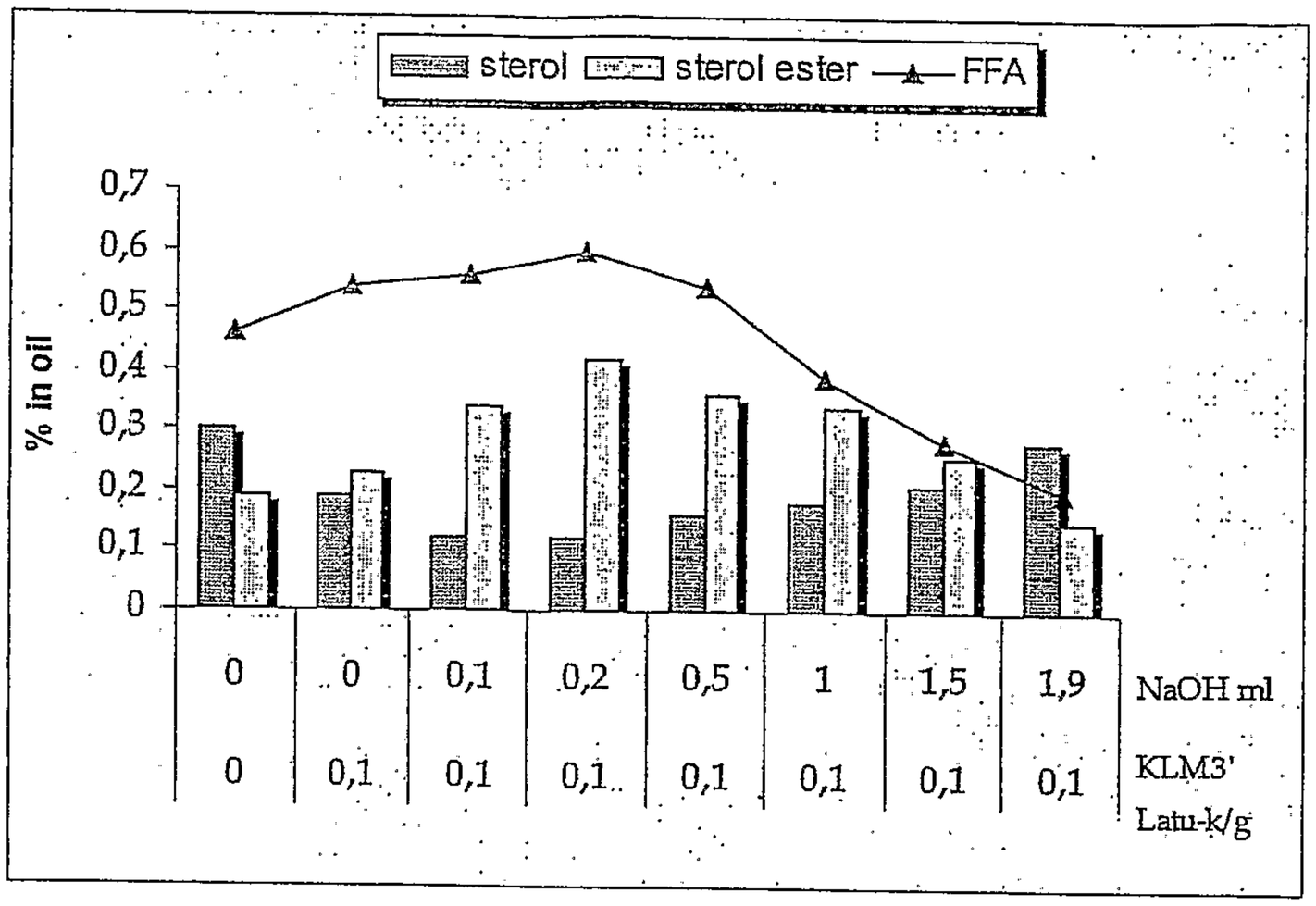

% TRI in gum
60
50
40
30
20
10
0
59,0
20,2
22,6
20,6
19,6
18,8
23,8
25,1
0
0
0,1
0,2
0,5
1
1,5
1,9
ml NaOH
0
0,1
0,1
0,1
0,1
0,1
0,1
0,1
KLM3'
LATU-K/g

FIGURE 90



	0	0	0,1	0,2	0,5	1	1,5	1,9	NaOH ml
	0	0,1	0,1	0,1	0,1	0,1	0,1	0,1	KLM3' Latu-k/g

FIGURE 91

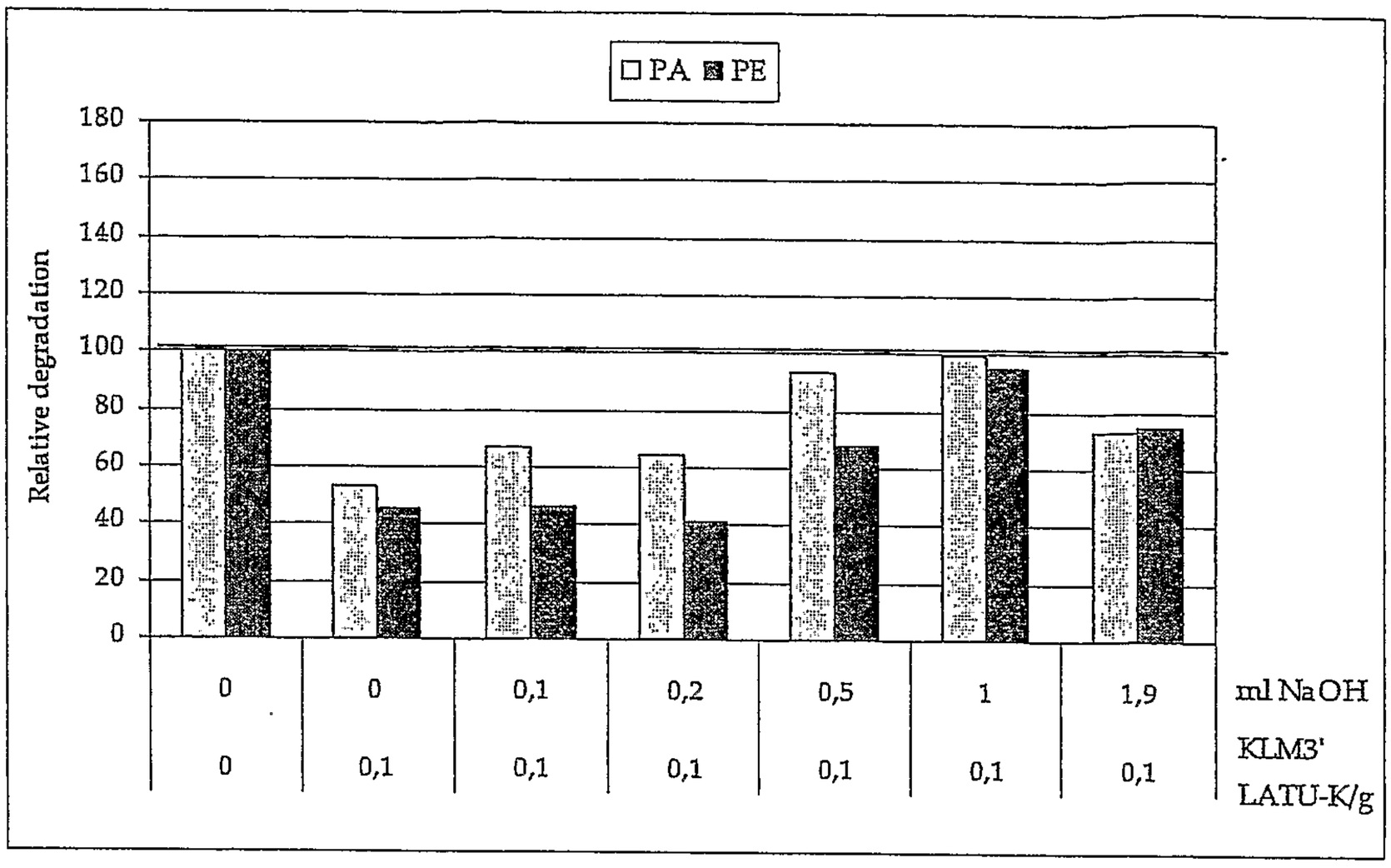
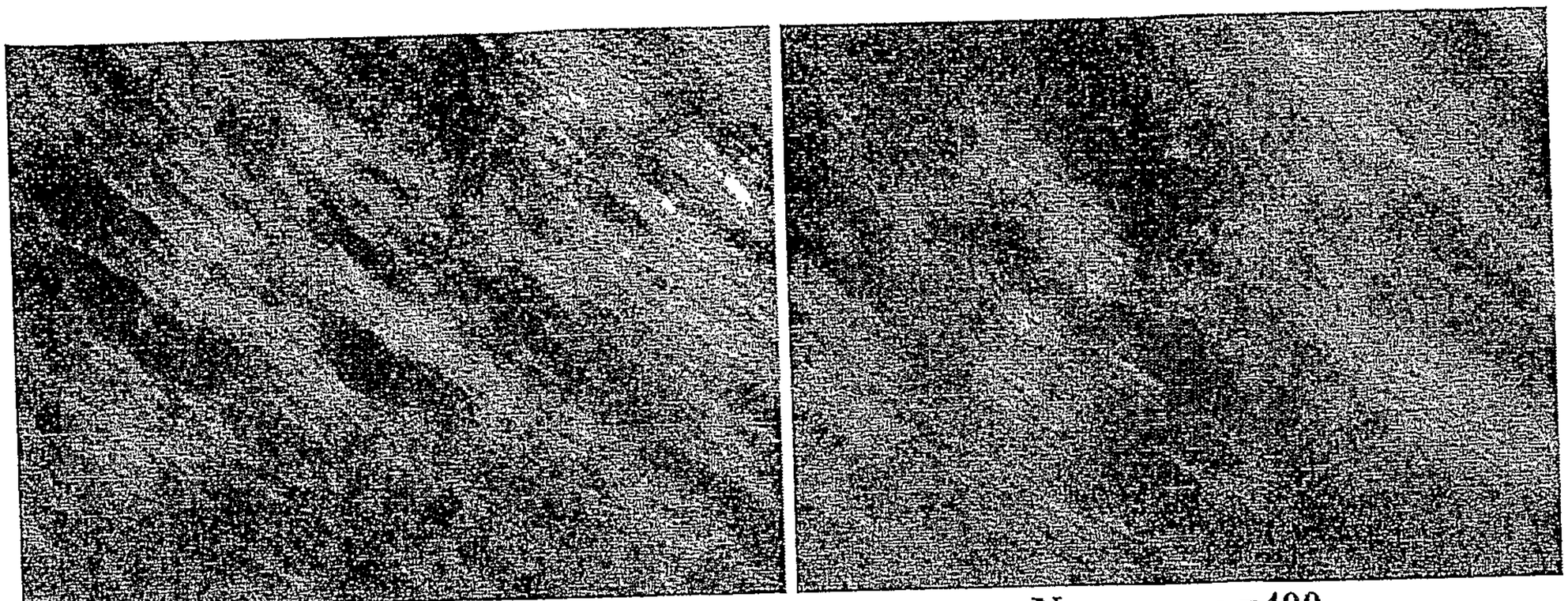

PA
PE
Relative degradation
180
160
140
120
100
80
60
40
20
0
0
0
0,1
0,2
0,5
1
1,9
ml NaOH
0
0,1
0,1
0,1
0,1
0,1
0,1
KLM3'
LATU-K/g

FIGURE 92

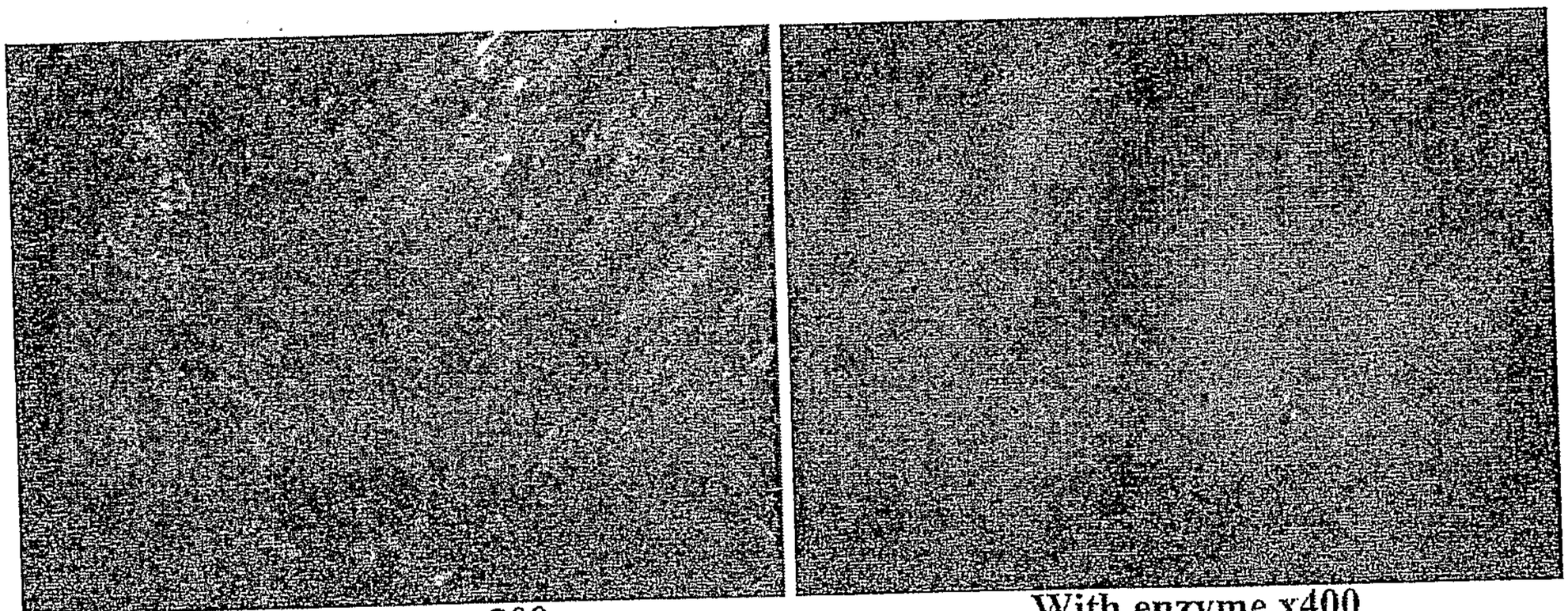


No enzyme x200

2460-183 ax20a 25°C

No enzyme x400

2460-183 ax40a 25°C



With enzyme x200

2460-183 bx20a 25°C

With enzyme x400

2460-183 bx40a 25°C

FIG. 93

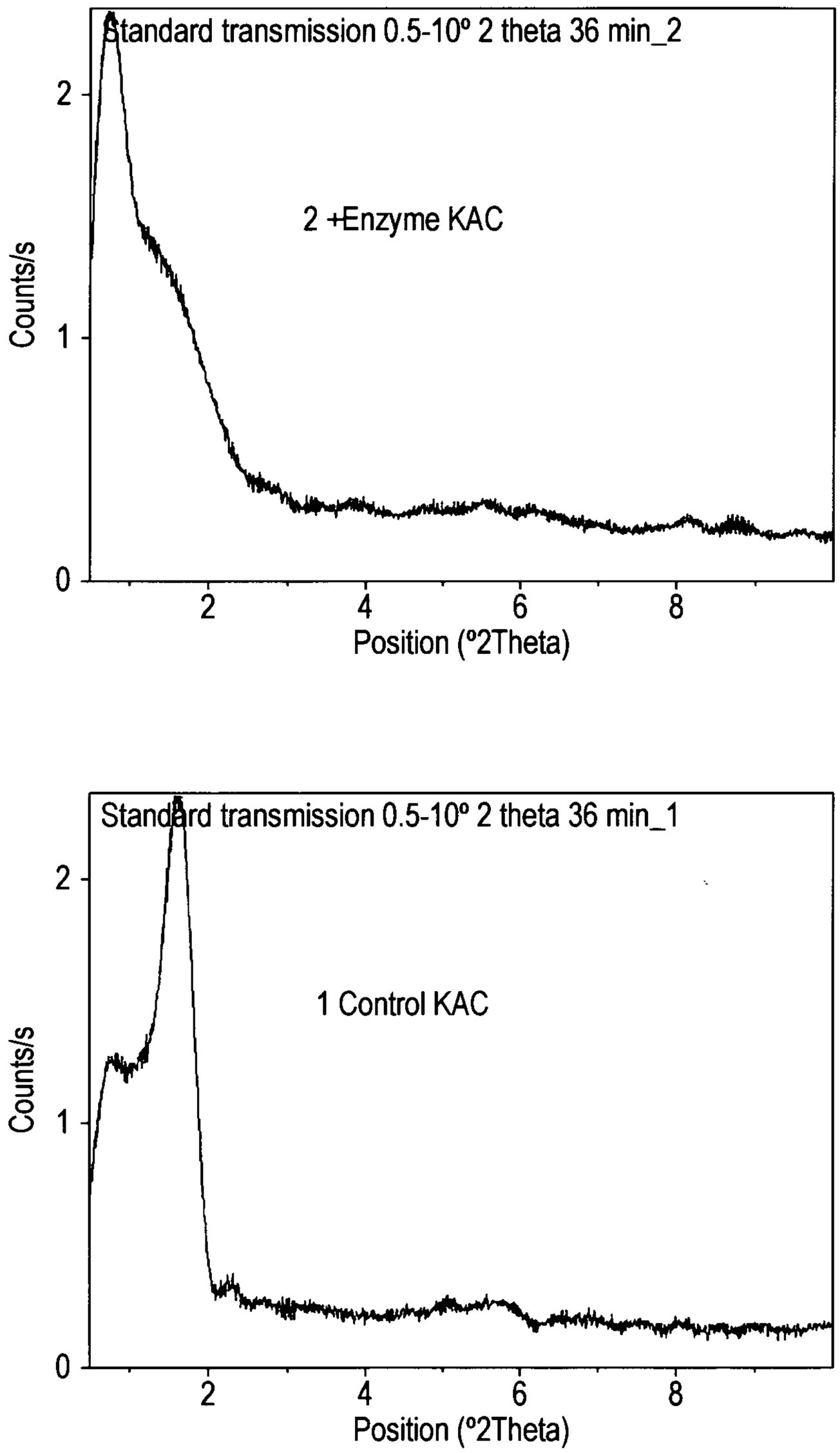

Standard transmission 0.5-10º 2 theta 36 min_2
2 +Enzyme KAC
Counts/s
0
1
2
2
4
6
8
Position (º2Theta)
Standard transmission 0.5-10º 2 theta 36 min_1
1 Control KAC
Counts/s
0
1
2
2
4
6
8
Position (º2Theta)

FIG. 94

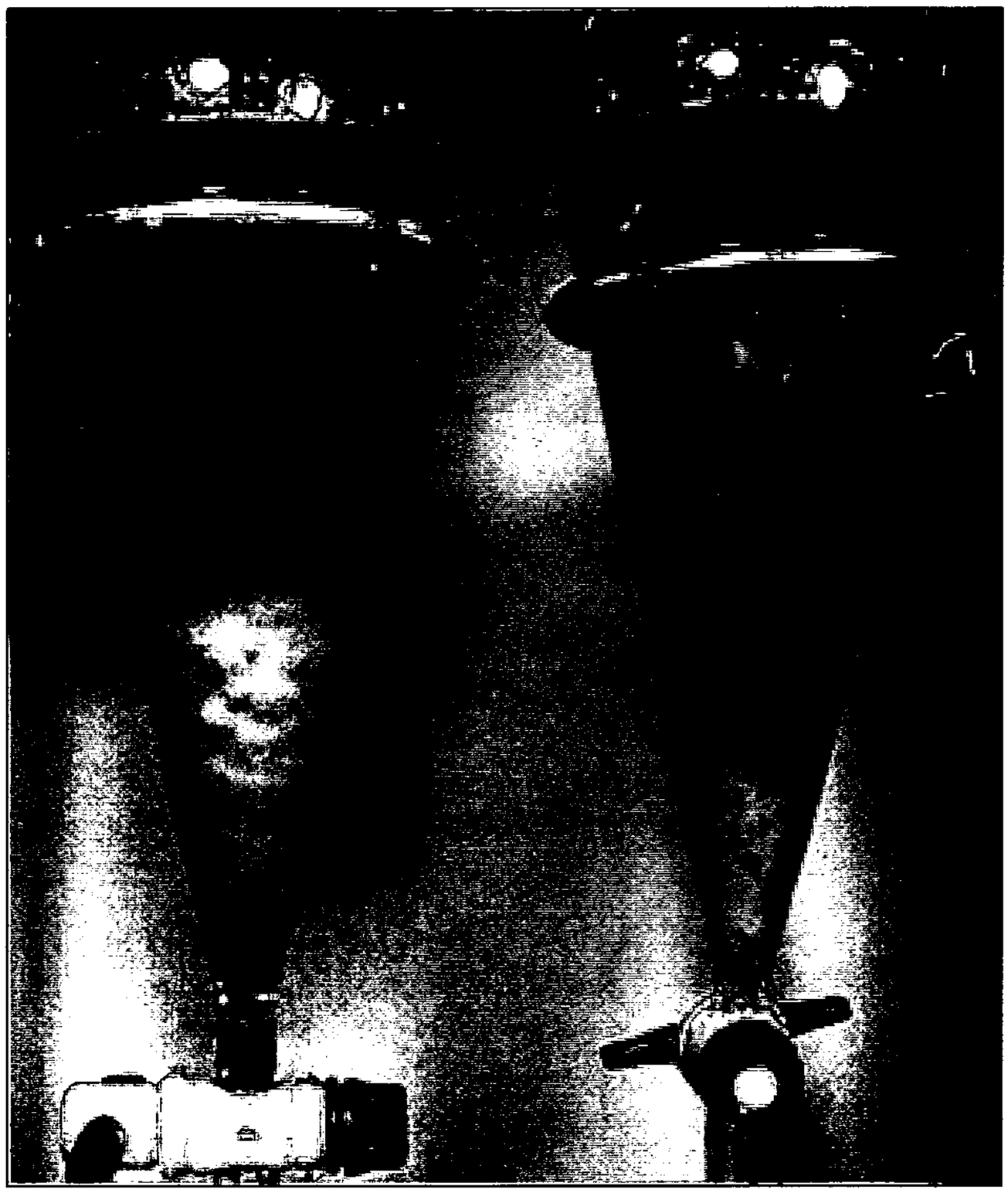
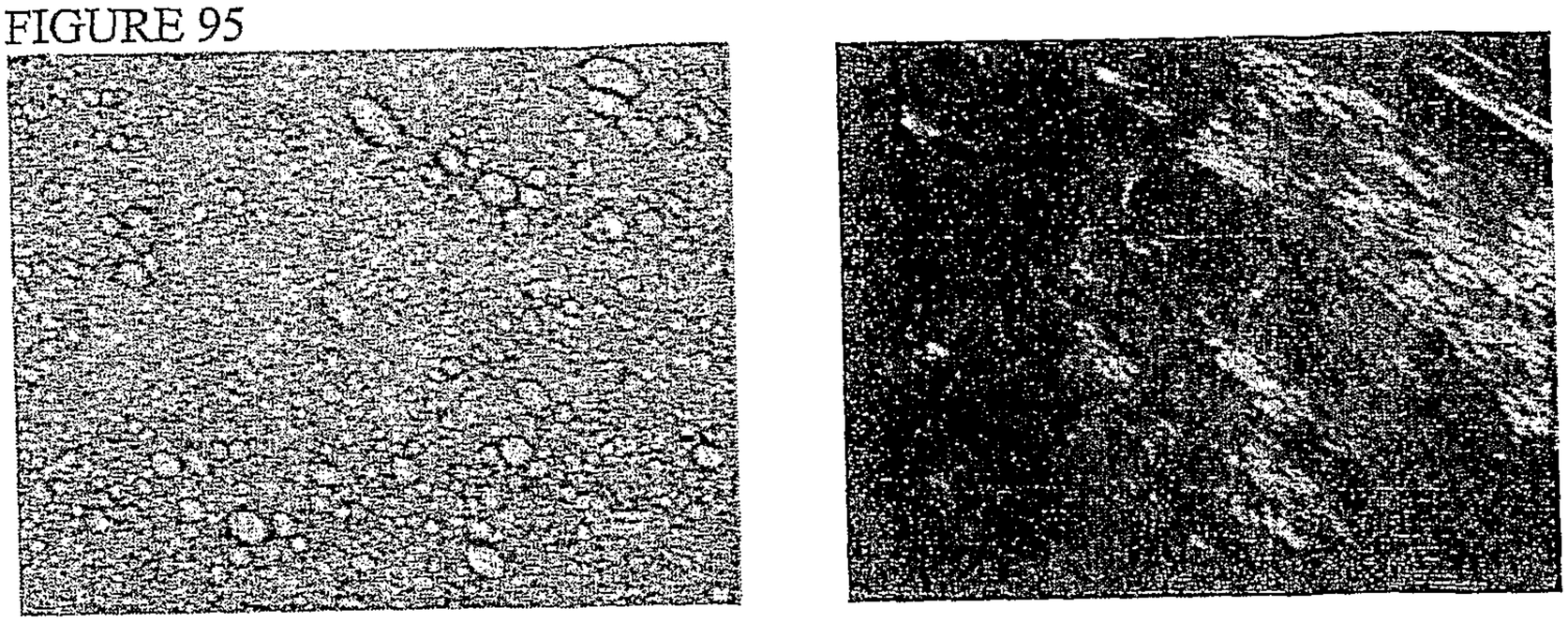


FIGURE 95



With enzyme No enzyme

FIGURE 96

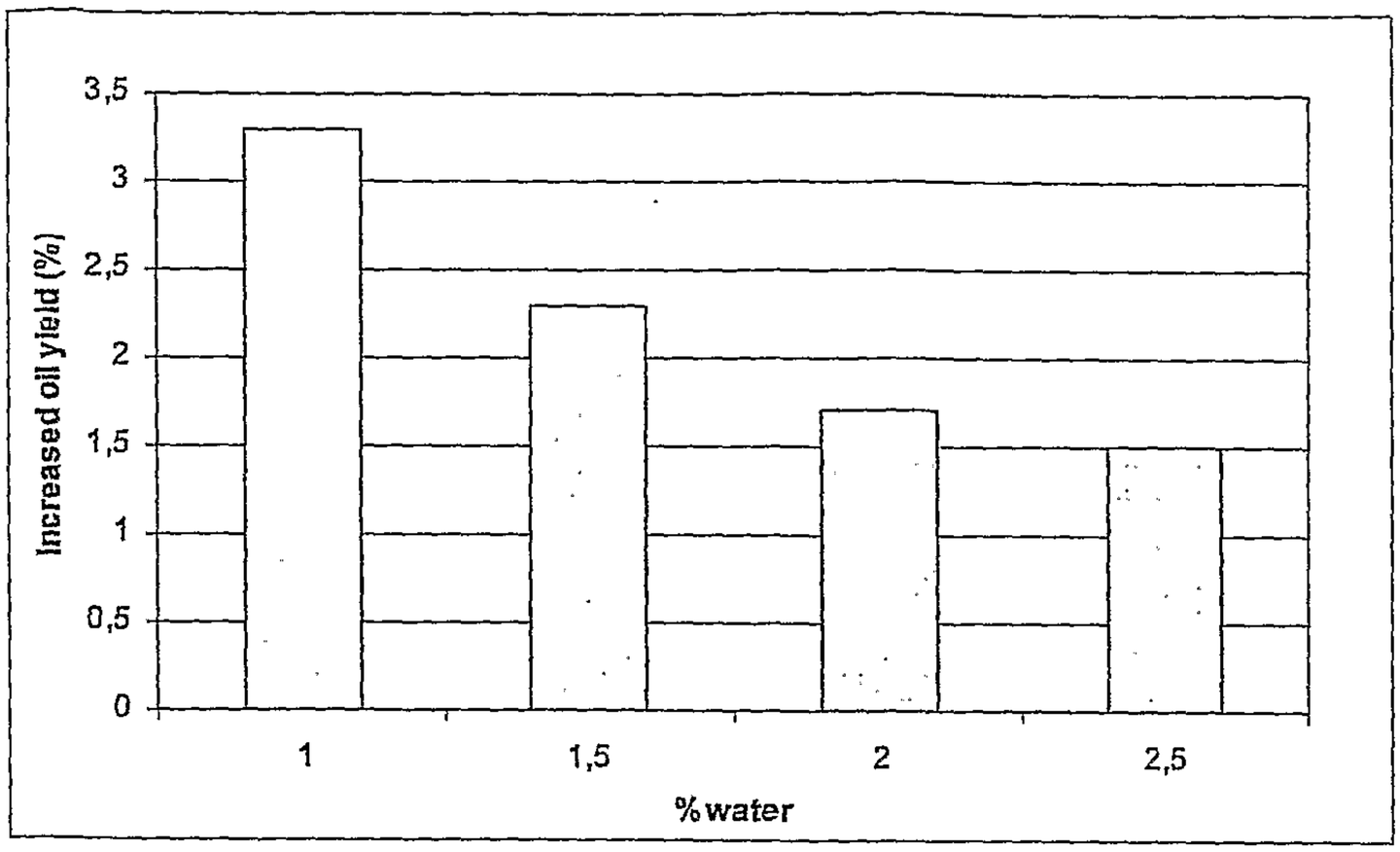


FIGURE 97

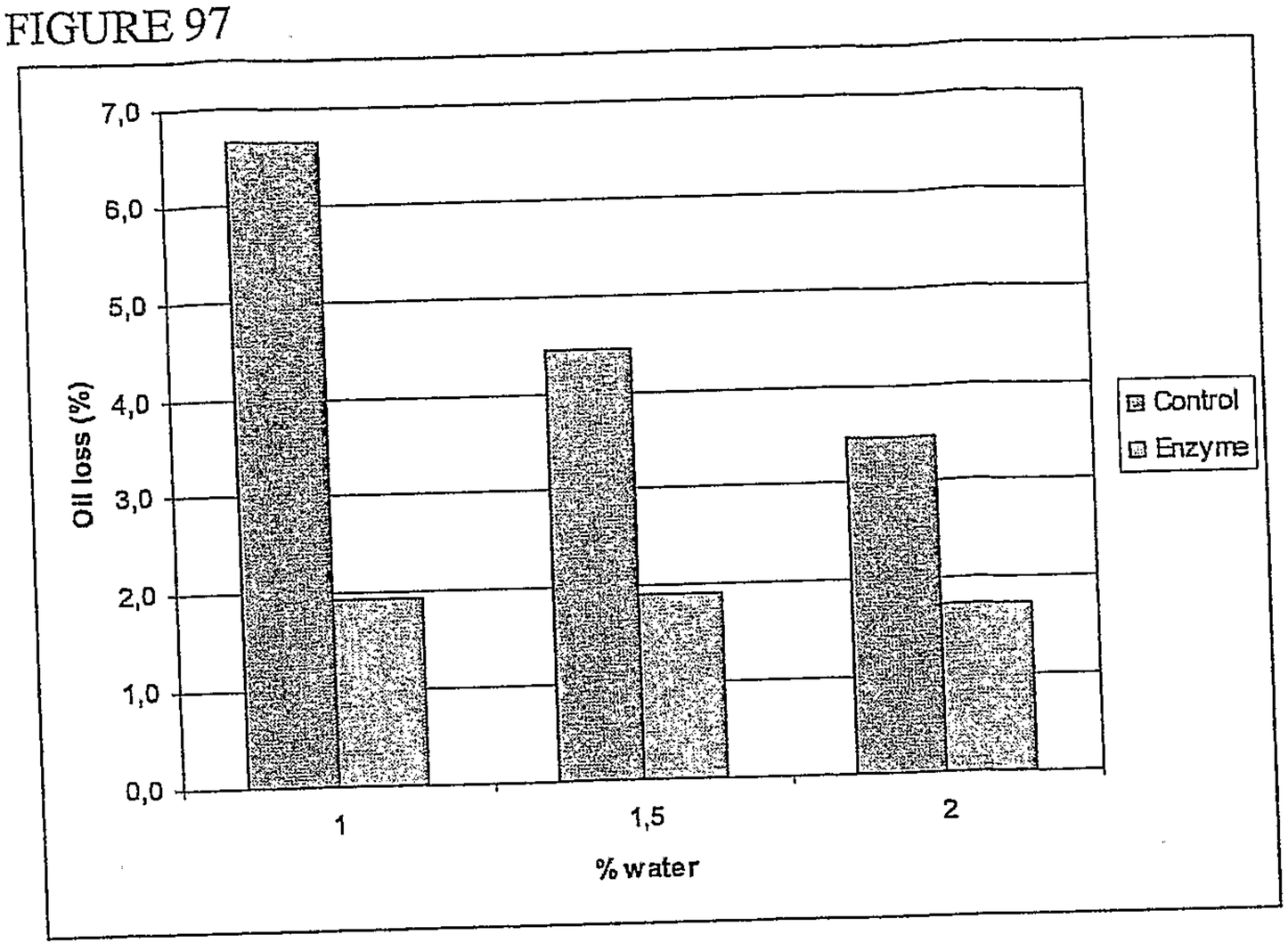
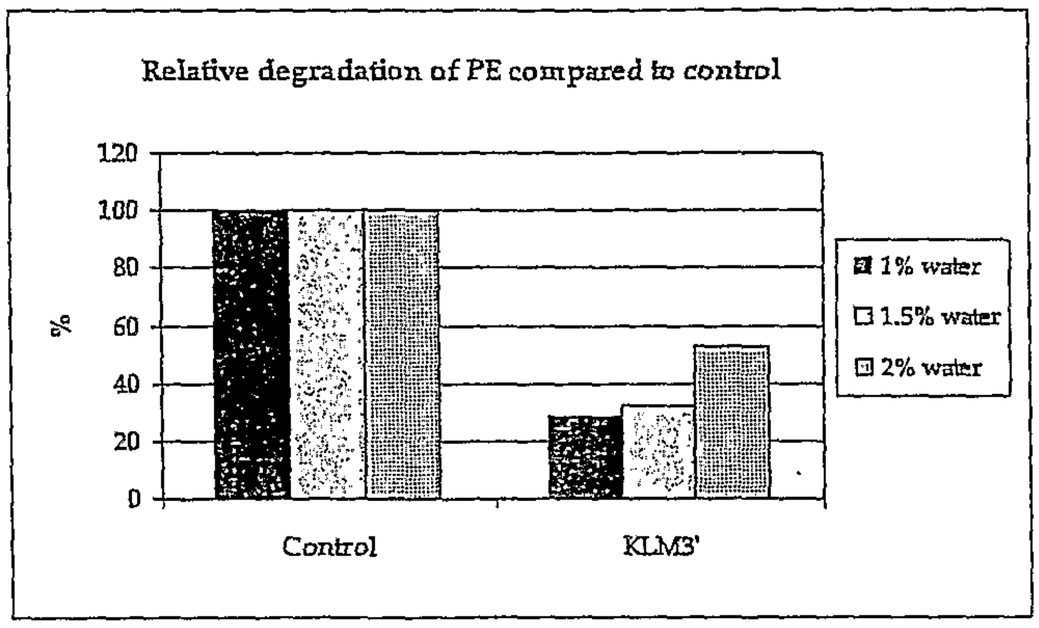


FIGURE 98


Relative degradation of PE compared to control
120
100
80
60
40
20
0
%
Control
KLM3'
1% water
1.5% water
2% water

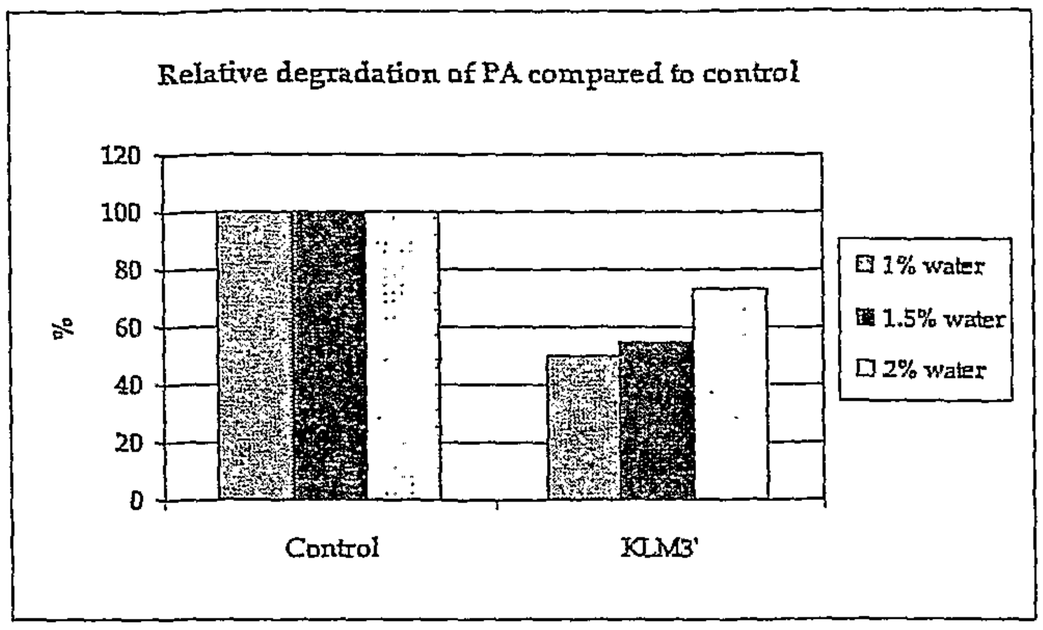

Relative degradation of PA compared to control
120
100
80
60
40
20
0
%
Control
KLM3'
1% water
1.5% water
2% water

FIGURE 99

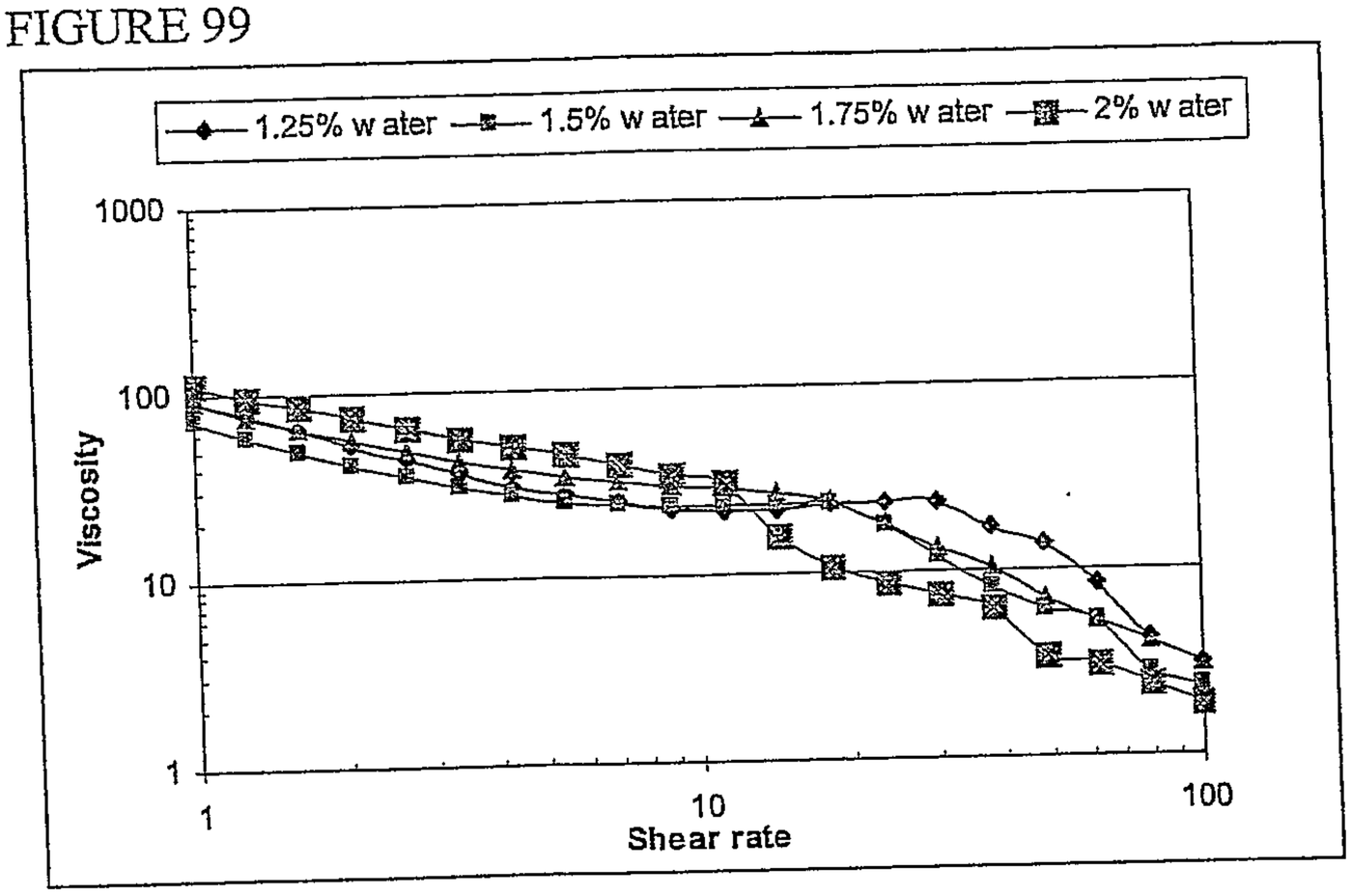

1.25% w ater
1.5% w ater
1.75% w ater
2% w ater
1000
100
10
1
Viscosity
1
10
100
Shear rate

FIGURE 100

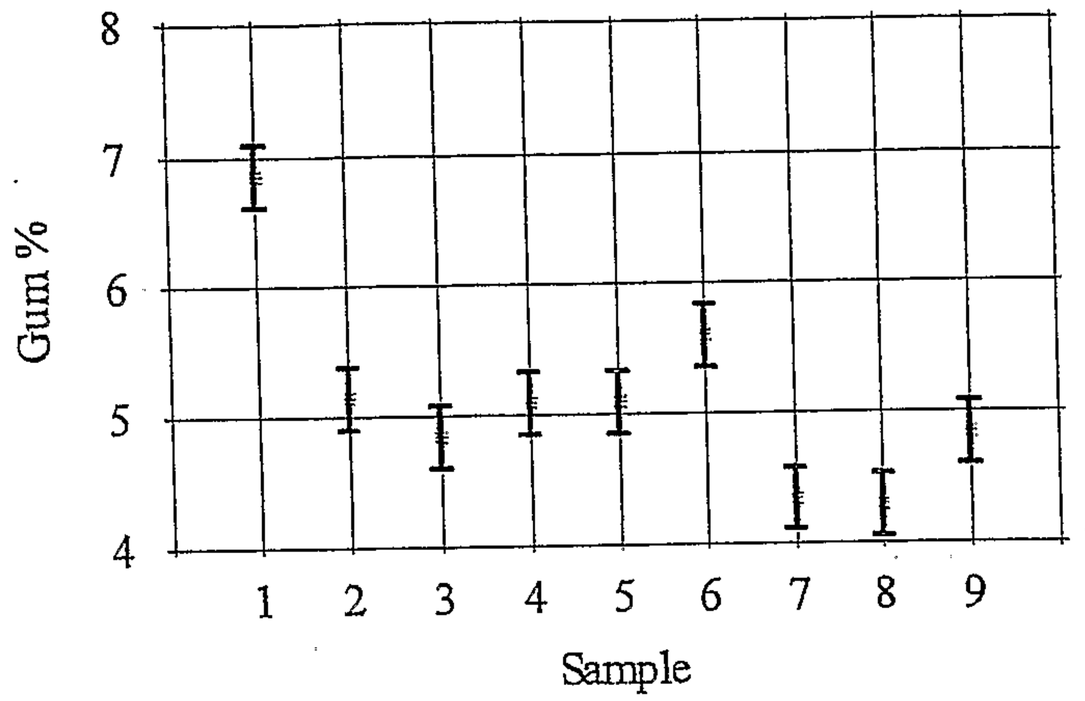
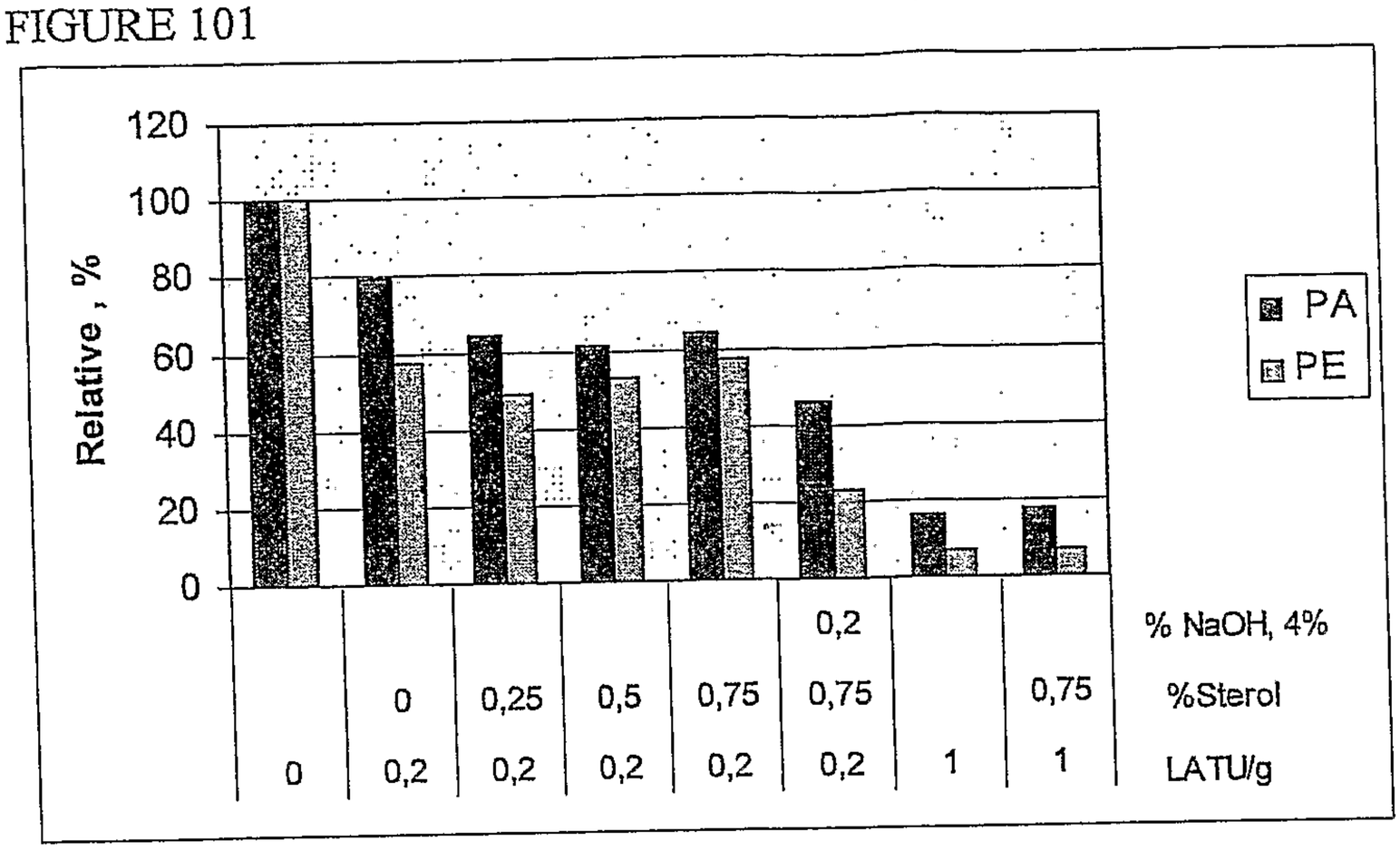
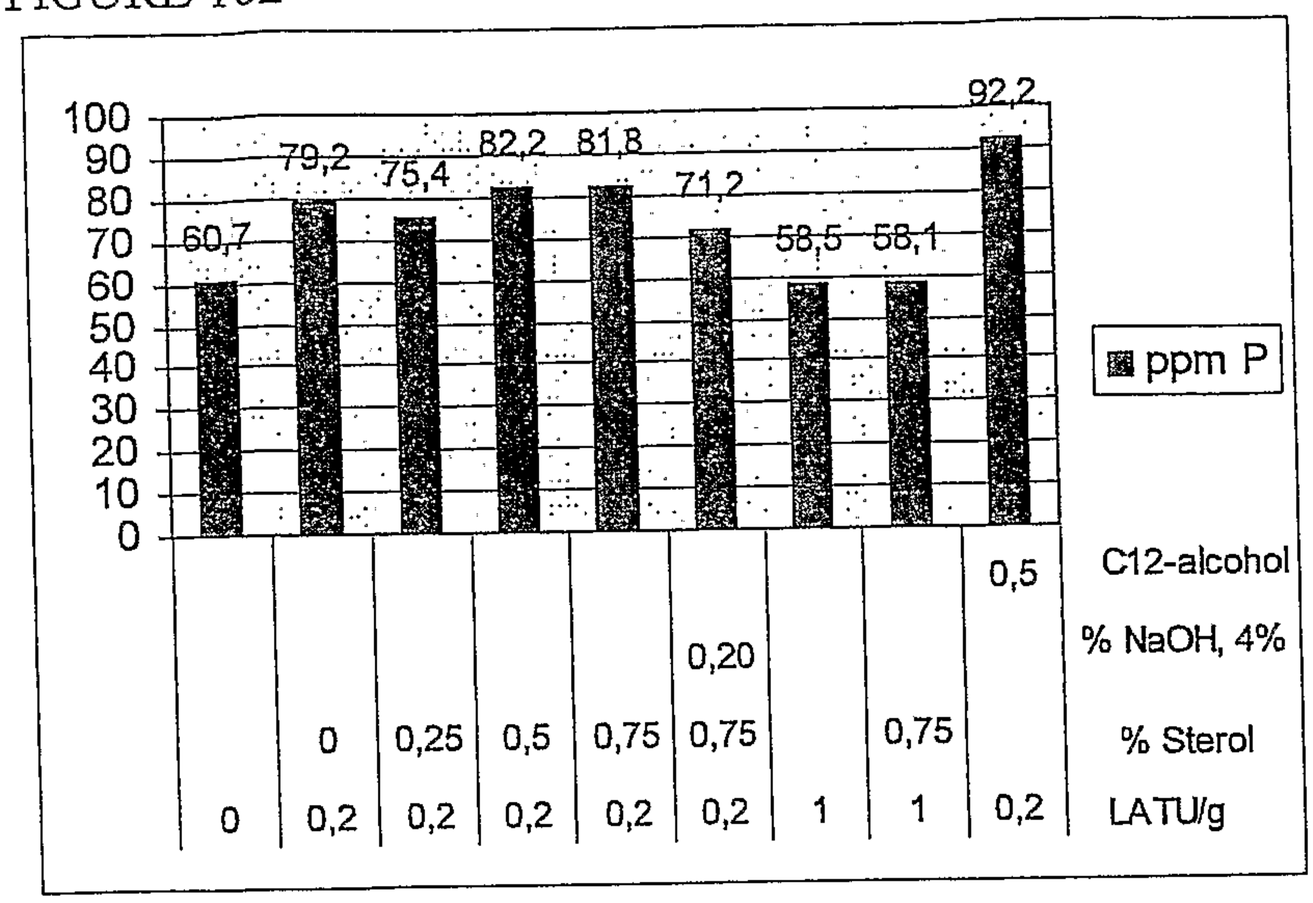


FIGURE 101



						0,2			% NaOH, 4%
		0	0,25	0,5	0,75	0,75		0,75	%Sterol
	0	0,2	0,2	0,2	0,2	0,2	1	1	LATU/g

FIGURE 102



									0,5	C12-alcohol
						0,20				% NaOH, 4%
		0	0,25	0,5	0,75	0,75		0,75		% Sterol
	0	0,2	0,2	0,2	0,2	0,2	1	1	0,2	LATU/g

FIGURE 103

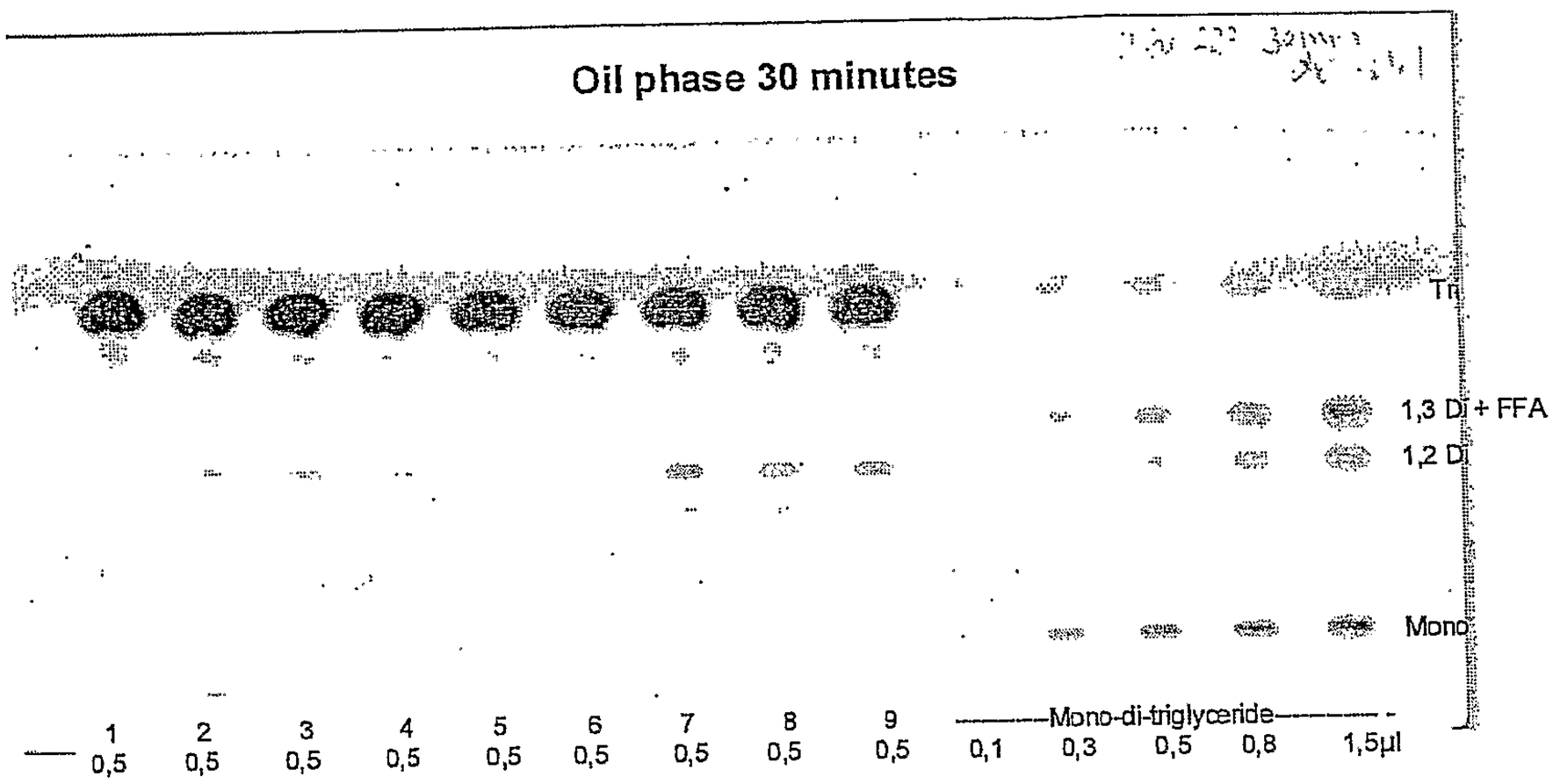

Oil phase 30 minutes
TG
1,3 DG + FFA
1,2 DG
Mono
Mono-di-triglyceride
1 0,5
2 0,5
3 0,5
4 0,5
5 0,5
6 0,5
7 0,5
8 0,5
9 0,5
0,1
0,3
0,5
0,8
1,5µl

FIGURE 104

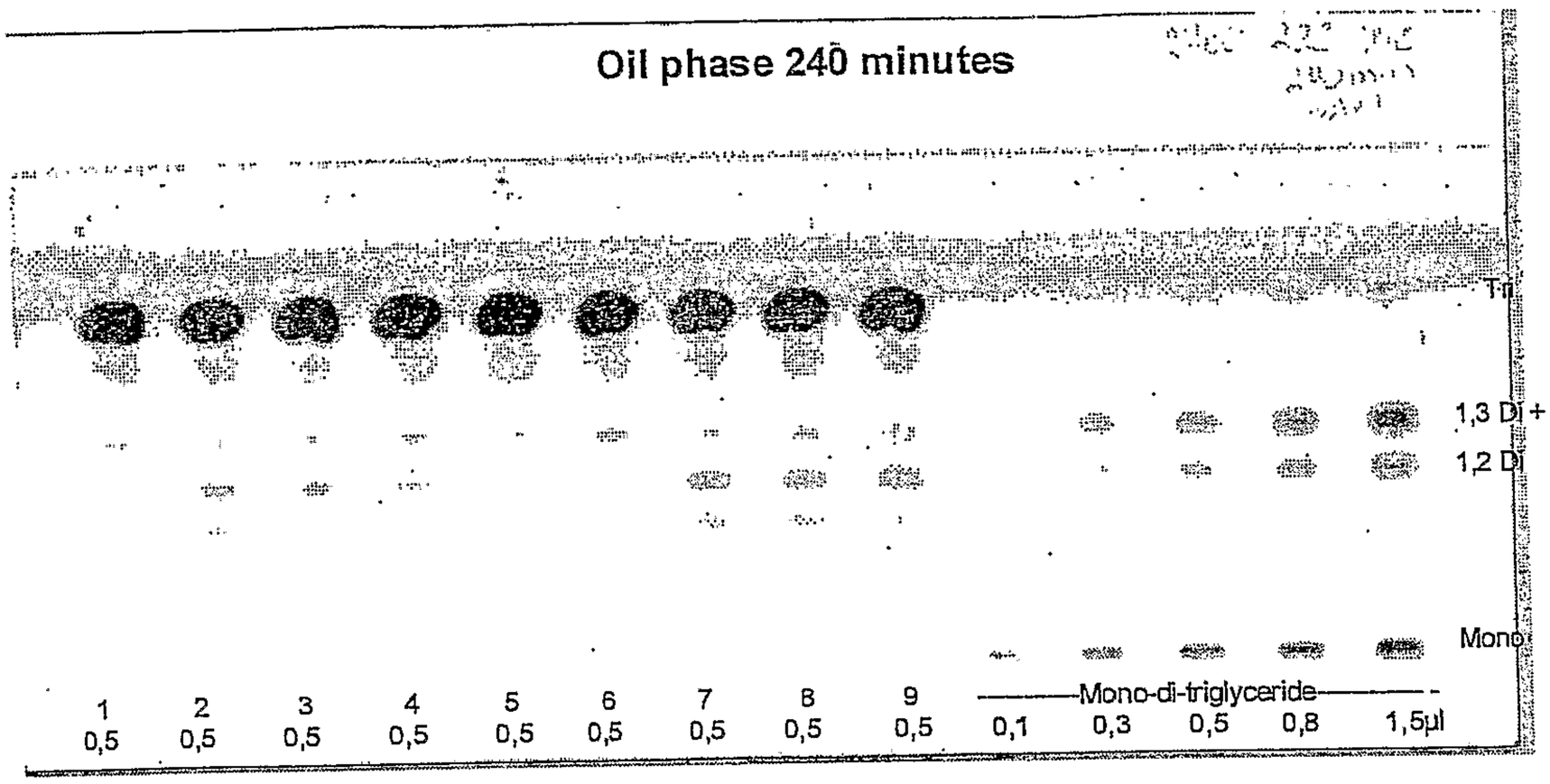

Oil phase 240 minutes
TG
1,3 DG + FFA
1,2 DG
MG
Mono-di-triglyceride
1 2 3 4 5 6 7 8 9 0,1 0,3 0,5 0,8 1,5µl
0,5 0,5 0,5 0,5 0,5 0,5 0,5 0,5 0,5

FIGURE 105

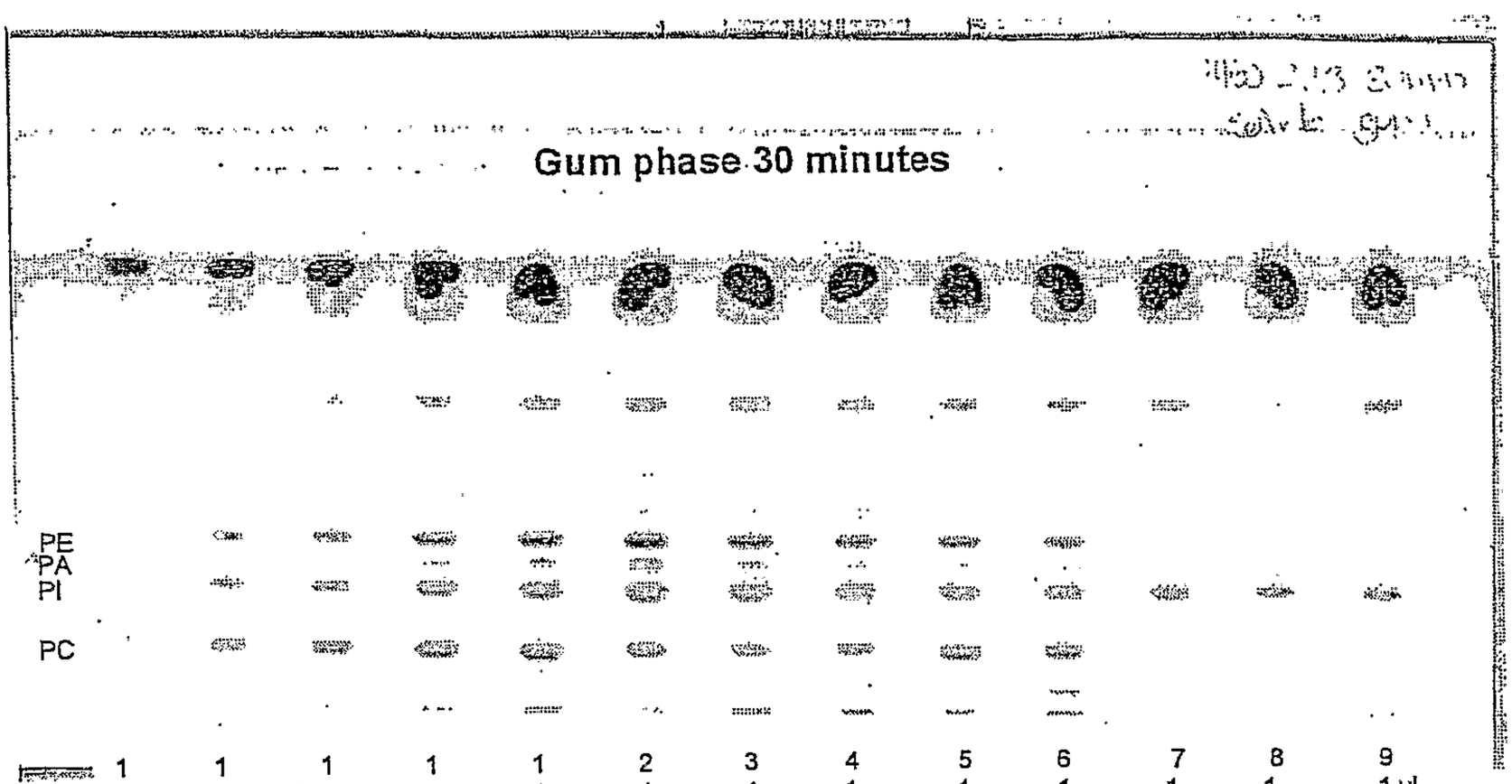

Gum phase 30 minutes
PE
PA
PI
PC
1 1 1 1 1 2 3 4 5 6 7 8 9
0,1 0,3 0,5 0,8 1 1 1 1 1 1 1 1 1 µl

FIGURE 106

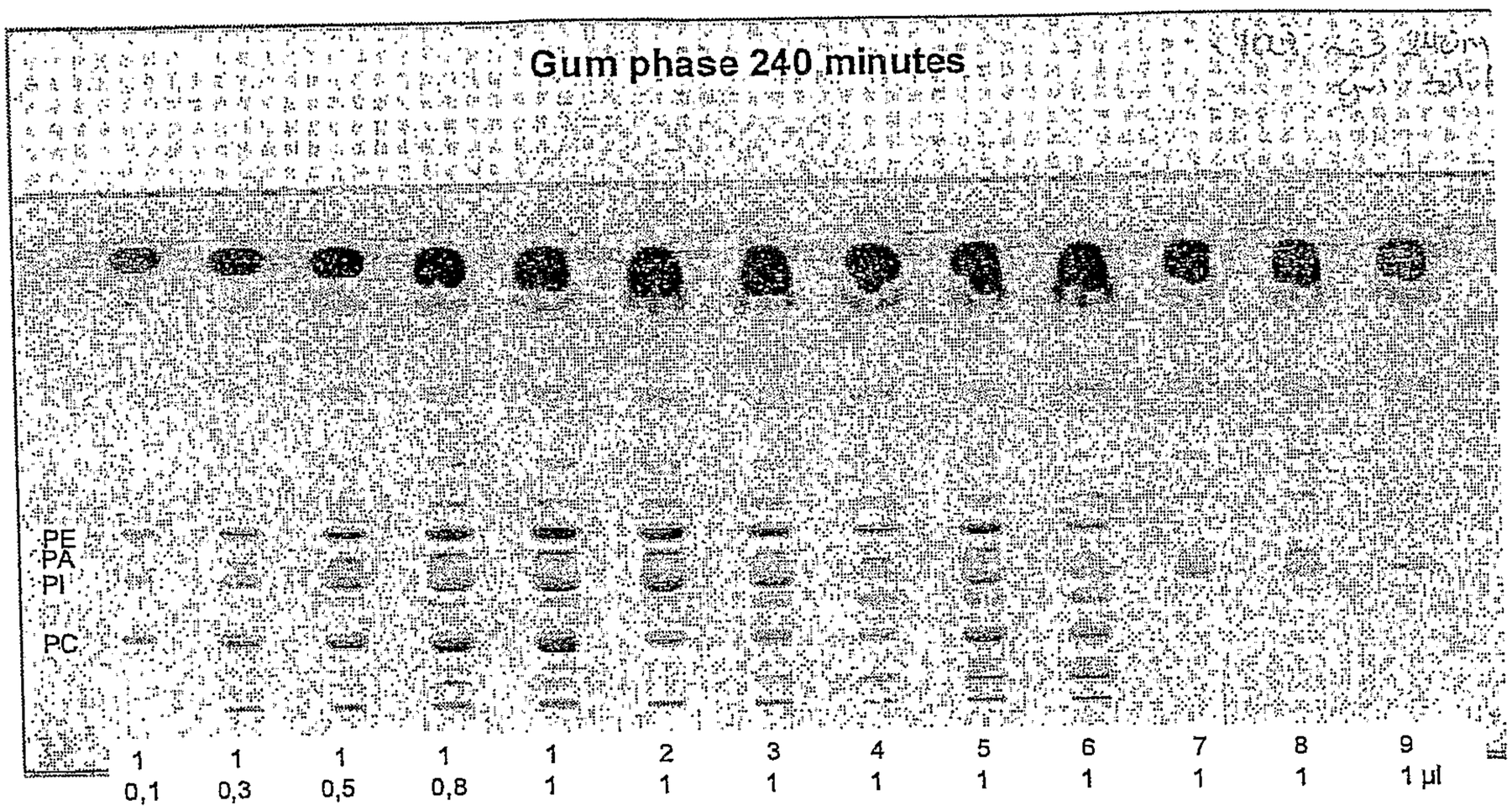
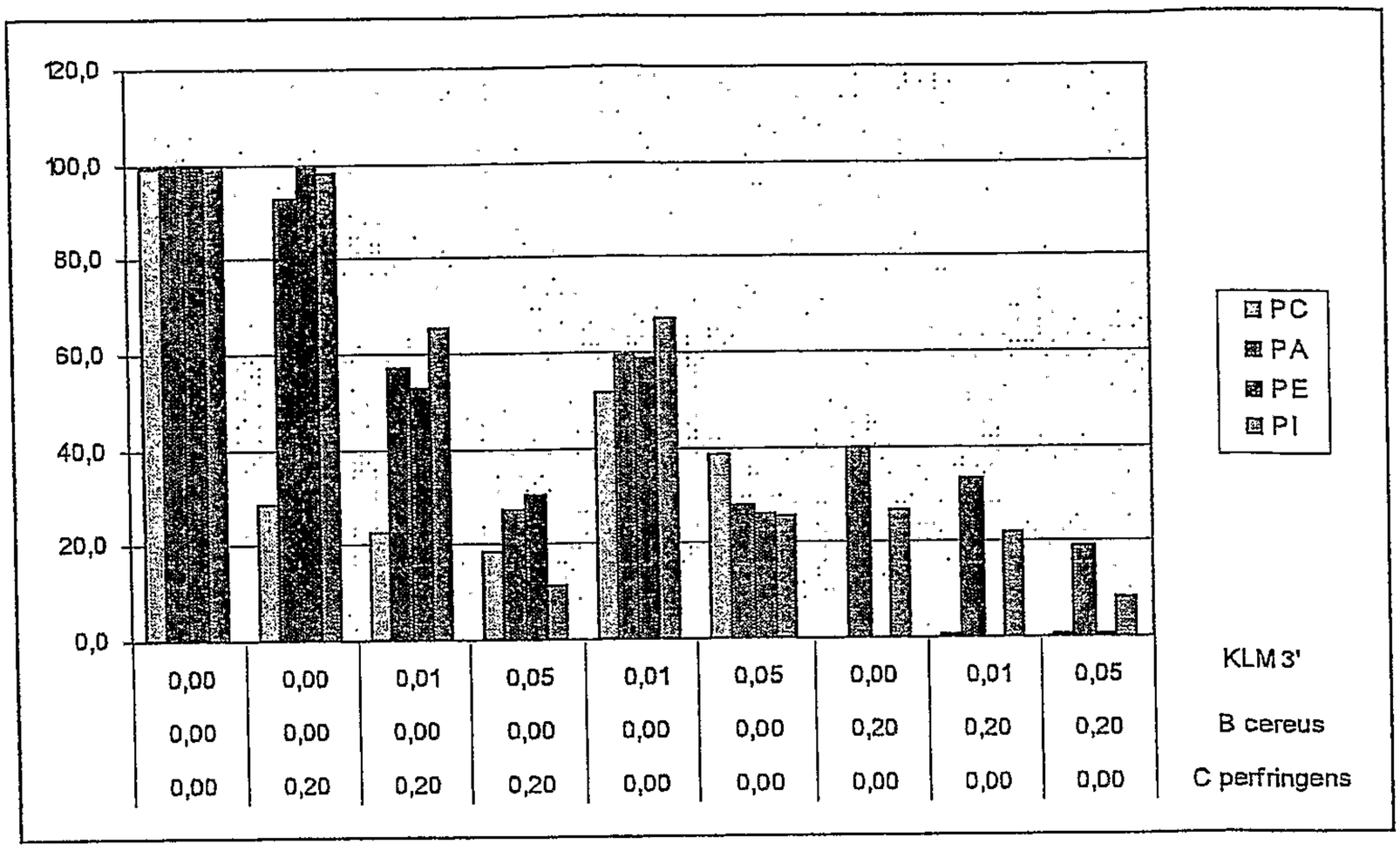
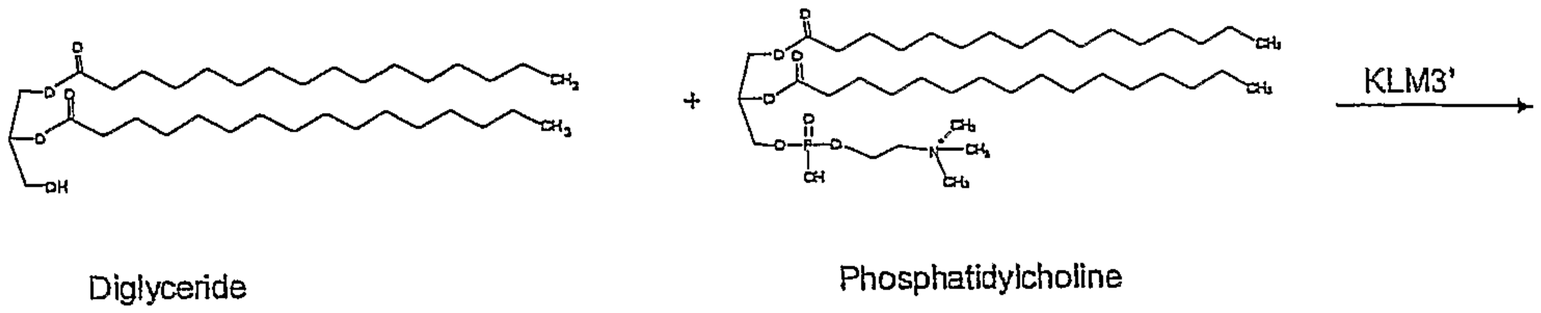

Gum phase 240 minutes
PE
PA
PI
PC
1 1 1 1 1 2 3 4 5 6 7 8 9
0,1 0,3 0,5 0,8 1 1 1 1 1 1 1 1 1 µl

FIGURE 107

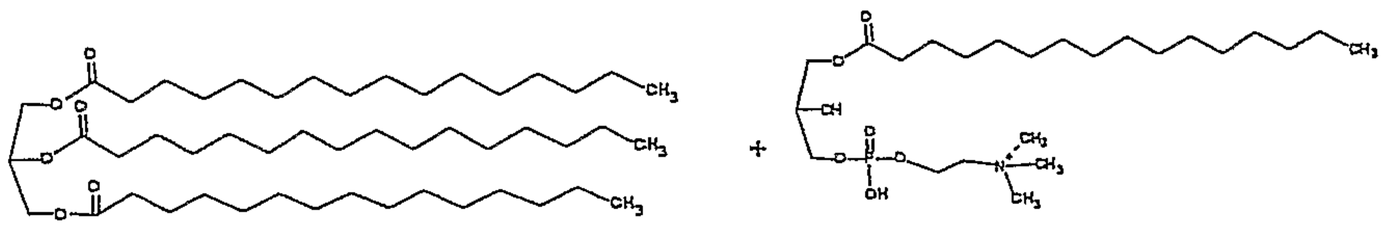


	0,00	0,00	0,01	0,05	0,01	0,05	0,00	0,01	0,05	KLM 3'
	0,00	0,00	0,00	0,00	0,00	0,00	0,20	0,20	0,20	B cereus
	0,00	0,20	0,20	0,20	0,00	0,00	0,00	0,00	0,00	C perfringens

FIGURE 108



Diglyceride + Phosphatidylcholine



Triglyceride + Lysophosphatidylcholine

FIGURE 109

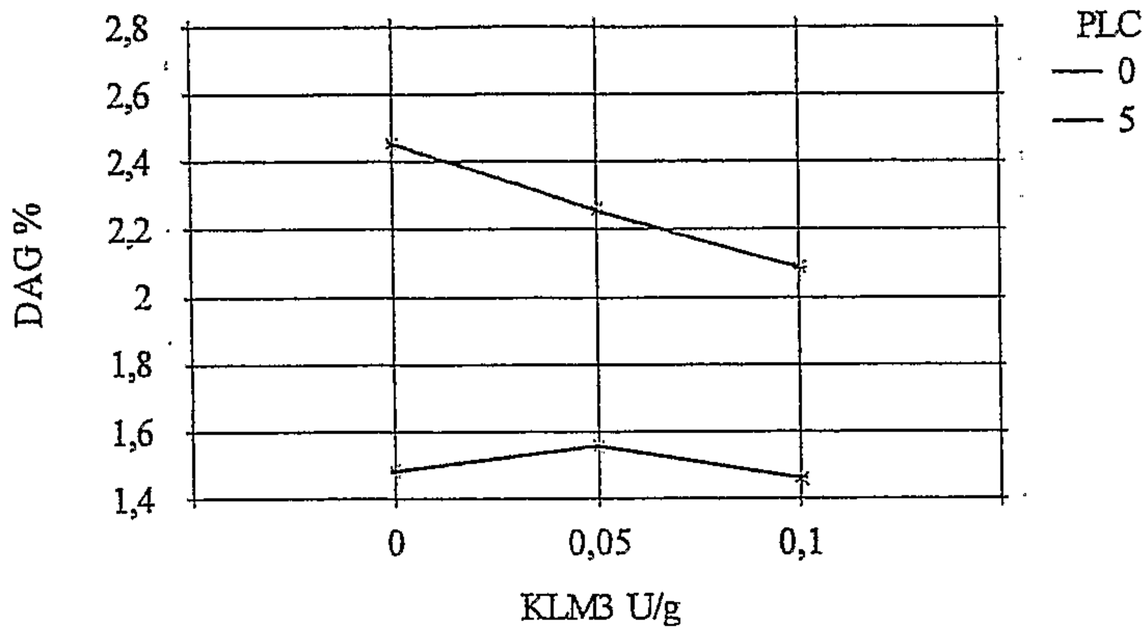

Interaction Plot
PLC
0
5
DAG %
2,8
2,6
2,4
2,2
2
1,8
1,6
1,4
0
0,05
0,1
KLM3 U/g

FIGURE 110

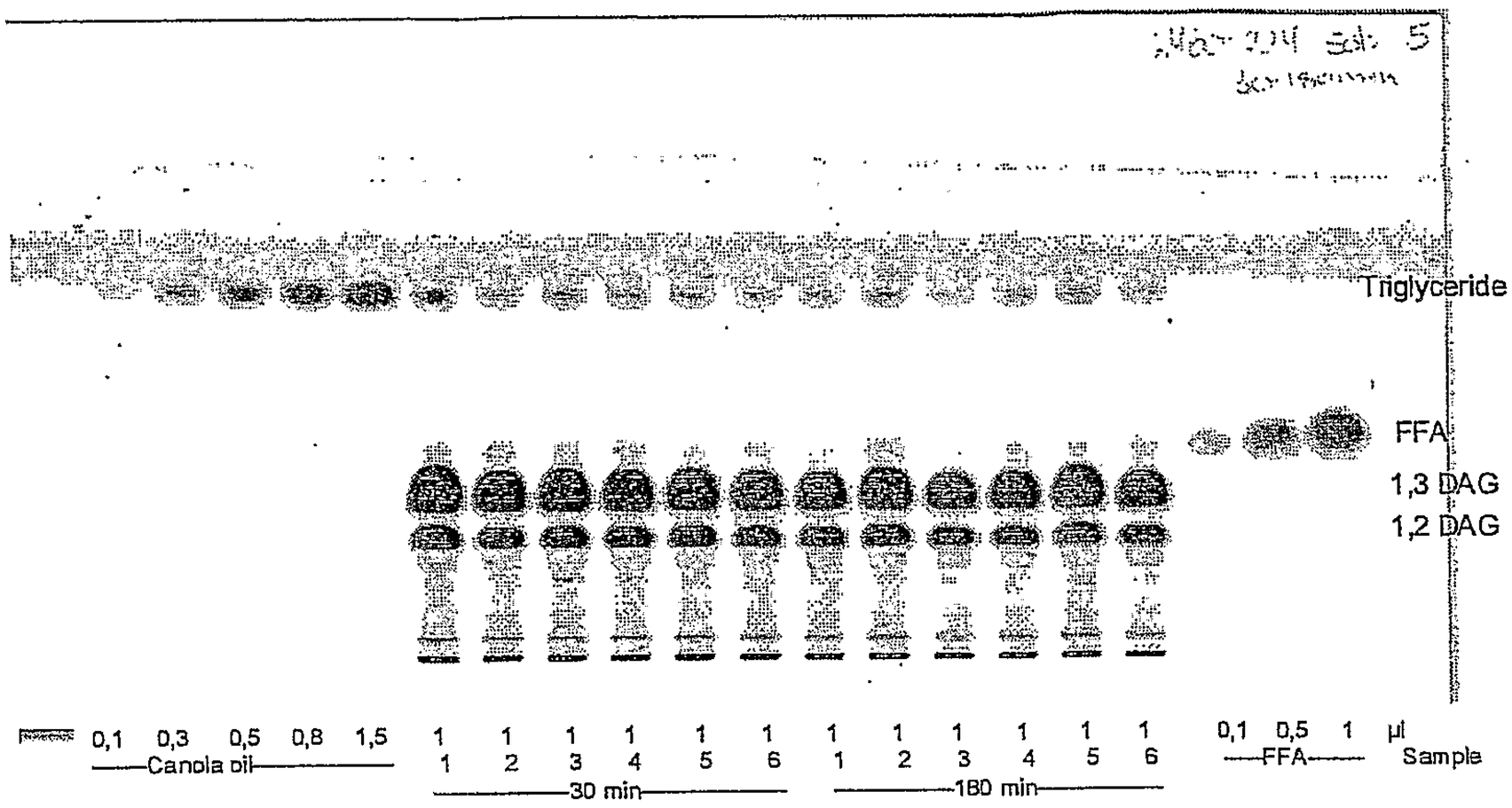

Triglyceride
FFA
1,3 DAG
1,2 DAG
0,1 0,3 0,5 0,8 1,5 1 1 1 1 1 1 1 1 1 1 1 1 0,1 0,5 1 µl
Canola oil
1 2 3 4 5 6 1 2 3 4 5 6
30 min
180 min
FFA
Sample

FIGURE 111

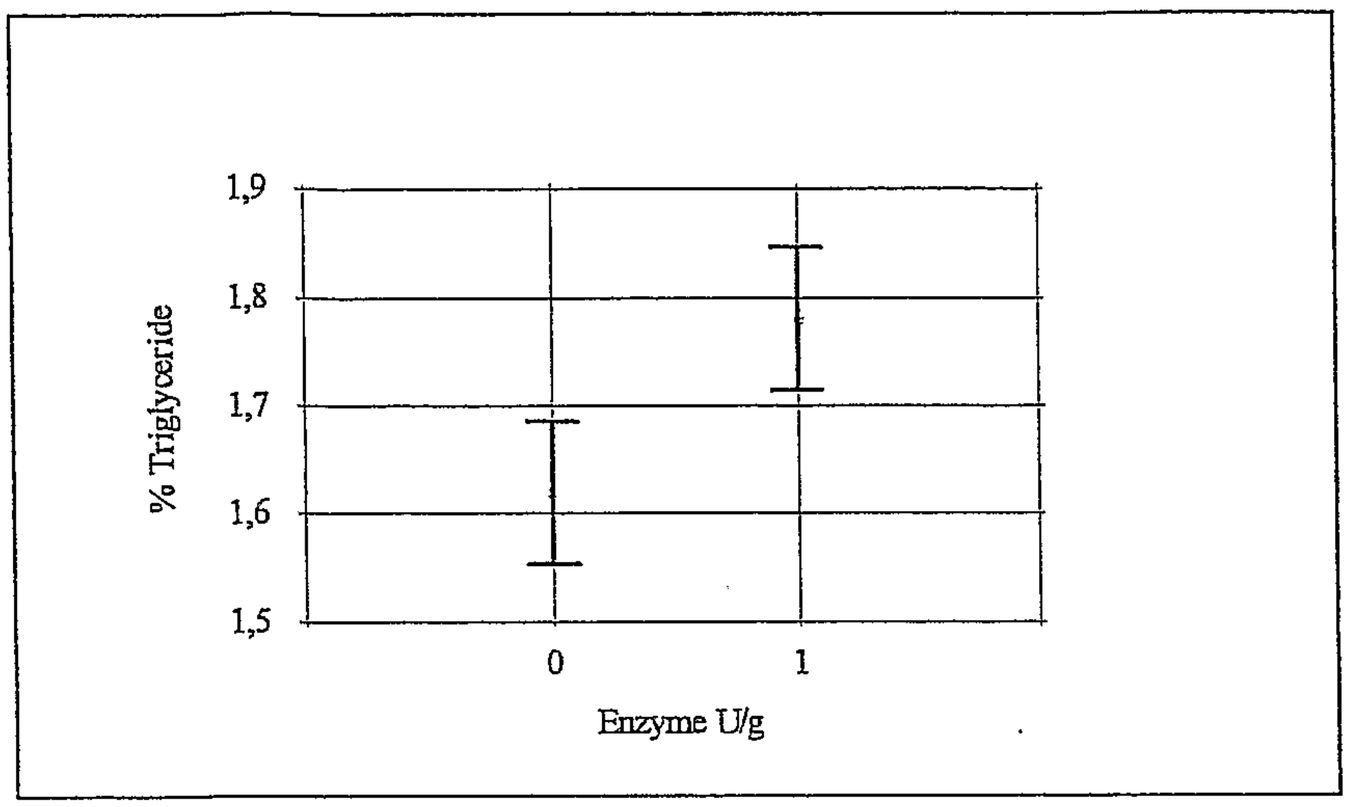


FIGURE 112

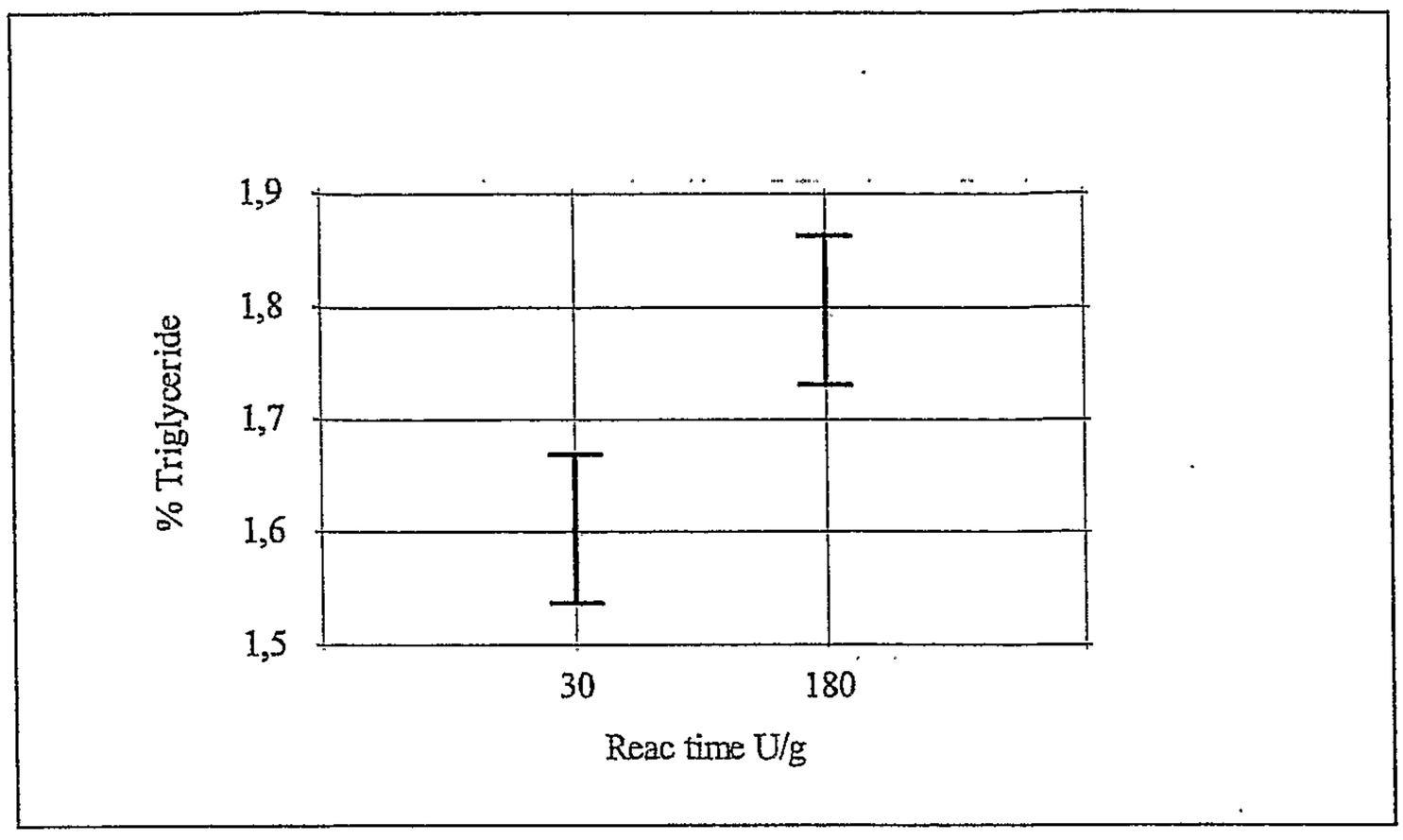


FIGURE 113

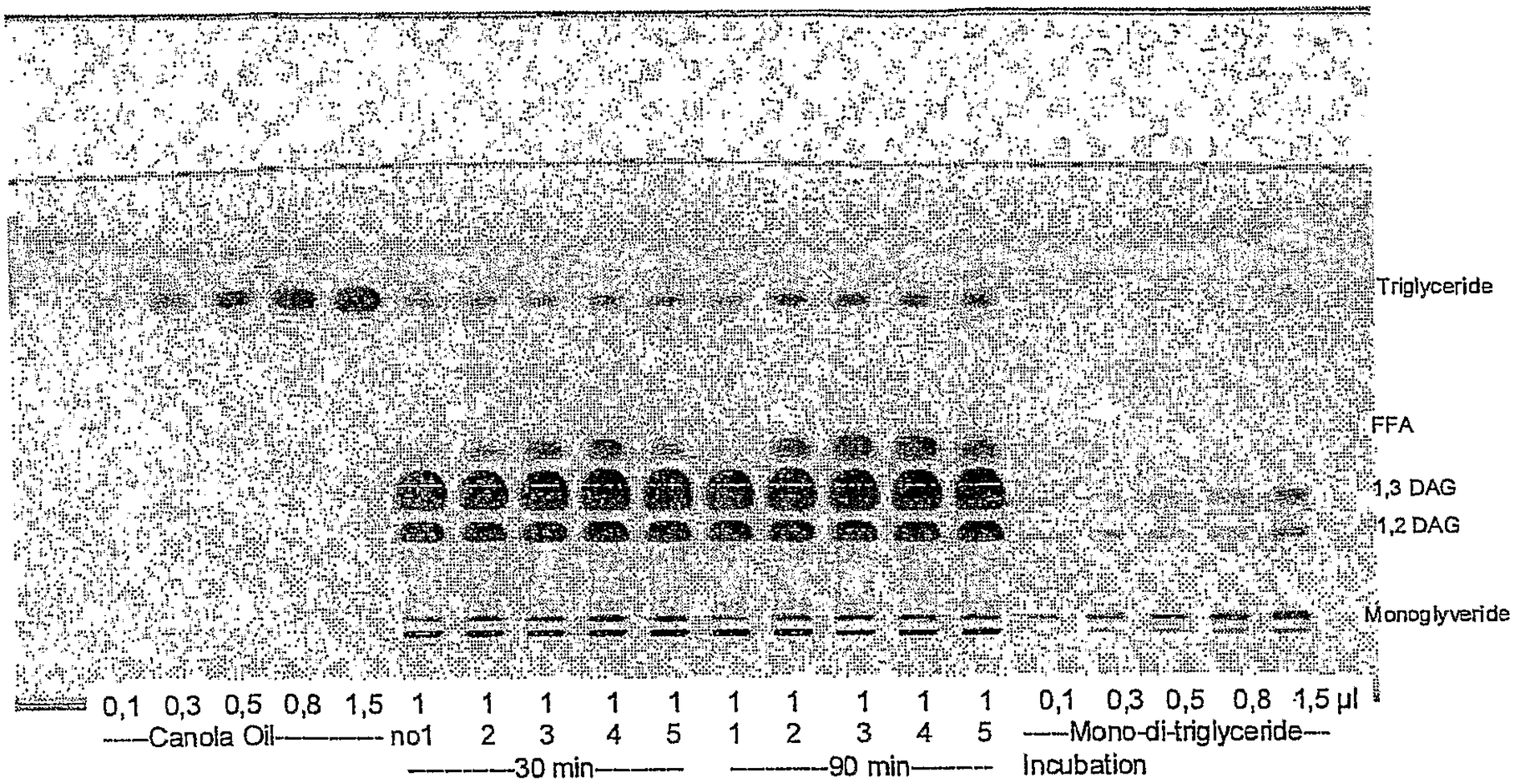

Triglyceride
FFA
1,3 DAG
1,2 DAG
Monoglyveride
0,1 0,3 0,5 0,8 1,5 1 1 1 1 1 1 1 1 1 1 0,1 0,3 0,5 0,8 1,5 µl
—Canola Oil— no1 2 3 4 5 1 2 3 4 5 —Mono-di-triglyceride—
30 min 90 min Incubation

FIGURE 114

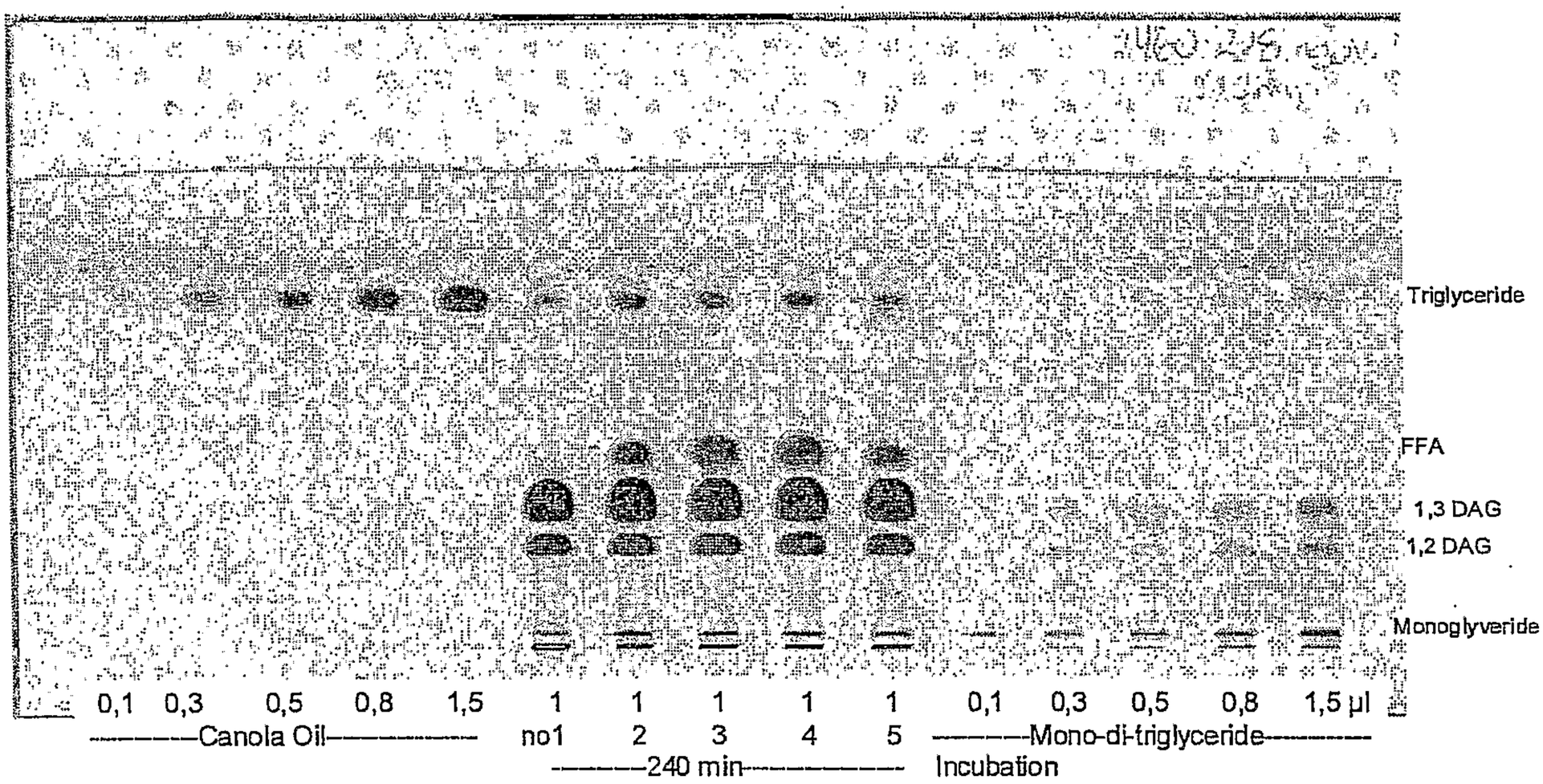

Triglyceride
FFA
1,3 DAG
1,2 DAG
Monoglyveride
0,1 0,3 0,5 0,8 1,5 1 1 1 1 1 0,1 0,3 0,5 0,8 1,5 µl
Canola Oil
no1 2 3 4 5
Mono-di-triglyceride
240 min
Incubation

FIGURE 115

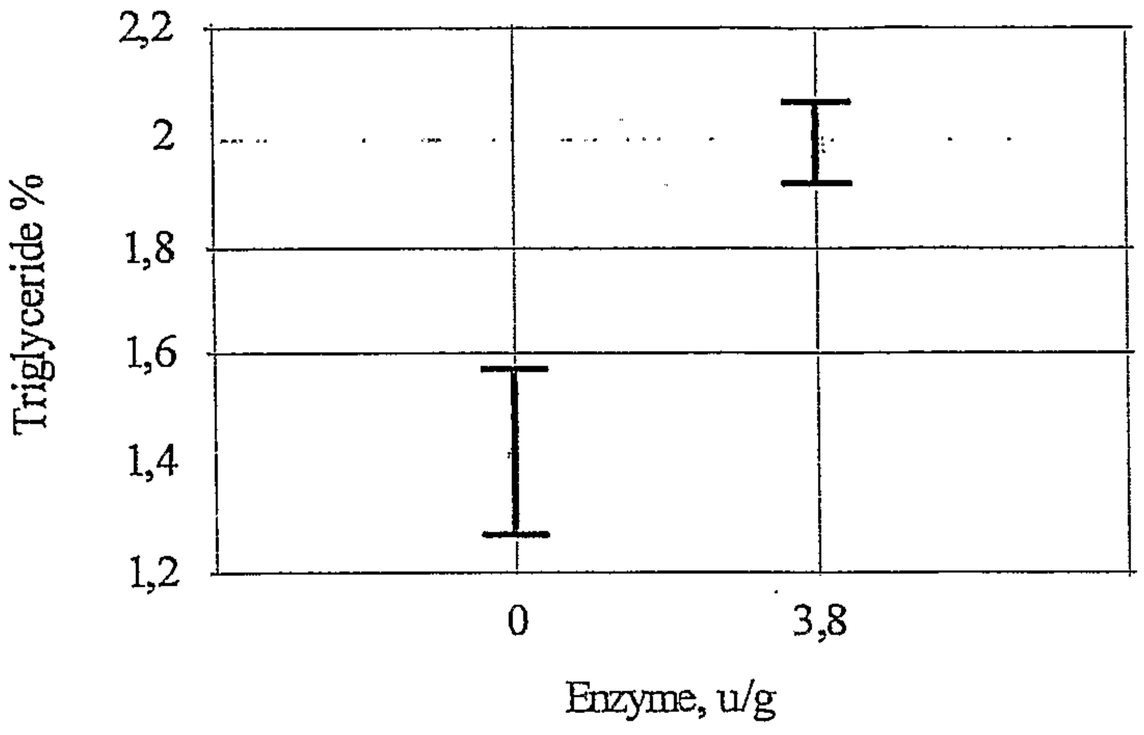


FIGURE 116

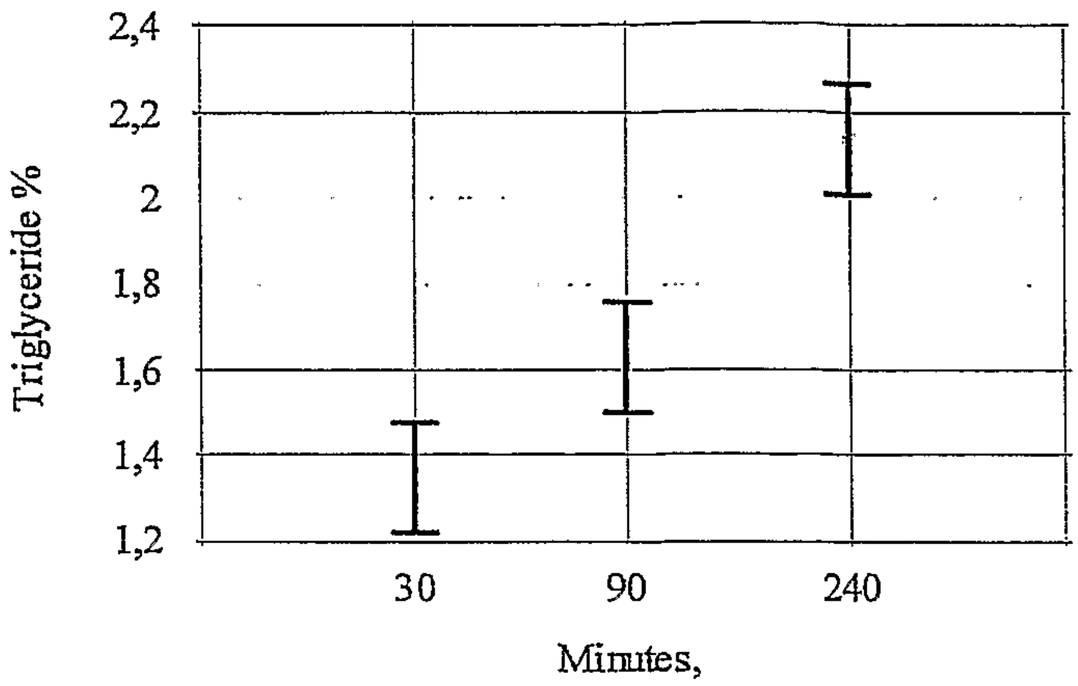


FIGURE 117

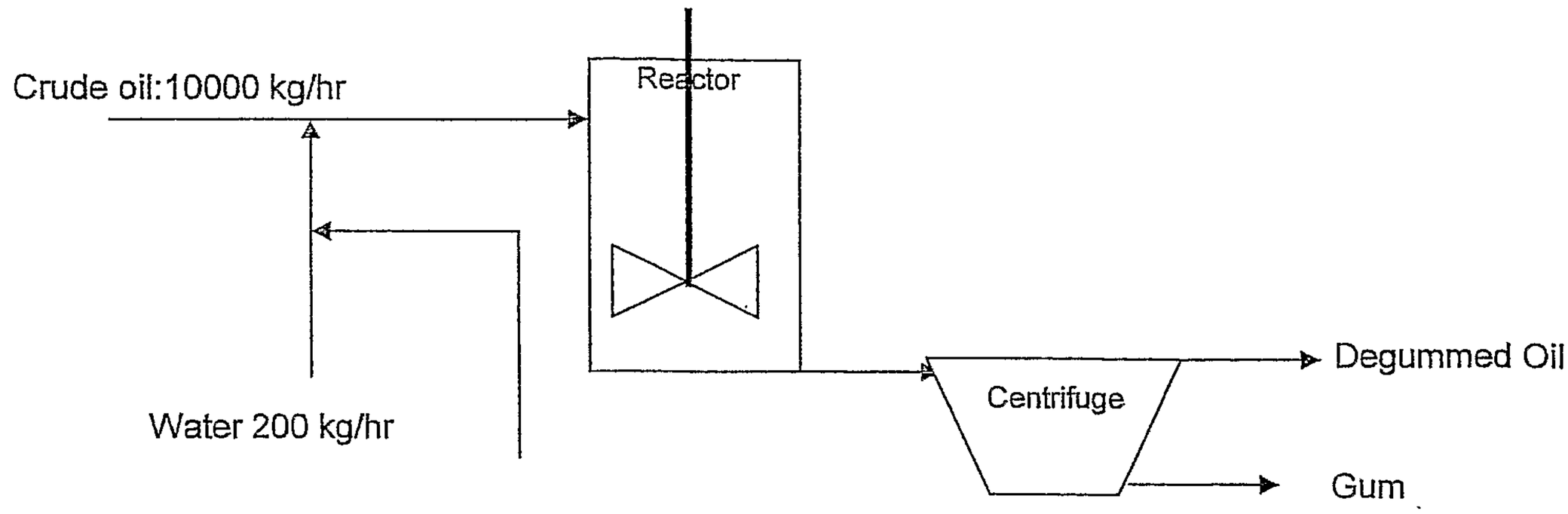
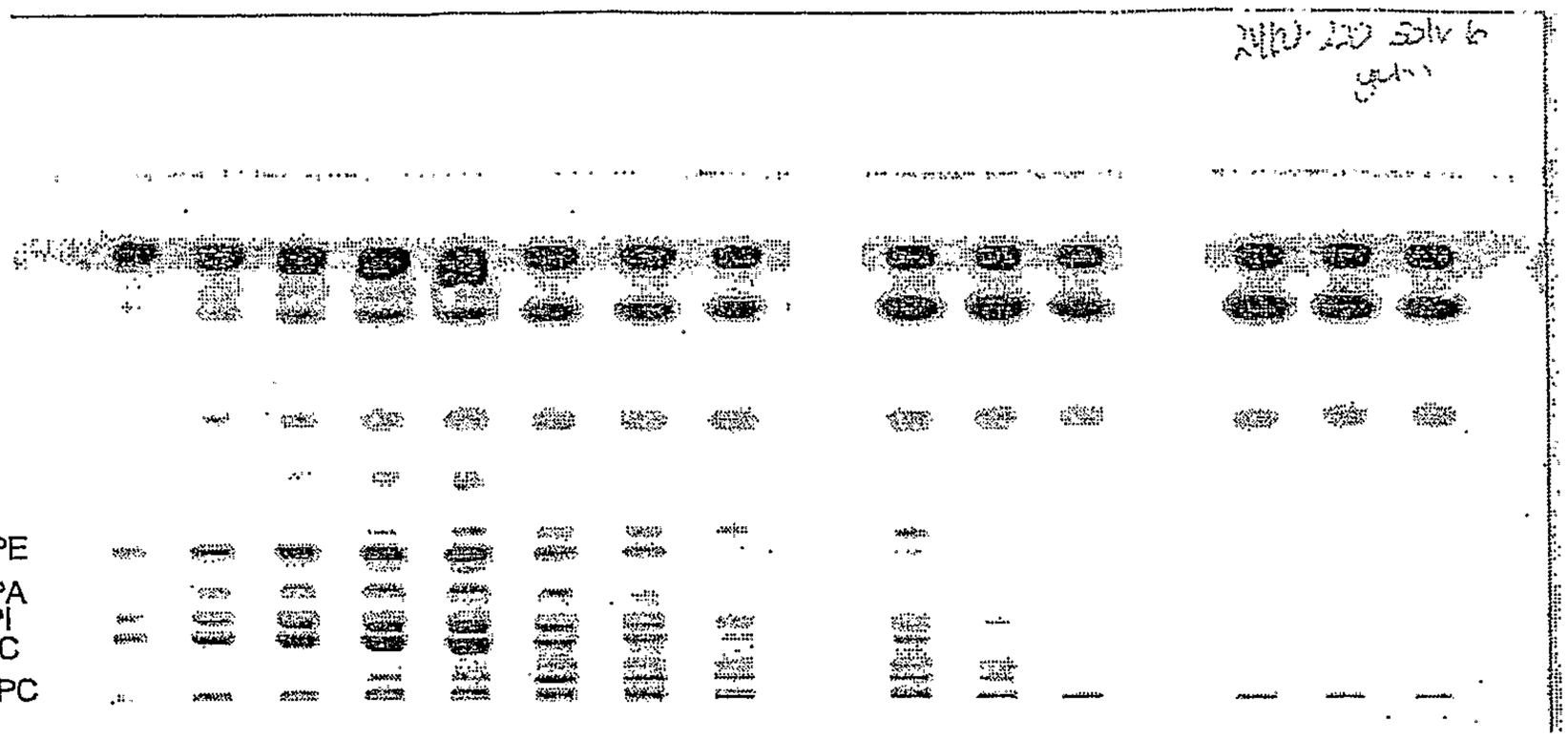
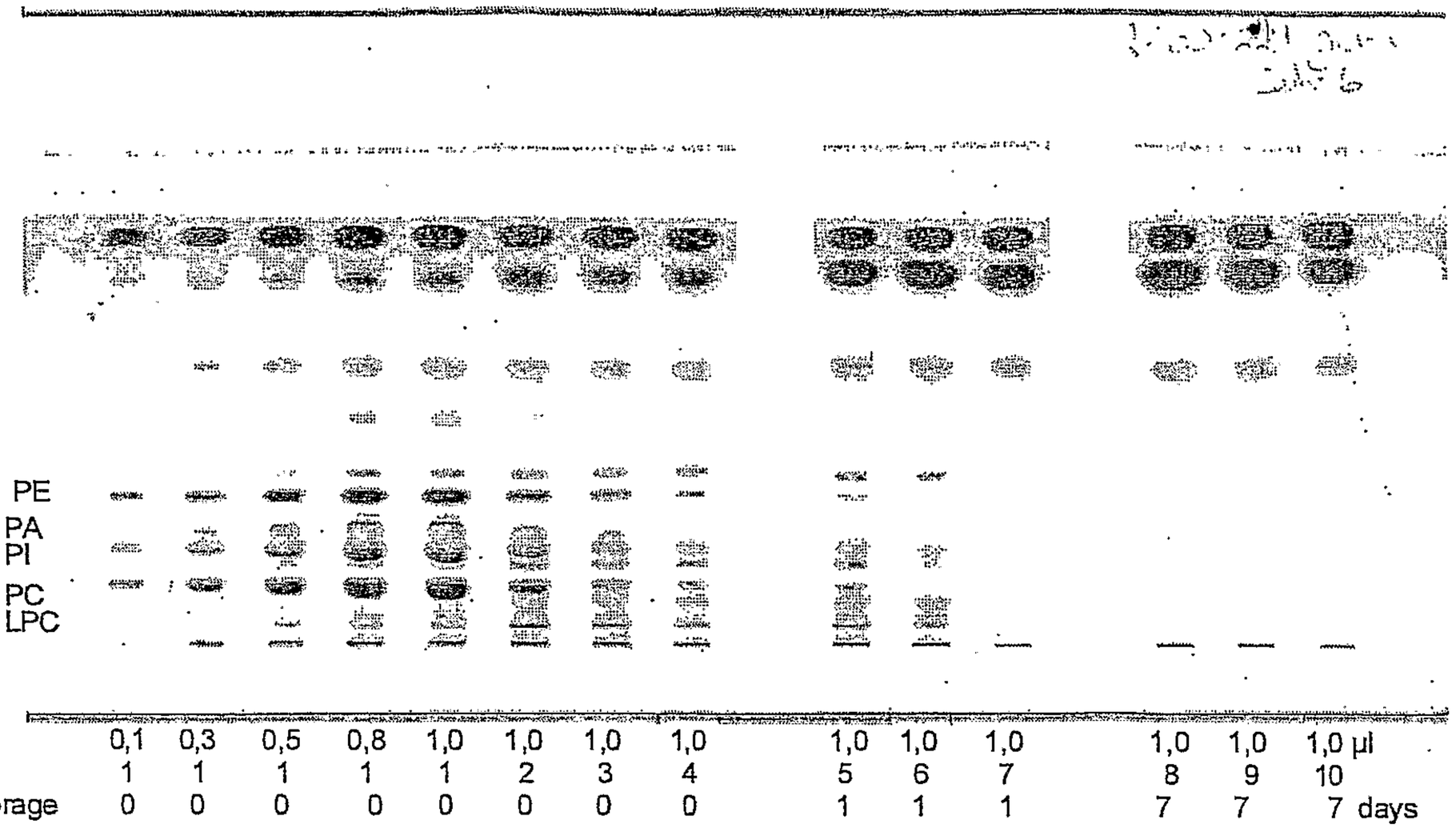

Crude oil:10000 kg/hr
Reactor
Water 200 kg/hr
Centrifuge
Degummed Oil
Gum
Enzyme: 200 LATU/kg oil

FIGURE 118



	0,1	0,3	0,5	0,8	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0	µl
No	1	1	1	1	1	2	3	4	5	6	7	8	9	10	
Storage	0	0	0	0	0	0	0	0	1	1	1	7	7	7	days

FIGURE 119


PE
PA
PI
PC
LPC
0,1 0,3 0,5 0,8 1,0 1,0 1,0 1,0 1,0 1,0 1,0 1,0 1,0 1,0 µl
No 1 1 1 1 1 2 3 4 5 6 7 8 9 10
Storage 0 0 0 0 0 0 0 0 1 1 1 7 7 7 days