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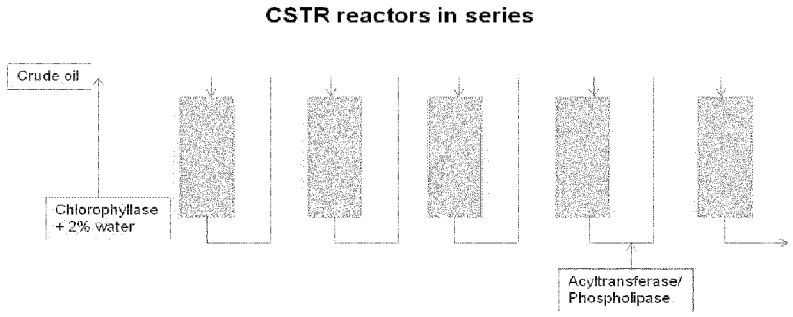
(54) Title: PROCESS FOR TREATING PLANT OIL INVOLVING ADDITION OF SERIAL DOSES OF CHLOROPHYLL OR CHLOROPHYLL DERIVATIVE DEGRADING ENZYME

Figure 36



(57) Abstract: In one aspect, provided herein is a process for treating a plant oil, comprising (i) adding a first dose of an enzyme which is capable of hydrolysing chlorophyll or a chlorophyll derivative to the oil; (ii) performing a first reaction step in which the enzyme hydrolyses chlorophyll or a chlorophyll derivative present in the oil; and (iii) adding one or more further doses of the enzyme to the oil, wherein a further reaction step is performed after addition of each further dose of the enzyme to the oil.

WO 2013/160372 A1

PROCESS FOR TREATING PLANT OIL INVOLVING ADDITION OF SERIAL DOSES OF CHLOROPHYLL OR CHLOROPHYLL DERIVATIVE DEGRADING ENZYME

FIELD

The present invention relates to the industrial processing of plant-derived food and feed products, especially vegetable oils. The invention may be employed to reduce or eliminate contamination by chlorophyll and chlorophyll derivatives.

BACKGROUND

Chlorophyll is a green-coloured pigment widely found throughout the plant kingdom. Chlorophyll is essential for photosynthesis and is one of the most abundant organic metal compounds found on earth. Thus many products derived from plants, including foods and feeds, contain significant amounts of chlorophyll.

For example, vegetable oils derived from oilseeds such as soybean, palm or rape seed (canola), cotton seed and peanut oil typically contain some chlorophyll. However the presence of high levels of chlorophyll pigments in vegetable oils is generally undesirable. This is because chlorophyll imparts an undesirable green colour and can induce oxidation of oil during storage, leading to a deterioration of the oil.

Various methods have been employed in order to remove chlorophyll from vegetable oils. Chlorophyll may be removed during many stages of the oil production process, including the seed crushing, oil extraction, degumming, caustic treatment and bleaching steps. However the bleaching step is usually the most significant for reducing chlorophyll residues to an acceptable level. During bleaching the oil is heated and passed through an adsorbent to remove chlorophyll and other colour-bearing compounds that impact the appearance and/or stability of the finished oil. The adsorbent used in the bleaching step is typically clay.

In the edible oil processing industry, the use of such steps typically reduces chlorophyll levels in processed oil to between 0.02 to 0.05 ppm. However the bleaching step increases processing cost and reduces oil yield due to entrainment in the bleaching clay. The use of clay may remove many desirable compounds such as carotenoids and tocopherol from the oil. Also the use of clay is expensive, this is particularly due to the treatment of the used clay (i.e. the waste) which can be difficult, dangerous (prone to self-ignition) and thus costly to handle.

Thus attempts have been made to remove chlorophyll from oil by other means, for instance using the enzyme chlorophyllase.

In plants, chlorophyllase (chlase) is thought to be involved in chlorophyll degradation and catalyzes the hydrolysis of an ester bond in chlorophyll to yield chlorophyllide and phytol. WO 2006009676 describes an industrial process in which chlorophyll contamination can be reduced in a composition such as a plant oil by treatment with chlorophyllase. The water-soluble chlorophyllide which is produced in this process is also green in colour but can be removed by an aqueous extraction or silica treatment.

Chlorophyll is often partly degraded in the seeds used for oil production as well as during extraction of the oil from the seeds. One common modification is the loss of the magnesium ion from the porphyrin (chlorin) ring to form the derivative known as pheophytin (see Figure 32). The loss of the highly polar magnesium ion from the porphyrin ring results in significantly different physico-chemical properties of pheophytin compared to chlorophyll. Typically pheophytin is more abundant in the oil during processing than chlorophyll. Pheophytin has a greenish colour and may be removed from the oil by an analogous process to that used for chlorophyll, for instance as described in WO 2006009676 by an esterase reaction catalyzed by an enzyme having a pheophytinase activity. Under certain conditions, some chlorophyllases are capable of hydrolyzing pheophytin as well as chlorophyll, and so are suitable for removing both of these contaminants. The products of pheophytin hydrolysis are the red/brown-colored pheophorbide and phytol. Pheophorbide can also be produced by the loss of a magnesium ion from chlorophyllide, i.e. following hydrolysis of chlorophyll (see Figure 32). WO 2006009676 teaches removal of pheophorbide by an analogous method to chlorophyllide, e.g. by aqueous extraction or silica adsorption.

Pheophytin may be further degraded to pyropheophytin, both by the activity of plant enzymes during harvest and storage of oil seeds or by processing conditions (e.g. heat) during oil refining (see "Behaviour of Chlorophyll Derivatives in Canola Oil Processing", JAOCS, Vol, no. 9 (Sept. 1993) pages 837-841). One possible mechanism is the enzymatic hydrolysis of the methyl ester bond of the isocyclic ring of pheophytin followed by the non-enzymatic conversion of the unstable intermediate to pyropheophytin. A 28-29 kDa enzyme from *Chenopodium album* named pheophorbidase is reportedly capable of catalyzing an analogous reaction on pheophorbide, to produce the phytol-free derivative of pyropheophytin known as

pyropheophorbide (see Figure 32). Pyropheophorbide is less polar than pheophorbide resulting in the pyropheophoribe having a decreased water solubility and an increased oil solubility compared with pheophorbide.

Depending on the processing conditions, pyropheophytin can be more abundant than both pheophytin and chlorophyll in vegetable oils during processing (see Table 9 in volume 2.2. of Bailey's Industrial Oil and Fat Products (2005), 6th edition, Ed. by Fereidoon Shahidi, John Wiley & Sons). This is partly because of the loss of magnesium from chlorophyll during harvest and storage of the plant material. If an extended heat treatment at 90°C or above is used, the amount of pyropheophytin in the oil is likely to increase and could be higher than the amount of pheophytin. Chlorophyll levels are also reduced by heating of oil seeds before pressing and extraction as well as the oil degumming and alkali treatment during the refining process. It has also been observed that phospholipids in the oil can complex with magnesium and thus reduce the amount of chlorophyll. Thus chlorophyll is a relatively minor contaminant compared to pyropheophytin (and pheophytin) in many plant oils.

However, there is a still a need for an improved process for removing chlorophyll and chlorophyll derivatives such as pheophytin and pyropheophytin from plant oils. In particular, there is a need for a process which is specifically optimised according to the kinetic properties of the reaction of chlorophyllases with their substrates. Moreover, during oil refining the oil is often present at an elevated temperature (e.g. 70 to 90°C). At these temperatures, enzymes often start to denature and become inactive. Therefore in one aspect there is a particular need for a process for oil refining using chlorophyllases which is capable of operating at high temperatures.

SUMMARY

In one aspect the present invention provides a process for treating a plant oil, comprising (i) adding a first dose of an enzyme which is capable of hydrolysing chlorophyll or a chlorophyll derivative to the oil; (ii) performing a first reaction step in which the enzyme hydrolyses chlorophyll or a chlorophyll derivative present in the oil; and (iii) adding one or more further doses of the enzyme to the oil, wherein a further reaction step is performed after addition of each further dose of the enzyme to the oil.

In one embodiment, each reaction step is performed at a temperature of 70 to 90°C. Preferably each reaction step is performed at a temperature of 75 to 80°C.

In one embodiment, the process is performed in a series of reaction vessels, such that each reaction step takes place in a different reaction vessel.

In one embodiment, at least two further doses of the enzyme are added to the oil. In another embodiment, at least three further doses of the enzyme are added to the oil.

In one embodiment, the process further comprises an enzymatic degumming step. The enzymatic degumming step may, for example, comprise contacting the oil with a phospholipase or an acyltransferase. Preferably the enzymatic degumming step is performed in one or more further reaction vessels.

In further embodiments, the enzyme comprises a polypeptide sequence as defined in any one of SEQ ID NOs: 1 to 31, or a functional fragment or variant thereof having at least 75% sequence identity to any one of SEQ ID NOs: 1 to 31 over at least 50 amino acid residues and having chlorophyllase, pheophytinase and/or pyropheophytinase activity.

In some embodiments, the process further comprises an acid treatment step. For instance, the oil may be treated with an acid before or after addition of the enzyme to the oil. In one embodiment, the oil is first treated with an acid, and then the pH of the oil is adjusted to 5.5 to 6.5 (e.g. addition of an alkali, such as in a caustic neutralisation step) before addition of the enzyme to the oil.

In a further aspect, the present invention provides a treated plant oil obtainable by a process as defined in any preceding claim.

In another aspect, the present invention provides an apparatus for plant oil refining, comprising (i) a first reaction vessel for performing a first enzymatic hydrolysis step; and (ii) one or more further reaction vessels for performing one or more further enzymatic hydrolysis steps; wherein the first and further reaction vessels are arranged in series and are in fluid communication with one another, such that the oil can be transferred from the first reaction vessel to each further reaction vessel in series; and wherein the apparatus comprises a plurality of enzyme input means, arranged such that a dose of an enzyme can be introduced into the oil between each reaction vessel.

Preferably the enzyme is capable of hydrolysing chlorophyll or a chlorophyll derivative.

In one embodiment, each reaction vessel further comprises a mixing means for mixing the oil during each enzymatic hydrolysis step.

In one embodiment the apparatus further comprises one or more reaction vessels for performing an enzymatic degumming step. The apparatus may further comprise one or more further enzyme input means for introducing a phospholipase or acyltransferase into the oil before the enzymatic degumming step.

In one embodiment, each reaction vessel comprises a continuous stirred tank reactor.

In one embodiment, the apparatus may further comprise one or more vessels for performing an acid treatment step.

It has been found that the hydrolysis of chlorophyll derivatives in plant oil by chlorophyllases can be described a first order reaction kinetic. Accordingly for an efficient degradation of chlorophyll derivatives, in embodiments of the present invention chlorophyllases are preferably added in multiple doses, for example such that a series of reactions is performed in a plurality of continuous stirred reactors.

Moreover, it has surprisingly been found that by adding serial doses of chlorophyllases, the hydrolysis reaction can be performed more efficiently at elevated temperature, e.g. at 70 to 90°C, leading to increased degradation of chlorophyll components. Although chlorophyllases are rapidly inactivated at such temperatures, the initial reaction speed is very high at 70 °C or above. Thus it has been found that at high temperature, chlorophyll derivatives can be degraded more effectively by adding multiple low doses of a chlorophyllase in series. This has the added advantage that because the oil typically needs to be at a high temperature both before and after the enzyme treatment step, energy need not be wasted in heating or cooling the oil if the chlorophyllase treatment can be performed at 70 to 90°C.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows the amino acid sequence of an *Arabidopsis thaliana* chlorophyllase (SEQ ID NO:1).

Figure 2 shows the amino acid sequence of an *Arabidopsis thaliana* chlorophyllase (SEQ ID NO:2).

Figure 3 shows the amino acid sequence of *Citrus sinensis* chlorophyllase (SEQ ID NO:3).

Figure 4 shows the amino acid sequence of a *Triticum aestivum* chlorophyllase (SEQ ID NO:4).

Figure 5 shows the amino acid sequence of a *Triticum aestivum* chlorophyllase (SEQ ID NO:5).

Figure 6 shows the amino acid sequence of a *Brassica oleracea* chlorophyllase (SEQ ID NO:6).

Figure 7 shows the amino acid sequence of a *Brassica oleracea* chlorophyllase (SEQ ID NO:7).

Figure 8 shows the amino acid sequence of a *Brassica oleracea* chlorophyllase (SEQ ID NO:8).

Figure 9 shows the amino acid sequence of a *Zea Mays* chlorophyllase (SEQ ID NO:9).

Figure 10 shows the amino acid sequence of a *Zea Mays* chlorophyllase (SEQ ID NO:10).

Figure 11 shows the amino acid sequence of a *Phyllostachys edulis* chlorophyllase (SEQ ID NO:11).

Figure 12 shows the amino acid sequence of a *Chenopodium album* chlorophyllase (SEQ ID NO:12).

Figure 13 shows the amino acid sequence of a *Ricinus communis* chlorophyllase (SEQ ID NO:13).

Figure 14 shows the amino acid sequence of a *Glycine max* chlorophyllase (SEQ ID NO:14).

Figure 15 shows the amino acid sequence of a *Ginkgo biloba* chlorophyllase (SEQ ID NO:15).

Figure 16 shows the amino acid sequence of a *Pachira macrocarpa* chlorophyllase (SEQ ID NO:16).

Figure 17 shows the amino acid sequence of a *Populus trichocarpa* chlorophyllase (SEQ ID NO:17).

Figure 18 shows the amino acid sequence of a *Sorghum bicolor* chlorophyllase (SEQ ID NO:18).

Figure 19 shows the amino acid sequence of a *Sorghum bicolor* chlorophyllase (SEQ ID NO:19).

Figure 20 shows the amino acid sequence of a *Vitis vinifera* chlorophyllase (SEQ ID NO:20).

Figure 21 shows the amino acid sequence of a *Physcomitrella patens* chlorophyllase (SEQ ID NO:21).

Figure 22 shows the amino acid sequence of a *Aquilegia* chlorophyllase (SEQ ID NO:22).

Figure 23 shows the amino acid sequence of a *Brachypodium distachyon* chlorophyllase (SEQ ID NO:23).

Figure 24 shows the amino acid sequence of a *Medicago truncatula* chlorophyllase (SEQ ID NO:24).

Figure 25 shows the amino acid sequence of a *Piper betle* chlorophyllase (SEQ ID NO:25).

Figure 26 shows the amino acid sequence of a *Lotus japonicus* chlorophyllase (SEQ ID NO:26).

Figure 27 shows the amino acid sequence of a *Oryza sativa Indica* chlorophyllase (SEQ ID NO:27).

Figure 28 shows the amino acid sequence of a *Oryza sativa Japonica* chlorophyllase (SEQ ID NO:28).

Figure 29 shows the amino acid sequence of a *Oryza sativa Japonica* chlorophyllase (SEQ ID NO:29).

Figure 30 shows the amino acid sequence of a *Picea sitchensis* chlorophyllase (SEQ ID NO:30).

Figure 31 shows the amino acid sequence of a *Chlamydomonas* chlorophyllase (SEQ ID NO:31).

Figure 32 shows the reactions involving chlorophyll and derivatives and enzymes used in the present invention.

Figure 33 shows shows amino acid and nucleotide sequences showing the fusion of a chlorophyllase gene to a His tag and thrombin site.

Figure 34 shows a schematic presentation of an *E. coli.* expression vector pET28-TRI_CHL containing the TRI_CHL gene encoding a chlorophyllase from *Triticum aestivum* (database acc. no. BT009214).

Figure 35 shows the temperature stability of chlorophyllases from *Arabidopsis thaliana* (ARA2_CHL) and *Triticum aestivum* (Tri_CHL). Enzyme samples were heated to indicated temperatures for 10 min followed by cooling to 4°C. Residual pheophytinase activity was measured.

Figure 36 shows a flow diagram for chlorophyllase reactions in continuous stirred tank reactors in series.

Figure 37 shows a diagrammatic representation of a chlorophyllase based oil refining plant according to one embodiment of the present invention.

Figure 38 shows a diagrammatic representation of a chlorophyllase based oil refining plant according to one embodiment, involving an initial acid treatment/caustic neutralization step.

Figure 39 shows a diagrammatic representation of a chlorophyllase based oil refining plant according to one embodiment, involving an acid treatment/caustic neutralization step after centrifugation and gum separation.

Figure 40 shows the results of liquid chromatography-mass spectrometry (LC-MS) analysis of pheophytin in oil treated with chlorophyllase at 75°C.

Figure 41 shows the natural logarithm of pheophytin concentration (in ppm) as a function of time in oil treated with chlorophyllase at 75°C. 0.2 U/g enzyme was added at the beginning of the method (exp no. 2), or 0.05 U/g enzyme was added 4 times (exp no. 3), the total enzyme amount added being the same in each case.

Figure 42 shows the results of LC-MS analysis of pheophytin in oil treated with chlorophyllase at 80°C.

Figure 43 shows the natural logarithm of pheophytin concentration (in ppm) as a function of time in oil treated with chlorophyllase at 80°C. 0.2 U/g enzyme was added at the beginning of the method (exp. no. 2) or 0.05 U/g enzyme was added 4 times (exp. no. 3), the total enzyme amount added being the same in each case.

Figure 44 shows a diagrammatic representation of a chlorophyllase based oil refining plant according to one embodiment of the present invention.

Figure 45 shows the amino acid sequence of a mutant *Aeromonas salmonicida* mature lipid acyltransferase (GCAT) with a mutation of Asn80Asp after undergoing post-translational modification (SEQ ID No. 40).

DETAILED DESCRIPTION

In one aspect the present invention relates to a process for refining a crude plant oil. Typically the process is used to remove chlorophyll and/or chlorophyll derivatives from the oil, or to reduce the level of chlorophyll and/or chlorophyll derivatives in the oil, for instance where the chlorophyll and/or chlorophyll derivatives are present as a contaminant.

Chlorophyll and chlorophyll derivatives

By "chlorophyll derivative" it is typically meant compounds which comprise both a porphyrin (chlorin) ring and a phytol group (tail), including magnesium-free phytol-containing derivatives such as pheophytin and pyropheophytin. Chlorophyll and (phytol-containing) chlorophyll derivatives are typically greenish is colour, as a result of the porphyrin (chlorin) ring present in the molecule. Loss of magnesium from the porphyrin ring means that pheophytin and pyropheophytin are more brownish in colour than chlorophyll. Thus the presence of chlorophyll and chlorophyll derivatives in an oil, can give such an oil an undesirable green, greenish or brownish colour. In one embodiment, the present process may

be performed in order to remove or reduce the green or brown colouring present in the oil. Accordingly the present process may be referred to as a bleaching or de-colorizing process.

Enzymes used in the process may hydrolyse chlorophyll and phytol-containing chlorophyll derivatives to cleave the phytol tail from the chlorin ring. Hydrolysis of chlorophyll and chlorophyll derivatives typically results in compounds such as chlorophyllide, pheophorbide and pyropheophorbide which are phytol-free derivatives of chlorophyll. These compounds still contain the colour-bearing porphyrin ring, with chlorophyllide being green and pheophorbide and pyropheophorbide a reddish brown colour. In some embodiments, it may also be desirable to remove these phytol-free derivatives and to reduce the green/red/brown colouring in the oil. Thus in one embodiment of the invention, the process may further comprise a step of removing or reducing the level of phytol-free chlorophyll derivatives in the oil. The process may involve bleaching or de-colorizing to remove the green and/or red/brown colouring of the oil.

The chlorophyll or chlorophyll derivative may be either a or b forms. Thus as used herein, the term "chlorophyll" includes chlorophyll a and chlorophyll b. In a similar way both a and b forms are covered when referring to pheophytin, pyropheophytin, chlorophyllide, pheophorbide and pyropheophorbide.

Chlorophyll and chlorophyll derivatives may exist as a pair of epimers determined by the stereochemistry around the carbon number 13^2 (numbering according to the IUPAC system). Thus chlorophyll a exists as the pair of epimers chlorophyll *a* and chlorophyll *a'*, and chlorophyll b comprises *b* and *b'* forms. Pheophytin a comprises the epimers *a* and *a'* and pheophytin b comprises *b* and *b'* forms. The prime (') forms have S-stereochemistry and non-prime forms have R-stereochemistry about the carbon 13^2 atom. When used generally herein, the term "chlorophyll and chlorophyll derivatives" includes both prime and non-prime forms.

Plant oils

Any plant oil may be treated according to the present process, in order to remove undesirable contamination by chlorophyll and/or chlorophyll derivatives. The oil may be derived from any type of plant, and from any part of a plant, including whole plants, leaves, stems, flowers, roots, plant protoplasts, seeds and plant cells and progeny of same. The class of plants from which products can be treated in the method of the invention includes higher plants, including

angiosperms (monocotyledonous and dicotyledonous plants), as well as gymnosperms. It includes plants of a variety of ploidy levels, including polyploid, diploid, haploid and hemizygous states.

In preferred embodiments, the oil may comprise a vegetable oil, including oils processed from oil seeds or oil fruits (e.g. seed oils such as canola (rapeseed) oil and fruit oils such as palm). Examples of suitable oils include rice bran, soy, canola (rape seed), palm, olive, cottonseed, corn, palm kernel, coconut, peanut, sesame or sunflower. The process of the invention can be used in conjunction with methods for processing essential oils, e.g., those from fruit seed oils, e.g. grapeseed, apricot, borage, etc. In some embodiments, the enzyme is contacted with a crude plant oil.

Chlorophyll and chlorophyll derivatives in oil

The chlorophyll and/or chlorophyll derivatives (e.g. chlorophyll, pheophytin and/or pyropheophytin) may be present in the oil naturally, as a contaminant, or as an undesired component in a processed product. The chlorophyll and/or chlorophyll derivatives (e.g. chlorophyll, pheophytin and/or pyropheophytin) may be present at any level in the oil. Typically chlorophyll, pheophytin and/or pyropheophytin may be present as a natural contaminant in the oil at a concentration of 0.001 to 1000 mg/kg (0.001 to 1000 ppm, 10^{-7} to 10^{-1} wt %), based on the total weight of the oil. In further embodiments, the chlorophyll and/or chlorophyll derivatives may be present in the oil at a concentration of 0.1 to 100, 0.5 to 50, 1 to 50, 1 to 30 or 1 to 10 mg/kg, based on the total weight of the oil.

Phytol-free chlorophyll derivatives may also be present in the oil. For instance, chlorophyllide, pyropheophorbide and/or pyropheophorbide may be present at any level in the oil. Typically chlorophyllide, pyropheophorbide and/or pyropheophorbide may be present in the oil, either before or after treatment with an enzyme according to the method of the present invention, at a concentration of 0.001 to 1000 mg/kg (0.001 to 1000 ppm, 10^{-7} to 10^{-1} wt %), based on the total weight of the oil. In further embodiments, the chlorophyllide, pyropheophorbide and/or pyropheophorbide may be present in the composition at a concentration of 0.1 to 100, 0.5 to 50, 1 to 50, 1 to 30 or 1 to 10 mg/kg, based on the total weight of the composition.

Enzymes hydrolysing chlorophyll or a chlorophyll derivative

The process of the present invention comprises a step of contacting the oil with an enzyme which is capable of hydrolysing chlorophyll or a chlorophyll derivative. Typically "hydrolyzing chlorophyll or a chlorophyll derivative" means hydrolysing an ester bond in chlorophyll or a (phytol-containing) chlorophyll derivative, e.g. to cleave a phytol group from the chlorin ring in the chlorophyll or chlorophyll derivative. Thus the enzyme typically has an esterase or hydrolase activity. Preferably the enzyme has esterase or hydrolase activity in an oil phase, and optionally also in an aqueous phase.

Thus the enzyme may, for example, be a chlorophyllase, pheophytinase or pyropheophytinase. Preferably, the enzyme is capable of hydrolysing at least one, at least two or all three of chlorophyll, pheophytin and pyropheophytin. In a particularly preferred embodiment, the enzyme has chlorophyllase, pheophytinase and pyropheophytinase activity. In further embodiments, two or more enzymes may be used in the method, each enzyme having a different substrate specificity. For instance, the method may comprise the combined use of two or three enzymes selected from a chlorophyllase, a pheophytinase and a pyropheophytinase.

Any polypeptide having an activity that can hydrolyse chlorophyll or a chlorophyll derivative can be used as the enzyme in the process of the invention. By "enzyme" it is intended to encompass any polypeptide having hydrolytic activity on chlorophyll or a chlorophyll derivative, including e.g. enzyme fragments, etc. Any isolated, recombinant or synthetic or chimeric (or a combination of synthetic and recombinant) polypeptide can be used.

Enzyme (chlorophyllase, pheophytinase or pyropheophytinase) activity assay

Hydrolytic activity on chlorophyll or a chlorophyll derivative may be detected using any suitable assay technique, for example based on an assay described herein. For example, hydrolytic activity may be detected using fluorescence-based techniques. In one suitable assay, a polypeptide to be tested for hydrolytic activity on chlorophyll or a chlorophyll derivative is incubated in the presence of a substrate, and product or substrate levels are monitored by fluorescence measurement. Suitable substrates include e.g. chlorophyll, pheophytin and/or pyropheophytin. Products which may be detected include chlorophyllide, pheophorbide, pyropheophorbide and/or phytol.

Assay methods for detecting hydrolysis of chlorophyll or a chlorophyll derivative are disclosed in, for example, Ali Khamessan et al. (1994), Journal of Chemical Technology & Biotechnology, 60(1), pages 73 – 81; Klein and Vishniac (1961), J. Biol. Chem. 236: 2544-2547; and Kiani et al. (2006), Analytical Biochemistry 353: 93–98.

Alternatively, a suitable assay may be based on HPLC detection and quantitation of substrate or product levels following addition of a putative enzyme, e.g. based on the techniques described below. In one embodiment, the assay may be performed as described in Hornero-Mendez et al. (2005), Food Research International 38(8-9): 1067-1072. In another embodiment, the following assay may be used:

170 µl mM HEPES, pH 7.0 is added 20 µl 0.3 mM chlorophyll, pheophytin or pyropheophytin dissolved in acetone. The enzyme is dissolved in 50 mM HEPES, pH 7.0. 10 µl enzyme solution is added to 190 µl substrate solution to initiate the reaction and incubated at 40°C for various time periods. The reaction was stopped by addition of 350 µl acetone. Following centrifugation (2 min at 18,000 g) the supernatant was analyzed by HPLC, and the amounts of (i) chlorophyll and chlorophyllide (ii) pheophytin and pheophorbide or (iii) pyropheophytin and pyropheophorbide determined.

In a further embodiment, enzyme activity may be determined using an assay as described in WO2011/125028.

One unit of enzyme activity is defined as the amount of enzyme which hydrolyzes one micromole of substrate (e.g. chlorophyll, pheophytin or pyropheophytin) per minute at 40°C, e.g. in an assay method as described herein.

In preferred embodiments, the enzyme used in the present method has chlorophyllase, pheophytinase and/or pyropheophytinase activity of at least 1000 U/g, at least 5000 U/g, at least 10000 U/g, or at least 50000 U/g, based on the units of activity per gram of the purified enzyme, e.g. as determined by an assay method described herein.

Chlorophyllases

In one embodiment, the enzyme is capable of hydrolyzing at least chlorophyll. Any polypeptide that catalyses the hydrolysis of a chlorophyll ester bond to yield chlorophyllide and phytol can be used in the process. For example, a chlorophyllase, chlase or chlorophyll

chlorophyllido-hydrolyase or polypeptide having a similar activity (e.g., chlorophyll-chlorophyllido hydrolase 1 or chlase 1, or, chlorophyll-chlorophyllido hydrolase 2 or chlase 2, see, e.g. NCBI P59677-1 and P59678, respectively) can be used in the process. Typically the chlorophyllase is also capable of hydrolyzing pheophytin and/or pyropheophytin at least to some extent.

In one embodiment the enzyme is a chlorophyllase classified under the Enzyme Nomenclature classification (E.C. 3.1.1.14). Any isolated, recombinant or synthetic or chimeric (a combination of synthetic and recombinant) polypeptide (e.g., enzyme or catalytic antibody) can be used, see e.g. Marchler-Bauer (2003) Nucleic Acids Res. 31: 383-387. In one aspect, the chlorophyllase may be an enzyme as described in WO 0229022 or WO 2006009676. In one embodiment, the *Arabidopsis thaliana* chlorophyllase can be used as described, e.g. in NCBI entry NP_199199. Thus the chlorophyllase may be a polypeptide comprising the sequence of SEQ ID NO:2 (see Figure 2). In another embodiment, the chlorophyllase is derived from algae, e.g. from *Phaeodactylum tricornutum*.

In another embodiment, the chlorophyllase is derived from wheat, e.g. from *Triticum sp.*, especially from *Triticum aestivum*. For example, the chlorophyllase may be polypeptide comprising the sequence of SEQ ID NO:4 (see Figure 4).

Further suitable chlorophyllase sequences are shown in Table 1 below, and in Figures 1 to 31 (SEQ ID NO:s 1 to 31):

Table 1. Chlorophyllases with accession numbers and names used herein.

Organism	**Database acc. no.**	**CHL name**
Arabidopsis thaliana	AAG12547	ARA_CHL
Arabidopsis thaliana	NP_199199	ARA_CHL2
Citrus sinensis	AAF59834	CIT_CHL
Triticum aestivum	BT009214	TRI_CHL
Triticum aestivum	BT008923	TRI_CHL2
Brassica oleracea	AAN51935	BRA_CHL
Brassica oleracea	AAN51933	BRA_CHL1
Brassica oleracea	AAN51934	Brass_CHL2
Zea Mays	ACN32030	ZEA_CHL
Zea Mays	ACG44273	ZEA_CHL2
Phyllostachys edulis	FP092915	BAM_CHL
Chenopodium album	Q9LE89	CHE_CHL
Ricinus communis	XP_002517075	CB_CHL
Glycine max	BAF43704	GlyMax_CHL

Ginkgo biloba	AAP44978	Gin_CHL
Pachira macrocarpa	ACO50429	PAC_CHL2
Populus trichocarpa	XP_002315752	POP_CHL
Sorghum bicolor	XP_002459848	Sor_CHL
Sorghum bicolor	XP_002445588	SORG_CHL
Vitis vinifera	XP_002273926	Vitis_CHL
Physcomitrella patens	EDQ81786	PHYS_CHL
Aquilegia		AQU_CHL
Brachypodium distachyon	ADDN01001446	BRACH_CHL
Medicago truncatula	ACJ85964	MED_CHL
Piper betle	ABI96085	PIP_CHL
Lotus japonicus	AK338339	LOTUS_CHL
Oryza sativa Indica	EEC66959	ORYI_CHL
Oryza sativa Japonica	NP_001064620	ORYJ1_CHL
Oryza sativa Japonica	EEE50970	ORYJ2_CHL
Picea sitchensis	ACN40275	PICEA_CHL
Chlamydomonas	XP_001695577	CHL_CHL

Multiple chlorophyllase amino acid sequences show the conserved sequence motif GHSRG (SEQ ID NO: 32). In particular, the serine residue at the active site of the enzyme, which is present in this motif, is highly conserved. In preferred embodiments, the enzyme used in the present invention comprises an enzyme as shown in Table 1 above and/or any of Figures 1 to 31, comprising any one of SEQ ID NOs 1 to 31, or comprising the sequence motif of SEQ ID NO:32, including fragments, variants and derivatives thereof. These chlorophyllases are typically capable of hydrolysing at least pheophytin (and optionally also pyropheophytin), in addition to chlorophyll.

Pheophytin pheophorbide hydrolase

In one embodiment, the enzyme is capable of hydrolyzing pheophytin and pyropheophytin. For example, the enzyme may be pheophytinase or pheophytin pheophorbide hydrolase (PPH), e.g. an enzyme as described in Schelbert *et al.*, The Plant Cell 21:767-785 (2009).

PPH and related enzymes are capable of hydrolyzing pyropheophytin in addition to pheophytin. However PPH is inactive on chlorophyll. As described in Schelbert *et al.*, PPH orthologs are commonly present in eukaryotic photosynthesizing organisms. PPHs represent a defined sub-group of α/β hydrolases which are phylogenetically distinct from chlorophyllases, the two groups being distinguished in terms of sequence homology and substrates.

In specific embodiments of the invention, the enzyme may be any known PPH derived from any species or a functional variant or fragment thereof or may be derived from any known PPH enzyme. In particular, the enzyme may be a PPH derived from any one of the following species: *Arabidopsis thaliana, Populus trichocarpa, Vitis vinifera, Oryza sativa, Zea mays, Nicotiana tabacum, Ostreococcus lucimarinus, Ostreococcus taurii, Physcomitrella patens, Phaeodactylum tricornutum, Chlamydomonas reinhardtii,* or *Micromonas sp. RCC299*. For example, the enzyme may be a polypeptide comprising an amino acid sequence, or encoded by a nucleotide sequence, defined in one of the following database entries shown in Table 2, or a functional fragment or variant thereof:

Table 2

Organism	**Accession**	**Genbank ID**
Arabidopsis thaliana	NP_196884	15240707
Populus trichocarpa	XP_002314066	224106163
Vitis vinifera	CAO40741	157350650
Oryza sativa (japonica)	NP_001057593	115467988
Zea mays	ACF87407	194706646
Nicotiana tabacum	CAO99125	156763846
Ostreococcus lucimarinus	XP_001415589	145340970
Ostreococcus tauri	CAL50341	116000661
Physcomitrella patens	XP_001761725	168018382
Phaeodactylum tricornutum	XP_002181821	219122997
Chlamydomonas reinhardtii	XP_001702982	159490010
Micromonas sp. RCC299	ACO62405	226516410

Variants and fragments

Functional variants and fragments of known sequences which hydrolyse chlorophyll or a chlorophyll derivative may also be employed in the present invention. By "functional" it is meant that the fragment or variant retains a detectable hydrolytic activity on chlorophyll or a chlorophyll derivative. Typically such variants and fragments show homology to a known chlorophyllase, pheophytinase or pyropheophytinase sequence, e.g. at least about 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a known chlorophyllase, pheophytinase or pyropheophytinase amino acid sequence, e.g. to any one of SEQ ID NOs: 1 to 31, e.g. over a region of at least about 10, 20, 30, 50, 100, 200, 300, 500, or 1000 or more residues, or over the entire length of the sequence.

The percentage of sequence identity may be determined by analysis with a sequence comparison algorithm or by a visual inspection. In one aspect, the sequence comparison algorithm is a BLAST algorithm, e.g., a BLAST version 2.2.2 algorithm.

Other enzymes having chlorophyllase, pheophytinase and/or pyropheophytinase activity suitable for use in the process may be identified by determining the presence of conserved sequence motifs present e.g. in known chlorophyllase, pheophytinase or pyropheophytinase sequences. For example, many chlorophyllases comprise the conserved sequence motif GHSRG (SEQ ID NO: 32), which as mentioned above comprises a serine residue present at the active site. Conserved sequence motifs found in PPH enzymes include the following: LPGFGVG (SEQ ID NO:33), DFLGQG (SEQ ID NO:34), GNSLGG (SEQ ID NO:35), LVKGVTLLNATPFW (SEQ ID NO:36), HPAA (SEQ ID NO:37), EDPW (SEQ ID NO:38), and SPAGHCPH (SEQ ID NO:39). In some embodiments, an enzyme for use in the present invention may comprise one or more of these sequences. The GNSLGG (SEQ ID NO:35) motif present in PPH enzymes contains an active site serine residue. Polypeptide sequences having suitable activity may be identified by searching genome databases, e.g. the microbiome metagenome database (JGI-DOE, USA), for the presence of these motifs.

Isolation and production of enzymes

Enzymes for use in the present invention may be isolated from their natural sources or may be, for example, produced using recombinant DNA techniques. Nucleotide sequences encoding polypeptides having chlorophyllase, pheophytinase and/or pyropheophytinase activity may be isolated or constructed and used to produce the corresponding polypeptides.

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).

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.

Typically, the nucleotide sequence encoding a polypeptide having chlorophyllase, pheophytinase and/or pyropheophytinase activity is prepared using recombinant DNA techniques. 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).

Modification of enzyme sequences

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 enzymes which hydrolyse chlorophyll and/or chlorophyll derivatives 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 enzymes with preferred characteristics. Suitable methods for performing 'shuffling' can be found in EP0752008, EP1138763, EP1103606. 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

chlorophyllase, pheophytinase or pyropheophytinase 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, a nucleotide sequence encoding an enzyme (e.g. a chlorophyllase, pheophytinase and/or pyropheophytinase) used in the invention may encode a variant enzyme, i.e. the variant enzyme 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%, or 99% identity with the parent enzyme. Suitable parent enzymes may include any enzyme with hydrolytic activity on chlorophyll and/or a chlorophyll derivative.

Polypeptide sequences

The present invention also encompasses the use of amino acid sequences encoded by a nucleotide sequence which encodes a chlorophyllase, pheophytinase or pyropheophytinase 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 comparison

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 Advance™ 11 (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 Advance™ 11 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; and FEMS Microbiol Lett 1999 177(1): 187-8.).

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 Advance™ 11 package.

Alternatively, percentage homologies may be calculated using the multiple alignment feature in Vector NTI Advance™ 11 (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 default parameters for the programme are used for pairwise alignment. For example, the following parameters are the current default parameters for pairwise alignment for BLAST 2:

FOR BLAST2	DNA	PROTEIN
EXPECT THRESHOLD	10	10
WORD SIZE	11	3
SCORING PARAMETERS		
Match/Mismatch Scores	2, -3	n/a
Matrix	n/a	BLOSUM62
Gap Costs	Existence: 5 Extension: 2	Existence: 11 Extension: 1

In one embodiment, preferably the sequence identity for the nucleotide sequences and/or amino acid sequences may be determined using BLAST2 (blastn) with the scoring parameters set as defined above.

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 Advance™ 11 (Invitrogen Corp.). For pairwise alignment the scoring parameters used are preferably BLOSUM62 with Gap existence penalty of 11and Gap extension penalty of 1.

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.

Amino acid mutations

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

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 plant 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 plant 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 pyropheophytinase sequence which it is desired to clone, bringing the primers into contact with mRNA or cDNA obtained from a plant 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.

Enzyme formulation

Enzymes used in the methods of the invention can be formulated or modified, e.g., chemically modified, to enhance oil solubility, stability, activity or for immobilization. For example, enzymes used in the methods of the invention can be formulated to be amphipathic or more lipophilic. For example, enzymes used in the methods of the invention can be encapsulated, e.g., in liposomes or gels, e.g., alginate hydrogels or alginate beads or equivalents. Enzymes used in the methods of the invention can be formulated in micellar systems, e.g., a ternary micellar (TMS) or reverse micellar system (RMS) medium. Enzymes used in the methods of the invention can be formulated as described in Yi (2002) J. of Molecular Catalysis B: Enzymatic, Vol. 19, pgs 319-325.

Enzyme dosing

In embodiments of the present invention, the enzyme is added to the oil in multiple doses. More particularly, the enzyme is typically added in a series of doses to the oil, wherein a reaction step is performed after each dose. By this it is meant that after addition of each dose

of the enzyme, the enzyme is contacted with the oil for a period of time to allow the enzyme to degrade at least some of the chlorophyll or chlorophyll derivatives present in the oil.

The enzyme may be dosed into the oil in any suitable amount. For example, the enzyme may be dosed in a range of about 0.001 to 10Units/g of the composition (e.g. based on the total weight of the oil), preferably 0.01 to 1 U/g, e.g. 0.01 to 0.1 U/g of the oil. One unit is defined as the amount of enzyme which hydrolyses 1 μmol of substrate (e.g. chlorophyll, pheophytin and/or pyropheophytin) per minute at 40°C, e.g. under assay conditions as described in J. Biol. Chem. (1961) 236: 2544-2547.

In preferred embodiments of the present invention, the enzyme may be added to the oil in a number of relatively low doses, instead a single large bolus dose. Thus in one embodiment, for example, each individual dose may comprise less than 0.1 Units chlorophyllase/g oil, or less than 0.08, 0.07, 0.06 or 0.05 U/g.

The enzyme may be added in two or more doses, e.g. 3, 4, 5 or more doses in total. Preferably the process involves adding a first dose and at least two or three further doses. In particularly preferred embodiments, each reaction step takes place in a different reaction vessel. Preferably each reaction vessel is a continuous stirred tank reactor.

Temperature

In embodiments of the present invention, after addition of each dose of the enzyme, a reaction step is performed. One or more or all of the reaction steps of the present process may be performed under the conditions described below.

In general the oil may be incubated (or admixed) with the enzyme at any temperature about 5°C to and about 100°C, more preferably between 10°C to about 90°C. However, in particularly preferred embodiments, each reaction step is preferably performed about 70°C to about 90°C, more preferably between about 75°C to about 80°C.

At higher temperatures, the enzymatic reaction rate is increased. However, above 65°C chlorophyllases become inactivated quite rapidly. As demonstrated herein, an efficient degradation of chlorophyll derivatives can be achieved by performing the reaction at high temperature but adding multiple aliquots of chlorophyllase, and performing a series of short reaction steps.

Accordingly, further preferred temperature ranges for the incubation of the enzyme with the oil include about 70°C to about 88°C, about 72°C to about 87°C, about 72°C to about 85°C, about 73°C to about 83°C, about 73°C to about 82°C, and about 74°C to about 81°C.

Preferably the temperature of the oil may be at the desired reaction temperature when the enzyme is admixed therewith. The oil 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. However, in preferred embodiments no heating or cooling step is necessary before addition of the enzyme, because the oil is already at a suitable temperature (e.g. about 70°C to about 90°C).

Reaction time

Suitably the reaction time (i.e. the time period in which the enzyme is incubated with the oil in each reaction step), preferably with agitation, is for a sufficient period of time to allow hydrolysis of at least some of the chlorophyll and chlorophyll derivatives present in the oil, e.g. to form phytol and chlorophyllide, pheophorbide and/or pyropheophorbide. For example, the reaction time may be at least about 1 minute, more preferable at least about 5 minutes, more preferably at least about 10 minutes. In some embodiments the reaction time may be between about 5 to 60 minutes, preferably between about 10 minutes to about 30 minutes, preferably about 15 to about 20 minutes. In embodiments of the present invention, each individual reaction step is preferably of relatively short duration, particularly where the process is performed at high temperatures.

pH and water content

Preferably the process is carried out between about pH 4.0 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 7.0, more preferably between about pH 5.5 and about pH 6.5.

Suitably the water content of the oil when incubated (or admixed) with the enzyme is between about 0.5 to about 5% water, more preferably between about 1 to about 3% and more preferably between about 1.5 and about 2% by weight. In specific embodiments, the water

content may be, for example, 0.7% to 1.2%, e.g. about 1% by weight; or 1.7% to 2.2%, e.g. about 2% by weight.

Chlorophyll and/or chlorophyll derivative removal

The process of the present invention involving an enzyme treatment typically reduces the level of chlorophyll and/or chlorophyll derivatives in the oil. For example, the process may reduce the concentration of chlorophyll, pheophytin and/or pyropheophytin by at least 5%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 99%, compared to the concentration of chlorophyll, pheophytin and/or pyropheophytin (by weight) present in the oil before treatment. Thus in particular embodiments, the concentration of chlorophyll and/or chlorophyll derivatives in the oil after treatment may be less than 100, less than 50, less than 30, less than 10, less than 5, less than 1, less than 0.5, less than 0.1 mg/kg or less than 0.02 mg/kg, based on the total weight of the oil.

Further processing steps

In a typical plant oil processing method, oil is extracted in hexane, the crude vegetable oil is degummed, optionally caustic neutralized, bleached using, e.g. clay adsorption with subsequent clay disposal, and deodorized to produce refined, bleached and deodorized or RBD oil. The need for the degumming step depends on phosphorus content and other factors. The process of the present invention can be used in conjunction with processes based on extraction with hexane and/or enzyme assisted oil extraction (see Journal of Americal Oil Chemists' Society (2006), 83 (11), 973-979). In general, the process of the invention may be performed using oil processing steps as described in Bailey's Industrial Oil and Fat Products (2005), 6th edition, Ed. by Fereidoon Shahidi, John Wiley & Sons. In preferred embodiments of the present invention, the enzyme is contacted with a crude oil, preferably before a degumming step.

Further processing steps, after treatment with the enzyme, may assist in removal of the products of enzymatic hydrolysis of chlorophyll and/or chlorophyll derivatives. For instance, further processing steps may remove chlorophyllide, pheophorbide, pyropheophorbide and/or phytol.

Degumming

The degumming step in oil refining serves to separate phosphatides by the addition of water. The material precipitated by degumming is separated and further processed to mixtures of lecithins. The commercial lecithins, such as soybean lecithin and sunflower lecithin, are semi-solid or very viscous materials. They consist of a mixture of polar lipids, primarily phospholipids such as phosphatidylcholine with a minor component of triglycerides (e.g. 30-50%). Lecithin may be deoiled to reduce the triglyceride content to below 5%. Thus as used herein, the term "degumming" means the refining of oil by removing phospholipids from the oil. In some embodiments, degumming may comprise a step of converting phosphatides (such as lecithin and phospholipids) into hydratable phosphatides.

The process of the invention can be used with any degumming procedure, particularly in embodiments where the chlorophyll- or chlorophyll derivative-hydrolyzing enzyme is contacted with the oil before the degumming step. Thus suitable degumming methods include water degumming, ALCON oil degumming (e.g., for soybeans), safinco degumming, "super degumming," UF degumming, TOP degumming, uni-degumming, dry degumming and ENZYMAX^{TM} degumming. See e.g. U.S. Patent Nos. 6,355,693; 6,162,623; 6,103,505; 6,001,640; 5,558,781; 5,264,367, 5,558,781; 5,288,619; 5,264,367; 6,001,640; 6,376,689; WO 0229022; WO 98118912; and the like. Various degumming procedures incorporated by the methods of the invention are described in Bockisch, M. (1998), Fats and Oils Handbook, The extraction of Vegetable Oils (Chapter 5), 345-445, AOCS Press, Champaign, Illinois.

Water degumming typically refers to a step in which the oil is incubated with water (e.g. 1 to 5% by weight) in order to remove phosphatides. Typically water degumming may be performed at elevated temperature, e.g. at 50 to 90°C. The oil/water mixture may be agitated for e.g. 5 to 60 minutes to allow separation of the phosphatides into the water phase, which is then removed from the oil.

Acid degumming may also be performed. For example, oil may be contacted with acid (e.g. 0.1 to 0.5% of a 50% solution of citric or malic acid) at 60 to 70°C, mixed, contacted with 1 to 5% water and cooled to 25 to 45 °C.

Further suitable degumming procedures for use with the process of the present invention are described in WO 2006/008508. In one embodiment the process comprises contacting the

chlorophyll- or chlorophyll derivative-hydrolyzing enzyme with the oil and subsequently performing an enzymatic degumming step using an acyltransferase as described in WO 2006/008508. Acyltransferases suitable for use in the process are also described in WO 2004/064537, WO 2004/064987 and WO 2009/024736. Any enzyme having acyltransferase activity (generally classified as E.C.2.3.1) may be used, particularly enzymes comprising 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. In one embodiment, acyltransferase is a mutant *Aeromonas salmonicida* mature lipid acyltransferase (GCAT) with a mutation of Asn80Asp, e.g. an acyltransferase comprising the amino acid sequence of SEQ ID NO:40 after undergoing post-translational modification (see Figure 45), or an enzyme having at least 80% sequence identity thereto.

In another embodiment, the process comprises a degumming step using a phospholipase. Any enzyme having e.g. a phospholipase A1 (E.C.3.1.1.32) or a phospholipase A2 (E.C.3.1.1.4) activity may be used, for example Lecitase Ultra® or pancreatic phospholipase A2 (Novozymes, Denmark). In one embodiment the process comprises contacting the chlorophyll- or chlorophyll derivative-hydrolyzing enzyme with the oil and subsequently performing an enzymatic degumming step using a phospholipase, for example using a degumming step as described in US 5,264,367, EP 0622446, WO 00/32758 or Clausen (2001) "Enzymatic oil degumming by a novel microbial phospholipase," Eur. J. Lipid Sci. Technol. 103:333-340.

In another such embodiment, an enzymatic degumming step using an enzyme such as phospholipase C (IUB 3.1.4.1) may be used. Polypeptides having phospholipase C activity which are may be used in a degumming step are disclosed, for example, in WO2008143679, WO2007092314, WO2007055735, WO2006009676 and WO03089620. A suitable phospholipase C for use in the present invention is Purifine®, available from Verenium Corporation, Cambridge, MA.

In one embodiment, an enzymatic degumming step is performed after the chlorophyllase treatment step, e.g. in one or more further reaction vessels.

Acid treatment/caustic neutralization

In some embodiments, an acid treatment/caustic neutralization step may be performed, e.g. in order to further reduce phospholipid levels in the oil. In another embodiment, a single degumming step comprising acid treatment/caustic neutralization may be performed. Such methods are typically referred to as total degumming or alkali refining. The acid treatment step may be performed at any stage in the process. For instance in one embodiment, the acid treatment is performed before addition of the enzyme, and is followed by a caustic neutralization step in order to adjust the pH to about 5.5 to 6.5 (before the enzyme treatment). In another embodiment, the acid treatment is performed after the enzyme treatment, and optionally after an enzymatic degumming step (and centrifugal separation of gum).

It has been found that an acid treatment/caustic neutralization step is particularly effective in removing products of the enzymatic hydrolysis of chlorophyll, e.g. chlorophyllide, pheophorbide and pyropheophorbide. For example, such a step may comprise addition of an acid such as phosphoric acid followed by neutralization with an alkali such as sodium hydroxide. Following an acid/caustic neutralization treatment compounds such as chlorophyllide, pheophorbide and pyropheophorbide are extracted from the oil in an aqueous phase.

In such methods, the oil is typically first contacted with 0.05 to 0.5% by weight of concentrated phosphoric acid, e.g. at a temperature of 50 to 90°C, and mixed to help precipitate phosphatides. The contact time may be, e.g. 10 seconds to 30 minutes. Subsequently an aqueous solution of an alkali (e.g. 1 to 20% aqueous sodium hydroxide) is added, e.g. at a temperature of 50 to 90°C, followed by incubation and mixing for 10 seconds to 30 minutes. The oil may then be heated to about 90°C and the aqueous soap phase separated from the oil by centrifugation.

Optionally, further wash steps with e.g. sodium hydroxide or water may also be performed.

Chlorophyllide, pheophorbide and pyropheophorbide removal

Thus the method of the present invention may optionally involve a step of removing phytol-free derivatives of chlorophyll such as chlorophyllide, pheophorbide and pyropheophorbide. Such products may be present in the composition due to the hydrolysis of chlorophyll or a chlorophyll derivative by the enzyme of the invention, or may be present naturally, as a

contaminant, or as an undesired component in a processed product. Pyropheophorbide may also be present in the composition due to the breakdown of pheophorbide, which may itself be produced by the activity of an enzyme having pheophytinase activity on pheophytin, or pheophorbide may be formed from chlorophyllide following the action of chlorophyllase on chlorophyll (see Figure 32). Processing conditions used in oil refining, in particular heat, may favour the formation of pyropheophorbide as a dominant component, for instance by favouring the conversion of pheophytin to pyropheophytin, which is subsequently hydrolysed to pyropheophorbide.

In one embodiment the process of the present invention reduces the level of chlorophyllide, pheophorbide and/or pyropheophorbide in the oil, compared to either or both of the levels before and after enzyme treatment. Thus in some embodiments the chlorophyllide, pheophorbide and/or pyropheophorbide concentration may increase after enzyme treatment. Typically the process involves a step of removing chlorophyllide, pheophorbide and/or pyropheophorbide such that the concentration of such products is lower than after enzyme treatment. Preferably the chlorophyllide, pheophorbide and/or pyropheophorbide produced by this enzymatic step is removed from the oil, such that the final level of these products in the oil is lower than before enzyme treatment.

For example, the process may reduce the concentration of chlorophyllide, pheophorbide and/or pyropheophorbide by at least 5%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 99%, compared to the concentration of chlorophyllide, pheophorbide and/or pyropheophorbide (by weight) present in the oil before the chlorophyllide, pheophorbide and/or pyropheophorbide removal step, i.e. before or after enzyme treatment. Thus in particular embodiments, the chlorophyllide, pheophorbide and/or pyropheophorbide concentration in the oil after the removal step may be less than 100, less than 50, less than 30, less than 10, less than 5, less than 1, less than 0.5, less than 0.1 mg/kg, or less than 0.02 mg/kg, based on the total weight of the composition (e.g a vegetable oil).

It is an advantage of the present process that reaction products such as chlorophyllide, pheophorbide and/or pyropheophorbide may be simply and easily removed from the oil by a step such as acid treatment/caustic neutralization. Thus in preferred embodiments chlorophyll

and chlorophyll derivatives may be substantially removed from the oil without the need for further processing steps such as clay and/or silica treatment and deodorization.

Oil separation

In some embodiments, the process comprises a step of separating the treated oil from an aqueous phase, e.g. following the addition of the enzyme and/or the degumming step. In one embodiment the treated liquid (e.g. oil) is separated with an appropriate means such as a centrifugal separator and the processed oil is obtained. Upon completion of the enzyme treatment, if necessary, the processed oil can be additionally washed with water or organic or inorganic acid such as, e.g., acetic acid, citric acid, phosphoric acid, succinic acid, and the like, or with salt solutions.

Clay treatment

It is particularly preferred that the process reduces the need for clay treatment of the oil. For instance, the process may reduce the amount of clay required by at least 50%, 70%, 80%, 90%, 95% or 99% by weight, e.g. compared to the amount of clay required to treat the oil in the absence of a chlorophyllase treatment step. In one embodiment, the process does not comprise a clay treatment step. Avoiding the use of clay is advantageous for the reasons described earlier, in particular the reduction in cost, the reduced losses of oil through adherence to the clay and the increased retention of useful compounds such as carotenoids and tocopherol.

In some embodiments, the process may be performed with no clay treatment step and no deodorization step, which results in an increased concentration of such useful compounds in the refined oil, compared to a process involving clay treatment.

Silica treatment

Although not always required, in some embodiments the process may comprise a step of silica treatment, preferably subsequent to the enzyme treatment. For example, the method may comprise use of an adsorbent-free or reduced adsorbent silica refining devices and processes, which are known in the art, e.g., using TriSyl Silica Refining Processes (Grace Davison, Columbia, MD), or, SORBSIL R^{TM} silicas (INEOS Silicas, Joliet, IL).

The silica treatment step may be used to remove any remaining chlorophyllide, pheophorbide and/or pyropheophorbide or other polar components in the oil. For example, in some embodiments a silica treatment step may be used as an alternative to an acid treatment/caustic neutralization (total degumming or alkali refining) step.

In one embodiment the process comprises a two-stage silica treatment, e.g. comprising two silica treatment steps separated by a separation step in which the silica is removed, e.g. a filtration step. The silica treatment may be performed at elevated temperature, e.g. at above about 30°C, more preferably about 50 to 150°C, about 70 to 110°C, about 80 to 100°C or about 85 to 95°C , most preferably about 90°C.

Deodorization

In some embodiments, the process may comprise a deodorization step, typically as the final refining step in the process. In one embodiment, deodorization refers to steam distillation of the oil, which typically removes volatile odor and flavor compounds, tocopherol, sterols, stanols, carotenoids and other nutrients. Typically the oil is heated to 220 to 260°C under low pressure (e.g. 0.1 to 1 kPa) to exclude air. Steam (e.g. 1-3% by weight) is blown through the oil to remove volatile compounds, for example for 15 to 120 minutes. The aqueous distillate may be collected.

In another embodiment, deodorization may be performed using an inert gas (e.g. nitrogen) instead of steam. Thus the deodoriztion step may comprise bubble refining or sparging with an inert gas (e.g. nitrogen), for example as described by A. V. Tsiadi *et al.* in "Nitrogen bubble refining of sunflower oil in shallow pools", Journal of the American Oil Chemists' Society (2001), Volume 78 (4), pages 381-385. The gaseous phase which has passed through the oil may be collected and optionally condensed, and/or volatile compounds extracted therefrom into an aqueous phase.

In some embodiments, the process of the present invention is performed with no clay treatment but comprising a deodorization step. Useful compounds (e.g. carotenoids, sterols, stanols and tocopherol) may be at least partially extracted from the oil in a distillate (e.g. an aqueous or nitrogenous distillate) obtained from the deodorization step. This distillate provides a valuable source of compounds such as carotenoids and tocopherol, which may be at least partially lost by entrainment in a process comprising clay treatment.

The loss of tocopherol during bleaching depends on bleaching conditions and the type of clay applied, but 20-40% removal of tocopherol in the bleaching step has been reported (K. Boki, M, Kubo, T. Wada, and T. Tamura, ibid., 69, 323 (1992)). During processing of soy bean oil a loss of 13% tocopherol in the bleaching step has been reported (S. Ramamurthi, A. R. McCurdy, and R. T. Tyler, in S. S. Koseoglu, K. C. Rhee, and R. F. Wilson, eds., Proc. World Conf. Oilseed Edible Oils Process, vol. 1, AOCS Press, Champaign, Illinois, 1998, pp. 130–134).

Carotenoids may be removed from the oil during deodorization in both clay-treated and non-clay-treated oil. Typically the removal of coloured carotenoids is controlled in order to produce an oil having a predetermined colour within a specified range of values. The level of carotenoids and other volatile compounds in the refined oil can be varied by modifying the deodorization step. For instance, in an embodiment where it is desired to retain a higher concentration of carotenoids in the oil, the deodorization step may be performed at a lower temperature (e.g. using steam at 200°C or below). In such embodiments it is particularly preferable to avoid a clay treatment step, since this will result in a higher concentration of carotenoids in the refined oil.

Further enzyme treatments

In further aspects, the processes of the invention further comprise use of lipid acyltransferases, phospholipases, proteases, phosphatases, phytases, xylanases, amylases (e.g. α-amylases), glucanases, polygalacturonases, galactolipases, cellulases, hemicellulases, pectinases and other plant cell wall degrading enzymes, as well as mixed enzyme preparations and cell lysates. In alternative aspects, the processes of the invention can be practiced in conjunction with other processes, e.g., enzymatic treatments, e.g., with carbohydrases, including cellulase, hemicellulase and other side degrading activities, or, chemical processes, e.g., hexane extraction of soybean oil. In one embodiment the method of the present invention can be practiced in combination with a method as defined in WO 2006031699.

Apparatus for oil refining

In some embodiments, the present invention provides an apparatus for performing the process described herein. Typically the apparatus comprises a plurality of reaction vessels arranged in series, such that each reaction step of the process may be performed in a different reaction

vessel. By each "reaction vessel" it is meant a distinct container, chamber or other vessel which contains a volume of liquid in which the reaction occurs. Thus each reaction vessel may comprise a separate container or vessel, e.g. operably connected to further reaction vessels by one or more connecting vessels, pipes, tubes or conduits. Alternatively each reaction vessel may comprise a single chamber within a larger vessel or container, provided that it is possible to regulate the flow of oil between chambers in the vessel. For instance, a vessel may be divided into 3 or 4 separate reaction chambers, each of which can be isolated from further chambers to perform a distinct reaction step therein, but which is operably connected to further chambers in order to permit transfer of oil between the chambers.

Typically the apparatus comprises a first reaction vessel for performing a first enzymatic hydrolysis step, and one or more further reaction vessels for performing one or more further enzymatic hydrolysis steps. Thus the apparatus comprises at least two distinct reaction vessels, each of which is suitable for performing an enzymatic reaction. The apparatus may comprise, for example, 2, 3, 4, 5 or more reaction vessels, each of which may be the same or different.

Various types of reaction vessels are known in the art for performing enzymatic reactions in oil refining plants. Preferably one or more (or each of) the reaction vessels comprises a continuous stirred tank reactor. Continuous stirred tank reactors are well known and their use is described, for example, by Uppal *et* al. in Chemical Engineering Science (1974), Volume 29(4), pages 967–985.

In embodiments of the present invention, the first and further reaction vessels are arranged in series. By this it is meant that the reaction vessels are arranged one after another, such that oil can flow from the first reaction vessel to a second reaction vessel, from the second reaction vessel to a third reaction vessel and so on. Thus each of the reaction vessels is in fluid communication with a preceding and/or succeeding reaction vessel in the series.

Typically the flow of oil between the reaction vessels may be controlled by one or more valves and/or pumps. For instance, each reaction vessel may comprise one or more inlet and/or outlet valves, or valves may be positioned in a connecting vessel or conduit which transports oil between reaction vessels. One or more pumps, e.g. positioned before the first reaction vessel and/or between reaction vessels, may be provided for propelling the oil

through the system. Transport of oil between the reaction vessels may be regulated by the opening and closing of the valves and/or operation of the pump(s).

In one embodiment, the apparatus comprises a plurality of enzyme input means. Each enzyme input means permits the introduction of a dose of the enzyme between each reaction vessel. By this it is meant that each enzyme input means provides a dose of the enzyme for enzymatic hydrolysis in a particular reaction vessel. For example, each enzyme input means may permit introduction of a dose of the enzyme directly into a particular reaction vessel. Alternatively, the enzyme input means may enable the enzyme to be introduced into a connecting vessel or conduit which transports oil from one reaction vessel to the next.

The enzyme input means may, for example, comprise an inlet or conduit which permits addition of the enzyme, typically in the form of an aqueous solution, into the plant oil being processed in the apparatus. The enzyme input means may further comprise one or more valves for pumps for regulating the entry of the enzyme (e.g. enzyme solution) into the oil. Addition of the enzyme may be followed by an inline mixing step, e.g. using a static mixer or high shear mixer.

In one embodiment, one or more (or all) of the reaction vessels may comprise a mixing means. The mixing means may comprise a mixer, stirrer, agitator or any other type of device which is capable of enabling mixing the contents of the reactor, i.e. the oil and enzyme. Continuous stirred tank reactors, as employed in one embodiment of the present invention, may use various mechanically-driven mixing devices in order to achieve continuous mixing of reactants.

In another embodiment, the apparatus further comprises one or more reaction vessels for performing an enzymatic degumming step. Typically the reaction vessel(s) for enzymatic degumming are located downstream of the chlorophyllase reaction vessels, i.e. the reaction vessels are arranged such that that oil flows into the enzymatic degumming vessels after the series of reaction vessels discussed above. The apparatus may comprise one or more enzyme input means for introducing a phospholipase or acyltransferase into the oil, e.g. an inlet or conduit, optionally together with one or more associated valves and/or pumps, which permits addition of the enzyme (in the form of an aqueous solution) into the oil being processed.

In further embodiments, the apparatus may comprise one or more further components typically found in vegetable oil refining units. For instance, the apparatus may comprise a centrifugal separator (e.g. a centrifuge for gum separation and/or separation of soap stock), one or more acid treatment and/or caustic neutralization tanks, a silica treatment vessel and/or a deodorizer. In some embodiments, the apparatus further comprises one or more oil heaters and/or coolers, for regulating the temperature of the oil at one or more points during the process.

The invention will now be further illustrated with reference to the following non-limiting examples.

EXAMPLE 1

Cloning and expression of chlorophyllase genes in *E. coli*

Synthetic genes encoding *Arabidopsis thaliana* (ARA_CHL2, SEQ ID NO:2) and *Triticum aestivum* (TRI_CHL, SEQ ID NO:4) chlorophyllases were prepared. Each gene was codon optimized for expression in *E. coli*. For cloning purposes the genes were extended in the 5'-end to contain a restriction site for NheI and in the 3'-end to contain a restriction site for XhoI.

Following digestion with NheI and XhoI restriction enzymes the synthetic DNA was ligated into the *E. coli* expression vector pET-28a(+) (Novagen) digested with the same restriction enzymes. This vector includes a T7 promoter with a Lac operator for controlling expression of inserted genes. The chlorophyllase genes were fused in frame to a His tag and a thrombin cleavage site for purification (example shown in Figure 33). The resulting constructs (an example pET28-TRI_CHL is shown in Figure 34), were transformed into competent *E. coli* TOP10 cells (Invitrogen), and plasmids were isolated from transformed colonies and subjected to nucleotide sequencing to verify the correct sequence and that all fusions were as expected.

For expression the plasmids were transformed into the expression host *E. coli* BL21(DE3) (Novagen). The cells were cultured at 37°C in LB containing carbenicillin (50mg/ml) until OD_{600} 0.6-0.8. For induction the culture was added 1 mM IPTG and incubated at 25°C for another 20-24 h before harvesting the cells by centrifugation. The recombinant

chlorophyllases were released from the cell pellet by sonication and cellular debris removed by centrifugation.

EXAMPLE 2

Temperature stability of *Arabidopsis* and *Triticum* chlorophyllases

Fermentation samples of *Arabidopsis thaliana* (ARA_CHL2, SEQ ID NO:2) and *Triticum aestivum* (TRI_CHL, SEQ ID NO:4) chlorophyllases were heated to temperatures between 40 and 85°C for 10 min and subsequently rapidly cooled to 4°C. Residual pheophytinase activity was measured using a pheophytinase activity assay as described in WO2011/125028. The results are shown in Figure 35.

As seen from Fig. 35, both enzymes show very similar heat inactivation profiles with 65°C being critical for the inactivation. After 10 minutes incubation at a temperature of 70°C or above, the residual activity of both enzymes is very low.

EXAMPLE 3

Treatment of rapeseed oil with serial addition of chlorophyllase

The kinetics of the hydrolysis of pheophytin by a chlorophyllase from *Arabidopsis* (ARA_CHL2, SEQ ID NO:2) was investigated by running a series of experiments, in which this enzyme was added to crude rapeseed oil at different enzyme dosages (see Table 1). Crude rapeseed oil was scaled in a Wheaton glass and heated to 60°C. Water and enzyme was added and mixed with a high speed Turrax mixer for 20 seconds followed by incubation at 60°C with magnetic stirring. Samples were taken out after ½, 1, 2 and 4 hours and analyzed by liquid chromatography-mass spectrometry (LC-MS) with results shown in Table 2.

Table 1: Recipe for chlorophyllase treatment of crude rapeseed oil

Sample no.		1	2	3	4
Crude rapeseed oil, (no. 22 from AarhusKarlsham, Denmark)	g	10	10	10	10
water	ml	0.183	0.158	0.116	0.075

Arabidopsis chlorophyllase (ARA_CHL2, SEQ ID NO:2, 5.98 Units/ml)	ml	0.017	0.042	0.084	0.125
Units chlorophyllase/g oil		0.0100	0.0250	0.0500	0.0750
% Water		2.000	2.000	2.000	2.000
Temperature	°C	60	60	60	60

Table 2: LC-MS analysis of pheophytin (initial conc. =8.65 ppm) in crude rapeseed oil treated with chlorophyllase

Dosage\time	0.5 hr.	1 hr.	2 hr.	4hr.	Velocity
	µg/g	µg/g	µg/g	µg/g	constant
0.01 U/g	5.33	4.83	2.31	0.55	-0.674
0.025 U/g	3.91	2.54	1.1	0.19	-0.864
0.05 U/g	3.63	2.18	0.66	0.1	-1.030
0.075 U/g	2.58	1.44	0.38	0.06	-1.074

Based on the results from Table 2, a first order decay for degradation of pheophytin as a function of time can be constructed and the velocity constant (as shown in Table 2) calculated:

$$Ca = Ca_o\, e^{(K \times T)}$$

wherein:

Ca= concentration of pheophytin, ppm

Ca_o= initial concentration of pheophytin, ppm

K= velocity constant

T = reaction time, hr

Based on the above results, in embodiments of the present invention the present process may be implemented in a continuous process line with continuous stirred tank reactors, for example in an oil refining plant having a capacity of more than 30 tonnes per day (typically 20 to 50 tonnes per hour). Because the reaction of chlorophyllase with pheophytin shows a first order decay, it is preferable to run the enzyme reaction in a series of reactors in order to reduce the level of pheophytin to approx. 50 ppb or lower.

Chlorophyllase activity may depend on the amount of phospholipids in the crude oil. In order to achieve good hydrolysis of chlorophyll derivatives it is therefore preferable to perform the chlorophyllase reaction before any phospholipases or acyltransferases are added to the oil. Provided that the phospholipase is added after the chlorophyllase reaction has ceased, it is therefore possible to use an acyltransferase/phospholipase for improvement of oil yield. Continuous stirred tank reactors for the chlorophyllase reaction can be followed by a reactor for acyltransferase/phospholipase treatments in one extra tank in series. A series of reactors for both the chlorophyllase and phospholipase/acyltransferase reactions can be implemented as indicated in Fig. 36.

Based on velocity constant (Table 2) for the chlorophyllase (0.075 U/g) reaction at 60°C in 4 stirred tank reactors in series, the pheophytin concentration in each reactor is calculated (see Table 3).

Table 3: Pheophytin concentration in 4 stirred tank reactors in series

Reactor	Conc. into reactor	flow	Volume	velocity constant	space time	Ca =Pheophytin
nr.	CA_o. ppm	m^3/hr	m^3	k= ppm/hr	tau . hr	ppm
1	8.65	8	20	1.07	2.5	2.35
2	2.35	8	20	1.07	2.5	0.64
3	0.64	8	20	1.07	2.5	0.17
4	0.17	8	20	1.07	2.5	0.05

In the fifth reactor (Fig. 36) the phospholipase reaction takes place and contributes to reduce the oil loss in the gum phase.

The integration of a combined chlorophyllase and phospholipase/acyltransferase reaction in a more sustainable oil refining process with a reduced amount of waste material and with improved oil yield can be implemented in a process line as shown in Fig. 37.

In some embodiments, an acid treatment may be applied to the crude oil, e.g. with phosphoric acid or citric acid in order to remove non-hydratable phospholipids. Typically the acid is added to the crude oil before the chlorophyllase treatment. In some embodiments, the pH is

adjusted to e.g. pH 5.5-6.5 before chlorophyllase treatment in order to secure good enzyme activity. The extension of the process line with an acid treatment is illustrated in the flow diagram in Fig. 38. In some embodiments, the acid treatment takes place after centrifugation and removal of the gum phase (see Fig. 39).

EXAMPLE 4

Chlorophyllase treatment of rapeseed oil at elevated temperature

The velocity constant of chlorophyll degradation by chlorophyllase in oil depends not only on the enzyme dosage but also depends on other parameters like temperature. It is known that enzyme reactions as well as other chemical reactions are dependent on temperature. As long as the enzyme is active and no denaturation occurs, the reaction velocity increases with an increase in temperature. It is however also known that most enzymes at higher temperatures start to denature and lose their activity.

Indeed, the chlorophyllase from *Arabidopsis* is stable in oil at a temperature up to approximate 60°C. At temperatures above 65°C the enzyme starts to denature and lose its activity as a function of time (see Example 2 above).

In normal oil refining, the oil after extraction is normally at a temperature of 75 to 85°C and therefore needs to be cooled down before chlorophyllase treatment. From a process point of view this creates some difficulties because a heat exchanger needs to be installed, and also energy is needed to cool down the oil. After the chlorophyllase reaction the oil should be heated up to 75-80°C before centrifugation because the oil separates better from the water phase at 75-85°C. Heating up the oil before centrifugation requires further energy.

Therefore in embodiments of the present invention, chlorophyllase treatment of crude oil may advantageously place at 75-85°C. Performing the enzyme reaction at these temperatures will both save installation costs on the heat exchanger and energy expenditure.

Although the chlorophyllase from *Arabidopsis* starts to denature above 65°C, it will initially show activity on pheophytin at higher temperatures. In the following experiments the initial velocity for pheophytin decay as a function of temperature above 60°C was investigated.

Crude rapeseed oil (no 22 from AarhusKarlshamn, Denmark) was treated with chlorophyllase according to the recipe in Table 4.

Table 4. Chlorophyllase treatment of crude rapeseed oil at elevated temperature

Sample no.		1	2	3	4
Crude rapeseed oil, (no. 22 from AarhusKarlsham, Denmark)	g	10	10	10	10
water	ml	0.200	0.183	0.158	0.116
Arabidopsis chlorophyllase (ARA_CHL2, SEQ ID NO:2, 5.98 Units/ml)	ml		0.017	0.042	0.084
Units chlorophyllase/g oil		0.000	0.0100	0.025	0.050
% Water		2.000	2.000	2.000	2.000

Crude rapeseed oil was scaled in a 20 ml Wheaton glass and a magnetic rod added. The oil was heated to temperature (65, 70, 75 or 80°C) and enzyme and water was added.

The sample was treated with Ultra Turrax mixer for 20 seconds followed by incubation with magnetic stirring (450 rpm) for 4 hours.

After ½, 1, 2 and 4 hours a 1 ml sample was taken out and centrifuged at 10000 rcf for 5 minutes. Pheophytin and pyropheophytin in the oil phase were analysed by LC-MS. For the experiment at 80°C, samples was taken out after ¼, ½, 1 and 2 hr. and analysed by LC-MS. Based on the LC-MS analysis of pheophytin the initial velocity constant was determined as the slope of the curve for ln(ppm pheophytin) as a function of time. The calculation of initial velocity constant is based on first order kinetic decay of pheophytin. The initial first order kinetic was confirmed by very high correlation coefficients for the relationship between ln(ppm pheophytin) and time.

The initial velocity constants of pheophytin decay at different dosage and temperature are shown in Table 5.

Table 5: Initial velocity constant (ppm/hr) for pheophytin decay at different temperature and enzyme dosages.

Dosage/temperature	65°C	70°C	75°C	80°C
0.01 U/g	-1.38	-1.66	-1.71	-1.81
0.025 U/g	-2.06	-2.71	-2.91	-3.83
0.05 U/g	-3.00	-3.75	-4.36	-5.27

EXAMPLE 5

Chlorophyllase treatment of rapeseed oil at 75°C

The results in Table 5 confirm a significant effect of temperature on the initial velocity constant. In embodiments of the present invention, the fact that initially high conversion of pheophytin by chlorophyllase is observed at higher temperatures may be used to conduct the enzyme treatment at a higher temperature than 65°C in stirred tank reactors in series, particularly by adding the enzyme between each tank.

The effect of adding chlorophyllase between each tank in a stirred tank reactor at 75°C is replicated by adding the enzyme several times in a batch process according to the recipe in Table 6.

Table 6

		Exp. 1	Exp. 2	Exp. 3
Crude rapeseed oil, (no. 22 from AarhusKarlsham, Denmark)	g	50	50	50
water	ml	1.100	0.036	0.834
Arabidopsis chlorophyllase (ARA_CHL2, SEQ ID NO:2, 9.4 Units/ml)	ml		1.064	0.266
Total Water	%	2.200	2.200	2.200
Extra chlorophyllase (ARA_CHL2, SEQ ID NO:2, 9.4 Units/ml) after 20min	ml			0.266
Extra chlorophyllase (ARA_CHL2, SEQ ID	ml			0.266

NO:2, 9.4 Units/ml) after 40min				
Extra chlorophyllase (ARA_CHL2, SEQ ID NO:2, 9.4 Units/ml) after 60min	ml			0.266
Extra water after 20min	ml	0.266	0.266	
Extra water after 40min	ml	0.266	0.266	
Extra water after 60min	ml	0.266	0.266	
Total Units chlorophyllase/g oil	U/g	0.000	0.200	4 x 0.05
Temperature	°C	75	75	75

Oil was scaled in a 20 ml Wheaton glass and a magnetic rod was added. The oil was heated to 75°C and enzyme and water was added. The sample was mixed with an Ultra Turrax mixer for 20 seconds followed by incubation at 75°C with magnetic stirring. After 20 minutes more enzyme and water was added and the sample was mixed with Ultra turrax for 20 seconds. This was repeated at 40 and 60 minutes reaction time. Samples were taken out after 20, 40, 60 and 80 minutes reaction time and centrifuged at 10000 rcf for 5 minutes. Pheophytin and pyropheophytin were analysed by LC-MS with results shown in Table 7.

Table 7 LC-MS analysis of pheophytin and pyropheophytin in oil treated with chlorophyllase at 75°C

Sample ID	Reaction Time, min	Pheophorbide ppm	Pheophytin ppm	Pyropheophytin ppm
Exp. 1	20	0.52	4.81	0.68
Exp. 2	20	3.39	0.72	0.16
Exp. 3	20	3.09	1.34	0.28
Exp. 1	40	0.64	4.91	0.65
Exp. 2	40	3.78	0.27	0.08
Exp. 3	40	3.66	0.27	0.09
Exp. 1	60	0.84	4.73	0.74
Exp. 2	60	3.98	0.13	0.04
Exp. 3	60	3.89	0.09	0.03
Exp. 1	80	0.79	4.33	0.65

Exp. 2	80	4.03	0.10	0.03
Exp. 3	80	4.01	0.07	0.02

The results are also illustrated graphically in Fig. 40.

The results from Table 7 and Fig. 40 indicate that initially the degradation of pheophytin in experiment no. 2 (0.2 U/g) is stronger than experiment no. 3 (0.05 U/g), but already after 40 minutes pheophytin content is lower in experiment no. 3 (3 x 0.05 U/g) than experiment no. 2. After 80 minutes reaction time the amount of pheophytin plus pyropheophytin is below 0.1 ppm in experiment no. 3, and significant lower than in experiment no. 2. It is therefore concluded that at 75°C chlorophyllase can advantageously be added in multiple portions to compensate for the thermal degradation of the enzyme.

The kinetics of pheophytin degradation by chlorophyllase can be illustrated by plotting ln(pheophytin) as a function of time (see Fig. 41). It is apparent from Fig. 41 that multiple additions of enzyme can compensate for the thermal degradation of the enzyme so that the first order kinetic remains valid.

EXAMPLE 6

Chlorophyllase treatment of rapeseed oil at 80°C

The experiment mentioned in Example 5 was repeated at 80°C. However, in this experiment the enzyme addition was conducted every 15 minutes, because degradation of the enzyme is faster at 80°C. The LC-MS analysis for the experiment is shown in Table 8.

Table 8 LC-MS analysis of pheophytin and pyropheophytin in oil treated with chlorophyllase at 80°C

Sample	Min.	Pheophorbide	Pheophytin	Pyropheophytin
ID		µg/g	µg/g	µg/g
Exp. 1	15	0.51	5.30	0.66
Exp. 2	15	2.84	1.47	0.26
Exp. 3	15	2.49	2.01	0.32

Exp. 1	30	0.58	5.04	0.66
Exp. 2	30	3.31	0.51	0.11
Exp. 3	30	3.19	0.37	0.12
Exp. 1	45	0.77	5.08	0.88
Exp. 2	45	3.92	0.26	0.08
Exp. 3	45	3.71	0.12	0.06
Exp. 1	60	0.88	4.63	0.66
Exp. 2	60	3.56	0.17	0.06
Exp. 3	60	3.19	0.08	0.04

The results are also illustrated graphically in Fig. 42.

The kinetics of pheophytin degradation by chlorophyllase can be illustrated by plotting ln(pheophytin) as a function of time (see Fig. 43). It is apparent from Fig. 43 that also at 80°C multiple addition of enzyme every 15 minutes can compensate for the thermal degradation of the enzyme, so that the first order kinetic remains valid.

Based on the results obtained by conducting chlorophyllase reactions at 75 or 80°C, where the enzyme is added 4 times at the same time interval of 20 or 15 minutes respectively, it is concluded that it is possible to conduct the chlorophyllase reaction in stirred tank reactors in series at temperatures of 75 to 80°C.

The enzyme can preferably be added in the pipeline between the stirred tanks as illustrated in Fig. 44. Thus Fig. 44 illustrates one embodiment of a plant oil refining apparatus according to the present invention. The apparatus comprises a plurality of continuous stirred tank reactors arranged in series (1, 2, 3, 4 and 5), and a plurality of enzyme input means (6, 7, 8, 9 and 10). The apparatus further comprises a centrifugal gum separator (11), a caustic neutralization tank (12), a centrifuge for soap stock separation (13), a silica treatment tank (14) and a deodorizer (15).

The apparatus is arranged such that crude oil enters the process, and a first enzyme input means (6) introduces a first dose of chlorophyllase into the oil. Following mixing the oil with added enzyme enters the first reaction vessel (1), where a first chlorophyllase reaction step

takes place with mixing. By virtue of valve and/or pump means regulating the flow, the oil then passes into a connecting vessel where a second enzyme input means (7) introduces a further dose of chlorophyllase into the oil. A second chlorophyllase reaction step then takes place in the second reaction vessel (2). Further doses of chlorophyllase are added to the oil by enzyme input means (8) and (9), and third and fourth chlorophyllase reaction steps take place in continuous stirred tank reactors (3) and (4). Approximately one quarter of the total dose of chlorophyllase is added to the oil in each of the four doses.

After exiting reactor (4), an acyltransferase or phospholipase is added to the oil by enzyme input means (10), and an enzymatic degumming step takes place in continuous stirred tank reactor (5). Separation of gum from the oil is performed by centrifuge (11). The oil then continues along the apparatus for further oil refining by components (12) to (15). The output of the process is a refined plant oil with reduced content of chlorophyll derivatives, phospholipids and other undesirable components.

Conclusion

Chlorophyllase reactions in crude oil can be described by a first order reaction kinetic. The chlorophyllase reaction preferably takes place in continuous stirred reactors in series.

The chlorophyllase reaction can be followed by an acyltransferase/phospholipase reaction to improve oil yield by the reduction of oil content in the gum phase. Very significantly, the combination of a chlorophyllase reaction followed by an acyltransferase/phospholipase reaction makes it possible to eliminate the use of bleaching clay and water wash in the oil refining process.

These enzyme reactions combined with silica treatment after neutralization opens up the possibility for a completely new and more sustainable oil refining plant design and process, which produces refined oil of edible quality in higher yield and with reduced amounts of waste because of the elimination the bleaching clay.

The industrial use of enzymes in the oil refining processes is often challenged by the fact that oil, during refining, is often present at 70 to 90°C. At these temperatures, enzymes often start

to denature and become inactive. Although enzymes are denatured at higher temperatures, the initial velocity of an enzyme reaction increases with increasing temperature. These features can be utilized to conduct chlorophyllase reactions in stirred tank reactors in series at higher temperatures than in batch processes, by adding the enzyme at different time intervals to the oil.

Experiments have shown that a chlorophyllase from *Arabidopsis*, which in a batch process is operated at maximum 65°C, can be applied to stirred tank reactors in series at 75-80°C and benefit from the higher reaction velocities at 75-80°C compared to 65°C, despite the denaturation of the enzyme at this temperature as a function of time. A further benefit of such a process is that the oil does not need to be cooled down before the enzyme reaction, and thus saves energy and investment costs for heat exchangers.

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

CLAIMS

1. A process for treating a plant oil, comprising:

(i) adding a first dose of an enzyme which is capable of hydrolysing chlorophyll or a chlorophyll derivative to the oil;

(ii) performing a first reaction step in which the enzyme hydrolyses chlorophyll or a chlorophyll derivative present in the oil; and

(iii) adding one or more further doses of the enzyme to the oil, wherein a further reaction step is performed after addition of each further dose of the enzyme to the oil.

2. A process according to claim 1, wherein each reaction step is performed at a temperature of 70 to 90°C.

3. A process according to claim 2, wherein each reaction step is performed at a temperature of 75 to 80°C.

4. A process according to any preceding claim, wherein the process is performed in a series of reaction vessels, such that each reaction step takes place in a different reaction vessel.

5. A process according to any preceding claim, wherein at least two further doses of the enzyme are added to the oil.

6. A process according to any preceding claim, wherein at least three further doses of the enzyme are added to the oil.

7. A process according to any preceding claim, further comprising an enzymatic degumming step.

8. A process according to claim 7, wherein the enzymatic degumming step comprises contacting the oil with a phospholipase or an acyltransferase.

9. A process according to claim 7 or claim 8, wherein the enzymatic degumming step is performed in one or more further reaction vessels.

10. A process according to any preceding claim, wherein the enzyme comprises a polypeptide sequence as defined in any one of SEQ ID NOs: 1 to 31, or a functional fragment or variant thereof having at least 75% sequence identity to any one of SEQ ID NOs: 1 to 31 over at least 50 amino acid residues and having chlorophyllase, pheophytinase and/or pyropheophytinase activity.

11. A process according to any preceding claim, further comprising an acid treatment step.

12. A process according to claim 11, wherein the oil is treated with an acid, and then the pH of the oil is adjusted to 5.5 to 6.5 by addition of an alkali, before addition of the enzyme to the oil.

13. A process according to claim 11, wherein the oil is treated with an acid after addition of the enzyme to the oil.

14. A treated plant oil obtainable by a process as defined in any preceding claim.

15. An apparatus for plant oil refining, comprising:

(i) a first reaction vessel for performing a first enzymatic hydrolysis step; and

(ii) one or more further reaction vessels for performing one or more further enzymatic hydrolysis steps;

wherein the first and further reaction vessels are arranged in series and are in fluid communication with one another, such that the oil can be transferred from the first reaction vessel to each further reaction vessel in series; and

wherein the apparatus comprises a plurality of enzyme input means, arranged such that a dose of an enzyme can be introduced into the oil between each reaction vessel.

16. An apparatus according to claim 15, wherein the enzyme is capable of hydrolysing chlorophyll or a chlorophyll derivative.

17. An apparatus according to claim 15 or claim 16, wherein each reaction vessel further comprises a mixing means for mixing the oil during each enzymatic hydrolysis step.

18. An apparatus according to any of claims 15 to 17, wherein the apparatus further comprises one or more reaction vessels for performing an enzymatic degumming step, and one or more further enzyme input means for introducing a phospholipase or acyltransferase into the oil before the enzymatic degumming step.

19. An apparatus according to any of claims 15 to 18, wherein each reaction vessel comprises a continuous stirred tank reactor.

20. An apparatus according to any of claims 15 to 19, further comprising one or more acid treatment vessels.

Figure 1 (SEQ ID NO:1)

```
ARA_CHL
       1   MAAIEDSPTF SSVVTPAAFE IGSLPTTEIP VDPVENDSTA PPKPVRITCP
      51   TVAGTYPVVL FFHGFYLRNY FYSDVLNHIA SHGYILVAPQ LCKLLPPGGQ
     101   VEVDDAGSVI NWASENLKAH LPTSVNANGK YTSLVGHSRG GKTAFAVALG
     151   HAATLDPSIT FSALIGIDPV AGTNKYIRTD PHILTYKPES FELDIPVAVV
     201   GTGLGPKWNN VMPPCAPTDL NHEEFYKECK ATKAHFVAAD YGHMDMLDDD
     251   LPGFVGFMAG CMCKNGQRKK SEMRSFVGGI VVAFLKYSLW GEKAEIRLIV
     301   KDPSVSPAKL DPSPELEEAS GIFV
```

Figure 2 (SEQ ID NO:2)

```
ARA_CHL2
       1   MSSSSSRNAF EDGKYKSNLL TLDSSSRCCK ITPSSRASPS PPKQLLVATP
      51   VEEGDYPVVM LLHGYLLYNS FYSQLMLHVS SHGFILIAPQ LYSIAGPDTM
     101   DEIKSTAEIM DWLSVGLNHF LPAQVTPNLS KFALSGHSRG GKTAFAVALK
     151   KFGYSSNLKI STLIGIDPVD GTGKGKQTPP PVLAYLPNSF DLDKTPILVI
     201   GSGLGETARN PLFPPCAPPG VNHREFFREC QGPAWHFVAK DYGHLDMLDD
     251   DTKGIRGKSS YCLCKNGEER RPMRRFVGGL VVSFLKAYLE GDDRELVKIK
     301   DGCHEDVPVE IQEFEVIM
```

Figure 3 (SEQ ID NO:3)

```
CIT_CHL
       1   MAAMVDAKPA ASVQGTPLLA TATLPVFTRG IYSTKRITLE TSSPSSPPPP
      51   KPLIIVTPAG KGTFNVILFL HGTSLSNKSY SKIFDHIASH GFIVVAPQLY
     101   TSIPPPSATN ELNSAAEVAE WLPQGLQQNL PENTEANVSL VAVMGHSRGG
     151   QTAFALSLRY GFGAVIGLDP VAGTSKTTGL DPSILSFDSF DFSIPVTVIG
     201   TGLGGVARCI TACAPEGANH EEFFNRCKNS SRAHFVATDY GHMDILDDNP
     251   SDVKSWALSK YFCKNGNESR DPMRRCVSGI VVAFLKDFFY GDAEDFRQIL
     301   KDPSFAPIKL DSVEYIDASS MLTTTHVKV
```

Figure 4 (SEQ ID NO:4)

```
TRI_CHL
       1   MAAAAPAETM NKSAAGAEVP EAFTSVFQPG KLAVEAIQVD ENAAPTPPIP
      51   VLIVAPKDAG TYPVAMLLHG FFLHNHFYEH LLRHVASHGF IIVAPQFSIS
     101   IIPSGDAEDI AAAAKVADWL PDGLPSVLPK GVEPELSKLA LAGHSRGGHT
     151   AFSLALGHAK TQLTFSALIG LDPVAGTGKS SQLQPKILTY EPSSFGMAMP
     201   VLVIGTGLGE EKKNIFFPPC APKDVNHAEF YRECRPPCYY FVTKDYGHLD
     251   MLDDDAPKFI TCVCKDGNGC KGKMRRCVAG IMVAFLNAAL GEKDADLEAI
     301   LRDPAVAPTT LDPVEHRVA
```

Figure 5 (SEQ ID NO:5)

```
TRI_CHL2
       1   MAAMATTVFQ AGPMEVDVKH VDKSMIPNLA RPLMVVAPKE TGAYPVIVFL
      51   HGWNMLNSWY EQLLTHVASH GFIAVAPQLY WMVSEPDADD IDATKRITNW
     101   LADHDKGLAH VLKDVLKLEH VEPDLSKLAL AGHSRGGQTA FAVALGLGDA
     151   KTKLELKFSA LIGVDPVAGV SRAQQLEPKV LTFEPDCLDV GMPVLVMGTG
     201   LGPKHIGGFP CAPVGVNHAE FYKECAPPRY HLVVKDYGHL DMLDDNVPYI
     251   INNCMCMRNQ HDTKDLARRT MGGAMVAFLR AKLRIDVRDL IAIYHNPEIA
     301   PAVLDQVDEF LPCFVGRPNP SSV
```

Figure 6 (SEQ ID NO:6)

```
BRA_CHL
       1   MSPSFLFFTL FLIKEMSSSS SANSFEDGKY KTDLLTVGLS SCCWKKPSSS
      51   PTPQSPPKRL LVATPVEEGE YPVVMLLHGY LLYNSFYSQL MLHVSSHGFI
     101   VIAPQLYSIA GPDTMDEIKS TAEIIDWLSV GLNHFLPPQV TPNLSKFALS
     151   GHSRGGKTAF ALALKKFGYS SDLKISALIG IDVGTVFWTN GYGQYSGEFF
     201   EQFDCRNDRI VES
```

Figure 7 (SEQ ID NO:7)

```
BRA_CHL1
       1   MAGKEDSETF FSAATPLAFE LGSLPTTVIP ADPSATDLTA PPKPVIITSP
      51   TVAGTYPVVL FFHGFYLRNY FYSDVINHVA SHGYIVVAPQ LCKILPPGGQ
     101   VEVDDAGKVI NWTSKNLKAH LPSSVNANGN YTALVGHSRG GKTAFAVALG
     151   HAATLDPSIK FSALVGIDPV AGISKCIRTD PEILTYKPES FDLDMPVAVI
     201   GTGLGPKSNM LMPPCAPAEV NHEEFYIECK ATKGHFVAAD YGHMDMLDDN
     251   LPGFVGFMAG CMCKNGKRKK SEMRSFVGGI VVAFLKYSIW GEMSEIRQIL
     301   KDPSVSPARL DPSPELEEAS GYLV
```

Figure 8 (SEQ ID NO:8)

```
Brass_CHL2
       1   MSSSSSRNAF VDGKYKPDLL TVDLASRCRC YKTTPSSSLT PPPPPKSLLV
      51   ATPVEEGEYP VVMLLHGYLL YNSFYSQLML HVSSYGFIVI APQLYNIAGP
     101   DTIDEIKSTA EIIDWLSVGL NHFLPPQVTP NLSKFALTGH SRGGKTAFAV
     151   ALKKFGYSSE LKISAIIGVD PVDGTGKGKQ TPPPVLTYEP NSFNLEKMPV
     201   LVIGSGLGEL ARNPLFPPCA PTGVNHREFF QECQGPAWHF VAKDYGHLDM
     251   LDDDTKGLRG KSSYCLCKNG EERKPMRRFI GGIVVSFLMA YLEDDDCELV
     301   KIKAGCHEGV PVEIQEFEVK K
```

Figure 9 (SEQ ID NO:9)

```
ZEA_CHL
       1   MAASPVAIGT AVFQRGPLRV EARHVDYSQV PSVPKPLMVV APTDAGVYPV
      51   AVFLHGCNTV NSWYESLLSH VASHGFIAVA PQLYCVTLNM NDLKDIDATR
     101   QVTAWLADKQ QGLAHVLANI LQLHGVRPDL SRLALAGHSR GGDTAFAVAL
     151   GLGPAASDDD DNNADAGTSP AALPLKFSAL IGVDPVAGLS KQAQVEPKVL
     201   TFRPRSLDPG MPALVVGTGL GPKHVGGPPC APAGVNHAEF YDECAPPRYH
     251   VVLRDYGHMD MLDDDGVPYV INNCMCMRNT KDTKDLARRA IGGAVVAFLR
     301   ATLEDDDEDL KVVLENRPGL SPAVLDPVGH DLA
```

Figure 10 (SEQ ID NO:10)

```
ZEA_CHL
       1   MNLASAVRVF LSYCLLLHRW MGSEQAGGVF DQGGHSVSLT RLDEARAPPR
      51   CAVQSSLSSA ASLPPKPLLV AAPRETGEYP VILFLHGYLA VNSFYSQLFE
     101   HVASHGFIVV GPQLYTISGA DTTEEINSAA AVIDWLATGL PSTLPLGVRA
     151   DLTKVSISGH SRGGKVAFAL ALGHAKAKLA VPLAAVVAVD PVDGMGVGKQ
     201   TPPPILTGRH GSLHVGAPTM VIGTGLGELP RGSLLPPCAP RGVSHAAFYD
     251   ELDGAAPACH LVARDYGHTD MMDDDTPGAR GMLTRTICRS GGARAPMRRF
     301   VAGATVAFLK KWVAGDAAAM DSITARPDQA PIALSVVEFG DEKAIA
```

Figure 11 (SEQ ID NO:11)

```
BAM_CHL
       1   MAATAEIKIP STEALEAVTS VFRPGKLAVE LVPVDHNAVP TPPIPILIVA
      51   PKDAGTYPVA MLLHGFFLQN HFYEHLLKHV ASHGFIMVAP QFHAICTGET
     101   EDIAAAAKVT DWLPEGLPSV LLKGVEADLS KLALAGHSRG GHTAFSLALG
     151   HGKTNLNFAA LIGLDPVAGT GKSSQLPPKI LTYKPSSFDV AMPVLVIGTG
     201   LGEEKKNVLF PPCAPKDVNH REFYYECKPP CYYFVTKDYG HLDMLDDDAP
     251   KFITCLCKDG DNCKDKMRRA VAGIMIAFLR AVLDEKDGDI KVILKDPGLA
     301   PVTLDPVECR LP
```

Figure 12 (SEQ ID NO:12)

```
CHE_CHL
       1   MAKLLLLIFG VFIFVNSQAQ TFPTILEKHN SEKITDVFHK GNFQVTNNPI
      51   RVKRYEFSAP EPLIIISPKE AGVYPVLLFI HGTMLSNEDY SLFFNYIASH
     101   GFIVVAPKLF RLFPPKLPSQ QDEIDMAASV ANWMPLYLQV VLQRYVTGVE
     151   GDLEKLAISG HSRGGKSAFA LALGFSNIKL DVTFSALIGV DPVAGRSVDD
     201   RTLPHVLTYK PNSFNLSIPV TVIGSGLGNH TISCAPNHVS HQQFYDECKE
     251   NSSHFVITKY GHMDMLNEFR LSPIAVTMSL MCAQSFRPKA TMRRTLGGIM
     301   VAFLNAYFRD DGRQYYAIIA NRSLAPTNLF AEKKGFNFGF ATTYAQL*
```

Figure 13 (SEQ ID NO:13)

```
CB_CHL
       1   MSSSCATVTN VYENGKYTTV VAKIESGSCA RSSLPLPLPP KPLLIAMPSE
      51   AGEFPVLIFL HGYLLYNSFY SLLIQHVASH GFIVIAPQLY TVAGADSADE
     101   IKCTAAITNW LSKGLHHVLP PHVQPKLSKL GLAGHSRGGK AAFALALQKA
     151   GISTALKFSA LIGVDPVDGM DKGKQTPPPV LTYTPHSFDL DMAAMVIGSG
     201   LGEVKRNPMF PPCAPKGVNH EDFFKECKKP AYYFVVKDYG HLDMLDDDTN
     251   GIRGKATYCL CVNGKSREPM RRFVGGVLVA FLKAYLGGDS SDLMTITDGQ
     301   TGPVELQAAE CYV
```

Figure 14 (SEQ ID NO:14)

```
GlyMax_CHL
       1   MAQRAQPALA TTDVFQKGDI HWKQFNVETS TASSSPPKPL LIFTPTVPGL
      51   YPVILFCHGF CIRTSYYSKL LAHIVSHGFI LVAPQLFSIG VPMFGPEEVK
     101   CEGRVVDWLD NGLQPLLPES VEAKLEKLVL VGHSKGGKTA FAVALGYCKT
     151   KLKFSALIGI DPVAGVSKCK PCRSLPDILT GVPRSFNLNI PVAVIGTGLG
     201   PEKANSLFPP CAPNGVNHKE FFSECKPPSA YFVATDYGHM DMLDDETPGV
     251   IGTMMSKCMC KNGKKGPRDL MRRTVGGLVV AFLRAQLNEQ WKDFDAILAS
     301   PNLAPAKLDD VRYLPT
```

Figure 15 (SEQ ID NO:15)

```
Gin_CHL
       1   MVLVKDVFSE GPLPVQILAI PQANSSPCSK LADKNGTATT PSPCRPPKPL
      51   LIALPSQHGD YPLILFFHGY VLLNSFYSQL LRHVASHGYI AIAPQMYSVI
     101   GPNTTPEIAD AAAITDWLRD GLSDNLPQAL NNHVRPNFEK FVLAGHSRGG
     151   KVAFALALGR VSQPSLKYSA LVGLDPVDGM GKDQQTSHPI LSYREHSFDL
     201   GMPTLVVGSG LGPCKRNPLF PPCAPQGVNH HDFFYECVAP AYHFVASDYG
     251   HLDFLDDDTK GIRGKATYCL CKNGEAREPM RKFSGGIVVA FLQAFLGDNR
     301   GALNDIMVYP SHAPVKIEPP ESLVTEDVKS PEVELLRRAV CR
```

Figure 16 (SEQ ID NO:16)

```
PAC_CHL
       1   MAQLLETKHD LSTVVPVFVT GKYHPTSVSV DPSNSSPSSP PKPLLIFTPS
      51   EQGTYPVILF FHGFYLRNNF YTGLLLHISS HGFIIVAPQL SNIIPPSGTE
     101   EVEHAAKVAD WLPSGLPSVL PGNVEANLAK LALVGHSRGG KTAFALALGR
     151   AKTAQNFSAL VGIDPVAGNR FGETSPKILT YTPGSFDLSI PVAVVGTGLG
     201   PESKGCMPCP CAPTQYNHEE FFNECKPPRV HFDAKNYGHM DTLDDNPSGF
     251   IGKLSDTICV NGEGPRDPMR RCVGGIVVAF LNYFFEAEKE DFMTIMNEPY
     301   VAPVTLDQVQ FNV
```

Figure 17 (SEQ ID NO:17)

```
POP_CHL
       1   MSSSSAIATV TTTVFEAGKY TTVLQKVESR TTCCTAKTSP PLPVPPPKPL
      51   LIVMPCEAGE FPLLVFLHGY LLYNSFYSQL LQHIASHGFI VIAPQLYLVA
     101   GQDSSDEIKS VAATTNWLSE GLHHLLPPHV KPNLSKLGLA GHSRGGKTAF
     151   ALALEKAAAT LKFSALIGVD PVDGMDKGKQ TPPPVLTYVP HSFDLDMAIM
     201   VIGSGLGELK KNPLFPPCAP EGVNHKDFFK ECKGPASYFV VKDYGHLDML
     251   DDDTEGIRGK TTYCLCKNGK SREPMRKFIG GVVVAFMKAY LGGDSSDLMA
     301   IKGGQTGPVE LQTVEYIL
```

Figure 18 (SEQ ID NO:18)

```
Sor_CHL
       1   MATTPKVLEE PPSAVITSVF QPGKLAVEVI SVEHDARPTP PPIPILIAAP
      51   KDAGTYPVAI LLHGFFLQNR YYEQLLKHVA SFGFIMVAPQ FHTSLISNSD
     101   ADDIAAAAKV TDWLPEGLPT VLPTGVEADL SKLALAGHSR GGHTAFSLAL
     151   GYAKTNTSSL LKFSALIGLD PVAGTGKNSQ LPPAILTYEP SSFDIAVPVL
     201   VIGTGLGDER ENALFPPCAP VEVNHAEFYR ECRAPCYHLV TKDYGHLDML
     251   DDDAPKLVTC LCKEGNTCKD VMRRTVAGIM VAFLKAVMGE DEDGDLKAIL
     301   QHPGLAPTIL DPVEYRLA
```

Figure 19 (SEQ ID NO:19)

```
SORG_CHL
       1   MASPVAISTT AVFKRGRHPV DTKHVDHSQV PGVPKPLMVV TPTDAGVYPV
      51   AVFLHGCSMY NSWYQTLLSH VASHGFIAVA PQLGGILPPL DMKDLKDIDA
     101   TRKVTAWLAD NLAHVLTNIL HLHGVTPDLS RLALAGHSRG GDTAFAVALG
     151   LGSSSSSSDT TPLKFSALIG VDPVAGLSKE LQLEPKVLTF EPRSLDPGMP
     201   ALVVGTGLGP KGLLPCAPAG VSHGEFYDEC APPRYHVVVR DYGHLDMLDD
     251   DGVPYVISNC MCKRNTNTTK DLARRAIGGA MVAFLRAKLE DDDEDLRAVL
     301   QNSPGLSPAV LDPVEYDDDE AMDGPGCAGN NGVAGASG
```

Figure 20 (SEQ ID NO:20)

```
Vitis_CHL
       1   MALLGGNPST QGIKLDLKTT TSVFEPGNLS VTCIRVETSN IASPPKPLLI
      51   VTPTIQGTYP VLLFLHGFEL RNTFYTQLLQ LISSHGYIVV APQLYGLLPP
     101   SGIQEIKSAA AVTNWLSSGL QSVLPENVKP DLLKLALSGH SRGGKTAFAL
     151   ALGYADTSLN FSALLGLDPV GGLSKCSQTV PKILTYVPHS FNLAIPVCVI
     201   GTGLGDEPRN CLTCPCAPDG VNHVEFFSEC KPPCSHFVTT EYGHLDMLDD
     251   HLSGCIGAIS GYICKSGKGP RDPMRRCVGG LFVAFLKAYL EGQTGDFKAI
     301   VDEPDLAPVK LDPVEFIEA
```

Figure 21 (SEQ ID NO:21)

```
PHYS_CHL
       1   MEDPIPNVHG GIYEDGPFKI EIVHVDDASS SSTCLKKSRA AVDRENLSPK
      51   PLVVALPKEE GVYPVIQFHH GFTLQNMFYS QIISHIASYG FIVVAPQMYK
     101   ISGSDATTEI EDAVQILNWM PTGLVAALPE TLSKHRPDFS KVALVGHSRG
     151   AKVVFGLALG VRNSILQYSA VVGLDPVDGM GIGQQTNPPI LQFSEGSLNL
     201   GVPTLIIGTG LGPLRKNFLF PACAPAGVSH EAFYYDSAAP AFHFVASKQG
     251   HMDFLNDDCS GPTGMFSYCL CKNGPTRKPM RRFSGGMVVA FLRAAFFGET
     301   APLVAALATP ELAPIPLDRP EFKGKLGDAF NKPMLAPALT P
```

Figure 22 (SEQ ID NO:22)

```
AQU_CHL
       1   MTTSLPPPKP LLIATPSEEG QFPVLIFLHG FLLFNKFYSQ LIQHIASHGF
      51   IVIAPQLYKV AGPDTTDEIK SAALVIDWLS NGLHSVLPPL VQPNLSKLGI
     101   GGHSRGGKVA FALALGHIKT SLKYSVLLGI DPVDGMGQGN QTPPPVLTYT
     151   PRSFDFNMPV LVIGSGLGET KKNSLFPPCA PKGVNHENFY SECCSPACYF
     201   VVKDYGHMDM LDDDTGGVRG KATYCTCSNG KAREPMRTFV GGIMVAFMKA
     251   YMENDSRDLM AIKETQGMAL IELQSVEFRL
```

Figure 23 (SEQ ID NO:23)

```
BRACH_CHL
       1   MAATAAAAEL KKNSGADVLE AVITSVFQPG KLAVEVIQVD HNAVPTPPIP
      51   VLIVAPKDAG TYPVAMLLHG FFLQNHYYKQ LLRHVASHGF IMVAPQFHLS
     101   MIPTGDTKDI EAAAKVSDWL PEGLPSVLPK GVEPELSKLA LAGHSRGGHT
     151   AFSLALGHAK SNLSFSALIG IDPVAGTGKS SQLAPKILTY EPSSFNMSAA
     201   MPVLVIGTGL GEEKKNIFTP PCAPKDVNHR EFYLECKPPC YYFVTKDYGH
     251   LDMLDDDAPM VITCLCKDGG SCKDKMRRCV AGIMVAFLNS ALGGKDNAAH
     301   DLEVIVKDPA LAPTTLDPVE CRLE
```

Figure 24 (SEQ ID NO:24)

```
MED_CHL
       1   MCSSVSNVFE TGNYTTKLLR VDSCSHAQNV PPPKSLLIAT PIEGGEFPLL
      51   LFLHGYLLLN SFYSQLIQHV ASHGFIVIAP QLYTVAGPDI TEEIYSVAAI
     101   TNWLSKGLSK ILPLNIKPNF HKLALGGHSR GGKTSFAVAL RKLNMTTDLK
     151   FSAIIGVDPV DGMDKGKQTS PPIFTYVPHS FDYDMATLVI GFGLGDVKKN
     201   PLFPPCAPKG VNHEDFFSEC EKPSWYFVAK DYGHVDMLDD DTKGVRGKVS
     251   YCLCKNGESR KPMRMFVGGV MVAFLKAYLH GDNVDLLAIR DKNLSVPIEM
     301   KFDYFV
```

Figure 25 (SEQ ID NO:25)

```
PIP_CHL
       1   MAASSVFEMG KLEVHVKSVN QSNSSSPPKS LLISYPSQKG DYGVVLFLHG
      51   FLISNSFYKE LISHISSHGY IVVAPRIIYP CLQDEINSAA QVANWLPEGL
     101   QAALPPNVQP NTSKLTLAGH SRGGKAAFCM LLGLAGSPLT VQFSGLIGVD
     151   PVAGFQIPGI NYKMEIPPKI ITNNSKPFDI NVPTLIIGTE LGEEAKGCLA
     201   PPYAPAGLNY EQFYEKSKEP SYQFVAKGYG HVDMLDDISK NDLMGKLTYC
     251   VCKNGKEREP MRRTAGGLMV AFLKAFSDGQ RDDLDAILND PELAPIQLDA
     301   GAKLSS
```

Figure 26 (SEQ ID NO:26)

```
LOTUS_CHL
       1   MSLSISSVTH PSSVMGSDAS TALTNVFDSG KYTTKFQRIE SNSCNGTHPD
      51   PPPPKSLLIA TPLEGGEFPV LLFLHGYLLY NSFYSQLIQH IASHGFIVIA
     101   PQLYAVAGPD VSGEIHSTAA IKNWLSEGLS KFLPPNVTPN SSKLALAGHS
     151   RGGKTAFAVA LRKLNITTDL KFSALVGVDP VDGLDRGKQT PPPVLTYVPH
     201   SFDFDMPAMV IGSGLGDVKR NPLFPPCAPK TVNHEDFFNE CNKPAWYFVA
     251   KDYGHVDMLD DDTNGIIGKA TYCLCKNGES RKPMRTFVGG LVVAFLKAYL
     301   QGDNRDSLAI KDKHLSAPVE LKFDYFV
```

Figure 27 (SEQ ID NO:27)

```
ORYI_CHL
       1   MIAFAAQILA FCLLLLLLLL LQLQTTMAGD SSFSGVFDHG SHGVTLVKVD
      51   EAPRKCSSAA AAKKTDDDTA PAGGAPPKPL LVAAPCDAGV YPVVVFLHGY
     101   LAYNSFYSQL FEHVASHGFV VVGPQVNQSI LIYYFSYIRC LDRIPPTRST
     151   RRAAVINWLA AGGLTSKLPP NVRADATKIS ISGHSRGGKV AFALALGHAN
     201   VSLRGGAGGA TIAALVAVDP VDGFATGKQT PPPILTYGGA NSLRVPAPVM
     251   VIGTGLGGLA RAAPLLPACA PPGVSHGEFY GECAAPACHL VARDYGHTDM
     301   VVDVTPGSWA SLRVPCAGAS APGRPCVGSS SAPWSRS
```

Figure 28 (SEQ ID NO:28)

```
ORYJ1_CHL
       1   MIAFAAQILA FCLLLLLLLL LQLQTTMAGD SSFSGVFDHG SHGVTLVKVD
      51   EAPRKCSSAA AAKKTDDDTA PAGGAPPKPL LVAAPCDAGV YPVVVFLHGY
     101   LAYNSFYSQL FEHVASHGFV VVGPQLYTMS GPDTTDEINS AAAVINWLAA
     151   GGLTSKLPPN VRADATKISI SGHSRGGKVA FALALGHANV SLRGGAGGAT
     201   IAALVAVDPV DGFAAGKQTP PPILTYGGAN SLRVPAPVMV IGTGLGGLAR
     251   AAPLLPACAP PGVSHGEFYG ECAAPACHLV ARDYGHTDMM DDVTPGARGL
     301   ATRAVCRSGG ARAPMRRFFG GAMVAFVKRW VEGEPELLDC VRARPETAPV
     351   VLSAVEFRDE AIANHSY
```

Figure 29 (SEQ ID NO:29)

```
ORYJ2_CHL
       1   MIAFAAQILA FCLLLLLLLL LQLQTTMAGD SSFSGVFDHG SHGVTLVKVD
      51   EAPRKCSSAA AAKKTDDDTA PAGGAPPKPL LVAAPCDAGV YPVVVFLHGY
     101   LAYNSFYSQL FEHVASHGFV VVGPQLFLGC ELILSNNFDA KMLYTMSGPD
     151   TTDEINSAAA VINWLAAGGL TSKLPPNVRA DATKISISGH SRGGKVAFAL
     201   ALGHANQTPR PILTYGGANS LRLPAPVMVI GTGLGGLARA APLLPACAPP
     251   GVSHGEFYGE CAAPACHLVA RDYGHTDMMD DVTPGARGLA TRAVCRSGGA
     301   RAPMRRFFGG AMVAFVKRWV EGEPELLDCV RARPETAPVV LSAVEFRDEA
     351   IANHSY
```

Figure 30 (SEQ ID NO:30)

```
PICEA_CHL
       1   MGQQGEEPWE DVFKPGRFPV RILKIPQRTT HGSTTAAAPK PLLLALPAQP
      51   GEYPVLLFFH GYLLLNSFYT QLLQHIASHG YIAIAPQMYC VTGADATPEI
     101   ADAAAICNWL LQGLSSYLPD DVRPDFQNVA MAGHSRGGKV AFGLALDRTS
     151   QTTELKFSAL VGVDPVDGMA RGRQTQPRIL TYKPHSFDSV IPTLIVGSGL
     201   GAVKRNPLFP PCAPEGVSHR EFFSECSAPA YHFVASDYGH MDFLDDETGG
     251   VKGQSSYCLC KNGVAREPMR RFCGGIIVAF LNVCLQNDSG AFNDLLVHPS
     301   HAPVKLEPPE SFVSEVEHQA VESLLPQTV
```

Figure 31 (SEQ ID NO:31)

```
CHL_CHL
       1   MPSTQFLGAS TLLLFGLRAV MSSDDYIKRG DLPTSKWSGR VTLRVDSAMA
      51   VPLDVVITYP SSGAAAYPVL VMYNGFQAKA PWYRGIVDHV SSWGYTVVQY
     101   TNGGLFPIVV DRVELTYLEP LLTWLETQSA DAKSPLYGRA DVSRLGTMGH
     151   SRGGKLAALQ FAGRTDVSGC VLFDPVDGSP MTPESADYPS ATKALAAAGR
     201   SAGLVGAAIT GSCNPVGQNY PKFWGALAPG SWQMVLSQAG HMQFARTGNP
     251   FLDWSLDRLC GRGTMMSSDV ITYSAAFTVA WFEGIFRPAQ SQMGISNFKT
     301   WANTQVAARS ITFDIKPMQS PQ
```

Figure 32

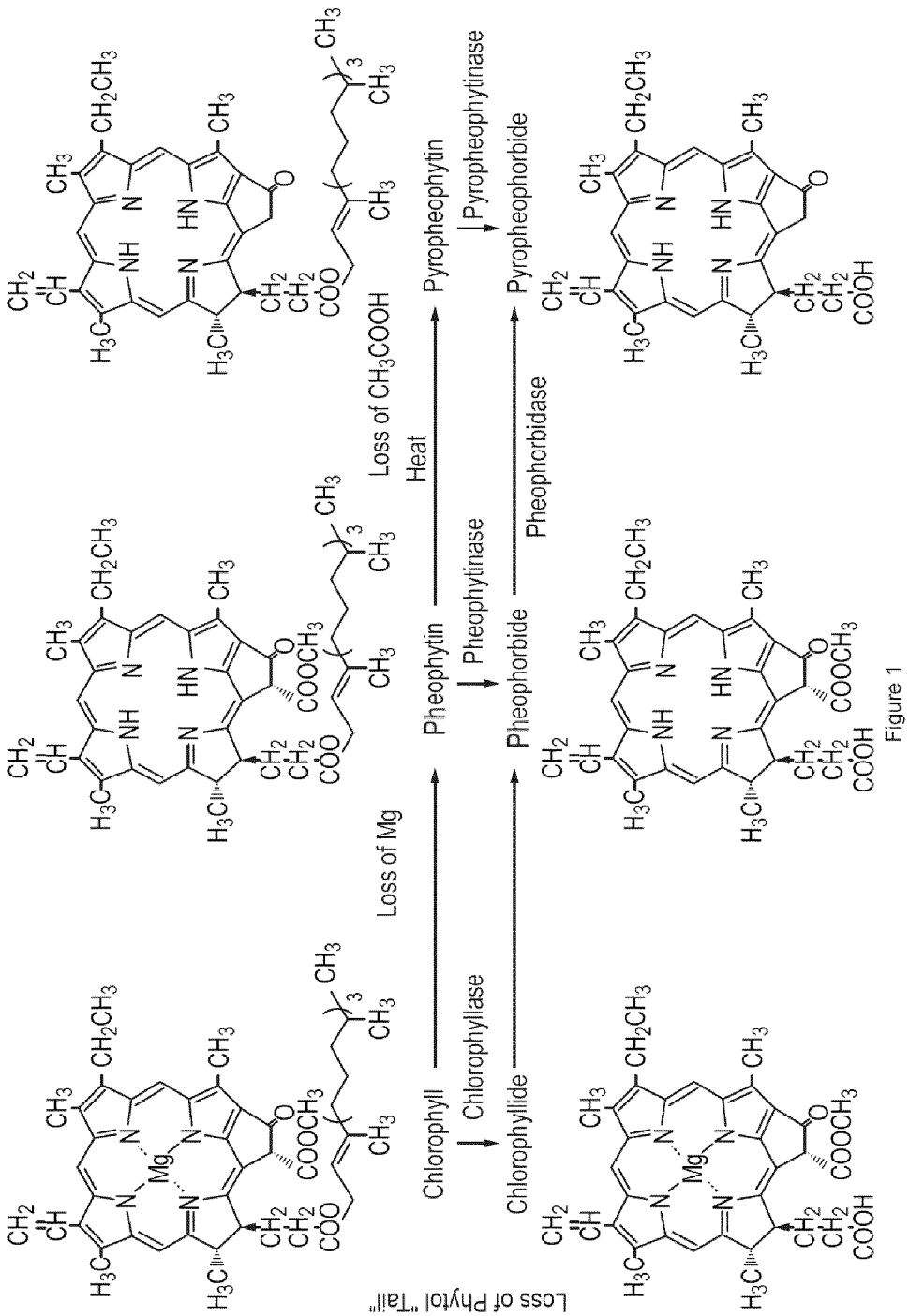


Figure 1

Figure 33

M G S S H H H H H H S S G L V P R G S H M A A A A P
CATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGGCAGCGGCTGCCCC
His tag Thrombin site CHL

Figure 34

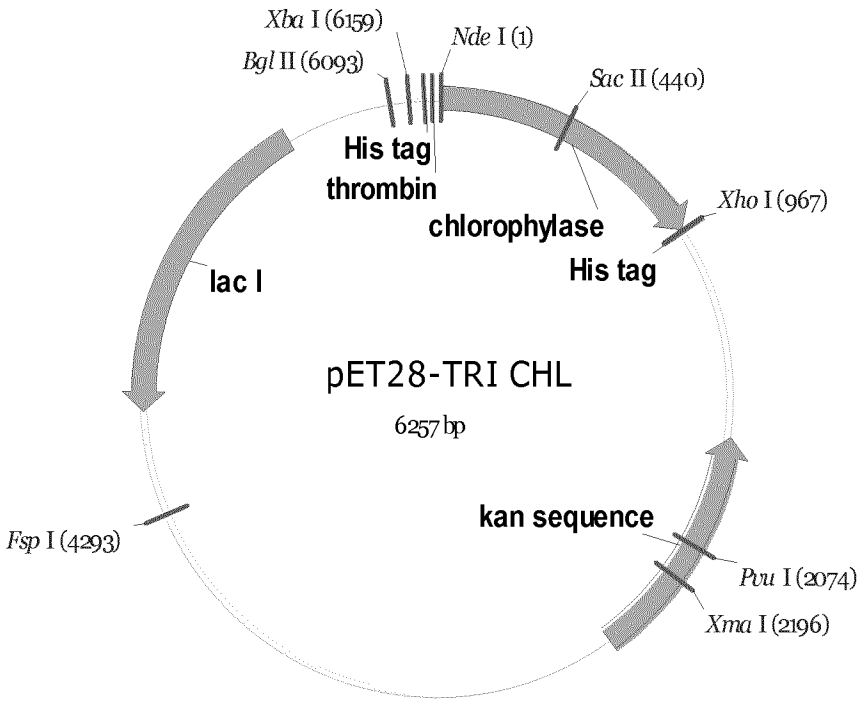


Figure 35

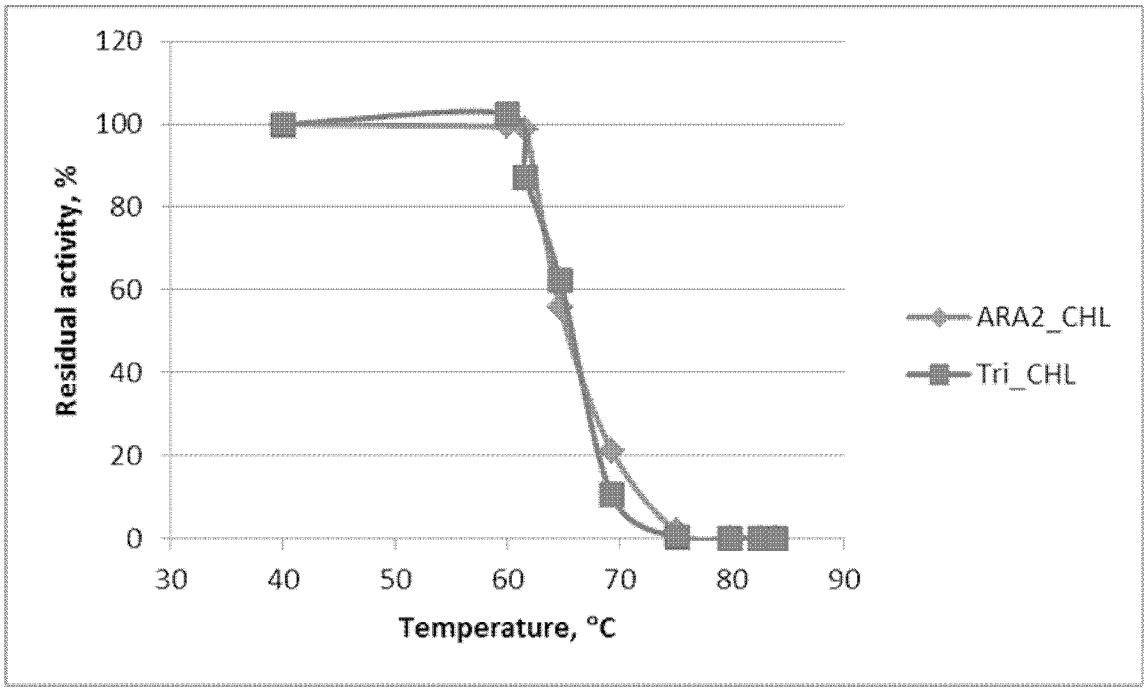


Figure 36

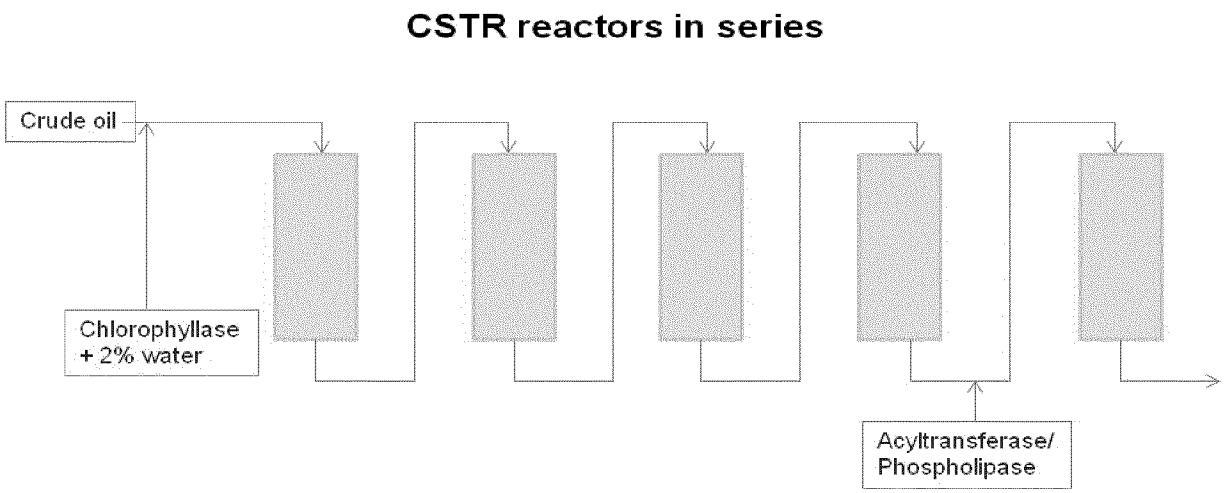


Figure 37

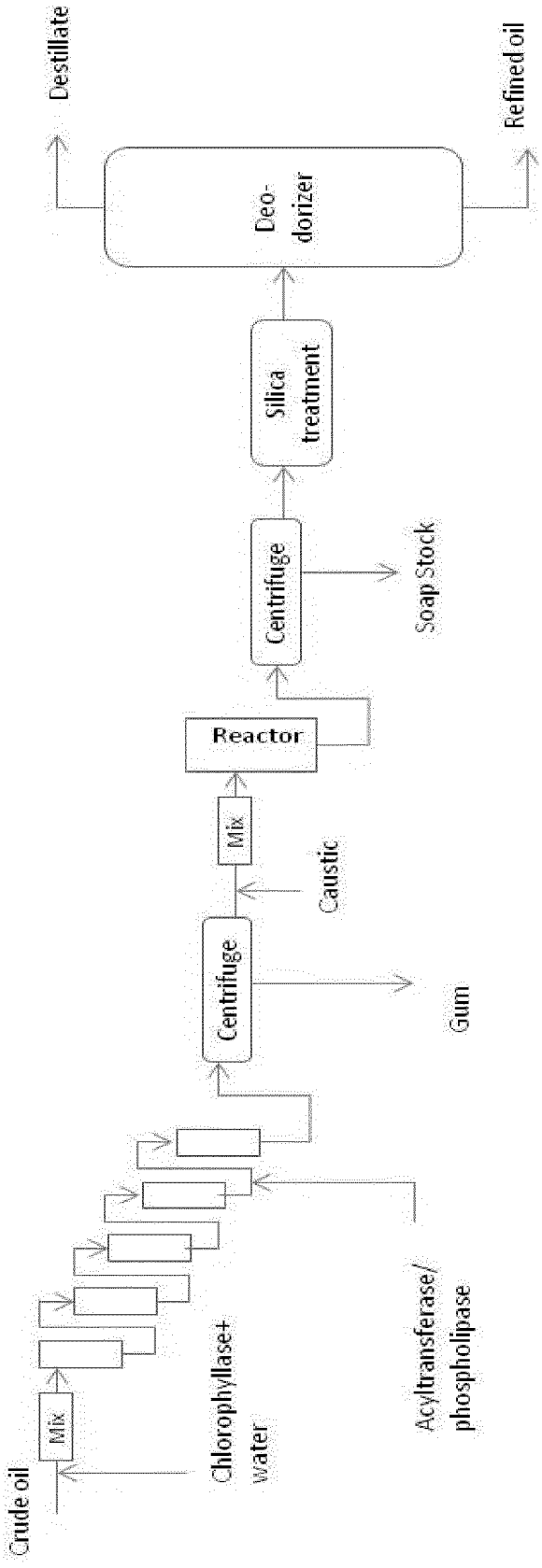

Crude oil
Mix
Chlorophyllase+ water
Acyltransferase/ phospholipase
Centrifuge
Gum
Mix
Caustic
Reactor
Centrifuge
Soap Stock
Silica treatment
Deo-dorizer
Destillate
Refined oil

Figure 38

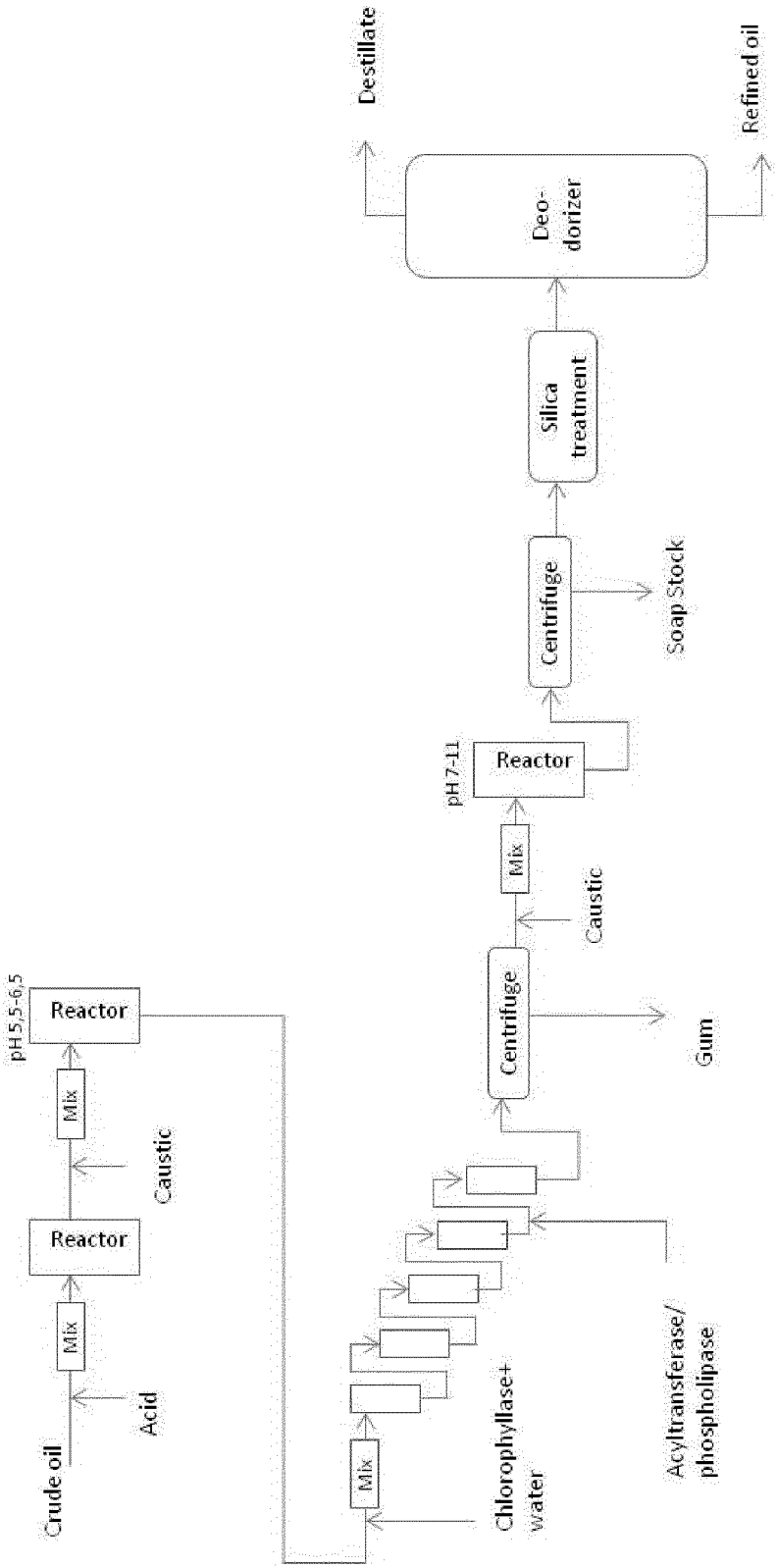

Crude oil
Acid
Mix
Reactor
Caustic
Mix
Reactor
pH 5,5-6,5
Chlorophyllase+ water
Mix
Acyltransferase/ phospholipase
Centrifuge
Gum
Caustic
Mix
Reactor
pH 7-11
Centrifuge
Soap Stock
Silica treatment
Deo-dorizer
Destillate
Refined oil

Figure 39

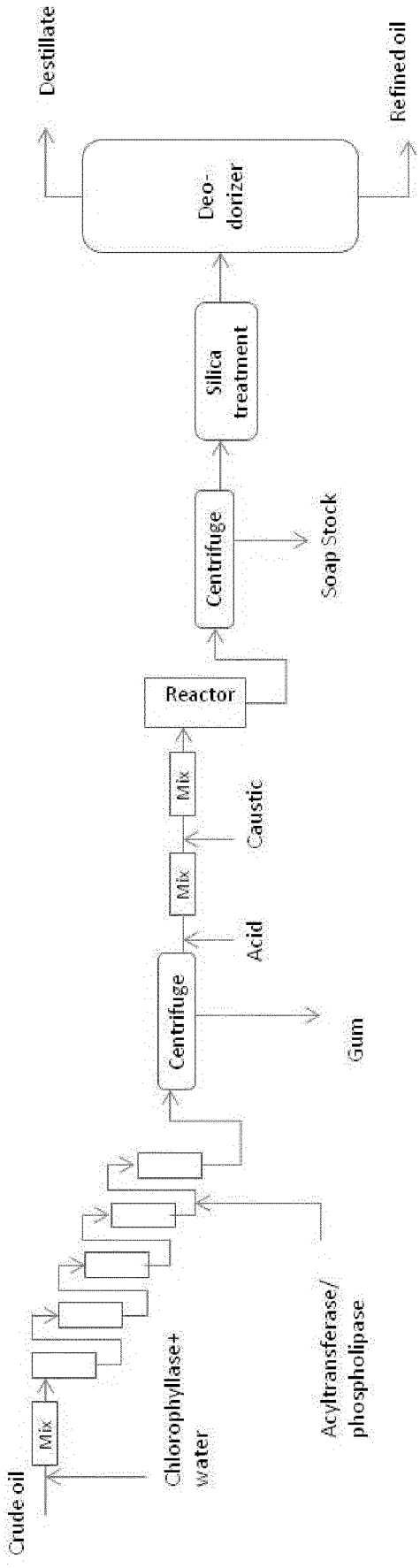

Destillate
Refined oil
Deo-dorizer
Silica treatment
Centrifuge
Soap Stock
Reactor
Mix
Caustic
Mix
Acid
Centrifuge
Gum
Crude oil
Mix
Chlorophyllase+ water
Acyltransferase/ phospholipase

Figure 40

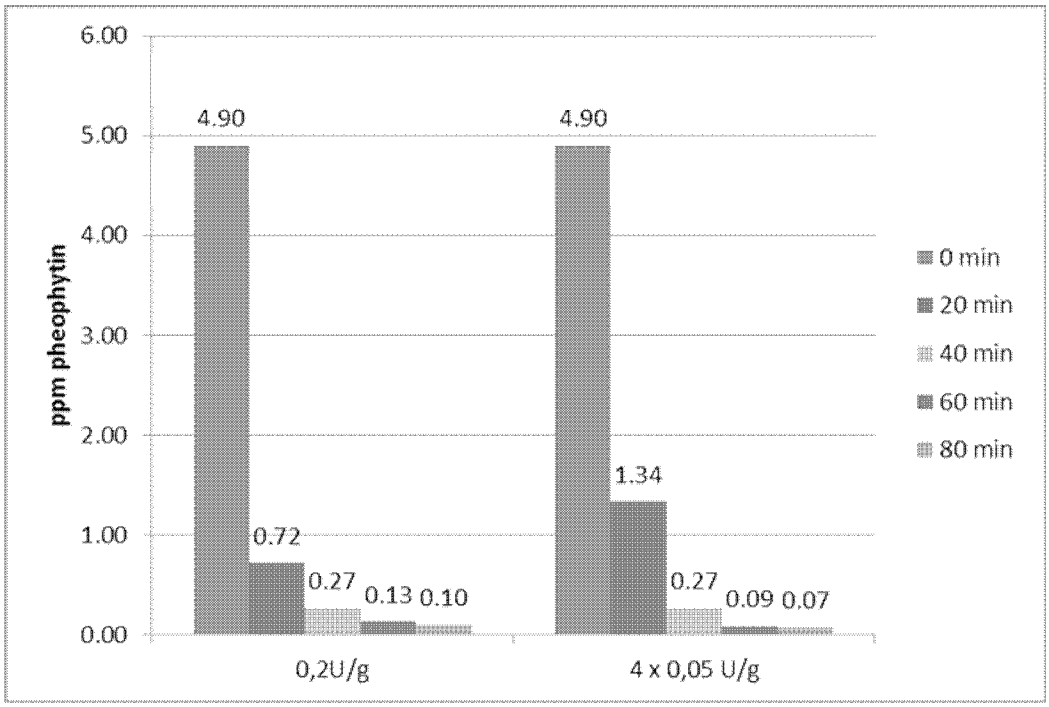


Figure 41

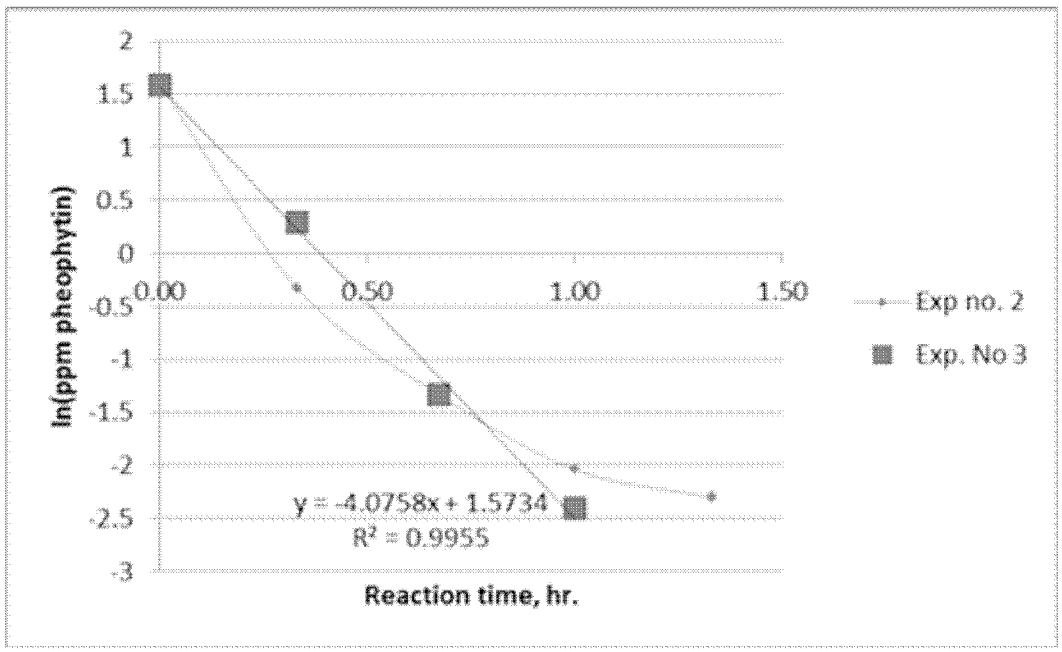


Figure 42

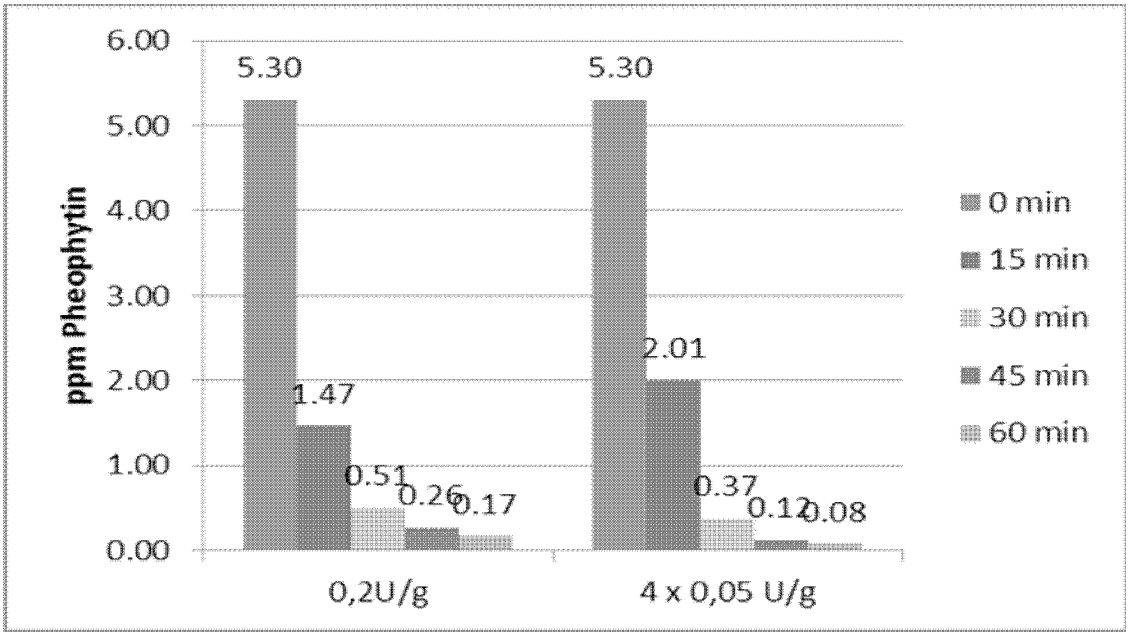


Figure 43

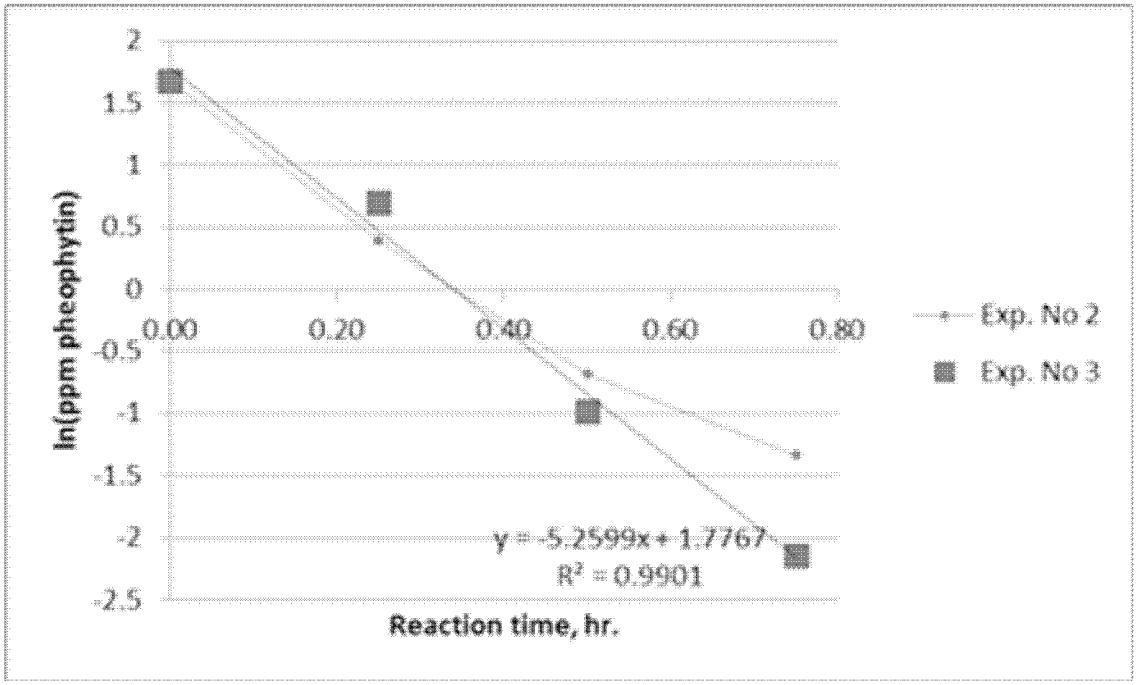


Figure 44

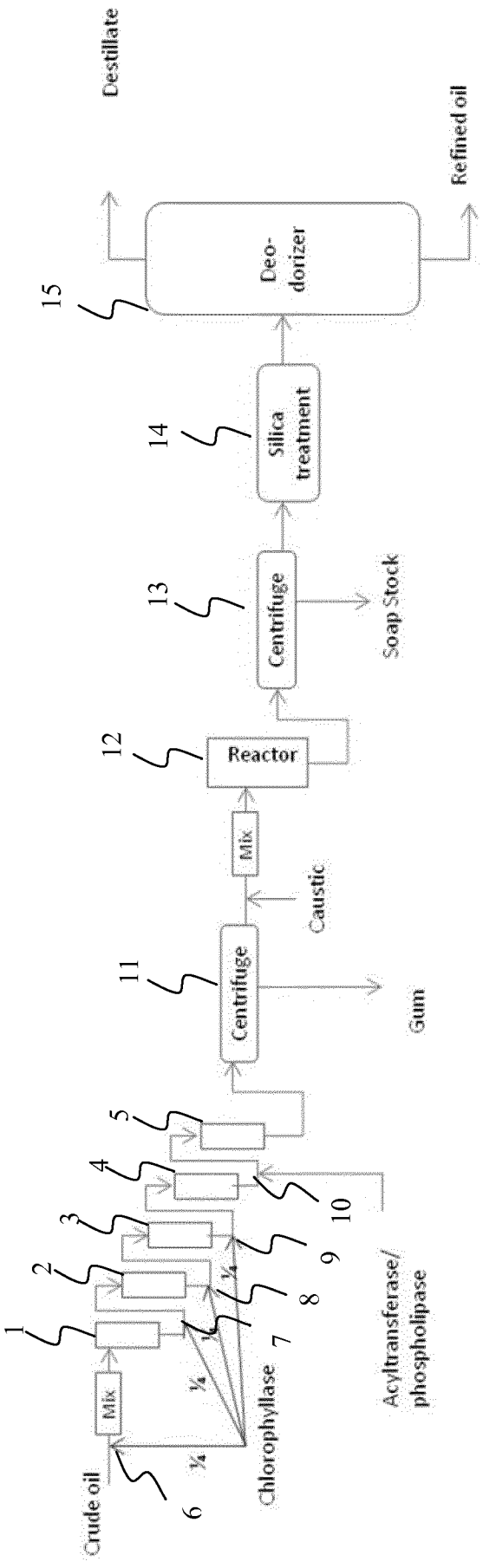

Destillate
Refined oil
Deo-dorizer
15
Silica treatment
14
Centrifuge
13
Soap Stock
Reactor
12
Mix
Caustic
Centrifuge
11
Gum
5
4
3
2
1
10
9
8
7
Acyltransferase/ phospholipase
Chlorophyllase
¼
¼
¼
Mix
Crude oil
6

Figure 45

SEQ ID NO:40

```
  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 VWKPFRSASP
241  RSASPLNCEG KMFWDQVHPT TVVHAALSER AATFIETQYE FLAHG
```

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/058540

A. CLASSIFICATION OF SUBJECT MATTER
INV. A23L1/277 C12M1/40 C12M1/00 C11B3/00 C12N9/18
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A23L C12M C11B C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2011/110967 A1 (DANISCO [DK]; MIKKELSEN RENE [DK]; JOERGENSEN TINA [DK]; SOEE JOERN BO) 15 September 2011 (2011-09-15)	14
Y	page 12, paragraph 4 page 28, paragraph 2 page 32, paragraph 5 - page 33, paragraph 2 page 35, paragraph 5 - page 36, paragraph 1 page 36, paragraph 2 - page 42, paragraph 2 claim 17	5

X	WO 2011/158203 A1 (DANISCO [DK]; MIKKELSEN RENE [DK]; JORGENSEN TINA LILIAN [DK]; SOE JOR) 22 December 2011 (2011-12-22)	14
Y	claim 17	5

	-/--	

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 July 2013	09/08/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Schlegel, Birgit

2

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	GB 1 539 680 A (MILES LAB) 31 January 1979 (1979-01-31) the whole document	15-20 1-9, 11-13

X Y	WO 2010/143149 A2 (DANISCO [DK]; SOEE JOERN BORCH [DK]; POULSEN CHARLOTTE HORSMANS [DK];) 16 December 2010 (2010-12-16) the whole document	1,4, 7-11,13 5,12

Y	WO 2006/009676 A2 (DIVERSA CORP [US]; LAM DAVID [US]; WEINER DAVID [US]; HITCHMAN TIMOTHY) 26 January 2006 (2006-01-26) cited in the application page 1 - page 29 page 149 - page 166 claims 155,167-237	1-9, 11-13

Y	BITAR MARIANNE ET AL: "Chlorophyllase biocatalysis in an aqueous/miscible organic solvent medium containing canola oil", JOURNAL OF THE AMERICAN OIL CHEMISTS' SOCIETY, vol. 81, no. 10, October 2004 (2004-10), pages 927-932, XP002543901, SPRINGER, DE ISSN: 0003-021X, DOI: 10.1007/S11746-004-1003-7 abstract	1-4,7-9, 11-13

Y	DATABASE WPI Week 201129 Thomson Scientific, London, GB; AN 2011-E17607 XP002702437, "Hydrating non hydratable phospholipids in edible oil involves mixing aqueous acid with the oil to form specific pH acidic mixture, and mixing base with the mixture to form specific pH reacted mixture, and creating emulsion of aqueous phase", & WO 2011/046815 A1 (BARTON N) 21 April 2011 (2011-04-21) abstract	12

A	US 4 451 566 A (SPENCER DONALD B [US]) 29 May 1984 (1984-05-29) abstract figure 1	7-9

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2013/058540

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2011110967 A1	15-09-2011	AR 080293 A1	28-03-2012
		CA 2788148 A1	15-09-2011
		CN 102844418 A	26-12-2012
		EA 201290903 A1	28-06-2013
		EP 2545150 A1	16-01-2013
		US 2013059036 A1	07-03-2013
		WO 2011110967 A1	15-09-2011
WO 2011158203 A1	22-12-2011	AR 081950 A1	31-10-2012
		CA 2798152 A1	22-12-2011
		CN 103003420 A	27-03-2013
		EP 2582809 A1	24-04-2013
		US 2013085287 A1	04-04-2013
		WO 2011158203 A1	22-12-2011
GB 1539680 A	31-01-1979	AU 498187 B2	15-02-1979
		BE 853216 A1	01-08-1977
		BR 7702127 A	08-11-1977
		DE 2715041 A1	06-10-1977
		DK 142277 A	06-10-1977
		ES 457508 A1	16-08-1978
		FR 2347091 A1	04-11-1977
		GB 1539680 A	31-01-1979
		IT 1073248 B	13-04-1985
		JP S52122689 A	15-10-1977
		NL 7703711 A	07-10-1977
		SE 7703926 A	06-10-1977
WO 2010143149 A2	16-12-2010	AR 077042 A1	27-07-2011
		CA 2764023 A1	16-12-2010
		CN 102803479 A	28-11-2012
		EP 2440655 A2	18-04-2012
		US 2012149927 A1	14-06-2012
		WO 2010143149 A2	16-12-2010
WO 2006009676 A2	26-01-2006	AU 2005264938 A1	26-01-2006
		BR PI0510939 A	17-07-2007
		CA 2570872 A1	26-01-2006
		CN 101432292 A	13-05-2009
		CN 103173282 A	26-06-2013
		EA 200700032 A1	29-08-2008
		EP 1791853 A2	06-06-2007
		EP 2468853 A1	27-06-2012
		JP 2008505619 A	28-02-2008
		WO 2006009676 A2	26-01-2006
WO 2011046815 A1	21-04-2011	AR 078582 A1	16-11-2011
		CA 2774660 A1	21-04-2011
		CN 102575243 A	11-07-2012
		EP 2488639 A1	22-08-2012
		US 2013011887 A1	10-01-2013
		WO 2011046815 A1	21-04-2011
US 4451566 A	29-05-1984	NONE	