

METHOD OF CONVERTING GREASE CONTAINING HIGH CONTENT OF FREE FATTY ACIDS TO FATTY ACID ESTERS AND CATALYSTS FOR USE IN SAID METHOD

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TECHNICAL FIELD

The present disclosure relates broadly to a method of converting free fatty
10 acids to fatty acid esters. Particularly, the disclosure includes a method of
converting grease containing high content of free fatty acids to fatty acid esters.
The disclosure also relates to catalysts for use in such methods.

BACKGROUND

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The increase in worldwide energy and fuel demand coupled with the rapid
depletion of non-renewable energy such as crude oil have led to a growing
interest in biodiesel as an alternate energy source.

20 Biodiesel comprises of long chain fatty acid alkyl ester derived from
triglycerides such as vegetable oil and animal fats and is considered as a
renewable and cleaner alternative to petroleum-based diesel. One of the more
common biodiesels comprises fatty acid methyl esters (FAME).

25 FAME are commonly produced by methanolysis of vegetable oils (refined
rapeseed oil, sunflower oil and soybean oil) and animal fats (beef, tallow, lard)
using base catalysts. However, the current price of FAME is still too high to
replace the traditional fossil fuels. The high price of FAME is mainly due to the
high cost of the feedstock which is used as the starting material to produce
30 FAME. For example, reports have shown that rapeseed oil and soybean oil cost
about 1.28 USD/L and about 0.70 USD/L respectively, which constitutes almost
80% of the typical biodiesel production cost.

On the other hand, the grease trap oil (GTO) such as brown grease (having 15-40 wt% of free fatty acid (FFA)) is a cheap (costs less than about 0.3USD/L, and in some reports as low as about 0.19USD/L) and non-edible resource. It is discharged in large amounts without any use (about 800-1000 tons/year in Singapore and about 1.69 million ton/year in US). Therefore, due to its low acquisition cost and abundant amount, GTO is seen to be an attractive alternative feedstock for biodiesel production. Prospective advantages that can be gleaned from using GTO as a starting material for biodiesel production include (i) reduction in the production cost of biodiesel and (ii) avoidance of the disposal problem of waste oil such as brown grease. In view thereof, attempts have been made to utilize waste grease such as brown grease to produce useful biodiesel.

However, these attempts are met with technical challenges that arise when using GTO to produce biodiesel. For example, the traditional alkaline catalysis method to convert triglycerides to FAME cannot work well for the GTO due to the elevated amounts of FFA present in GTO, which often results in soap formation. Therefore, it is desirable to reduce the level of FFA presented in GTO before performing a transesterification process on the triglycerides by base catalysts. Thus, two-step reactions consisting of acid catalyzed esterification of FFA and subsequent transesterification of the remaining triglycerides with base catalyst have been conceived.

Homogeneous acid catalysts such as sulfuric acid have been used for the esterification of FFA and methanol, but suffer from several problems such as complex neutralization of the remaining acid with base which produces large quantity of salts for subsequent disposal, corrosion of equipment used, and environment issues relating to the use of highly concentrated acids.

While the use of heterogeneous acid catalysts may avoid some of the above problems of homogeneous acid catalysts, reported heterogeneous acid catalysts such as acidic ion-exchange resins, zeolites, sulfated zirconia, and niobic acids were found to be unsatisfactory for the pretreatment of grease *via* esterification. For example, zeolites and niobic acid have low densities of

effective acid sites and thus give unsatisfactory performance in the esterification; acidic ion-exchange resins such as Amberlyst 15 and Nafion suffer from the high cost, low thermal stability, and lower catalytic activity; sulfated zirconia is extremely expensive due to zirconium being a rare and expensive metal; a series of diarylammonium catalyst incorporated into insoluble porous polymer and porous silica structure can give improved biodiesel conversion, but they require the use of expensive triflic acid in the catalyst synthesis and regeneration step, as well as the use of high reaction temperature (95-125°C) and high pressure equipment for accommodating pressures that are higher than atmospheric pressure. For example, when the reaction using diarylammonium catalyst was employed at 95-125°C, which is much higher than the boiling point of methanol (65°C), the corresponding vapour pressure of methanol is as high as 3.0-7.2 atm. As such, the process necessitated the use of high-pressure equipment. Moreover, most of the catalysts that are currently used in the above two-step process are not sufficiently reusable or recyclable.

In summary, currently, the use of acid catalysts in the above mentioned two-step reaction is still laden with numerous drawbacks. Such two-step reactions can also result in environmental pollution and corrosion. Furthermore, the conversion achieved with heterogeneous acid catalysts is not sufficiently high, even when high temperature (of more than 100°C) and long reaction time are applied. Therefore, the currently used two-step acid-base catalytic process for transformation of grease to biodiesel is not as efficient and not environmental friendly as desired.

In view of the above, there have also been attempts to rely on the use of biocatalyst such as enzymes in place of acid catalyst. One known example is the use of the enzyme lipases. Nevertheless, these isolated enzymes are relatively expensive and unstable. Also, the efficiency and conversion of FAME using isolated enzymes are considerably low, especially for high FFA-containing grease that have more than 15% by weight (15 wt%) of FFA. The use of immobilized enzymes for biodiesel production has also been reported. The reported biodiesel production from GTO containing more than 10 wt% of FFA using immobilized enzymes is still not satisfactory. For example, *Candida*

antartica Lipase B (CALB) and *Thermomyces lanuginose* Lipase (TLL) immobilized on the granulated silica (Grant-CA and Gran-TL) and the commercially available immobilized enzyme Novozyme SP435 afforded only 30%, 5% and 60% conversion of grease (containing 6.8 wt% of FFA) to FAME after 48 hour reaction time, respectively. Furthermore, it is reported that *Pseudomonas cepacia* Lipase immobilized on a phyllosilicate sol-gel matrix (PS-30) can produce FAME at reasonably acceptable yields from restaurant grease containing only 6.8 wt% of FFA after about 48 hours of incubation time, but when the FFA in the grease is increased to 8.5 wt% FFA, the FAME yield is decreased dramatically. Consequently, it can be seen that increasing the amount of FFA decreases the performance of the biocatalyst, and the present methods using such biocatalysts are not able to reach desirable FAME conversion levels from brown grease containing more than 10 wt% of FFA (for e.g. 15-40% by weight of FFA). This is further evidence by reports that show that the use of *Candida antartica* Lipase (Chirazyme L-2) for the conversion of grease containing 10.6% of FFA to biodiesel gave only 25% yield after 24 hours of incubation time.

In addition to isolated and immobilized enzymes, over the years, some whole cell systems have been developed for the biodiesel production from soybean oil or *Jatropha* oil. However, there is still no successful example of using whole cell biocatalysts for FAME production from GTO containing more than 10 wt% of FFA. For instance, it has been reported that when using the *R. oryzae* whole-cell biocatalysts to transform the pre-acidified Rapeseed oil (67 mol% FFA content) in *tert*-butanol system with methanol for the FAME production, an unsatisfactory yield of 70% was obtained.

Therefore, in view of the above, the current methods for converting GTO containing high FFA, (for example, more than 10 wt% of FFA), to FAME is far from satisfactory or desirable.

There is a need to provide a method that addresses or at least ameliorates the above drawbacks. There is also a need to provide a catalyst for use in such method.

SUMMARY

According to one aspect, there is provided a method of producing fatty acid esters from a composition comprising glycerides and at least 10% by weight of free fatty acids (FFA), the method comprising incubating a plurality of micro- or nano-sized catalysing particles with the composition, to catalyse the conversion of FFA to fatty acid esters, such that at least 80% of the FFA are converted to fatty acid esters.

In one embodiment, the incubation step is carried out in the presence of an alcohol.

In one embodiment, the micro- or nano-sized catalysing particles have an average particle size or diameter of no more than 800 μm .

In one embodiment, the micro- or nano-sized catalysing particles is capable of additionally catalysing the conversion of the glycerides to fatty acid esters.

In one embodiment, the micro- or nano-sized catalysing particles are selected from a group consisting of polymer particles, silica particles, micro-organism cells and mixtures thereof.

In one embodiment, at least one of the polymer particles and silica particles are magnetic particles, the magnetic particle comprising an outer shell; a magnetic core at least partially encapsulated by the outer shell; and a catalyzing entity selected from at least one of an inorganic acid group or an enzyme, the catalyzing entity being immobilized on the outer shell, for catalyzing the conversion of FFA to fatty acid esters, optionally for additionally catalysing the conversion of glycerides to fatty acid esters.

In one embodiment, the micro-organism is a wild type strain of micro-organism expressing hydrolase or a recombinant micro-organism expressing hydrolase.

According to another aspect, there is provided a micro- or nano-sized catalysing particle for producing fatty acid esters from a composition comprising glycerides and at least 10% by weight of free fatty acids (FFA), the particle
5 comprising a body having dimensions in the micrometer or nanometer range; and catalytic means associated with the body for catalysing the conversion of free fatty acids (FFA) to fatty acid esters, and optionally for additionally catalysing the conversion of glycerides to fatty acid esters, wherein the catalysing particle is adapted to catalyse the conversion of at least 80% of the
10 FFA in a composition comprising at least 10% by weight of FFA, to fatty acid esters.

In one embodiment, the particle is a magnetic particle and the body comprises an outer shell; and a magnetic core at least partially encapsulated by
15 the outer shell, wherein the catalytic means associated with the body is a catalyzing entity immobilized on the outer shell, for catalyzing the conversion of FFA to fatty acid esters, and optionally for additionally catalysing the conversion of glycerides to fatty acid esters.

20 In one embodiment, the magnetic core comprises a plurality of magnetic nano-sized particles having an average size or diameter of from 5 to 20 nm.

In one embodiment, the outer shell is at least one of a silica shell or polymer shell made from the group of polymers consisting of poly(glycidyl methacrylate) (PGMA), polystyrene (PS) and poly(methyl methacrylate) (PMMA).

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In one embodiment, the catalyzing entity is selected from at least one of an inorganic acid group or an enzyme.

In one embodiment, the catalyzing entity is immobilized on the polymer
30 shell *via* a linker selected from the group comprising epoxide functional group containing linker, amine functional group containing linker, aldehyde functional group containing linker, benzene functional group containing linker, ester functional group containing linker and mixtures thereof.

In one embodiment, the particle has a specific loading of from 10 to 500 mg enzyme per particle.

5 In one embodiment, the particle has a specific loading of from 0.1 to 3 mmol H⁺ per gram of particle.

10 In one embodiment, the micro- or nano-sized catalysing particle is a cell of a micro-organism, the body of the particle is the body of the cell, and the catalytic means associated with the body is an enzyme produced in or from the body of the cell for catalyzing the conversion of FFA to fatty acid esters and optionally for additionally catalysing the conversion of glycerides to fatty acid esters.

15 In one embodiment, the recombinant micro-organism comprises a nucleic acid sequence that encodes for the enzyme, the nucleic acid sequence having at least 80% homology/identity to at least one of SEQ ID No. 1 or SEQ ID No. 3 or SEQ ID No. 5.

20 According to another aspect, there is provided a method of producing a micro- or nano-sized catalysing particle, the method comprising forming a magnetic core; encapsulating at least part of the magnetic core with an outer shell; and immobilizing a catalyzing entity on the outer shell, wherein the catalysing entity is adapted to catalyse the conversion of at least 80% of the FFA in a composition comprising at least 10% by weight of FFA, to fatty acid esters, 25 and optionally for additionally catalysing the conversion of glycerides to fatty acid esters.

30 In one embodiment, the step of forming a magnetic core comprises forming a plurality of magnetic nano-sized particles having an average size or diameter of from 5 to 20 nm.

In one embodiment, the step of forming a plurality of magnetic nano-sized particles comprises co-precipitation to obtain the magnetic nano-sized particles.

In one embodiment, the outer shell comprises a polymer shell and the step of encapsulating at least part of the magnetic core with the polymer shell comprises mixing the magnetic core with one or more monomer precursor of the polymer shell; and polymerizing the monomer precursors to form a polymer shell that encapsulates at least part of the magnetic core. In one embodiment, the polymer shell encapsulates substantially the whole magnetic core.

In one embodiment, the step of mixing the magnetic core with one or more monomer precursor of the polymer shell is carried out in the presence of an initiator. In one embodiment, the step of mixing the magnetic core with one or more monomer precursor of the polymer shell and the initiator is carried out in the presence of water.

In one embodiment, the outer shell comprises a silica shell and the step of encapsulating at least part of the magnetic core with the silica shell comprises mixing the magnetic core with an alkyl silicate, an alkali and an alcohol to obtain a mixture; and form a silica shell that encapsulates at least part of the magnetic core from precipitation of the mixture. In one embodiment, the alkali is ammonia.

In one embodiment, the outer shell comprises a silica shell and the step of encapsulating at least part of the magnetic core with the silica shell comprises mixing the magnetic core with tetraethyl orthosilicate in water containing an alcohol and ammonia to obtain a mixture; and precipitating silica from the mixture to form a silica shell that encapsulates the magnetic core

In one embodiment, the step of immobilizing a catalyzing entity to the outer shell comprising: covalently coupling the catalyzing entity with the functional group of the outer shell which is chemically reactive to the catalyzing entity, to immobilize the catalyzing entity to the outer shell; or functionalizing the surface of the outer shell with a functional group chemically reactive to the catalyzing entity; and covalently or non covalently coupling the catalyzing entity with the functional group to immobilize the catalyzing entity to the outer shell.

According to another aspect, there is provided a method of obtaining a cell catalyst for producing fatty acid esters from a composition comprising glycerides and at least 10% by weight of free fatty acids (FFA), the method comprising:

- a. identifying a strain of micro-organism from a repertoire of hydrolase
5 producing micro-organisms, the identified strain of micro-organism capable of catalysing the conversion of more FFA to fatty acid esters than at least 50% of the other strains of micro-organisms in the repertoire, and optionally capable of additionally catalysing the conversion of glycerides to fatty acid esters;
 - b. identifying a gene sequence of the hydrolase that is capable of catalysing
10 the conversion of FFA to fatty acid esters and optionally capable of additionally catalysing the conversion of glycerides to fatty acid esters, from the identified strain in step a); and
 - c. introducing the gene sequence in a host cell to obtain a recombinant host cell that is capable of expressing the hydrolase in an amount that is more than
15 the strain of micro-organism identified in step a) under substantially similar conditions,
- wherein the recombinant host cell is capable of catalysing the conversion of at least 80% of the FFA in a composition comprising at least 10% by weight of FFA, to fatty acid esters, and optionally capable of additionally catalysing the
20 conversion of glycerides to fatty acid esters.

According to another aspect, there is provided a method of separating a plurality of micro- or nano-sized catalysing particles disclosed above from a mixture, the method comprising applying an external magnetic field or a
25 centrifugal force to consolidate the catalysing particles together; and removing the rest of the mixture from the consolidated catalysing particles.

According to another aspect, there is provided a method of producing fatty acid esters from a composition comprising glycerides and at least 10% by weight
30 of free fatty acids (FFA), the method comprising incubating a plurality of micro- or nano-sized catalysing particles recycled from the method of separating a plurality of magnetic catalysing particles disclosed above with the composition, to catalyse the conversion of FFA to fatty acid esters, such that at least 80% of the

FFA are converted to fatty acid esters, and optionally to additionally catalyse the conversion of glycerides to fatty acid esters.

DEFINITIONS

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The term "micro" as used herein is to be interpreted broadly to include dimensions from about 1 micron to about 1000 microns.

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The term "nano" as used herein is to be interpreted broadly to include dimensions less than about 1000 nm.

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The term "particle" as used herein broadly refers to a discrete entity or a discrete body. The particle described herein can include an organic, an inorganic or a biological particle. Biological particles can include biological particle mammalian cell, blood cell, bacterial cell, cell organelle and virus. The particle used described herein may also be a macro-particle that is formed by an aggregate of a plurality of sub-particles or a fragment of a small object. The particle of the present disclosure may be spherical, substantially spherical, or non-spherical, such as irregularly shaped particles or ellipsoidally shaped particles. The term "size" when used to refer to the particle broadly refers to the largest dimension of the particle. For example, when the particle is substantially spherical, the term "size" can refer to the diameter of the particle; or when the particle is substantially non-spherical, the term "size" can refer to the largest length of the particle.

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The term "fatty acid" as used herein, broadly refers to a non-esterified carboxylic acid having an aliphatic tail that is saturated or unsaturated, or its corresponding carboxylate anion, and can be denoted as RCOOH or RCOO^- respectively, where R is an aliphatic tail. The aliphatic tail may for example contain 3 to 25 carbon atoms.

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The terms "free fatty acids" or "FFA" used herein are intended to include any fatty acid which is substantially not associated with other molecules. For example, a free fatty acid includes a fatty acid whose carboxyl group is not covalently bonded to another compound.

The term "fatty acid esters" used herein broadly refers to esters of fatty acids and includes monoesters, diesters or triesters of fatty acids. The term "fatty acid esters" is also intended to include fatty acid alkyl esters such as fatty acid methyl esters (FAME).

The term "alkyl" includes straight chain or branched chain saturated aliphatic groups having from 1 to 50 carbon atoms, eg, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or 50 carbon atoms. For example, the term alkyl includes, but is not limited to, methyl, ethyl, 1-propyl, isopropyl, 1-butyl, 2-butyl, isobutyl, *tert*-butyl, amyl, 1,2-dimethylpropyl, 1,1-dimethylpropyl, pentyl, isopentyl, hexyl, 4-methylpentyl, 1-methylpentyl, 2-methylpentyl, 3-methylpentyl, 2,2-dimethylbutyl, 3,3-dimethylbutyl, 1,2-dimethylbutyl, 1,3-dimethylbutyl, 1,2,2-trimethylpropyl, 1,1,2-trimethylpropyl, 2-ethylpentyl, 3-ethylpentyl, heptyl, 1-methylhexyl, 2,2-dimethylpentyl, 3,3-dimethylpentyl, 4,4-dimethylpentyl, 1,2-dimethylpentyl, 1,3-dimethylpentyl, 1,4-dimethylpentyl, 1,2,3-trimethylbutyl, 1,1,2-trimethylbutyl, 1,1,3-trimethylbutyl, 5-methylheptyl, 1-methylheptyl, octyl, nonyl, decyl, and the like.

The terms "coupled" or "connected" as used in this description are intended to cover both directly connected or connected through one or more intermediate means, unless otherwise stated.

The terms "microorganism", "microbial cell" and "microbe" used herein broadly refer to prokaryotic and eukaryotic microscopic organism such as a bacterium or protozoa, a virus or any kind of higher organism, such as a fungus, algae, a plant, or an animal, which can be maintained in the form of a cell suspension or cell culture.

The term "bacteria" used herein refers to a domain of prokaryotic organisms. Bacteria can include, but are not limited to at least 11 distinct groups, as follows: (1) Gram-positive (gram+) bacteria, of which there are two major subdivisions: (1) high G+C group (Actinomycetes, Mycobacteria, Micrococcus, others) (2) low G+C group (Bacillus, Clostridia, Lactobacillus, Staphylococci, Streptococci, Mycoplasmas); (2) Proteobacteria, e.g., Purple photosynthetic+non-photosynthetic Gram-negative

bacteria (includes most "common" Gram-negative bacteria); (3) Cyanobacteria, e.g., oxygenic phototrophs; (4) Spirochetes and related species; (5) Planctomyces; (6) Bacteroides, Flavobacteria; (7) Chlamydia; (8) Green sulfur bacteria; (9) Green non-sulfur bacteria (also anaerobic phototrophs); (10) Radioresistant micrococci and relatives; (11) Thermotoga and Thermosipho thermophiles.

"Gram-negative bacteria" can include but are not limited to cocci, nonenteric rods, and enteric rods. The genera of Gram-negative bacteria include, for example, *Neisseria*, *Spirillum*, *Pasteurella*, *Brucella*, *Yersinia*, *Francisella*, *Haemophilus*, *Bordetella*, *Escherichia*, *Salmonella*, *Shigella*, *Klebsiella*, *Proteus*, *Vibrio*, *Pseudomonas*, *Bacteroides*, *Acetobacter*, *Aerobacter*, *Agrobacterium*, *Azotobacter*, *Spirilla*, *Serratia*, *Vibrio*, *Rhizobium*, *Chlamydia*, *Rickettsia*, *Treponema*, and *Fusobacterium*.

"Gram positive bacteria" can include but are not limited to cocci, nonsporulating rods, and *sporulating rods*. The genera of gram positive bacteria include, for example, *Actinomyces*, *Bacillus*, *Clostridium*, *Corynebacterium*, *Erysipelothrix*, *Lactobacillus*, *Listeria*, *Mycobacterium*, *Myxococcus*, *Nocardia*, *Staphylococcus*, *Streptococcus*, and *Streptomyces*.

The term "recombinant microorganism" and "recombinant host cell" used herein are interchangeable and includes microorganisms that have been genetically modified to express or over-express endogenous polynucleotides, or to express non-endogenous sequences, such as those included in a vector, or which have a reduction in expression of an endogenous gene. The polynucleotide generally encodes a target enzyme involved in a metabolic pathway for producing a desired metabolite as described above. Accordingly, recombinant microorganisms described herein include those that have been genetically engineered to express or over-express target enzymes not previously expressed or over-expressed by a parental microorganism. It will be appreciated that the terms "recombinant microorganism" and "recombinant host cell" refer not only to the particular recombinant microorganism but to the progeny or potential progeny of such a microorganism.

The term "parental microorganism" used above refers to a cell used to generate a recombinant microorganism. The term "parental microorganism" includes both a cell that has not been genetically modified and a cell that has been genetically modified but which does not express or over-express a target enzyme.

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The term "wild-type" when referring to a micro-organism, refers broadly to a cell that occurs in nature, i.e. a cell that has not been genetically modified.

The term "enzyme" when used herein refers broadly to any substance, composed wholly or largely of protein or polypeptides, that catalyzes or promotes one or more chemical or biochemical reactions.

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The term "gene" when used herein refers to a polynucleotide that codes for a particular sequence of amino acids, which comprise all or part of one or more proteins or enzymes, and may include regulatory (non-transcribed) DNA sequences, such as promoter sequences, which determine for example the conditions under which the gene is expressed. The transcribed region of the gene may include untranslated regions, including introns, 5'-untranslated region (UTR), and 3'-UTR, as well as the coding sequence.

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The term "nucleic acid" or "recombinant nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA).

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The term "expression" and the like when used in relation to a gene sequence refers to transcription of the gene and, as appropriate, translation of the resulting mRNA transcript to a protein.

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The term "vector" used herein refers broadly to any means by which a nucleic acid can be propagated and/or transferred between organisms, cells, or cellular components. Vectors can include, but is not limited to, viruses, bacteriophage, proviruses, plasmids, phagemids, transposons, and artificial chromosomes such as YACs (yeast artificial chromosomes), BACs (bacterial artificial chromosomes), and PLACs (plant artificial chromosomes), and the like, and can be "episomes," that is,

that replicate autonomously or can integrate into a chromosome of a host cell. A vector can also be a naked RNA polynucleotide, a naked DNA polynucleotide, a polynucleotide composed of both DNA and RNA within the same strand, a polylysine-conjugated DNA or RNA, a peptide-conjugated DNA or RNA, a liposome-conjugated DNA, or the like, that are not episomal in nature, or it can be an organism which comprises one or more of the above polynucleotide constructs such as an agrobacterium or a bacterium.

The term "transformation", "transfection" and the like, when used in the context of genetic engineering refers to the process by which a vector is introduced into a host cell., Transformation (or transduction, or transfection), can be achieved by any one of a number of means including electroporation, microinjection, biolistics (or particle bombardment-mediated delivery), or agrobacterium mediated transformation.

The term "optionally substituted" as used herein means the group to which this term refers may be unsubstituted, or may be substituted with one or more groups. Exemplary substituent groups include alkyls, alkenyl, alkynyl, thioalkyl, cycloalkyl, cycloalkenyl, heterocycloalkyl, halo, carboxyl, haloalkyl, haloalkynyl, hydroxyl, alkoxy, thioalkoxy, alkenyloxy, haloalkoxy, haloalkenyloxy, nitro, amino, nitroalkyl, nitroalkenyl, nitroalkynyl, nitroheterocyclyl, alkylamino, dialkylamino, alkenylamine, alkynylamino, acyl, alkenoyl, alkynoyl, acylamino, diacylamino, acyloxy, alkylsulfonyloxy, heterocycloxy, heterocycloamino, haloheterocycloalkyl, alkylsulfenyl, alkylcarbonyloxy, alkylthio, acylthio, phosphorus-containing groups such as phosphono and phosphinyl, aryl, heteroaryl, alkylaryl, alkylheteroaryl, cyano, cyanate, and isocyanate.

The terms "homology" and "identity" used herein, refer to the overall relatedness between polymeric molecules, e.g. between nucleic acid molecules (e.g. DNA molecules and/or RNA molecules) and/or between polypeptide molecules.

The term "associated with", used herein when referring to two elements refers to a broad relationship between the two elements. The relationship includes, but is not limited to a physical, a chemical or a biological relationship. For example, when element A is associated with element B, elements A and B may be directly or

indirectly attached to each other or element A may be produced by element B or vice versa.

The term "and/or", e.g., "X and/or Y" is understood to mean either "X and Y" or "X or Y" and should be taken to provide explicit support for both meanings or for either meaning.

Further, in the description herein, the word "substantially" whenever used is understood to include, but not restricted to, "entirely" or "completely" and the like. In addition, terms such as "comprising", "comprise", and the like whenever used, are intended to be non-restricting descriptive language in that they broadly include elements/components recited after such terms, in addition to other components not explicitly recited. Further, terms such as "about", "approximately" and the like whenever used, typically means a reasonable variation, for example a variation of +/- 5% of the disclosed value, or a variance of 4% of the disclosed value, or a variance of 3% of the disclosed value, a variance of 2% of the disclosed value or a variance of 1% of the disclosed value.

Furthermore, in the description herein, certain values may be disclosed in a range. The values showing the end points of a range are intended to illustrate a preferred range. Whenever a range has been described, it is intended that the range covers and teaches all possible sub-ranges as well as individual numerical values within that range. That is, the end points of a range should not be interpreted as inflexible limitations. For example, a description of a range of 1% to 5% is intended to have specifically disclosed sub-ranges 1% to 2%, 1% to 3%, 1% to 4%, 2% to 3% etc., as well as individually, values within that range such as 1%, 2%, 3%, 4% and 5%. The intention of the above specific disclosure is applicable to any depth/breadth of a range.

Where applicable, conventional techniques of chemistry, molecular biology, genetic engineering, recombinant DNA, which are within the capabilities of a person of ordinary skill in the art may be applied to assist the practice of one or more steps disclosed herein. The techniques may be available in texts such as Ausubel, F. M. et al. (1995 and periodic supplements; Current Protocols in Molecular Biology, ch. 9,

13, and 16, John Wiley & Sons, New York, N.Y.); B. Roe, J. Crabtree, and A. Kahn, 1996, DNA Isolation and Sequencing: Essential Techniques, John Wiley & Sons J. M. Polak and James O'D. McGee, 1990, In Situ Hybridization: Principles and Practice; Oxford University Press; J. Sambrook, E. F. Fritsch, and T. Maniatis, 1989, 5 Molecular Cloning: A Laboratory Manual, Second Edition, Books 1-3, Cold Spring Harbor Laboratory Press; M. J. Gait (Editor), 1984, Oligonucleotide Synthesis: A Practical Approach, Irl Press; D. M. J. Lilley and J. E. Dahlberg, 1992, Methods of Enzymology: DNA Structure Part A: Synthesis and Physical Analysis of DNA Methods in Enzymology, Academic Press; and Lab Ref: A Handbook of Recipes, 10 Reagents, and Other Reference Tools for Use at the Bench, Edited Jane Roskams and Linda Rodgers, 2002, Cold Spring Harbor Laboratory, ISBN 0-87969-630-3.

DETAILED DESCRIPTION

15 Exemplary, non-limiting embodiments of a method of producing fatty acid esters from free fatty acids and catalysts for use in the method are disclosed hereinafter.

The method of producing fatty acid esters from a composition comprising at 20 least about 10% by weight of free fatty acids (FFA) may comprise incubating a plurality of micro- or nano-sized catalysing particles with the composition, to catalyse the conversion of at least about 80% of the FFA to fatty acid esters. The method may be carried out in the presence of an alcohol.

25 In one embodiment, the fatty acid esters comprise fatty acid alkyl esters. The fatty acid alkyl esters can be straight or branched chain fatty acid alkyl esters. The alkyl esters may be selected from a group comprising methyl esters, ethyl esters, propyl esters, butyl esters, pentyl esters, hexyl esters and mixtures thereof. In one embodiment, the fatty acid alkyl esters comprise fatty acid methyl esters (FAME). 30 The fatty acids disclosed herein may be selected from the group consisting of octanoic acid; decanoic acid; dodecanoic acid; tetradecanoic acid; hexadecanoic acid; heptadecanoic acid; *cis*, *cis*-9,12-octadecadienoic acid; *cis*-9-octadecenoic acid; and octadecanoic acid.

The composition disclosed herein may comprise at least about 10% by weight of free fatty acids, at least about 11% by weight of free fatty acids, at least about 12% by weight of free fatty acids, at least about 13% by weight of free fatty acids, at least about 14% by weight of free fatty acids, at least about 15% by weight of free fatty acids, at least about 20% by weight of free fatty acids, at least about 21% by weight of free fatty acids, at least about 22% by weight of free fatty acids, at least about 23% by weight of free fatty acids, at least about 24% by weight of free fatty acids, at least about 25% by weight of free fatty acids, at least about 30% of weight by free fatty acids, at least about 35% of weight by free fatty acids, at least about 40% by weight of free fatty acids, at least about 45% by weight of free fatty acids, at least about 50% by weight of free fatty acids, at least about 60% by weight of free fatty acids, at least about 70% by weight of free fatty acids or at least about 80% by weight of free fatty acids. In one embodiment, the composition disclosed herein comprise from about 12% to about 22% by weight of free fatty acids.

In one embodiment, the composition disclosed herein further comprises glycerides. Accordingly, in one embodiment, the micro- or nano-sized catalysing particles is capable of additionally catalysing the conversion of the glycerides to fatty acid esters. The glycerides may be selected from the group consisting of triglycerides, diglycerides, and monoglycerides

The composition disclosed herein may be grease. The grease may also be brown grease or waste oil that comprises animal and/or vegetable oil. The grease may be obtained as a by-product from industrial or manufacturing processes, such as from food industries (for e.g. restaurants).

The alcohol disclosed herein may be an alcohol selected from the group consisting of methanol, ethanol, propanol, butanol, amyl alcohol and glycerol. In one embodiment, the alcohol comprises a monoalcohol. In some embodiments, the alcohol has the general formula $R-OH$ wherein R represents $C_n(H_{2n+1})$ and n is an integer ranging from 1 to 10. Examples of monoalcohols suitable for use include methanol, ethanol, *n*-propanol (1-propanol), isopropanol (2-propanol), *n*-butanol (1-butanol), *sec*-butanol (2-butanol), isobutanol (2-methyl-1-propanol), *tert*-butanol (2-methyl-2-propanol), *n*-pentanol (1-pentanol), 2-pentanol, 3-pentanol, 2-methyl-1-

butanol, *tert*-pentanol (2-methyl-2-butanol), 3-methyl-1-butanol, 3-methyl-2-butanol, neo-pentanol (2,2-dimethyl-1-propanol), 1-hexanol, 1-heptanol, 1-octanol, or combinations thereof. In some embodiments, the alcohol can be methanol, ethanol, propanol, butanol or combinations thereof.

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In some embodiments, the alcohol comprises a polyol such as a diol, triol and the like. In one embodiment, the alcohol comprises at least one of a methanol, a glycerol and mixtures thereof. The molar ratio of the alcohol to the FFA may be from about 2:1 to about 50:1, from about 3:1 to about 40:1, from about 4:1 to about 30:1, 10 from about 5:1 to about 20:1, from about 6:1 to about 15:1, from about 7:1 to about 10:1, from about 7:1 to about 9:1, or from about 2:1 to about 8:1. The molar ratio of the alcohol to the GTO may be from about 2:1 to about 50:1, from about 3:1 to about 40:1, from about 4:1 to about 30:1, from about 5:1 to about 20:1, from about 6:1 to about 15:1, from about 7:1 to about 10:1, from about 7:1 to about 9:1, or from about 15 2:1 to about 8:1. In an embodiment, the alcohol is used in an excess amount that is more than the theoretical amount required to complete the reaction.

The incubation step may be carried out under conditions suitable for the conversion of at least 80% of the FFA to fatty acid esters. The conditions may also 20 be pre-determined before the start of the method or determined ad-hoc during the reaction process. Such conditions may comprise at least one of an incubation time, incubation temperature and incubation pressure.

The incubation temperature may be a temperature of less than about 150°C, 25 less than about 140°C, less than about 130°C, less than about 125°C, less than about 120°C, less than about 100°C, less than about 90°C, less than about 80°C, less than about 70°C, less than about 60°C, less than about 50°C or less than about 40°C. In one embodiment, the incubation temperature is no more than about 100°C or no more than about 70°C. In another embodiment, the incubation temperature is 30 no more than about 40°C or no more than about 30°C. In one embodiment, when the alcohol used is methanol, the incubation temperature is no more than 70°C, no more than 80°C or no more than 90°C. In one embodiment, when the alcohol used is glycerol, the incubation temperature is no more than 100°C, no more than 110°C, no more than 120°C, no more than 125°C or no more than 130°C.

The incubation pressure may be a pressure of no more than about 150 KPa, no more than about 140 KPa, no more than about 130 KPa, no more than about 125 KPa, no more than about 121 KPa, no more than about 120 KPa, no more than about 115 KPa, no more than about 110 KPa, or no more than about 105 KPa. In one embodiment, the incubation pressure is substantially at standard atmospheric pressure, that is 1 atm (101.325 KPa). In one embodiment, the incubation pressure is less than about 7.2 atm (729.54 KPa), 7.0 atm (709.275 KPa), 6.0 atm (607.95 KPa), 5.0 atm (506.625 KPa), 4.0 atm (405.3 KPa), 3.0 atm (303.975 KPa).

The incubation time may be from about 0.5 hours to about 100 hours. In one embodiment, the incubation time is from about 0.5 hours to about 12 hours. In one embodiment, the incubation time is from about 0.5 hours to about 26 hours. In one embodiment, the incubation time is from about 84 hours to about 96 hours.

In one embodiment, the incubation step is carried out under conditions suitable for the conversion of at least 80% of the FFA to alkyl esters, wherein said conditions comprise at least one of an incubation temperature of no more than about 50°C, an incubation pressure of no more than 121 KPa or an incubation time of from about 0.5 hours to about 8 hours.

The amount of micro- or nano-sized catalysing particles added to the composition for incubation may be from about 0.01% to about 20%, from about 0.01% to about 15%, from about 0.01% to about 10%, from about 0.05% to about 10%, from about 0.1% to about 5%, from about 0.15% to about 4.5%, from about 0.2% to about 4.4%, from about 0.25% to about 4.3%, from about 0.3% to about 4.2%, from about 0.35% to about 4.1%, from about 0.4% to about 4.0%, from about 0.45% to about 3.9%, from about 0.5% to about 3.8%, from about 1% to about 3.7%, from about 1% to about 3.5%, from about 1% to about 3%, or from about 1% to about 2% by weight of the composition. In one embodiment, the amount of enzyme immobilized on micro- or nano-sized catalysing particles added to the composition for incubation is from about 0.01% to about 1% by weight of the composition. In one embodiment, the amount of micro- or nano-sized catalysing particles added to the composition for incubation is from about 3.5% to about 10% by weight of the

composition. In one embodiment, the amount of micro- or nano-sized catalysing particles added to the composition for incubation is from about 0.1% to about 20% by weight of the composition.

5 The incubation step may further comprise a step of stirring the mixture of the composition comprising the free fatty acids (FFA), the micro- or nano-sized catalysing particles and the alcohol. The stirring step may be carried out at about 20 rpm to about 1200 rpm. In one embodiment, the stirring step may be carried out at about 30 rpm. In one embodiment, the stirring step may be carried out at about 1000
10 rpm. In one embodiment, the stirring step may be carried out at from about 250 rpm to about 500 rpm.

 In one embodiment, the method disclosed herein is a "one-pot" reaction, wherein the composition comprising the free fatty acids (FFA), the micro- or nano-
15 sized catalysing particles and the alcohol are incubated altogether in one reaction chamber to produce fatty acid esters from the composition. Thus, the production of fatty acid esters may be carried out in one single step. Therefore in one embodiment, when the composition comprises glycerides, the conversion of FFA to fatty acid esters and the conversion of glycerides to said fatty acid esters are carried out
20 simultaneously. In one embodiment, the method disclosed herein is substantially free from solvent such as *tert*-butanol or *n*-hexane.

 In another embodiment, the method may further comprise exposing the glyceride to a base to catalyse the conversion of the glycerides to the fatty acid
25 esters. The base may be added to the composition after at least about 80% of the FFA to have been converted to fatty acid esters, to further convert glycerides to the fatty acid esters. The base catalyst may be an alkali containing OH⁻ groups. In one embodiment, the base catalyst may be a metal hydroxide. The metal hydroxide may be selected from the group consisting of NaOH, KOH and the like. The base catalyst
30 may also be selected from the group consisting of CH₃ONa, CH₃OK, Na₂CO₃, K₂CO₃ and the like. The chemical reaction to convert FFA to fatty acid esters may comprise an esterification process. The chemical reaction to convert glycerides to fatty acid esters may comprise a transesterification process.

The method disclosed herein may be capable of catalysing the conversion of at least about 80% of the FFA to fatty acid esters, at least about 85% of the FFA to fatty acid esters, at least about 90% of the FFA to fatty acid esters, at least about 95% of the FFA to fatty acid esters, at least about 96% of the FFA to fatty acid esters, at least about 97% of the FFA to fatty acid esters, at least about 98% of the FFA to fatty acid esters, at least about 99% of the FFA to fatty acid esters, or at least about 99.5% of the FFA to fatty acid esters.

The method disclosed herein may have a fatty acid ester, for example a fatty acid methyl ester (FAME) production yield of at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or at least about 99.5%. The FAME yield may be calculated by the following equation:

FAME yield = [(Total amount of FAME produced by the disclosed catalyst) / (FAME standard)] x 100 (%). The FAME standard may be the FAME yield obtained by the two-step reaction catalysed by Novozyme 435 and KOH. In certain embodiments, such FAME standards may be obtained by third party calibration curves that use the two-step reaction catalysed by Novozyme 435 and KOH.

The micro- or nano-sized catalysing particles may have an average size or diameter of no more than about 800 μm , no more than about 700 μm , no more than about 600 μm , no more than about 500 μm , no more than about 400 μm , no more than about 300 μm , no more than about 200 μm , no more than about 100 μm , no more than about 50 μm , no more than about 20 μm , no more than about 10 μm , no more than about 1 μm , no more than about 800 nm, no more than about 700 nm, no more than about 600 nm, no more than about 500 nm, no more than about 400 nm, no more than about 300 nm, no more than about 200 nm, no more than about 100 nm, no more than about 50 nm or no more than about 40 μm . In one embodiment, the micro- or nano-sized catalysing particles have an average size or diameter of from about 40 nm to about 100 μm . In one embodiment, the micro- or nano-sized catalysing particles have an average size or diameter of from about 80 nm to about 500 μm . In one embodiment, the micro- or nano-sized catalysing particles have an average size or diameter of from about 500 nm to about 10 μm . In some

embodiments, the micro-sized catalysing particles are comprised of clusters of nano-sized catalysing particles. Advantageously, the high surface area to volume ratio of the nano- and micro-sized catalysts allows for high catalyst loading on the particle surface, and the small size reduces the mass transfer limitations leading to high conversion and yield.

The micro- or nano-sized catalysing particles can be substantially spherical, substantially elongate or substantially irregularly shaped. Accordingly, when the micro- or nano-sized catalysing particles are substantially spherical, the average size may be provided by the average diameter. When the micro- or nano-sized catalysing particles are substantially elongate or substantially irregularly shaped, the average size of the particles may be provided by the average length.

The micro- or nano-sized catalysing particle may comprise a body having dimensions in the micrometer or nanometer range; and catalytic means associated with the body for catalysing the conversion of free fatty acids (FFA) to fatty acid esters, wherein the catalysing particle is adapted to catalyse the conversion of at least 80% of the FFA in a composition comprising at least 10% by weight of FFA, to fatty acid esters. In one embodiment, the micro- or nano-sized catalysing particles are selected from a group consisting of polymer particles, silica particles, micro-organism cells and mixtures thereof.

The catalytic means may comprise at least one of an enzyme and an acid group. The catalytic means may comprise more than one enzyme. The enzymes may be lipases. In one embodiment, the enzyme comprises a hydrolase. The hydrolase may be a lipase selected from the group consisting of, *Thermomyces lanuginosus* lipase (TLL), *Candida antarctica* lipase B (CALB), *Candida antarctica* lipase A (CALA), *Serratia marcescens* lipase (SML), *Pseudomonas cepacia* lipase, *Candida rugosa* lipase, *Burkholderia* lipase, *Rhizomucor miehei* lipase, *Aspergillus niger* lipase, *Penicillium roqueforti* lipase, *Rhizopus niveus* lipase, *Rhizopus oryzae* lipase, *Alcaligenes sp.* lipase, *Achromo-bacter sp.* lipase, *Pseudomonas stutzeri* lipase.

The acid group may be an inorganic acid group. The acid group may be selected from a group consisting of sulfonic acid, heteropoly acid and mixtures thereof. In one embodiment, the sulfonic acid is derived from at least one of methanesulfonic acid, benzenesulfonic acid, or propanesulfonic acid. In one
5 embodiment, the acid group comprises a sulfonic acid group. In one embodiment when the micro- or nano-sized catalysing particles comprises poly(glycidyl methacrylate) (PGMA) particles, the sulfonic acid group is derived from methanesulfonic acid. In one embodiment when the micro- or nano-sized catalysing
10 particles comprises polystyrene (PS) particles, the sulfonic acid group is derived from benzenesulfonic acid. In one embodiment when the micro or nano-sized catalysing particles comprise silica particles, the sulfonic acid group is derived from propanesulfonic acid. When the catalytic means is an acid group, the micro- or nano-sized catalysing particle may have a specific H^+ loading of about 0.1 to about 3 mmol
15 H^+ per gram of particle. The specific H^+ loading may be about 0.1 mmol H^+ , about 0.2 mmol H^+ , about 0.3 mmol H^+ , about 0.4 mmol H^+ , about 0.5 mmol H^+ , about 0.6 mmol H^+ , about 0.7 mmol H^+ , about 0.8 mmol H^+ , about 0.9 mmol H^+ , about 1.10 mmol H^+ , about 1.20 mmol H^+ , about 1.30 mmol H^+ , about 1.40 mmol H^+ , about 1.50 mmol H^+ , about 1.60 mmol H^+ , about 1.70 mmol H^+ , about 1.80 mmol H^+ , about 1.90 mmol H^+ , about 2.0 mmol H^+ , about 2.10 mmol H^+ , about 2.20 mmol H^+ , about 2.30
20 mmol H^+ , about 2.40 mmol H^+ , about 2.50 mmol H^+ , about 2.60 mmol H^+ , about 2.70 mmol H^+ , about 2.80 mmol H^+ , about 2.90 mmol H^+ , or about 3.0 mmol H^+ per gram of particle. In one embodiment, the micro- or nano-sized catalyzing particle has a specific H^+ loading of about 0.5 to about 2.3 mmol H^+ per gram of particles. In one embodiment, the micro- or nano-sized catalyzing particle has a specific H^+ loading of
25 about 2.3 mmol H^+ per gram of particles. In one embodiment, the micro- or nano-sized catalyzing particle has a specific H^+ loading of about 2.25 mmol H^+ per gram of particles. In one embodiment, the micro- or nano-sized catalyzing particle has a specific H^+ loading of about 0.54 mmol H^+ per gram of particles. In one embodiment, the micro- or nano-sized catalyzing particle has a specific H^+ loading of about 1.11
30 mmol H^+ per gram of particles.

The micro- or nano-sized catalysing particles may be magnetic particles. The magnetic particles may be paramagnetic particles. The body of the magnetic particle may comprise an outer shell; and a magnetic core at least partially encapsulated by

the outer shell, wherein the catalytic means associated with the body is a catalyzing entity immobilized on the outer shell, for catalyzing the conversion of FFA to fatty acid esters. In one embodiment, the polymer shell encapsulates substantially the whole magnetic core. In one embodiment, the magnetic core comprises a plurality of magnetic nano-sized particles. The magnetic core may be formed by clusters of magnetic nano-sized particles that are non-crosslinked and can be reversibly separated from one another. The magnetic nano-sized particles may have a substantially uniform size distribution with diameter from about 40 nm to about 800 nm. The magnetic nano-sized particles may have an average size or diameter of from about 5 nm to about 30 nm, from about 5 nm to about 20 nm, from about 5 nm to about 15 nm or from about 8 nm to about 20 nm. The magnetic nano-sized particles may be coated with a capping agent. The capping agent may be an acid. Without being bound by theory, it is believed that the capping agent may provide steric hindrance among the magnetic particle to improve stability. The capping agent may be selected from a group consisting of oleic acid, polyvinylpyrrolidone (PVP), trisodium citrate, cetyltrimethylammonium bromide (CTAB), and oleylamine. The magnetic property of the magnetic core may be provided by a metal oxide. The metal of the metal oxide may be selected from transition metals. In one embodiment, the metal of the metal oxide is selected from the metals of Group 6, Group 7 or Group 8 of the periodic table of elements. The metal oxide may be selected from a group consisting of Fe_3O_4 , $\gamma\text{-Fe}_2\text{O}_3$, FeCo , MnFe_2O_4 and CoFe_2O_4 . In one embodiment, the metal oxide comprises iron oxide (Fe_3O_4). Accordingly, in one embodiment, the magnetic nano-sized particle is an iron oxide nano-sized particle coated with oleic acid.

The outer shell is at least one of a polymer shell or a silica shell. The polymer may be selected from a group consisting of poly(glycidyl methacrylate) (PGMA), polystyrene (PS), poly(methyl methacrylate) (PMMA), poly(vinyl acetate) (PVAc), poly(vinyl alcohol) (PVA) and poly(ethylene glycol) (PEG). In one embodiment, the outer shell comprises a material selected from the group consisting of poly(glycidyl methacrylate) (PGMA), polystyrene (PS), poly(methyl methacrylate) (PMMA), Silica and mixtures thereof. When the catalyzing entity comprises an enzyme, the enzyme may be immobilized on the outer shell *via* a linker selected from the group

comprising epoxide functional group containing linker, amine functional group containing linker, aldehyde functional group containing linker, benzene functional group containing linker, ester functional group containing linker, anhydride functional group containing linker, carbonate functional group containing linker, acylazide functional group containing linker, isocyanate functional group containing linker, carboxylic acid phenyl ester functional group containing linker and mixtures thereof. In one embodiment, the linker is 4, 7, 10-trioxa-1,13-tridecanediamine. In one embodiment, the linker is obtained by reacting the outer shell of the particles with at least one of an amine and an aldehyde. The amine may be ethylene diamine (EDA) and the aldehyde may be glutaraldehyde (GA). In one embodiment, the aldehyde group on the surface of the outer shell is introduced *via* the reaction of an amine surface group with glutaraldehyde.

The micro- or nano-sized catalysing particle may have a specific loading of about 5mg to about 500 mg enzyme per gram of particle. The specific loading may be about 10mg, about 20mg, about 30mg, about 40mg, about 50mg, about 60mg, about 70mg, about 80mg, about 90mg, about 100mg, about 110mg, about 120mg, about 130mg, about 140mg, about 150mg, about 160mg, about 170mg, about 180mg, about 190mg, about 200mg, about 210mg, about 220mg, about 230mg, about 240mg, about 250mg, about 260mg, about 270mg, about 280mg, about 290mg, about 300mg enzyme, about 310mg, about 320mg, about 330mg, about 340mg, about 350mg, about 360mg, about 370mg, about 380mg, about 390mg, about 400mg, about 410mg, about 420mg, about 430mg, about 440mg, about 450mg, about 460mg, about 470mg, about 480mg, about 490mg, or about 500mg enzyme per gram of particle. In one embodiment, the micro-sized catalyzing particle has a specific loading of about 10 to about 100 mg enzyme per gram of particles. In another embodiment, the nano-sized catalyzing particle has a specific loading of about 10 to about 300 mg enzyme per gram of particles.

In view of the above, in one embodiment, there is also provided a method of producing a micro- or nano-sized catalysing particle described above. The method may comprise forming a magnetic core; encapsulating at least part of the magnetic core with an outer shell; and immobilizing a catalyzing entity on the outer shell, wherein the catalysing entity is adapted to catalyse the conversion of at least 80% of

the FFA in a composition comprising at least 10% by weight of FFA, to fatty acid esters. The step of forming a magnetic core may comprise forming a plurality of magnetic nano-sized particles. The step of forming a plurality of magnetic nano-sized particles can comprises co-precipitation to obtain the magnetic nano-sized particles.

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In one embodiment, when the outer shell is a polymer shell, the step of encapsulating at least part of the magnetic core with the polymer shell comprises mixing the magnetic core with monomer precursors of the polymer shell; and polymerizing the monomer precursors to form a polymer shell that encapsulates at least part of the magnetic core. The polymerization reaction may be a free radical polymerization reaction. The polymerization reaction may be carried out by adding an initiator such as ammonium persulfate (APS) with the relevant monomers in the presence of the magnetic core to obtain magnetic micro or nano particles.

15 In another embodiment, when the outer shell is a silica shell, the step of encapsulating the magnetic core with the silica shell comprises mixing the magnetic core with tetraethyl orthosilicate in water containing an alcohol and ammonia to obtain a mixture; and precipitating silica from the mixture to form a silica shell that encapsulates the magnetic core. In one embodiment, the step of encapsulating the magnetic core with the silica shell comprises the use of the Stöber method.

The step of immobilizing a catalyzing entity to the outer shell may comprise at least one of chemically coupling the catalyzing entity to the outer shell or physically adsorbing the catalyzing entity to the outer shell. The step of chemical coupling the catalyzing entity to the outer shell may comprise adding polymer or silica particles to an acid solution to immobilize the catalyzing entity derived from the acid solution to the outer shell. The step of adding polymer or silica particles to an acid solution may be carried out under ambient conditions. The chemical coupling the catalyzing entity to the outer shell may comprise covalently coupling the catalyzing entity with the functional group of the outer shell which is chemically reactive to the catalyzing entity, to immobilize the catalyzing entity to the outer shell; or functionalizing the surface of the outer shell with a functional group chemically reactive to the catalyzing entity; and covalently or non covalently coupling the catalyzing entity with the functional group to immobilize the catalyzing entity to the outer shell.

The step of functionalizing the surface of the outer shell may comprise (i) adding an organic amine group containing solution to the polymer particles; (ii) incubating the mixture under a first incubation temperature ; (iii) adding an aldehyde to the mixture after step (ii); and (iv) incubating the mixture of step (iii) under a second incubation temperature . The first incubation temperature may be higher than room temperature and may be more than about 50°C. The first incubation temperature may be at about room temperature and may be less than about 50°C. The step of chemically coupling the catalyzing entity with the functional group to immobilize the catalyzing entity to the outer shell may comprise adding an enzyme to the outer shell comprising the functional group that is chemically reactive to the catalyzing entity, in the presence of a suitable buffer and incubating the mixture at about 40°C or less.

In one embodiment, the micro- or nano-sized catalysing magnetic particles is adapted for being used repeatedly for a plurality of cycles, wherein at least about 80% of the FFA in a composition comprising about 10% by weight of (FFA), is converted to fatty acid in each cycle. The plurality of cycles may be 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 cycles. The micro- or nano-sized catalysing magnetic particles may be collected for recycling or reusing by applying a magnetic field for collection.

Therefore, there is also provided a method of separating the magnetic catalysing particles disclosed herein from a mixture, the method comprising applying an external magnetic field to consolidate the magnetic catalysing particles together; and removing the rest of the mixture from the consolidated particles magnetic catalysing particles. There is also provided a method of producing fatty acid esters from a composition comprising at least 10% by weight of free fatty acids (FFA), the method comprising incubating a plurality of recycled micro- or nano-sized catalysing particles obtained from the above separation method with the composition, to catalyse the conversion of FFA to fatty acid esters, such that at least 80% of the FFA are converted to fatty acid esters.

In one embodiment, the micro-sized particles are MNA TL particles which comprises a non-crosslinked cluster of CHO- magnetic nanoparticles (CHO-MNPs) having *Thermomyces Lanuginosus* Lipase (TLL) immobilized on the surface of the nanoparticles, the nanoparticles comprising an iron oxide core,

poly(glycidyl methacrylate) shell, and an aldehyde surface group. The MNA TL (Magnetic nanobiocatalyst Aggregates immobilized with *Thermomyces Lanuginosus* Lipase) particles may be prepared by shaking of TLL and CHO-MNPs in a phosphate buffer at a concentration of 7mM and at a pH of 5-8 at 4-30°C for 0.5-12h.

In another embodiment, the micro-sized particles are poly(glycidyl methacrylate) microparticles which comprise non-crosslinked clusters of CHO functionalized magnetic nanoparticles (CHO-MNPs) having *Candida Antarctica* Lipase B (CALB) immobilized on the surface of the nanoparticles, the nanoparticles comprising an iron oxide core, poly(glycidyl methacrylate) shell, and an aldehyde surface group. The MNA CA (Magnetic nanobiocatalyst Aggregates immobilized with CALB) particles may be prepared by shaking CALB and CHO-MNPs in phosphate buffer at a concentration of 7mM and at a pH of 5-8 at 4-30°C for 0.5-12h.

In another embodiment, the nano-sized particles comprises poly(glycidyl methacrylate) magnetic nanoparticles (GA-MNPs) having *Candida Antarctica* Lipase B (CALB) immobilized on the surface of the nanoparticles, the nanoparticles comprising an iron oxide core, poly(glycidyl methacrylate) shell, and an aldehyde surface group. The GAP CA particles may be prepared by shaking CALB and GA-MNPs in phosphate buffer at pH of 7 and 4-30°C for 4-12h.

In another embodiment, the nano-sized particles comprises ethylene diamine functionalized magnetic nanoparticles (EDA-MNPs) having *Candida Antarctica* Lipase B (CALB) immobilized on the surface of the nanoparticles, the nanoparticles comprising an iron oxide core, poly(glycidyl methacrylate) shell, and an amine surface group. The EDAP CA particles may be prepared by shaking CALB, glutaraldehyde, and EDA-MNPs in phosphate salt buffer at pH of 7 and 4-30°C for 4-12h.

In another embodiment, the nano-sized particles comprises polystyrene magnetic nanoparticles (PS-MNPs) having *Candida Antarctica* Lipase B (CALB)

immobilized on the surface of the nanoparticles, the nanoparticles comprising an iron oxide core, a polystyrene shell, and a benzene surface group. The PSP CA particles may be prepared via physical adsorption of CALB on the nanoparticles by shaking CALB and PS-MNPs in phosphate salt buffer at pH of 7 and 4-30°C for 4-12h.

In another embodiment, the nano-sized particles comprises poly(methyl methacrylate) magnetic nanoparticles (PMMA-MNPs) having *Candida Antarctica* Lipase B (CALB) immobilized on the surface of the nanoparticles, the nanoparticles comprising an iron oxide core, a poly(methyl methacrylate) shell, and ester surface group. The PSP CA particles may be prepared via physical adsorption of CALB on the nanoparticles by shaking CALB and PMMA-MNPs in phosphate salt buffer at pH of 7 and 4-30°C for 4-12h.

In some embodiments, the method may be carried out in presence of silica gel microbeads in addition to the micro- or nano-sized catalysing particles.

In one embodiment, the micro- or nano-sized catalysing particle is a cell of a micro-organism, wherein the body of the particle is the body of the cell, and the catalytic means associated with the body is an enzyme produced in or from the body of the cell for catalyzing the conversion of FFA to fatty acid esters. The catalytic means associated with the body may be a plurality of enzymes that may be the same or different. The enzymes may be lipases such as triacylglycerol lipase (EC 3.1.1.3). In one embodiment, the enzyme comprises a hydrolase. The hydrolase may be a lipase that is from or derived from the group consisting of *Serratia marcescens* lipase (SML), *Thermomyces lanuginosus* lipase (TLL), *Candida antarctica* lipase A (CALA), *Candida antarctica* lipase B (CALB), *Rhizomucor miehei* lipase, *Aspergillus niger* lipase, *Candida rugosa* lipase, *Penicillium roqueforti* lipase, *Rhizopus niveus* lipase, *Rhizopus oryzae* lipase, *Alcaligenes* sp. lipase, *Achromo-bacter* sp. lipase, *Burkholderia cepacia* lipase, *Pseudomonas stutzeri* lipase, *Mucor miehei* lipase, *Pseudomonas cepacia* lipase and *Aspergillus oryzae* lipase, and any variant which has an amino sequence with at least about 70% homology/identity, at least about 80% homology/identity, at least about 90% homology/identity, at least about 95% homology/identity, at least about 96% homology/identity, at least about 97%

homology/identity, at least about 98% homology/identity, at least about 99% homology/identity or about 100% homology/identity to one of these. In some embodiments, the variant can have up to 10, up to 9, up to 8, up to 7, up to 6, or up to 5 amino acid alterations to one of the lipases listed above, wherein each amino acid alteration is an amino acid substitution, deletion or addition, in any combination. In one embodiment, the amino acid substitutions are conservative substitutions.

The micro-organism may be a wild type strain of micro-organism expressing the hydrolase. In one embodiment, the wild type strain of micro-organism is or is derived from *Serratia marcescens*, *Thermomyces lanuginosus*, *Candida antarctica*, *Rhizomucor miehei*, *Aspergillus niger*, *Aspergillus oryzae*, *Mucor miehei*, *Candida rugosa*, *Penicillium roqueforti*, *Rhizopus niveus*, *Rhizopus oryzae*, *Rhizomucor miehei*, *Alcaligenes sp.*, *Achromobacter sp.*, *Burkholderia cepacia*, *Pseudomonas stutzeri*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas cepacia*, *Bacillus pumilus*, *B. thermocatenulatus*, *B. subtilis*, *B. licheniformis*, *B. coagulans*, *B. Cereus*, *Burkholderia cepacia* and *B. halodurans*. Other genera like *Acinetobacter*, *Staphylococcus*, *Streptococcus*, *Burkholderia*, *Serratia*, *Achromobacter*, *Arthrobacter*, *Alcaligenes* and *Chromobacterium*. The micro-organism may also be a recombinant micro-organism expressing one or more hydrolases that is/are derived from one or more wild type strain(s). Accordingly, the recombinant microorganism may comprise one or more exogenous nucleic acid sequence(s) encoding one or more lipases derived from the list of micro-organisms provided above.

In one embodiment, the recombinant micro-organism is or is derived from at least one of *Escherichia coli* (*E. coli*), *Rhizopus oryzae*, *Pichia pastoris* or *Saccharomyces cerevisiae*. In one embodiment, a hydrolase derived from a wild type strain (which gene sequences are already reported) is cloned and expressed in *E. coli*. In one embodiment, the hydrolase is derived from the lipase CALB from *Candida antarctica* (Genbank accession number Z30645.1). Thus, in some embodiments, the gene sequence from a wild type strain may also be cloned and expressed in recombinant *E. coli*.

The nucleotide sequence encoding the lipase CALB from *Candida antarctica* (Genbank accession number Z30645.1) is shown in SEQ ID No 1 below.

5 SEQ ID No 1: *Candida antarctica* nucleic acid sequence for lipase B (Genbank accession number Z30645.1)

atgaagctac tctctctgac cgggtgtggct ggtgtgcttg cgacttgcgt tgcagccact cctttggtga
agcgtctacc ttccggttcg gaccctgcct ttctgcagcc caagtcggtg ctcatgacgg gtctgacctg
10 ccagggtgct tcgccatcct cgggtctcaa acccatcctt ctctgtcccg gaaccggcac cacagggtcca
cagtcgttcg actcgaactg gatccccctc tcaacgcagt tgggttacac accctgctgg atctcaccac
cgccgttcac gctcaacgac acccagggtca acacggagta catggtcaac gccatcaccg
cgctctacgc tgggtcgggc aacaacaagc ttcccgctgt tacctgggtcc cagggtgggtc tgggtgcaca
gtgggggtctg acctcttcc ccagtatcag gtccaaggtc gatcgactta tggcctttgc gcccgactac
15 aagggcacccg tctctgcccg ccctctcgat gactcgcgg ttagtgcacc ctccgtatgg cagcaaacca
ccggttcggc actcaccacc gactccgaa acgcagggtg tctgaccag atcgtgcccc
ccaccaacct ctactcggcg accgacgaga tcttcagcc tcagggtgtc aactcggcac tcgactcatc
ctacctctc aacggaaaga acgtccaggc acaggccgtg tgtgggccgc tgttcgtcat cgaccatgca
ggctcgtcct cctcgcagtt ctctacgtc gtcggtcgat ccgccctgcg ctccaccacg ggccaggctc
20 gtagtgcaga ctatggcatt acggactgca accctcttcc cgccaatgat ctgactcccg agcaaaagggt
cgccgcggct gcgctcctgg cgccggcagc tgcagccatc gtggcgggtc caaagcagaa
ctgcgagccc gacctcatgc cctacgcccg cccctttgca gtaggcaaaa ggacctgctc cggcacgtgc
acccctga

25 The polypeptide sequence for the lipase CALB from *Candida antarctica* (Genbank accession number Z30645.1) is shown in SEQ ID No 2 below.

SEQ ID No 2: *Candida antarctica* amino acid sequence for lipase B (Genbank accession number Z30645.1)

30

MKLLSLTGVAGVLATCVAATPLVKRLPSGSDPAFSQPKSVLDAGLTCQGASPSVS
KPILLVPGTGTTGPQSFDSNWIPSTQLGYTPCWISPPFMLNDTQVNT EYMVNAIT
ALYAGSGNKKLPVLTWSQGGLVAQWGLTFFPSIRSKVDRLMAFAPDYKGTVLGAP
LDALAVSAPSVWQQTTGSALTALRNAGGLTQIVPTTNLYSATDEIVQPQVSN SPLD

SSYLFNGKNVQAQAVCGPLFVIDHAGSLTSQFSYVVGRSALRSTTGQARSADYGIT
DCNPLPANDLTPEQKVAAAALLAPAAAAIVAGPKQNCEPDLMPYARPFVAVGKR
TCSGIVTP

- 5 The nucleotide sequence encoding the lipase TLL from *Thermomyces lanuginosus* (Genbank accession number AF054513.1) is shown in SEQ ID No 3 below.

10 SEQ ID No 3: *Thermomyces lanuginosus* nucleic acid sequence for lipase TLL
(Genbank accession number EU022703.1)

agcgacgata tgaggagctc ccttgctgctg ttctttgtct ctgcgtggac ggccttgccc agtcctattc
gtcgaggtat gtggccacgc aatactctca tgcattgcct ttcgacctgc tgtactaaga ctgcacatac
agaggtctcg caggatctgt ttaaccagtt caatctcttt gcacagtatt ctgcagccgc atactgcgga
15 aaaaacaatg atgccccagc tggtaaaaac attacgtgca cgggaaatgc ctgccccgag
gtagagaagg cggatgcaac gtttctctac tcgtttgaag agtaagtgtc gacataagtg caggcactcg
ccgtggaaat agcagactga ccgggaagtg cagctctgga gtgggcgatg tcaccggctt ccttgctctc
gacaacacga acaaattgat cgtcctctct ttccgtggct ctcggtccat agagaactgg atcgggaatc
ttaacttcga cttgaaagaa ataaatgaca ttgctccgg ctgcagggga catgacggct tcacttcgct
20 ctggaggctt gtagccgata cgtaaggca gaaggaggag gatgctgtga gggagcatcc
cgactatcgc gtggtgttta ccggacatag cttgggtggg gcattggcaa ctgttgccgg agcagacctg
cgtggaaatg ggtatgatat cgacgtggtg ttagtagtaac gagatcatgc gggaaagtgc aggaagtctg
atacacgcat attagttttc atatggcgcc ccccgagtcg gaaacagggc tttgcagaa ttctgacctg
tacagaccgg cggaacactc taccgcatta cccacaccaa tgatattgtc cctagactcc cgccgcgcga
25 attcgggttac agccattcta gcccagagta ctggatcaaa tctggaacct ttgtccccgt caccgaaac
gatatcgtga agatagaagg catcgatgcc accggcggca ataaccagcc taacattccg
gatatccctg cgcacctatg gtacttcggg ttaattggga catgtcttta gtgcgcggcg cggctgggtc
cgcaatgaca gtttgataa agtttgaggt taggcaggat catgatgttc gtcacttggg gtcatttgac
ggtaaatca agatgacact ctccaagcat tgatgagttg aatcaaaatg gatcagtggt acat

30

The polypeptide sequence for the lipase TLL from *Thermomyces lanuginosus* (Genbank accession number EU022703.1) is shown in SEQ ID No 4 below.

SEQ ID No 4: *Thermomyces lanuginosus* amino acid sequence for lipase TLL
(Genbank accession number EU022703.1)

MRSSLVLFFVSAWTALASPIRREVSQDLFNQFNLFAQYSAAAYCGKNNDAPAGTNI
 5 TCTGNACPEVEKADATFLYSFEDSGVGDTVGFALDNTNKLIVLSFRGSRSIENWIG
 NLNFDLKEINDICSGCRGHDGFTSSWRSVADTLRQKVEDAVREHPDYRVVFTGHS
 LGGALATVAGADLRGNGYDIDVFSYGAPRVGNRAFAEFLTVQTGGTLYRITHTNDIV
 PRLPPREFGYSHSSPEYWIKSGTLVPVTRNDIVKIEGIDATGGNNQPNIPDIPAHW
 YFGLIGTCL

10

In view of the above, in one embodiment, there is also provided a method of a obtaining a cell catalyst described above. In one embodiment, the method comprises a) identifying a strain of micro-organism from a repertoire of hydrolase producing micro-organisms, the identified strain of micro-organism capable of catalysing the
 15 conversion of more FFA to fatty acid esters than at least about 50% of the other strains of micro-organisms in the repertoire; b) identifying a gene sequence of the hydrolase that is capable of catalysing the conversion of FFA to fatty acid esters from the identified strain in step a); and c) introducing the gene sequence in a host cell to obtain a recombinant host cell that is capable of expressing the hydrolase in
 20 an amount that is more than the strain of micro-organism identified in step a) under substantially similar conditions, wherein the recombinant host cell is capable of catalysing the conversion of at least 80% of the FFA in a composition comprising at least 10% by weight of FFA, to fatty acid esters. The hydrolase may be a lipase selected from those described above. The identified strain of micro-organism may
 25 be one that is capable of catalysing the conversion of more FFA to fatty acid esters than at least about 50%, than at least about 60%, than at least about 70%, than at least about 80%, than at least about 90%, than at least about 95% of the other strains of micro-organisms of the repertoire. In one embodiment, the identified strain of micro-organism may be one that is capable of catalysing the conversion of more
 30 FFA to fatty acid than all the other strains of micro-organisms of the repertoire. Advantageously, the disclosed method provides a simple and efficient way to find new strain with high lipase activity, for example, by applying a selection pressure. The method can also be used to diversify the enzyme sources for biodiesel production. By using the developed whole cell biocatalysts, the

production cost of biodiesel can potentially be greatly reduced due to their ready availability in large quantity at low cost.

In one embodiment, the recombinant host cell obtained in step c) is capable of
5 converting more FFA to fatty acid esters than the strain of micro-organism identified in step a) under substantially similar conditions. The percentage conversion of FFA to fatty acid esters by the recombinant microorganism obtained in step c) may be at least 1.2-fold, 1.3-fold, 1.4-fold, 1.5-fold, 1.6-fold, 1.7-fold, 1.8-fold, 1.9-fold, 2-fold, 3-fold, 4-fold, 5-fold or 10-fold of the strain of micro-organism identified in step a). The
10 amount of hydrolase produced for converting of FFA to fatty acid esters by the recombinant microorganism obtained in step c) may be at least 1.2-fold, 1.3-fold, 1.4-fold, 1.5-fold, 1.6-fold, 1.7-fold, 1.8-fold, 1.9-fold, 2-fold, 3-fold, 4-fold, 5-fold or 10-fold of the strain of micro-organism identified in step a). The conversion activity of FFA to fatty acid esters by the recombinant microorganism obtained in step c) may
15 be at least 1.2-fold, 1.3-fold, 1.4-fold, 1.5-fold, 1.6-fold, 1.7-fold, 1.8-fold, 1.9-fold, 2-fold, 3-fold, 4-fold, 5-fold or 10-fold of the strain of micro-organism identified in step a).

The step c) of introducing may comprise the use of vectors. Accordingly, the
20 identified exogenous gene may be prepared and inserted into an expression vector, which may be then transfected into a host cell, which may be then grown under culture conditions suitable for expressing the exogenous gene. Appropriate culture conditions are conditions of culture medium pH, ionic strength, nutritive content, etc.; temperature; oxygen/CO₂/nitrogen content; humidity; and other culture conditions
25 that permit production of the compound by the host microorganism, i.e., by the metabolic action of the microorganism.

In some embodiments, the recombinant microorganisms can be cultured in a bioreactor. The bioreactor can be used to culture microorganism cells through the
30 various phases of their physiological cycle. Bioreactors may also allow for the control of one or more culture conditions such as temperature, pH, oxygen tension, carbon dioxide levels, and the like, as well as combinations thereof. Cells may also be cultured in shake flasks, test tubes, vials, microtiter dishes, petri dishes, or the like, or combinations thereof.

In one embodiment, the step a) of identifying a strain of micro-organism that is capable of catalysing the conversion of FFA to fatty acid esters comprises a1) obtaining a sample of soil; a2) screening the soil for micro-organisms exhibiting hydrolase activity; and a3) screening from a repertoire of micro-organisms identified to exhibit hydrolase activity in step a2), a strain of micro-organism that is capable of catalysing the conversion of more FFA to fatty acid esters than at least about 50% of the other strains of micro-organisms of the repertoire.

In one embodiment, the nucleotide sequence of the identified gene is represented by SEQ ID No. 5

SEQ ID No 5: *Serratia marcescens* nucleic acid sequence for lipase TLL (Genbank accession number DQ841349)

ccaagcgccg cataccaata acgtttcatc aatcagtctc ctaaatgtct atgcagagct atcagtatag
gagagccagc gccggcactg ttaaccaacg cacaatctcg ccaattgat tcgcacgcct aatatttagg
gctaatacta ttctaccga tgttggtcct ctgaccagct gtcgttcggc taacgttgtt tcctgtttc
caccgccgac gcatgagagt tactccccg gccaggcggc ataattcata aggaactgat atgggcatct
ttagctataa ggatttgac gaaaacgcgt cgaaagcgct gtttccgac gccttggcca tctccaccta
cgcttaccac aatatcgata acggcttcga cgaaggctac caccagaccg gttcgggtct tggcctgccg
ctgacgctga tcaccgcgt gatcggcagc acccaatcgc agggcggcct gccccgcatt
ccctggaacc ccgactccga acaggccgcg caggagacgg tgaacaatgc cggctggctg
gtcatcagcg ccgcgcagct gggttacgcc ggcaaaaccg atgcacgcgg cacctattac
ggcgagaccg ccggttacac caccgcgcag gccgaggtgc tgggcaaata tgacagcgaa
ggcaatctca ccgccattgg tatctcattt ccggttacca gcggcccgcg cgagtcgctg atcggcgata
ccatcggcga tgtgattaac gatctgctgg ccggtttcgg gccgaaaggc tacgctgacg gctacacgct
gaacgccttc ggcaatctgc tgggcgacgt ggcgaaattc gcgcaggcgc acgggctgag
cggcgaggac gtagtggtca gcggccacag cctcggcggg ctggcggta acagcatggc
ggcgagagc gacgccaact ggggcggctt ctacgcgcag tccaactatg tcgccttcgc
ctcggcgacc cagtacgaag ccggcggcaa ggtgatcaac atcggctacg agaacgaccc
ggtgttcgc gcgctcgacg gcacctcgt aacctgccg tactgggcg tacacgatgc
gccgcacgcc tccgccacca acaatatcgt caacttaac gaccactacg cgctggacgc
ctggaacctg ctgccgtttt ccatttcaa cattccgacc tggctgtcgc acctgccgtt ctctatcag

gacgggctga tgcgggtgct gaactccgag tttattcgc tgaccgacaa ggactcgacc atcatcgtct
 ccaacctgtc gaacgtgacg cgcggaata cctgggtgga agacctgaac cgcaacgcgg
 aaacgcacag cggaccgacg tttatcatcg gcagcgacgg caatgatttg atcaagggcg
 gcaaaggcaa cgactatctc gagggccgcg acggcgacga tatcttcgc gacgccggcg
 5 gctataacct gatcgccggc ggcaaaggcc acaatatctt cgatacccaa caggcggtga
 aaaacaccga ggtcgcctac gacggcaata cgctttacct gcgcgacgcc aaaggcggtg
 ttacgctggc agacgacatc agcaccctgc gcagcaaaga aacctcctgg ctgattttca
 gcaaagaggt ggatcatcag gtgaccgctg cgggattgaa atcggactcg ggcctcaaag
 cctatgccgc cgccaccacc ggcggcgacg gcgatgacgt cctgcaggct cgagccacg
 10 acgcctggct gttcggcaac gccggcaacg acacgctgat cggccatgcc ggcggcaacc
 tgaccttctg cggcggcagc ggcatgaca tctgaaggc cgccggcaac ggtaataacct tctgttcag
 cggcgatttc ggccgcgacc agctgtatgg ttcaacgcc accgataaac tgggtttat cggtagcgaa
 ggcgccagcg ggaatatccg cgactatgcc acacagcaaa acgacgatct ggtgctggcc
 ttcggccacg gccaggtcac gctgatcggc gtctcgtcg atcactcaa caccgatcgg gtggtgttg
 15 cctaagggc ggcgtaaaaa aagccgggcg ctttcgccgc cgggcttcc tctttttt gtcgccctt
 acggcacgtc ataccacgac gccgccttgc gaatgcggaa cactgctgg cggttcagca ccagtttctg
 cgccttcagc gccgagcgca cccgctcaat ttgcccggag ccgataatcg gcagcggcga
 tgacggcagg cgcacacccc aggcgtacac cacctgctcg atggtctcg cgccgatctc ttgcgccacc
 cgttcagct cgtcgcgcag cggctggaac tcggcgtcgt taaacaggcg cccgcccccc
 20 aggcaggacc aggcag

In one embodiment, the identified gene encodes for a polypeptide comprising an amino acid sequence represented by SEQ ID No. 6

25 SEQ ID No 6: *Serratia marcescens* amino acid sequence for lipase (Genbank accession number DQ841349)

MGIFSYKDLDENASKALFSDALAISTYAYHNIDNGFDEGYHQTGFGLGLPLTLITALI
 GSTQSQGGLPRIPWNPDSEQAAQETVNNAGWSVISAAQLGYAGKTDARGTTYGE
 30 TAGYTТАQAEVLGKYDSEGNLTAIGISFRGTSGPRESLIGDTIGDVINDLLAGFGPKG
 YADGYTLNAFGNLLGDVAKFAQAHGLSGEDVVVSGHSLGGLAVNSMAAQSDANW
 GGFYAQSNYVAFASPTQYEAGGKVINIGYENDPVFRALDGTSLTLPGLVHDAPHA
 SATNNIVNFNDHYASDAWNLLPFSILNIPTWLSHLPFFYQDGLMRVLNSEFYSLTDK
 DSTIIVSNLSNVTRGNTWVEDLNRNAETHSGPTFIIGSDGNDLIKGGKGN DYLEGRD

GDDIFRDAGGYNLIAGGKGHNIFDTQQALKNTEVAYDGNTLYLRDAKGGITLADDIS
TLRSKETSWLIFSKEVDHQVTAAGLKSDSGLKAYAAATTGGDGDDVLQARSHDAW
LFGNAGNDTLIGHAGGNLTFVGGSGDDILKGAGNGNTFLFSGDFGRDQLYGFNAT
DKLVFIGTEGASGNIRDYATQQNDDLVLAFGHGQVTLIGVSLDHFNTDRVVLA

5

Due to the degenerate nature of the genetic code, it will be appreciated that a variety of DNA compounds differing in their nucleotide sequences can be used to encode a given amino acid sequence of the disclosure. The native DNA sequence encoding the biosynthetic enzymes described herein merely illustrates an embodiment of the disclosure, and the disclosure includes DNA compounds of any sequence that encode the amino acid sequences of the polypeptides and proteins of the enzymes utilized in the methods of the disclosure. Therefore, it will be appreciated that an isolated nucleic acid molecule encoding a polypeptide homologous to the polypeptides/enzymes described herein can also be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleotide sequence encoding the particular polypeptide, such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein, without loss or significant loss of a desired activity. Mutations can be introduced into the polynucleotide by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis.

20

Accordingly, some embodiments of the micro-organism disclosed herein may comprise a DNA sequence for encoding a hydrolase that is capable of catalysing the conversion of FFA to fatty acid esters, the DNA sequence having at least about 70% homology/identity, at least about 80% homology/identity, at least about 90% homology/identity, at least about 95% homology/identity, at least about 96% homology/identity, at least about 97% homology/identity, at least about 98% homology/identity, at least about 99% homology/identity or about 100% homology/identity to at least one of SEQ ID No. 1, SEQ ID No. 3 or SEQ ID No. 5. In one embodiment, the micro-organism is a recombinant micro-organism comprising a DNA sequence having at least 80% homology/identity to SEQ ID 1 and a DNA sequence having at least 80% homology/identity to SEQ ID 3. In one embodiment, the micro-organism is a recombinant micro-organism comprising a DNA sequence having at least 80% homology/identity to SEQ ID 5.

30

Likewise, a polypeptide can typically tolerate one or more amino acid substitutions, deletions, and insertions in its amino acid sequence without loss or significant loss of a desired activity. Thus, some embodiments of the micro-
5 organism disclosed herein may also express a polypeptide that is capable of catalysing the conversion of FFA to fatty acid esters, the polypeptide comprising an amino acid sequence that has at least about 70% homology/identity, at least about 80% homology/identity, at least about 90% homology/identity, at least about 95% homology/identity, at least about 96% homology/identity, at least about 97%
10 homology/identity, at least about 98% homology/identity, at least about 99% homology/identity or about 100% homology/identity to SEQ ID No. 2 and/or SEQ ID No. 4 and/or SEQ ID No. 6. In some embodiments, the polypeptide can have up to 10, up to 9, up to 8, up to 7, up to 6, or up to 5 amino acid alterations to SEQ ID No. 2 or SEQ ID No. 4 or SEQ ID No. 6, wherein each amino acid alteration is an amino
15 acid substitution, deletion or addition, in any combination. In one embodiment, the amino acid substitutions are conservative substitutions. In one embodiment, the micro-organism is a recombinant micro-organism expressing a hydrolase having at least 80% homology/identity to SEQ ID No. 2 and a hydrolase having at least 80% homology/identity to SEQ ID No. 4. In one embodiment, the micro-organism is a
20 recombinant micro-organism expressing a hydrolase having at least 80% homology/identity to SEQ ID No. 6.

In one embodiment, the catalytic activity of the modified/altered polypeptide or enzyme may be reduced by no more than about 5%, no more than about 10%, no
25 more than about 15%, or no more than about 20% with respect to the catalytic activity unmodified polypeptide or enzyme. The disclosure includes such polypeptides with alternate amino acid sequences, and the amino acid sequences encoded by the DNA described herein merely illustrate embodiments of the disclosure.

30

For optimal expression of a recombinant protein, it may be beneficial to employ coding sequences that produce mRNA with codons preferentially used by the host cell to be transformed. Thus, for an enhanced expression of transgenes, the codon usage of the transgene can be matched with the specific codon bias of the

organism in which the transgene is desired to be expressed. Accordingly, the present disclosure also provides, in some embodiments, for recombinant microorganisms transformed with an isolated nucleic acid molecule including a nucleic acid sequence that is codon-optimized for expression in the recombinant
5 microorganism. In some embodiments, the present disclosure also provides recombinant microorganisms transformed with an isolated nucleic acid molecule including a nucleic acid sequence that is operably linked to one or more expression control elements.

10 In some embodiments, the recombinant microorganisms of the present disclosure are transformed with exogenous genes by the introduction of appropriate expression vectors. The expression vector may be a plasmid, a part of a plasmid, a viral construct, a nucleic acid fragment, or the like, or a combination thereof. Vectors may be introduced into prokaryotic and eukaryotic cells *via* transformation and/or
15 transfection techniques within the capabilities of a person skilled in the art reading the present disclosure. Methods for the introduction of foreign nucleic acid (for example, exogenous DNA) into a host cell may include calcium phosphate and/or calcium chloride coprecipitation, DEAE-dextran-mediated transfection, lipofection, natural competence, chemically mediated transfer, electroporation, particle
20 bombardment, or the like, or combinations thereof.

The vector may also include sequences that promote expression of the transgene of interest (e.g., an exogenous lipase gene), such as a promoter, and may optionally include, for expression in eukaryotic cells, an intron sequence, a sequence
25 having a polyadenylation signal, or the like, or combinations thereof. Alternatively, if the vector does not contain a promoter in operable linkage with the gene of interest, the gene can be transformed into the cells such that it becomes operably linked to an endogenous promoter by homologous recombination, site specific integration, and/or vector integration.

30 As the disclosure provides methods for the heterologous expression of one or more genes involved in hydrolase expression, included within the scope of the disclosure are recombinant expression vectors that include such nucleic acids.

The polynucleotide of the disclosure may be amplified using cDNA, mRNA or alternatively, genomic DNA, as a template and appropriate oligonucleotide primers according to PCR amplification techniques within the capabilities of a person skilled in the art and those procedures described in the Examples section below. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. Furthermore, oligonucleotides corresponding to nucleotide sequences can be prepared by synthetic techniques within the capabilities of a person skilled in the art, e.g., using an automated DNA synthesizer.

In one embodiment, the micro- or nano-sized catalysing particle described herein is a lyophilized cell of a micro-organism. The lyophilisation of the cell of the micro-organism may be carried out by vacuum freeze drying for about 24 hours to about 72 hours.

In one embodiment, the micro- or nano-sized catalysing particle described herein is a lyophilized cell of a micro-organism and is adapted for being used repeatedly for a plurality of cycles, wherein at least about 80% of the FFA in a composition comprising about 10% by weight of (FFA), is converted to fatty acid in each cycle. The plurality of cycles may be 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 cycles. The lyophilized cells may be collected for recycling or reusing by applying a centrifugal force for collection.

Therefore, there is also provided a method of separating the catalysing particles disclosed herein from a mixture, the method comprising applying a centrifugal force to consolidate the catalysing particles together; and removing the rest of the mixture from the consolidated catalysing particles. There is also provided a method of producing fatty acid esters from a composition comprising at least 10% by weight of free fatty acids (FFA), the method comprising incubating a plurality of recycled micro- or nano-sized catalysing particles obtained from the above separation method with the composition, to catalyse the conversion of FFA to fatty acid esters, such that at least 80% of the FFA are converted to fatty acid esters.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic showing a chemical reaction occurring in the production of fatty acid methyl ester (FAME) from grease trap oil (GTO) using nano- and micro-biocatalysts with esterification and transesterification being carried out simultaneously in one pot, in accordance with one embodiment disclosed herein. R_x , R_1 , R_2 , and R_3 in Fig. 1 represent aliphatic groups which can be the same or different from each other.

Fig. 2 is a schematic showing a chemical reaction flowchart occurring in the production of fatty acid methyl ester (FAME) from grease trap oil (GTO) via two steps: a) Esterification of free fatty acids (FFA) with methanol using nano- and micro-biocatalysts; b) Transesterification of triglyceride (TG) with methanol using base-catalyst, in accordance with one embodiment disclosed herein. R_x , R_1 , R_2 , and R_3 in Fig. 2 represent aliphatic groups which can be the same or different from each other.

Fig. 3 is a schematic showing a flowchart for the synthesis of magnetic nano- and micro- biocatalyst containing hydrolase MNA (Magnetic nanobiocatalyst Aggregates), where the hydrolase can be MNA CA (CA: *Candida Antarctica* Lipase B) or MNA TL (TL: *Thermomyces Lanuginosus* Lipase), in accordance with one embodiment disclosed herein.

Fig. 4 is a transmission electron microscopy (TEM) image of CHO-MNPs, in accordance with one embodiment disclosed herein.

Fig. 5a is a microscopic image of MNA CA, in accordance with one embodiment disclosed herein.

Fig. 5b is a microscopic image of MNA TL after being freeze-dried and redispersed in GTO, in accordance with one embodiment disclosed herein.

Fig. 6 shows pictures of separation of MNA CA from a mixture by using magnet at time a) $t=0$ and b) $t=1\text{min}$, in accordance with one embodiment disclosed

herein and separation of MNA TL using a magnet at c) $t=0$ and d) $t=1\text{min}$ GTO in accordance with another embodiment disclosed herein.

Fig. 7 is a schematic showing a synthesis scheme of magnetic nano- and micro- biocatalyst particles, namely Hydrolase PMMP, Hydrolase PSP, Hydrolase EDAP and Hydrolase GAP, in accordance with some embodiments disclosed herein. As described herein, the hydrolase can be *Candida antarctica* Lipase B (CA) such that the catalytic particles include PMMP CA, PSP CA, EDAP CA and GAP CA in accordance with some embodiments disclosed herein.

Fig. 8a is a transmission electron microscopy (TEM) image of PMMA-MNPs, in accordance with some embodiments disclosed herein.

Fig. 8b is a transmission electron microscopy (TEM) image of PS-MNPs, in accordance with one embodiment disclosed herein.

Fig. 8c is a transmission electron microscopy (TEM) image of EDA-MNPs, in accordance with one embodiment disclosed herein.

Fig. 9a is a GC chromatogram of a FAME standard production, in accordance with one embodiment disclosed herein.

Fig. 9b is a GC chromatogram of FAME produced from GTO in one-pot reaction catalyzed by MNA TL, in accordance with one embodiment disclosed herein.

Fig. 10 is a graph of FAME production from GTO in one-pot reaction, where the FFA conversion and FAME yield after 12 hours are plotted for different loadings of MNA TL, in accordance with some embodiments disclosed herein. (●) represents FFA conversion and (■) represents FAME yield.

Fig. 11 is a graph showing the time course of FAME production by biotransformation of grease with methanol in one-pot reaction with different biocatalysts on a 1g scale: (■) represents MNA TL at 3.3 wt% (TLL at 0.2 wt%), (●)

represents Lipozyme[®] TL IM at 3.3 wt%, (▼) represents free TLL at 0.2 wt%; and on a 30g scale: (▲) represents MNA TL at 3.3 wt%, in accordance with some embodiments disclosed herein.

5 Fig. 12 is a bar chart showing the FAME conversion from GTO in one-pot reaction when MNA TL is recycled for a different number of cycles, in accordance with some embodiments disclosed herein.

10 Fig. 13 is a graph showing the esterification conversion of FFA in GTO after 4 hours with different amounts of MNA CA, in accordance with some embodiments disclosed herein.

Fig. 14 is a graph showing the time course of FFA conversion in the biotransformation of grease with methanol catalyzed by MNA CA at 0.45 wt% (CALB at 0.01 wt%) represented by (■); free CALB at 0.01 wt% represented by (●), and Novozyme 435[®] at 0.45 wt% represented by (▲), in accordance with an embodiment disclosed herein.

20 Fig. 15 is a bar chart showing the esterification conversion of FFA from GTO when MNA CA is recycled for a different number of cycles, in accordance with some embodiments disclosed herein.

Fig. 16 is a graph showing the time course of esterification of FFA in GTO catalyzed by PMMAP-30 CA and PMMAP-4 CA, in accordance with some 25 embodiments disclosed herein.

Fig. 17 is a graph showing the time course of esterification of FFA in GTO catalyzed by PSP-30 CA and PSP-4 CA, in accordance with some embodiments disclosed herein.

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Fig. 18 is a graph showing the time course of esterification of FFA in GTO catalyzed by GAP-30 CA, GAP-4 CA and EDAP-30 CA, in accordance with some embodiments disclosed herein.

Fig. 19 is a bar chart showing the esterification conversion of FFA from GTO when PMMAP CA is recycled for a different number of cycles, in accordance with some embodiments disclosed herein.

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Fig. 20 is a schematic showing a chemical reaction occurring in the production of the biodiesel (in this case, FAME) from grease *via* two steps: a) I) esterification of FFA in grease with methanol using recyclable nano- or micro-size paramagnetic solid acid catalyst; II) transesterification of the remaining triglyceride in grease with methanol using base catalyst.; b) I) esterification of FFA in grease with glycerol using recyclable nano- or micro-size paramagnetic solid acid catalyst; II) transesterification of the triglyceride in grease with methanol using base catalyst, in accordance with one embodiment disclosed herein. R_x , R_1 , R_2 , and R_3 in Fig. 20 represent aliphatic groups which can be the same or different from each other.

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Fig. 21 is a schematic showing a synthesis scheme of recyclable nano- and micro-size paramagnetic solid acid catalysts, in accordance with some embodiments disclosed herein.

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Fig. 22 is a transmission electron microscopy (TEM) image of nano-size SO_3H -PGMA-MNPs, in accordance with some embodiments disclosed herein.

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Fig. 23 is a graph showing the FFA content in grease after esterification of the FFA with methanol by using nano- and micro-size solid acid catalysts in comparison with Amberlyst 15, in accordance with some embodiments disclosed herein.

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Fig. 24 shows pictures of separation of nano-size solid acid catalyst SO_3H -PGMA-MNPs from a mixture by using magnet at time $t=0$ and time $t=5\text{s}$, in accordance with one embodiment disclosed herein.

Fig. 25 is a bar chart showing the esterification conversion of FFA in grease in the presence of methanol when nano-size solid acid catalyst SO_3H -PGMA-MNPs is

recycled for a different number of cycles, in accordance with some embodiments disclosed herein.

Fig. 26 is a transmission electron microscopy (TEM) image of nano-size SO_3H -PS-MNPs, in accordance with some embodiments disclosed herein.

Fig. 27 is a bar chart showing the esterification conversion of FFA in grease in the presence of methanol when nano-size solid acid catalyst SO_3H -PS-MNPs is recycled for a different number of cycles, in accordance with some embodiments disclosed herein.

Fig. 28 is a scanning electron microscopy (SEM) image of micro-size SO_3H -PS-MNPs, in accordance with some embodiments disclosed herein.

Fig. 29 is a graph showing the FFA content in grease after esterification of the FFA with methanol by using micro-size solid acid catalyst SO_3H -PS-MNPs in a small-scale system and in a large-scale system, in accordance with some embodiments disclosed herein.

Fig. 30 is a graph showing the FFA content in grease after esterification of the FFA with glycerol by using micro-size solid acid catalyst SO_3H -PS-MNPs in comparison with Amberlyst 15, in accordance with one embodiment disclosed herein.

Fig. 31 is a bar chart showing the esterification conversion of FFA in grease in the presence of methanol when micro-size solid acid catalyst SO_3H -PS-MNPs is recycled for a different number of cycles, in accordance with some embodiments disclosed herein.

Fig. 32 is a transmission electron microscopy (TEM) image of nano-size SO_3H -Si-MNPs, in accordance with some embodiments disclosed herein.

Fig. 33 shows pictures of separation of nano-size solid acid catalyst SO_3H -Si-MNPs from a mixture by using magnet at time a) 0 min, b) 15 min, c) 30 min and d) 1 hour, in accordance with one embodiment disclosed herein.

Fig. 34 is a bar chart showing the esterification conversion of FFA in grease in the presence of methanol when nano-size solid acid catalyst $\text{SO}_3\text{H-Si-MNPs}$ is recycled for a different number of cycles, in accordance with some embodiments disclosed herein.

Fig. 35 is a schematic showing a chemical reaction occurring in the production of FAME from grease trap oil (GTO) or waste grease using whole cell biocatalyst *via* esterification and transesterification in one-pot reaction, in accordance with some embodiments disclosed herein.

Fig. 36 is a schematic showing a chemical reaction occurring in the production of FAME from grease trap oil (GTO) or waste grease *via* two-step synthesis strategy. 1) Esterification of FFA with methanol using whole cell biocatalyst; 2) Transesterification of triglyceride with methanol using base-catalyst, in accordance with some embodiments disclosed herein.

Fig. 37 is a schematic of an isolation and screening process lipase-producing strains from soil, in accordance with one embodiment disclosed herein.

Fig. 38 is a bar chart showing the FAME yield obtained during catalysis with methanol using six different isolated strains, in accordance with some embodiments disclosed herein. The reactions were carried out at 30°C and 250 rpm for 72 h, with 2 wt% of catalyst loading based on GTO and molar ratio of methanol to GTO of 3:1, and methanol was added in three equal amounts at 24-h intervals.

Fig. 39 is a bar chart showing the FFA conversion obtained during esterification of FFA in GTO with methanol using six isolated strains as the first step in the two-step reaction shown in Fig. 38, in accordance some embodiments disclosed herein. The reactions were carried out at 30°C and 250 rpm for 72 h, with 2 wt% of catalyst loading based on GTO and molar ratio of methanol to GTO of 3:1, and methanol was added in three equal amounts at 24-h intervals.

Fig. 40 is a graph showing the time course of FAME production from GTO in one-pot reaction with methanol using the recombinant *E.coli* expressing lipase SML, in accordance with one embodiment disclosed herein. The reactions were carried out at 30°C and 500 rpm under magnetic stirring for 96 h, with 5 wt% of catalyst loading based on GTO and molar ratio of methanol to GTO of 4:1, and methanol was added in four equal amounts at 24-h intervals.

Fig. 41 is a graph showing the time course of FFA conversion obtained during esterification of FFA in GTO with methanol using recombinant *E.coli* expressing the lipase SML as the first step in the two-step strategy shown in Fig. 40, in accordance with some embodiments disclosed herein. The reactions were carried out at 30°C and 500 rpm under magnetic stirring system for 96 h, with 5 wt% of catalyst loading based on GTO and molar ratio of methanol to GTO of 4:1, and methanol was added in four equal amounts at 24-h intervals.

Fig. 42 is a graph showing the time course of FAME production from GTO in one-pot reaction with methanol using different amounts of recombinant *E.coli* whole cells expressing lipase SML, in accordance with one embodiment disclosed herein. The reactions were carried out at 30°C and 500 rpm under magnetic stirring for 96 h at a molar ratio of methanol to GTO of 4:1, and methanol was added in four equal amounts at 24-h intervals.

Fig. 43 is a graph showing the time course of FAME production from GTO with methanol using recombinant *E.coli* expressing lipase CALB, in accordance with one embodiment disclosed herein. The reaction was conducted at 40°C and 250 rpm, with 4 wt% of catalyst loading based on GTO and molar ratio of methanol to GTO of 3:1, and methanol was added in three equal amounts at 12-h intervals.

Fig. 44 is a graph showing the time course of FFA conversion obtained during esterification of FFA in GTO with methanol using recombinant *E.coli* expressing lipase CALB as the first step in the two-step strategy shown in Fig. 36, in accordance with some embodiments disclosed herein. The reaction was conducted at 40°C and 250 rpm, with 4wt% of catalyst loading based on GTO and molar ratio of methanol to GTO of 3:1, and methanol was added in three equal amounts at 12-h intervals.

Fig. 45 is a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) image of cell free extracts of recombinant *E. coli* strains. M: marker, 1: *E. coli* (CALB/TLL) expressing two enzymes with one plasmid, 2: *E. coli* (CALB), 3: *E. coli* (TLL), 4: *E. coli* (CALB-TLL) expressing two enzymes with two plasmids.

Fig. 46 is a graph showing the time course of FAME production from GTO with methanol using recombinant *E. coli* expressing lipase CALB and TLL in one plasmid, in accordance with one embodiment disclosed herein. The reaction was conducted at 30°C and 500 rpm, with 4 wt% of catalyst loading based on GTO and molar ratio of methanol to GTO of 3:1, and methanol was added in three equal amounts at 6-h intervals. (♦) represents FAME yield with wet cells, (■) represents FAME yield with dry cells, (▲) represents FFA content with wet cells, (●) represents FFA content with dry cells.

Fig. 47 is a bar chart showing the relative FAME yield produced from GTO with methanol when recombinant *E. coli* expressing lipase CALB and TLL in one plasmid is recycled for a number of cycles, in accordance with some embodiments disclosed herein.

Fig. 48 is an exemplary representation of a magnetic micro- or nano-catalyzing particle in accordance with one embodiment disclosed herein.

Fig. 49 is an exemplary representation of a cell catalyzing particle in accordance with one embodiment disclosed herein.

EXAMPLES

Example embodiments of the disclosure will be better understood and readily apparent to one of ordinary skill in the art from the following examples, and if applicable, in conjunction with the figures.

Example 1. Preparation of magnetic nano- and micro- biocatalyst MNA TL

Oleic acid coated superparamagnetic iron oxide magnetic nanoparticles (OA-MNPs) having diameters of 5-20 nm were synthesized by co-precipitation method.

An example of the co-precipitation method may be carried out as follows: Into 100 mL of de-ionized (DI) water, 0.01 mol (2.703 g) ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) and 0.005 mol (0.994 g) ferrous chloride tetrahydrate ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$) were added under mechanical stirring at 80°C and argon bubbling for 30 min. Then, 0.01 mol (8.014 g) of potassium oleate was added into the above solution and the mixture was continuously stirred for another 30 min. The iron oxide precipitation was initiated by rapidly injecting 35 mL of ammonium hydroxide (4% solution) to above mixture and reaction was continued for another 30 min. The oleic acid-stabilized iron oxide magnetic nanoparticles (OA-MNPs) were collected by centrifugation at 16700g and 20°C for 10 min. After further purification by subjecting it to high gradient magnetic separator (HGMS), the OA-MNPs were ready for coating.

Subsequently, after the OA-MNPs were obtained by the co-precipitation method, 9 mg of ammonium persulfate (APS) and 0.126 mL of glycidyl methacrylate (GMA) were added to 25 mL suspension of OA-MNPs at a concentration of 0.27-1mg/mL and the mixture was reacted for 1.5h to obtain GMA-MNPs containing poly(glycidyl methacrylate) shell with a diameter of 40-300 nm. 0.011 mol (2.4 mL) of 4,7,10-trioxa-1,13-tridecanediamine was then added to 48mL of aqueous suspension containing 0.112g of GMA-MNPs, and the mixtures were stirred at 80°C for 24h. The resultant Trioxa-MNPs containing amino group on the surface were collected by centrifugation at 12000-21000g for 10-30 min and washed by de-ionized water several times. 0.05-0.2g of Trioxa-

MNPs was stirred in 135 mL of glutaraldehyde (10% solution) at room temperature for 18h. The generated aldehyde-containing nanoparticles (CHO-MNPs) were washed several times in de-ionised (DI) water to give a mean diameter of 65 nm (Fig. 4).

For enzyme immobilization, 3.5-21 mg of *Thermomyces lanuginose* Lipase (TLL or TL) and 0.07 g of CHO-MNPs in 35 mL phosphate buffer (7mM, pH 5-8) were shaken at 4°C (or room temperature) for 0.5-12h. The obtained biocatalyst MNA TL (Magnetic nanobiocatalyst Aggregates immobilised with *Thermomyces lanuginose* Lipase) comprises clustered magnetic nanoparticles (MNPs) containing enzymes on the surface of individual MNPs. The micro-biocatalysts were collected by applying a magnetic field, and then subsequently washed and freeze-dried. The MNA TL obtained showed regular shape with sizes at 60 nm-100µm (Fig. 5b), and having a specific loading of 20-100mg TL per gram particles.

Example 2. Preparation of magnetic nano- and micro- biocatalyst MNA CA

5.85-35.1 mg of CALB and 0.117 g of CHO-MNPs (prepared as based on the steps described in example 1) in 35 mL phosphate buffer (7mM, pH 5-8) were shaken at 4°C (or room temperature) in 0.5-12h to give MNA CA (Magnetic nanobiocatalyst Aggregates immobilised with CALB) as non-crosslinked clusters with a diameter of 60nm to 100µm and enzyme loading of 10-100 mg/g particles.

Example 3. Preparation of magnetic nano-biocatalyst PMMAP CA

0.025 g of ammonium persulfate and 0.316 mL methyl methacrylate monomer were added into 30 mL DI water containing OA-MNPs (0.4-0.8 mg/mL), and the mixture was stirred at 80°C for 1h. After centrifugation at 16700 g for 10 min and washing by DI water for several times, PMMA-MNPs (poly(methyl methacrylate) magnetic nanoparticles) with a diameter of 50-500 nm were obtained (Fig. 8a). To immobilize *Candida Antarctica* Lipase B (CALB), 1-20 mL of phosphate buffer having a concentration of 0.75-1 mg/mL of enzyme and

PMMA-MNPs (which are present at ratio of 400mg CALB per gram of particles) at different pHs (pH from 5 to 8) was incubated at 4-30°C, 12 hours, 30rpm. The resultant nanobiocatalyst particles were collected under magnetic field after centrifugation at 5000 rpm for 5 min. 45-147 mg of enzymes were immobilized on one gram of PMMA-MNPs to result in PMMAP CA (poly(methyl methacrylate) particles immobilized with CALB)

Example 4. Preparation of magnetic nano-biocatalyst PSP CA

0.025 g of ammonium persulfate and 0.316 mL styrene were added to 30 mL of DI water containing OA-MNPs (0.4-0.8 mg/mL), and the mixture was stirred at 80°C for 1h. After centrifugation at 16700 g for 10 mins and washing with DI water for several times, PS-MNPs with diameters of 80-800 nm were obtained (Figure 8b). To immobilize CALB, 1-20 mL phosphate buffer having a concentration of 0.75-1 mg/mL enzyme and PS-MNPs (which are at a ratio of 400mg CALB per gram of particles) at different pHs (pH from 5 to 8) was incubated at 30°C, 12 h, 30rpm. The resultant nanobiocatalyst particles were collected under magnetic field after centrifugation at 5000 rpm for 5 min. 100-300mg enzymes were immobilized on one gram of PS-MNPs.

Example 5. Preparation of magnetic nano-biocatalyst EDAP CA

0.025 g of ammonium persulfate and 0.316 mL glycidyl methacrylate (GMA) were added into 30 mL DI water containing OA-MNPs (0.4-0.8 mg/mL), and the mixture was stirred at 80°C for 1h. After centrifugation at 16700 g for 10 min and washing by DI water for several times, the produced GMA-MNPs was re-dispersed in 30mL DI water. 3mL ethylene diamine (EDA) was added, and the mixture was stirred at 80°C for 12 hours. After centrifugation at 16700 g for 10 min and washing by DI water for several times, EDA-MNPs (ethylene diamine magnetic nanoparticles) with the sizes of 50-500 nm were obtained (Fig. 8c). To immobilize CALB, 1-20 mL phosphate buffer having a concentration of 0.75-1 mg/mL enzyme, glutaraldehyde, and EDA-MNP (which are at a ratio of 400mg CALB per gram of particles) at different pH was incubated at 30°C, 12h, 30rpm. The resultant nanobiocatalyst particles were collected under magnetic field after

centrifugation at 5000 rpm for 5 min. 10-30mg of enzymes were immobilized on one gram of EDA-MNPs.

Example 6. Preparation of magnetic nano-biocatalyst GAP CA

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30mL of EDA-MNPs solution (prepared in example 5) was slowly added into 30mL of glutaraldehyde (GA) solution to avoid any unwanted aggregation. The mixture was stirred at 80°C for 3 hours, followed by centrifugation at 12000 g for 10 min and washing with DI water for several times. The collected GA-MNPs (glutaraldehyde magnetic nanoparticles) were used for the immobilization of CALB. 1-20 mL phosphate buffer having concentration of 0.75-1 mg/mL of enzyme and GA-MNP (which are at ratio of 400mg CALB per gram of particles) at different pH was incubated at 30°C, 12h, 30rpm. The resultant nanobiocatalyst particles were collected under magnetic field after centrifugation at 5000 rpm for 15 5 min. 10-30mg of enzymes were immobilized on one gram of GA-MNPs.

Example 7. Preparation of FAME from GTO and methanol by one-pot reactions with nano- and micro-biocatalyst MNA TL

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A mixture of freeze-dried MNA TL containing 0.1-300 mg of TLL, 1-30 g of GTO (17wt% of FFA), MeOH (7.6 mol of MeOH :1 mol of FFA), and 0.1-9 g of silica gel micro-beads were shaken at 30°C and 30 rpm for 8-12 h. After the reaction, MNA TL was separated by applying an external magnetic field. Silica gel micro-bead and glycerol were then removed from biodiesel by centrifugation at 21500g in 10 min. The concentration of FAME was analyzed by using an Agilent gas chromatography (GC) with INNOWAX column at an inlet temperature of 220°C and FID temperature of 275°C. 5 µL of the sample was dissolved in 995 µL of *n*-hexane containing 2 mmol/L of *n*-hexadecane as internal standard. The temperature program was set as follows: increase from 150 to 225°C at a rate of 15°C/min, from 225°C to 260°C at the rate of 5°C/min, and keep at 260°C for 3 min. The GC chromatograms were shown in Fig. 9b. The FAME concentration was calculated based on the calibration curve with FAME standards (Fig. 9a). The biodiesel (FAME) standard was prepared from grease in a two-step process. Firstly, 0.75 g Novozyme 435® was used for esterification of

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30g waste grease with 2.08 g MeOH (3.5:1 mol of MeOH to FFA) were stirred at 30°C and 500rpm for esterification of FFA. After 2h, the Novozyme 435® was separated by centrifugation and the FFA content of grease was determined by titration. Only 0.5 wt% FFA left in the pre-treated grease. In the second step,
5 0.17 g KOH was used for transesterification of 10g pre-treated grease with 2.22 g MeOH (6:1 mol of MeOH to grease) were stirred at 65°C and 500 rpm in 18 h. The biodiesel produced was purified before analyzed by GC. The sample was firstly centrifuged at 13,500 rpm and 25°C for 5 min and the bottom layer was discharged to remove the by-product glycerol. Then, the excess MeOH in the top
10 layer was removed by rotary evaporator. After that, the biodiesel was washed with HCl solution (0.2%), followed by DI water until the pH of the washing solution was neutral. Biodiesel was dried by addition of magnesium sulfate overnight and the final product was obtained after filtration.

15 In this example, the total FAME yield of the nano- and micro-biocatalyst MNA TL reached 90-99% (Fig. 10). For comparison, the free enzyme TLL and commercially available immobilized enzyme (Lipozyme TLL IM) were used to perform the same reactions, but the conversions achieved were very low as compared to the MNA TL described above (Fig. 11).

20 Example 8. Recycling of MNA TL in the production of FAME from GTO by one-pot reactions

Transformation of grease trapped oil (GTO having 17wt% of FFA) with
25 methanol was performed with MNA TL as catalyst using the same procedure described for Example 7. After the reaction, the catalysts were separated under an external magnetic field, washed with *t*-butanol (or *n*-hexane), freeze-dried, and then added to the new reaction medium containing GTO (17wt% of FFA), MeOH and silica gel micro-beads. The new batch of reaction was carried out
30 under same conditions as the first batch. The biocatalysts were recycled and reused again. In total, 10 times recycling was performed, and high FAME yield (85-96%) was achieved in each reaction cycle (Fig. 12).

Example 9. Conversion of FFA in GTO to FAME by esterification with methanol using nano- and micro-biocatalyst MNA CA

A mixture of MNA CA containing 0.1-1mg of CALB, 1 g of GTO (17% wt FFA), MeOH (3.8 mol of MeOH : 1 mol of FFA) was shaken at 30°C and 30 rpm for 4 or 8h. After separation of MNA CA under external magnetic field, the amount of FFA in the remaining GTO was determined by titration. The acid value of GTO was decreased to <0.5% (Fig. 13 and Fig. 14).

Example 10. Recycling of MNA CA in the esterification of FFA in GTO with methanol to prepare FAME

Esterification of GTO (17% wt of FFA) with methanol was performed with MNA CA as catalyst using similar procedures described in Example 9. After the reaction, the catalysts were separated under an external magnetic field, washed with *t*-butanol (or *n*-hexane), freeze-dried, and then added to the new reaction medium containing GTO (17wt% of FFA) and MeOH. The new batch of reaction was carried out under same conditions as the first batch. The biocatalysts were recycled and reused again. In total, 11 times recycling was performed, and the acid value dropped to <2% in each cycle (Fig. 15).

Example 11. Conversion of FFA in GTO to FAME by esterification with methanol

Esterification of GTO using nano-biocatalyst (PMMAP CA, PSP CA, EDAP CA, or GAP CA): a mixture of 1g of GTO (14 wt% FFA), methanol (6 mol of MeOH : 1 mol of FFA) and magnetic nanobiocatalyst (PMMAP CA, PSP CA, EDAP CA or GAP CA at 1wt% of particles to GTO) were shaken at 30°C and 30 rpm. The fatty acid value dropped to <2% within 0.5-1h (Fig. 16-18). For comparison, the commercially available immobilized enzyme, Novozym 435, was used for the esterification reaction under same conditions. The specific activity of the magnetic nanobiocatalysts was about two times higher than that of Novozym 435.

Example 12. Recycling of nano-biocatalyst PMMAP CA in the esterification of FFA in GTO with methanol to prepare FAME

Esterification of GTO (15 wt% FFA) with methanol was performed with PMMAP CA as catalyst (1wt% loading) using similar procedures described in Example 11. After the reaction, the catalysts were separated under an external magnetic field, washed with *n*-hexane, freeze-dried, and then added to the new reaction medium containing GTO (15 wt% of FFA) and MeOH. The new batch of reaction was carried out under same conditions as the first batch. The biocatalysts were recycled and reused again. In total, 5 times recycling was performed, and the fatty acid value dropped to <2% in each cycle (Fig. 19) showing that the efficiency of FFA conversion is still maintained at a high level even after several cycles of recycling.

Overview of Examples 1-12

The following provides an overview and/or additional information in accordance with some embodiments disclosed herein, which may better help understand Examples 1-12 described above.

An example route for preparing nano- and micro-biocatalysts is shown in Fig. 3. Oleic acid coated iron oxide magnetic nanoparticles with a diameter of 5-20nm were first synthesized by a co-precipitation method and then reacted with poly(glycidyl methacrylate) to give magnetic core-shell structured nanoparticles containing epoxy surface function (GMA-MNPs) with a diameter 40-200 nm.

Reaction of GMA-MNPs with 4,7,10-trioxa-1,13-tridecanediamine afforded Trioxa-MNPs containing amino surface group. Further treatment of these particles with glutaraldehyde gave CHO-MNPs with aldehyde groups on particle surface. TEM measurements showed that the GMA-MNPs have substantially the same shape and size (Fig. 4).

Hydrolase such as CALB and TLL were immobilized on the CHO-MNP, respectively, by mild shaking of enzymes and CHO-MNPs in phosphate buffer, to form MNA CA and MNA TL, respectively.

5 After washing and freeze-drying, nano- and micro- biocatalyst MNA CA (Fig. 5a) and MNA TL (Fig. 5b) with size of 60nm-100 μ m were obtained. The micro-biocatalysts were formed by the non-crosslinked clustering of the individual nano-biocatalysts *via* physical interaction among the enzymes on the particles. The overall synthesis of both biocatalysts is simple and highly
10 reproducible with 70-90% yield.

Specific enzymes loading of 10-100mg CALB per gram particles for MNA CA and 20-100mg TL per gram particles for MNA TL were achieved, respectively. There was no leaching of enzymes during the washing process which confirmed
15 the stable immobilization *via* covalent bonding.

Fig. 6 shows the separation of the biocatalysts in the mixture of GTO and FAME under external magnetic field within 1 min. The separation was fast and complete.
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Other possible routes of preparing nano-biocatalysts are outlined in Fig. 7. MNPs with iron oxide core and polymer shell such as polystyrene (PS) and poly(methyl methacrylate) (PMMA) were synthesized by the treatment of OA MNPs with the corresponding monomer such as methyl methacrylate or styrene,
25 respectively. The resultant PS-MNPs (Fig. 8b) or PMMA-MNPs (Fig. 8a) showed a diameter of 50-800 nm.

Physical adsorption of CALB on PMMA-MNPs and PS-MNPs were carried out respectively, by mixing enzymes and particles in phosphate buffer. This
30 resulted in the nano-biocatalysts PMMAP CA containing 45-147 mg enzymes/g particle and PSP CA containing 100-300mg enzymes/g particle, respectively.

On the other hand, reaction of GMA-MNPs with ethylene diamine gave EDA-MNPs with amine group on the surface, and further treatment of EDA-MNPs with glutaraldehyde resulted in GA-MNPs containing surface aldehyde functional groups. Covalent binding of CALB on GA-MNPs gave the nano-biocatalyst GAP CA. Alternatively, the reaction of CALB with glutaraldehyde, and EDA-MNPs yielded EDAP CA. In both cases, the enzyme loading was 10-30mg/g of particles.

The efficient transformation of GTO to FAME *via* one-pot esterification and transesterification with methanol was achieved by using the nano- and micro-biocatalyst particles.

As a demonstrative example, freeze-dried MNA TL was used to catalyze the biotransformation of GTO containing 17% of FFA with MeOH (7.6 mol of MeOH:1 mol of FFA) in the presence of silica gel micro-beads at catalyst loading of 0.2wt% of TLL to GTO. The reaction was performed at 30°C for 12 h, and the biodiesel formation was followed by GC analysis (Fig. 9b). By using 0.1-0.5 wt% of enzyme loading (based on TLL), the FAME yield reached 90-99% (Fig. 10). The addition of silica gel helped to remove the water present, thus driving the esterification to the formation of biodiesel. The high yield achieved with the nano- and micro-biocatalyst is much better than that with the free enzyme TLL and the immobilized TLL on large solid carrier such as the commercially available TLL IM, as demonstrated in Fig. 11.

The recyclability of MNA TL was shown in Fig. 12. After each run of biotransformation, MNA TL was quickly separated under external magnetic field, washed, freeze-dried and then added to the new batch of reaction medium containing GTO, MeOH and Silica for next run of biotransformation. The catalysts were recycled for 10 times, with >85% yields of FAME even in run 11. Evidently, in this case, the efficient recycling of the biocatalysts can effectively reduce the cost of catalysts and thus the production cost of biodiesel.

The developed nano- and micro-biocatalysts are also useful for the high-yielding transformation of FFA in GTO to FAME *via* esterification with methanol. This is the first step in the two-step preparation of biodiesel from grease shown in Fig. 2. In a representative example, MNA CA was used for the esterification of FFA in GTO containing 16% FFA with MeOH. The reaction was examined at 0.01-0.1wt% of CA to GTO, 3.8:1 (mol:mol) of MeOH to FFA, and 30°C. The reduced amount of FFA in GTO was determined by titration. As shown in Fig. 13, high conversion for the esterification (corresponding to a FFA concentration of < 0.5 %) was achieved in 4 h when catalyst loading of 0.03-0.1wt% (based on CALB) was used. The acid value of GTO were also reduced to <0.5% with even only 0.01wt% of CALB after 8h reaction (Fig. 14). The recycling of MNA CA was demonstrated for 11 times (Fig. 15). Even in the run 12, the FFA in GTO was reduced to less than 2 %, an acid concentration that can be easily handled with the conversional process for biodiesel production with base catalysts.

Other types of nano-biocatalysts PMMA CA, PSP CA, EDAP CA and GAP CA were also proven to be efficient for the esterification of FFA in GTO with methanol to FAME. The acid value was drop from 15% to <2% within 0.5-1h reaction at low catalyst loading (1-2wt%; based on the nano-catalysts) (Fig. 16-18). The specific activity of these nanobiocatalysts was two times higher than that achieved with commercialized immobilized lipase Novozym 435 for the same transformation.

As shown in Fig. 19, the biocatalysts were also recycled and reused for six times, reducing the acid to <2% in each run of the reaction.

The micro- or nano-biocatalysts may be used in a one-pot reaction for esterification of free fatty acids to FAME and the transesterification of triglycerides to FAME as shown in Fig. 1 or as a pre-treatment step esterification of free fatty acids to FAME in the two-step reaction shown in Fig. 2.

As a further illustrative example, Fig. 48 provides an exemplary graphical representation of a magnetic micro- or nano- catalyzing particle that may be adopted

by some of the particles described in Examples 1-12. Referring to Fig. 48, the particle 420 comprises an outer shell 424 having a magnetic core 422 encapsulated by the outer shell 424. Catalysing entities 426 are immobilized on the outer shell 424 by linkers 428.

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Example 13. Synthesis of nano-size paramagnetic solid acid catalyst SO₃H-PGMA-MNPs

Oleic acid-stabilized superparamagnetic iron oxide magnetic nanoparticles (OA-MNPs) were synthesized by co-precipitation method, yielding particles with diameters of 5-20 nm. 25 mg ammonium persulfate and 0.316 mL glycidyl methacrylate were added into 30 mL deionised (DI) water containing 6.75 mg OA-MNPs, and the mixture was stirred at 80 °C for 1 h. The resultant poly(glycidyl methacrylate)-coated magnetic nanoparticles (PGMA-MNPs) have a core-shell structure with diameter of 90 nm. The PGMA-MNPs were centrifuged at 16 700 g for 10 min, washed with DI water several times, and re-suspended in DI water. 7.28 g Na₂SO₃ was then added into the PGMA-MNPs solution and the volume was adjusted to 72 mL by adding DI water. The mixture was stirred at 80 °C for 24 h. The resultant sulfonated particles were repeatedly washed with DI water. The particles were then protonated with 48 mL HCl solution (4%). After repeated washing by DI water and freeze drying, nano-size solid acid catalyst SO₃H-PGMA-MNPs with diameter of 90 nm were obtained (Fig. 22). By titration against 0.02 M KOH solution, the catalyst is tested to have an acid loading of 2.3 mmol H⁺/g.

Example 14. Esterification of FFA in grease with methanol by using nano-size paramagnetic solid acid catalyst SO₃H-PGMA-MNPs

A mixture of 8 mg catalyst, 0.195 g grease, and 0.18 mL methanol were shaken at 70°C and 1000 rpm. After 2 h, the catalyst and excess methanol were separated from the pretreated grease. By titration against 0.02 M KOH solution, the FFA content in pretreated grease was tested and the results showed that the FFA was reduced from 16 wt.% to 0.63 wt.% (corresponds to 96% conversion) as shown in Fig. 23.

Example 15. Recycling of nano-size solid acid catalyst SO₃H-PGMA-MNPs for esterification of FFA in grease with methanol

After esterification as described in Example 14, the catalyst was separated
5 from the product mixture, washed with *n*-hexane, and freeze-dried. Subsequently,
the catalyst was used in the new reaction round carried out in similar condition as the
first batch. The magnetic property of the SO₃H-PGMA-MNPs allows the catalyst to
be separated from product mixture within 5 s (Fig. 24). The nano-size SO₃H-PGMA-
MNPs can be reused at least for 10 cycles without any regeneration needed, in
10 which 96% conversion was achieved in each reaction round (Fig. 25).

Example 16. Synthesis of nano-size paramagnetic solid acid catalyst SO₃H-PS-MNPs

15 25 mg ammonium persulfate and 0.316 mL styrene were added into 40 mL DI
water containing 16 mg OA-MNPs, and the mixture was stirred at 80°C for 2.5 h. The
resultant polystyrene-coated magnetic nanoparticles (PS-MNPs) have a core-shell
structure with diameter of 80 nm. The PS-MNPs were purified by using high-gradient
magnetic separator (HGMS) and freeze-dried. Lastly, 150 mg PS-MNPs powder
20 were added into 7.5 mL of sulfuric acid and the mixture was stirred at 25°C for 15
min. After repeated washing by DI water and freeze drying, nano-size solid acid
catalyst SO₃H-PS-MNPs with diameter of 80 nm were obtained (Fig. 26). By titration
against 0.02 M KOH solution, the catalyst is tested to have an acid loading of 1.11
mmol H⁺/g.

Example 17. Esterification of FFA in grease with methanol by using nano-size paramagnetic solid acid catalyst SO₃H-PS-MNPs

30 A mixture of 8 mg catalyst, 0.195 g grease, and 0.18 mL methanol were
shaken at 70°C and 1000 rpm. After 30 min, the catalyst and excess methanol were
separated from the pretreated grease. By titration against 0.02 M KOH solution, the
FFA content in pretreated grease was tested and the results showed that the FFA

was reduced from 16 wt.% to 0.32 wt.% (corresponds to 98% conversion) as shown in Fig. 23.

Example 18. Recycling of nano-size paramagnetic solid acid catalyst SO₃H-PS-MNPs for esterification of FFA in grease with methanol

After esterification as described in Example 17, the catalyst was separated from the product mixture, washed with *n*-hexane, and dried. Subsequently, the catalyst was used in the new reaction round carried out in similar condition as the first batch. The nano-size SO₃H-PS-MNPs can be reused for at least for 8 cycles without any regeneration needed, in which conversion as high as 72-95% was achieved in each reaction round (Fig. 27).

Example 19. Synthesis of micro-size solid acid catalyst SO₃H-PS-MNPs

The PS-MNPs were prepared using the procedures in Example 16 and were purified by using HGMS and vacuum-dried to obtain micro-size PS-MNPs granules. Next, 150 mg of the granules were added into 7.5 mL sulfuric acid and the mixture was stirred at 39°C for 1.5 hours. After repeated washing by DI water and vacuum drying, micro-size solid acid catalysts SO₃H-PS-MNPs with sizes 60-500 μm were obtained (Fig. 28). By titration against 0.02 M KOH solution, the catalyst is tested to have an acid loading of 2.25 mmol H⁺/g.

Example 20. Esterification of FFA in grease with methanol by using micro-size solid acid catalyst SO₃H-PS-MNPs

A mixture of 16 mg catalyst, 0.39 g grease, and 0.35 mL methanol was shaken at 70°C and 1000 rpm. After 2 hours, the catalyst and excess methanol were separated from the pretreated grease. By titration against 0.02 M KOH solution, the FFA content in pretreated grease was tested and the results showed that the FFA was reduced from 16 wt.% to 0.4 wt.% (corresponds to 97% conversion). For comparison, millimeter-size ion-exchange resin Amberlyst 15 was used as solid acid catalyst for the pretreatment of grease. In the identical reaction condition and with

the same amount of catalysts, Amberlyst 15 was only able to decrease the FFA content to 5.3 wt% (corresponds to 67% conversion) after 2 hours reaction (Fig. 23).

Example 21. Scaled-up esterification of FFA in grease with methanol by using micro-size solid acid catalyst SO₃H-PS-MNPs

A mixture of 310 mg catalyst, 7.6 g grease, and 6.8 mL methanol were stirred at 70°C and 1000 rpm. After 2 hours, the catalyst and excess methanol were separated from the pretreated grease. By titration against 0.02 M KOH solution, the FFA content in pretreated grease was tested and the results showed that the FFA was reduced from 16 wt.% to 0.4 wt.% (corresponds to 97% conversion) as shown in Fig. 29.

Example 22. Esterification of FFA in grease with glycerol by using micro-size solid acid catalyst SO₃H-PS-MNPs

A mixture of 260 mg catalyst, 2.6 g grease, and 0.53 g glycerol were stirred at 100°C and 1000 rpm. After 26 hours, the catalyst and excess glycerol were separated from the pretreated grease. By titration against 0.02 M KOH solution, the FFA content in pretreated grease was tested and the results showed that the FFA was reduced from 16 wt.% to 1 wt.% (corresponds to 93% conversion) as shown in Fig. 30.

Example 23. Recycling of micro-size solid acid catalyst SO₃H-PS-MNPs for esterification of FFA in grease with methanol

After esterification as described in Example 20, the catalyst was separated from the product mixture, washed with *n*-hexane, and dried. Subsequently, the catalyst was used in the new reaction round carried out in similar condition as the first batch. The micro-size SO₃H-PS-MNPs can be reused for at least for 6 cycles without any regeneration needed, in which conversion as high as 74-97% was achieved in each reaction round (Fig. 31).

Example 24. Synthesis of nano-size paramagnetic solid acid catalyst SO₃H-Si-MNPs

OA-MNPs were synthesized using similar protocol described in Example 13. Thereafter, 2 mL OA-MNPs, 5 mL aqueous ammonia, 160 mL ethanol, and 1 mL
5 TEOS (tetraethylorthosilicate) were added into 40 mL DI water, and the mixture was stirred at room temperature and 400 rpm for 24 hours. The resultant silica-coated magnetic nanoparticles (Si-MNPs) had a multicore-shell structure with mean diameter of 200 nm. The Si-MNPs were repeatedly washed by DI water and freeze-dried. 60 mg Si-MNPs, 10 mL ethanol, and 1 mL 3-mercaptopropyl trimethoxysilane
10 (MPTMS) were then added into 10 mL of DI water, and the mixture was stirred at room temperature and 400 rpm for 24 hours. The resultant thiol-functionalized silica-coated magnetic nanoparticles (SH-Si-MNPs) were repeatedly washed by DI water. Subsequently, the SH-Si-MNPs, 10 mL methanol, and 10 mL hydrogen peroxide were added into 10 mL DI water, and the mixture was stirred at room temperature
15 and 400 rpm for 24 hours. Lastly, the recovered particles from previous step were added into 10 mL of 1 M H₂SO₄, and the mixture was stirred at room temperature and 400 rpm for 6 hours. After repeated washing by DI water and freeze drying, nano-size solid acid catalyst SO₃H-Si-MNPs with a mean diameter of 200 nm were obtained (Fig. 32). By titration against 0.02 M KOH solution, the catalyst is tested to
20 have an acid loading of 0.54 mmol H⁺/g.

Example 25. Esterification of FFA in grease with methanol by using nano-size paramagnetic solid acid catalyst SO₃H-Si-MNPs

25 A mixture of 8 mg catalyst, 0.195 g grease, and 0.18 mL methanol were stirred at 70°C and 1000 rpm. After 5 hours, the catalyst and excess methanol were separated from the pretreated grease. By titration against 0.02 M KOH solution, the FFA content in pretreated grease was tested and the results showed that the FFA was reduced from 16 wt.% to 1 wt.% (corresponds to 94% conversion) as shown in
30 Fig. 23.

Example 26. Recycling of nano-size solid acid catalyst SO₃H-Si-MNPs for esterification of FFA in grease with methanol

After esterification as described in Example 25, the catalyst was separated from the product mixture, washed with *n*-hexane and methanol, and freeze-dried. Subsequently, the catalyst was used in the new reaction round carried out in similar condition as the first batch. The magnetic property of the SO₃H-Si-MNPs allows the catalyst to be separated from water within 30 mins (Fig. 33). The nano-size SO₃H-Si-MNPs can be reused at least for 3 cycles without any regeneration needed (Fig. 34).

Overview of Examples 13 to 26

The following provides an overview and/or additional information in accordance with some embodiments disclosed herein, which may better help understand Examples 13-26 described above.

In these Examples, the nano-size solid acid catalysts are paramagnetic core-shell nanoparticles which comprise of magnetic iron oxide (Fe₃O₄) cores, external coating shell to protect the Fe₃O₄ cores, and acid functional group grafted onto the surface of the coating shell (Fig. 21).

The magnetic cores were synthesized by co-precipitation method, yielding Fe₃O₄ nanoparticles with size of 5-20 nm in diameter, which is in the range of size required for the particles to exhibit superparamagnetic property.

By harnessing free-radical polymerization method or Stöber method, three types of coatings were successfully applied onto the Fe₃O₄ cores, namely poly(glycidyl methacrylate) (PGMA), polystyrene (PS), and silica (Si). In a particular embodiment, the polymerization reaction was carried out by adding about 6.75-16 mg OA-MNPs in a total of 30-40 mL mixture volume to 25 mg ammonium persulfate (APS) initiator and 0.316 mL monomer. The mixture was stirred at 80°C for 1-2.5 h to obtain polymer coated-MNPs. In a particular embodiment, the Stöber method was carried out by mixing 2 mL OA-MNPs, 5 mL aqueous ammonia, 160 mL ethanol, and 1 mL TEOS (tetraethylorthosilicate)

with 40 mL DI water, and the mixture was stirred at room temperature and 400 rpm for 24 hours to afford silica-coated MNPs.

The coating methods employed facilitate the synthesis of perfect core-shell structure, which is advantageous to prevent the leaching of Fe_3O_4 and to enhance the stability of the nano-support. PGMA chains contain epoxide groups, which are chemically active and thus allow for the facile attachment of sulfonic acids *via* sulfonation reaction, thus yielding nanoparticles with acid function on the surface. PS chains contain reactive benzene rings which can be grafted with sulfonic acid moieties. Si is terminated by silanol functional groups which can be modified into thiols and subsequently oxidized to sulfonic acids.

Subsequently, sulfonic acid functional groups were grafted onto the surface of each Fe_3O_4 -coated nanoparticles. Sulfonic acid was selected as the acid functional groups due to its inexpensive cost and high catalytic activity. The sulfonic acid groups were covalently attached on the nano-support, yielding stable nano-size solid acid catalyst which can be recycled and reused without any regeneration step. The micro-size solid acid catalysts obtained were the aggregated nano-size solid acid catalysts achieved by drying under vacuum. The fabricated catalysts, namely nano-size sulfonated poly(glycidyl methacrylate)-coated magnetic nanoparticles (nano-size SO_3H -PGMA-MNPs), nano-size sulfonated polystyrene-coated magnetic nanoparticles (nano-size SO_3H -PS-MNPs), micro-size sulfonated polystyrene-coated magnetic nanoparticles (micro-size SO_3H -PS-MNPs), and nano-size sulfonated silica-coated magnetic nanoparticles (SO_3H -Si-MNPs), have diameters of 90 nm, 80 nm, 60-500 μm , and 200 nm, respectively (Fig. 22, 26, 28, and 32, respectively).

The four types of catalyst were successfully used for the pretreatment step in biodiesel production from grease, to reduce high FFA content. Methanol was used for esterification owing to its low cost. Alternatively, glycerol, a byproduct of base-catalyzed transesterification process, was also used for the pretreatment step to reduce FFA in grease. The use of cheap glycerol can further decrease the biodiesel production cost.

Esterification of grease with methanol by using 4 wt% (based on grease weight) of the nano-size SO_3H -PGMA-MNPs solid acid catalyst with 40:1 mole ratio of methanol to FFA at 70°C for 2 h reduced the FFA content in grease from 16 wt.% to 0.63 wt.% (corresponds to 96% conversion) as presented in Fig. 23.

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The paramagnetic property of the SO_3H -PGMA-MNPs allows the catalyst to be separated within 5 s (Fig. 24). The easily separated catalysts can be reused for at least for 10 cycles without any regeneration needed, thus potentially being able to lower the production cost of biodiesel (Fig. 25). In each cycle, the acid concentration was sufficiently low for the next step biodiesel synthesis.

Esterification of grease with methanol by using 4 wt% (based on grease weight) of the nano-size SO_3H -PS-MNPs solid acid catalyst with 40:1 mole ratio of methanol to FFA at 70°C for 30 min reduced the FFA content in grease from 16 wt.% to 0.32 wt.% (corresponds to 98% conversion) as presented in Fig. 23.

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For the same reaction with micro-size SO_3H -PS-MNPs solid acid catalyst, the FFA content in grease was reduced from 16 wt.% to 0.4 wt.% (corresponds to 97% conversion) within 2 hours as presented in Fig. 23.

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For comparison, mm-size ion-exchange resin Amberlyst 15 was used as solid acid catalyst for the pretreatment of grease under the identical reaction condition, decreasing the FFA content only to 5.3 wt% (corresponds to 67% conversion) after 2 hours reaction. Therefore, the nano-size and micro-size SO_3H -PS-MNPs solid acid catalysts developed here are much better than Amberlyst 15 for the esterification reaction (Fig. 23).

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In a large-scale experiment with micro-size solid acid catalyst SO_3H -PS-MNPs, where the amount of reactant was increased by 19.5 times, the catalyst activity was similar to that in small-scale experiment. After two hours reaction, the FFA content in grease decreased from 16 wt.% to 0.4 wt.% (corresponds to 97% conversion) (Fig. 29). This result demonstrated the feasibility of scaling-up with the catalysts.

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The nano- and micro-size solid acid catalyst $\text{SO}_3\text{H-PS-MNPs}$ can be simply separated and reused for at least for 8 and 6 cycles, respectively, without any regeneration needed, thus making it feasible to lower the production cost of biodiesel (Fig. 27 and 31, respectively). In each cycle, the acid concentration was sufficiently low for the next step biodiesel synthesis.

In the esterification of FFA in grease with glycerol by using 10 wt.% (based on grease weight) of the micro-size $\text{SO}_3\text{H-PS-MNPs}$ solid acid catalyst with 4:1 mole ratio of glycerol to FFA at 100°C , the FFA content can be reduced from 16 wt.% to 1 wt.% (corresponds to 93% conversion) within 26 hours. Whereas by using Amberlyst 15 under an identical reaction condition, 5.3 wt% FFA still remained in grease (corresponds to 67% conversion). Thus, the micro-size $\text{SO}_3\text{H-PS-MNPs}$ solid acid catalyst has higher activity compared to Amberlyst 15 for the esterification with glycerol (Fig. 30).

Esterification of grease with methanol by using 4 wt% (based on grease weight) of the nano-size $\text{SO}_3\text{H-Si-MNPs}$ solid acid catalyst with 40:1 mole ratio of methanol to FFA at 70°C reduced the FFA content in grease from 16 wt.% to 1 wt.% (corresponds to 94% conversion) within 5 hours (Fig. 23). For comparison, mm-size ion-exchange resin Purolite CT-275 was used as solid acid catalyst for the pretreatment of grease under even harsher condition of 75°C , 40:1 methanol to FFA mole ratio, and 10 wt.% catalyst loading (based on grease weight), and it was only able to decrease the FFA content from 18 wt.% to 7.6 wt. % (corresponds to 58% conversion) within 4 hours. Therefore, the $\text{SO}_3\text{H-Si-MNPs}$ solid acid catalyst has higher activity compared to Purolite CT-275 for the esterification reaction.

The paramagnetic property of the $\text{SO}_3\text{H-Si-MNPs}$ allows the catalyst to be separated within 30 mins (Fig. 33). The easily separated catalysts can be reused for at least for 3 cycles without any regeneration needed, thus potentially being able to lower the production cost of biodiesel (Fig. 34).

As a further illustrative example, Fig. 48 provides an exemplary representation of a magnetic micro- or nano- catalyzing particle that may be adopted by some of the particles described in Examples 13-26. Referring to Fig. 48, the particle 400 comprises an outer shell 424 having a magnetic core 422 encapsulated by the outer shell 424. Catalysing entities 426 are immobilized on the outer shell 424 by linkers 428.

Example 27. Screening for lipase-producing strains from soil

Two grams of soil sample was suspended in 10 mL of M9 medium containing, per liter of tap water, 8.5 g of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 3.0 g of KH_2PO_4 , 0.5 g of NaCl , 1.0 g of NH_4Cl , 2 mL of MgSO_4 (1M) and 1 mL of Tris-methyl (MT) solution. The suspension was supplemented with 0.1 g of olive oil as the sole carbon source. The enrichment culture was conducted at 30°C and 300 rpm for 2-3 days. After the enrichment culture, a sample of 100 μL of the mixture was withdrawn and diluted 10^6 times, 100 μL of the diluted solution was then plated onto a Rhodamine B selection agar medium composed of, per liter of tap water, 1 g of $(\text{NH}_4)_2\text{SO}_4$, 1 g of K_2HPO_4 , 5 g of KCl , 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 5 g of yeast extract, 5 g of tryptone, 120 mL of olive oil emulsion (olive oil:polyvinyl alcohol=1:3, v/v), 0.01 g of Rhodamine B, and 2 g of agar. The colonies developed on agar with clear fluorescence zones under UV-illumination were selected for final screening. The strains isolated with obvious lipase activity by preliminary screening were individually inoculated into 3 mL of Lysogeny Broth (LB) medium and cultivated for 12 h, the preculture of 2.5 mL was inoculated to 50 mL of fermentation medium (glucose 8.0 g/L, yeast extract 5.0 g/L, peptone 5.0 g/L, K_2HPO_4 0.5 g/L, KH_2PO_4 0.5 g/L, NaCl 1.0 g/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.4 g/L, Olive oil 5 g/L, pH 7.0). The cultivation was then cultivated for another 12 h at 30°C and 250 rpm. It will be appreciated that in other examples, the cultivation can be carried out at about 20°C to about 40°C and about 150 to about 400 rpm for about 6 to 18 hours. It will also be appreciated that in other examples, the enzyme expression can be carried out at about 15°C to about 30°C and about 150 to about 400 rpm for about 6 to 18 hours. After cultivation, the cells were harvested by centrifugation at 8000 rpm for 5 min and washed for 2 times with de-ionized water, the wet cells were then stored in -80°C for 6 h, and subjected to lyophilization using vacuum freeze dryer for 1 day to obtain the lyophilized cells. The

lyophilized cells were used as catalysts for biodiesel production from GTO or esterification of FFA in GTO.

Example 28. FAME production from GTO using isolated strains from soil

2.0 g of GTO and 0.04 g of lyophilized cells (2 wt% catalyst loading) were added together to form a mixture. Methanol was added in equal amounts at 24-h intervals (molar ratio of methanol to GTO, 3:1). The reaction was conducted at 30°C and 300 rpm for 72 h. After reaction, 50 µL of reaction mixture was taken and washed with 50 µL de-ionized water, and then centrifuged at 15,000 rpm for 10 min. 5 µL of the upper layer was mixed with 995 µL *n*-hexane containing 2 mM *n*-hexadecane as an internal standard. The mixture was subjected to GC analysis to quantify the FAME using an Agilent GC with INNOWAX column and an inlet temperature of 220°C, Flame Ionization Detector (FID) temperature of 275°C. The temperature program was as follows: the temperature was increased from 150 to 225°C at a rate of 15°C/min, from 225°C to 260°C with the rate of 5°C/min and kept at 260°C for 3 min. The FAME conversion was calculated based on the FAME produced from the reaction described above and the FAME standard produced from the two-step reaction catalyzed by Novozyme 435 and KOH as described in Example 7 above. Among all the strains isolated from soil, the strain P1-28 displayed the highest FAME yield, and the FAME yield from GTO reached 32% (Fig. 38).

Example 29. Esterification of FFA in GTO using isolated strains from soil

2.0 g of GTO and 0.04 g of lyophilized cells (2 wt% catalyst loading) were added together to form a mixture. Methanol was added in equal amounts at 24-h intervals (molar ratio of methanol to FFA, 3:1). The reaction was conducted at 30°C and 300 rpm for 72 h. After reaction, 150 µL of reaction mixture was withdrawn and centrifuged at 1,000 rpm for 10 min, the supernatant was mixed with isopropyl alcohol (5 mL) and then titrated using the KOH solution (20 mM) to determine the acid content left in GTO. Among all the strains isolated from soil, the strain P1-28 exhibited the highest esterification activity, and the FFA conversion reached 55% (Fig. 39).

Example 30. Preparation of E.coli whole cell biocatalyst expressing lipase SML

Recombinant *E.coli* T7 express expressing lipase SML (*Serratia marcescens* lipase) was inoculated to 5 mL of Lysogeny Broth (LB) medium after cultivation at 37 °C and 250 rpm for 12 h, 1% (v/v) of the preculture was transferred to 250 mL Erlenmeyer flask containing 50 mL Terrific Broth (TB) medium containing, per liter of tap water, 12 g of tryptone, 24 g of yeast extract, 4 mL of glycerol, 2.3 g of KH_2PO_4 , 16.37 g of $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 50 mg of kanamycin. The culture was incubated at 37 °C and 250 rpm up to an OD (Optical Density) 600nm of 0.6-0.8. Subsequently, lipase expression was induced by addition of isopropylthiogalactoside (IPTG, final concentration 0.1 mM). It will be appreciated that in other examples, the IPTG concentration used can vary from about 0.05 to 5 mM. The culture was grown for an additional 12 h at 20 °C and 250 rpm and then cells were harvested by centrifugation at 6000 rpm for 5 min. After washing two times with de-ionized water, the harvested wet cells were kept in -80°C for 6 h, and subjected to lyophilization using vacuum freeze dryer for 24-36 h to obtain the lyophilized cells. The lyophilized *E.coli* whole cells were then used for biodiesel production from GTO or esterification of FFA in GTO.

Example 31. FAME production from GTO in one-pot reaction using recombinant E.coli expressing lipase SML

2.0g of GTO and 0.1g of lyophilized cells of recombinant *E.coli* expressing lipase SML (5 wt% catalyst loading) were added together to form a mixture. Methanol was added in equal amounts with at 24-h intervals (molar ratio of methanol to GTO, 4:1). The reaction was performed at 30°C and 500 rpm under magnetic stirring. At designed time intervals, the reaction sample was taken for GC analysis as described in Example 24. After 96 h of reaction time, FAME yield reached 82.1% (Fig. 40). When the catalyst loading increased to 10 wt%, higher FAME yield (92.0%) was achieved, as shown in Fig. 42.

Example 32. Esterification of FFA in GTO using recombinant *E.coli* expressing lipase SML

2.0g of GTO and 0.1g of lyophilized cells of recombinant *E.coli* expressing lipase SML (5 wt% catalyst loading) were added together to form a mixture. Methanol was added in equal amounts with 24-h intervals (molar ratio of methanol to GTO, 4:1). The reaction was performed at 30°C and 500 rpm under magnetic stirring. At designed time intervals, the reaction sample was taken for the determination of acid content in GTO as described in Example 25. After 96 h reaction, the FFA conversion in GTO reached >95%, reducing the acid level in GTO to below 1.0% (Fig. 41).

Example 33. Preparation of *E.coli* whole cell biocatalyst expressing lipase CALB

Recombinant *E. coli* BL 21 was inoculated to 5 mL of LB medium, after cultivation for 12 h, 2% (v/v) of the preculture was transferred to 500 mL Erlenmeyer flask containing 100 mL LB medium supplemented with 50 mg/L of kanamycin. The culture was incubated at 37 °C and 250 rpm up to an OD600nm of 0.6-0.8. Subsequently, lipase expression was induced by addition of isopropylthiogalactoside (IPTG, final concentration 1 mM). The culture was grown for an additional 6 h at 30 °C and 250 rpm and cells were harvested by centrifugation at 6000 rpm for 5 min. After washing two times with de-ionized water, the harvested wet cells were kept in -80°C for 6 h, and subjected to lyophilization using vacuum freeze dryer for 24-36 h to obtain the lyophilized cells. The lyophilized *E.coli* whole cells were then used for biodiesel production from GTO or esterification of FFA in GTO.

Example 34. FAME production from GTO using recombinant *E.coli* expressing lipase (CALB)

2.0 g of GTO and 0.08 g of lyophilized cells of recombinant *E.coli* expressing lipase CALB (4 wt% catalyst loading) were added together to form a mixture. Methanol was added in equal amounts at 12-h intervals (molar ratio of methanol to GTO, 3:1). The reaction was performed at 40°C and 250 rpm in the shaker. At

designed time intervals, the FAME yield was determined as described in example 2. After 84 h reaction, 34% FAME yield was obtained (Fig. 43).

Example 35. Esterification of FFA in GTO using recombinant *E.coli* expressing lipase (CALB)

2.0 g of GTO and 0.08 g of lyophilized cells of recombinant *E.coli* expressing lipase CALB (4 wt% catalyst loading) were added together to form a mixture. Methanol was added in equal amounts with 12-h intervals (molar ratio of methanol to GTO, 3:1). The reaction was performed at 40°C and 250 rpm in the shaker. As shown in Figure 44, 96% of FFA in grease was converted to FAME.

Example 36. Preparation of *E.coli* whole cell biocatalyst expressing lipases CALB and TLL

Recombinant *E.coli* BL21(DE3) expressing lipases CALB and TLL in one plasmid was inoculated to 5 mL of Lysogeny Broth (LB) medium, after cultivation at 37 °C and 250 rpm for 12 h, 1% (v/v) of the preculture was transferred to a 500 mL Erlenmeyer flask containing 100 mL LB medium supplemented with 50 mg of kanamycin. The culture was incubated at 37 °C and 250 rpm up to an OD (Optical Density) 600nm of 0.6. Subsequently, lipase expression was induced by addition of isopropylthiogalactoside (IPTG, final concentration 0.2 mM). It will be appreciated that in other examples, the IPTG concentration used can vary from about 0.05 to 5 mM. The culture was grown for an additional 4 h at 30 °C and 250 rpm to an OD 600nm of 3, and then cells were harvested by centrifugation at 6000 rpm for 5 min. The *E. coli* whole cells were then used for biodiesel production from GTO or esterification of FFA in GTO. The expressing of the lipases CALB and TLL was analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 45). Cell-free extract was prepared by the disruption of the harvested cells with homogenizer under 20 psi followed by centrifugation. Soluble cell lysate (corresponding to 0.5 mL cell culture) were loaded to SDS-PAGE, and the gels were stained with Coomassie brilliant blue.

Example 37. FAME production from GTO using recombinant *E. coli* expressing lipases CALB and TLL

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2.0 g of GTO and 0.08 g of wet cells (4% loading) of recombinant *E. coli* expressing lipases CALB and TLL in one plasmid were added together to form a mixture. Methanol was added in equal amounts at 6-h intervals (molar ratio of methanol to GTO, 3:1). The reaction was performed at 30°C and 500 rpm. At
10 designed time intervals, the FAME yield was determined as described in example 2. After 72 h reaction, 87% FAME yield was obtained (Fig. 46).

Example 38. Recycling of recombinant *E. coli* expressing lipases CALB and TLL for FAME production from GTO

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After FAME production as described in Example 33, the whole cell *E. coli* was separated from the product mixture, washed with *tert*-butanol, and dried at 30°C for 2 h. Subsequently, the whole cell *E. coli* was used in a new reaction round carried out in same conditions as the first batch. The cells retained 75% of the original
20 productivity (FAME yield) after five reaction cycles (Fig. 47).

Overview of Examples 27 to 38

The following provides an overview and/or additional information in
25 accordance with some embodiments disclosed herein, which may better help understand Examples 27-38 described above.

The screening process for lipase-producing strains from soil adopted by some of the Examples is shown in Fig. 37. Here, two-step screening strategy
30 was developed: the preliminary screening for lipase activity and the final screening for esterification or transesterification activities.

An exemplary procedure for preliminary screening was as follows: Soil samples were collected from different places in Singapore, each soil sample (1-5 g) was suspended in 10 mL mineral salts medium. The suspension was supplemented with oil or fatty acid ester as carbon source to give a final concentration of 0.5-5% (v/v), and the enrichment culture was carried out at 20-30°C and 150-300 rpm for 2~3 days. After the enrichment culture, samples of the mixtures were plated onto rich medium agar containing the olive oil and Rhodamine B, which is used for the screening of lipase-producing strains. If the strain developed on the plate agar can produce lipase, it will hydrolyze the olive oil to produce free fatty acid, which can react with Rhodamine B to form a compound with fluorescence under ultraviolet (UV) light. The colonies with clear fluorescence zones under UV- illumination were selected for esterification and transesterification activities test using the GTO as substrate.

An exemplary procedure for final screening was as follows: 71 strains with obvious lipase activity were employed for esterification and transesterification activities test using the GTO as substrate. The strains were individually inoculated into 3-5 mL of LB medium and cultivated for 8-16 h, the preculture of 1-5% (v/v) was inoculated to fermentation medium for further cultivation at 20-30°C and 150-300 rpm. After 12 h cultivation, the cells were harvested by centrifugation and washed for 2 times with de-ionized water. The wet cells were then stored in -80 °C for 4-12 h and subjected to lyophilization using vacuum freeze dryer for 1-3 days to obtain the lyophilized cells. The lyophilized cells were used as catalyst for esterification or transesterification activities test, respectively. Among all the strains tested, six strains displayed obvious esterification or transesterification activities. FAME production and FFA conversion using GTO as substrate with lyophilized cells (1-10 wt% based on the GTO) of six strains were shown in Fig. 38 & 39. The strain P1-28 displayed the highest FAME yield (32%) and FFA conversion (55%), respectively.

The strain P1-28 was identified as *Serratia marcescens* according to 16S rDNA gene sequencing and taxonomic analyses. The hydrolase of strain p1-28 (*Serratia marcescens* YXJ-1002) was then cloned. The method for construction of recombinant *E.coli* expressing hydrolases from *Serratia marcescens* YXJ-

1002 was as follows: The enzyme gene *sml* was amplified by polymerase chain reaction (PCR) using DNA polymerase and a combination of forward (5'-ACTCATATGGGCATCTTTAGCTATAAGGATCTG-3') and reverse (5'-TGCAAGCTTTTAGGCCAACACCACTGATCGG-3') primers, where the underlines represent the NdeI and Hind III sites, respectively. The DNA genome from *Serratia marcescens* YXJ-1002 was used as a template. The PCR product was digested with NdeI and Hind III and inserted into corresponding restriction enzyme sites of plasmid pET-28a to construct recombinant expression plasmid pET28a-sml. The recombinant expression plasmid was transformed into *E. coli* T7 Express, and kanamycin-resistant transformants were subsequently selected using LB-agar plates supplemented with kanamycin. After the enzyme was cloned and expressed in *E. coli*, the hydrolytic activity of recombinant *E. coli* cell was greatly enhanced. As shown in Table 1 below, compared with wild type strain *Serratia marcescens* YXJ-1002, the lipase production in recombinant *E. coli* was greatly increased to 2304 U/l from an initial level of 457U/l, and the specific activity was also increased by about 3 times.

Table 1. Cell growth and hydrolytic activity of wild type strain *Serratia marcescens* YXJ-1002 (P1-28) and recombinant *E. coli* expressing lipase SML.

Entry	Time (h)	DCW (g/l)	Activity (U/l) ^a	Specific activity (U/g DCW) ^a
Wild type	12	8.8 ± 0.3	457 ± 22	52.3 ± 4.4
Recombinant <i>E. coli</i>	12	10.7 ± 0.5	2304 ± 40	215.3 ± 0.1

^a The reaction, consisting of 0.03 g lyophilized cells, 2 mL olive oil emulsion and 3 mL phosphate buffer (pH 7.5, 25 mM), was conducted at 30°C and 250 rpm for 20 min. 5 mL of 95% ethanol was used to terminate the reaction. The amount of free fatty acid produced was quantified by titration using KOH solution. One unit was defined as the amount of enzyme releasing 1.0 micromole (10⁻⁶ mole) of fatty acid per minute under the conditions above.

The method for construction of recombinant *E. coli* expressing hydrolases such as the lipase CALB from *Candida antarctica* in the Examples was as follows: Optimized CALB gene (accession number Z30645.1) was synthesized by GenScript. CALB gene was digested with BamH I and Kpn I and inserted into corresponding restriction enzyme sites of plasmid pRSFDuet to construct

recombinant expression plasmid pRSFDuet-Calb. The recombinant expression plasmid was transformed into *E. coli* BL 21 (DE3), and kanamycin-resistant transformants were subsequently selected using LB-agar plates supplemented with kanamycin. Then the recombinant *E. coli* cells expressing the lipase CALB
5 was prepared by cultivation, the cells harvested by centrifugation were washed three times with DI water, and immediately lyophilized to obtain cell powder as described above, then used as whole cell biocatalyst for biodiesel production.

The efficient transformation of GTO to FAME *via* one-pot esterification and
10 transesterification with methanol was achieved by using the whole cell biocatalyst. As a demonstrative example, the lyophilized *E. coli* whole cells expressing lipase SML was used as a catalyst to convert the GTO containing 17.4% of FFA with MeOH (4 mol of MeOH : 1 mol of GTO) at a catalyst loading of 5 wt% based on GTO. The reaction was carried out at 30°C and 500 rpm
15 under magnetic stirring in solvent-free system in the absence of a solvent such as *tert*-butanol, *n*-hexane and the like, methanol was added in four equal amounts with 24-h interval, and the biodiesel formation was detected by GC. After reaction for 96 h, the total FAME yield from grease reached 82.1% (Fig. 40). By increasing the catalyst loading to 10 wt%, as shown in Fig. 42, the total yield
20 of grease to FAME reached up to 92% at 96 h. The whole cell catalyst developed here displayed a very good performance, indicating the great potential in practical production of biodiesel from GTO.

The developed whole cell biocatalysts are also useful for the high-yielding
25 transformation of FFA in GTO to FAMEs *via* esterification with methanol. This is the first step in the two-step strategy for biodiesel production from grease shown in Fig. 36. In a representative example, *E. coli* whole cells expressing lipase SML was used for the esterification of FFA in GTO containing 17.4% of FFA with MeOH. The reaction was examined at 30°C and 500 rpm under magnetic stirring
30 with 5 wt% of catalyst to GTO, 4:1 (mol:mol) of MeOH to GTO, and methanol was added in four equal amounts with 24-h interval. The reduced amount of FFA in GTO was determined by titration. As shown in Fig. 41, high FFA conversion (>95%) was achieved after reaction for 96 h (corresponding to acid value <1.0%).

As another representative example, the *E.coli* whole cells expressing lipase CALB was also used for esterification of FFA in GTO containing 21.4% of FFA with MeOH. As shown in Fig. 44, the reaction was conducted at 40°C and 250 rpm, 4 wt% of catalyst loading based on GTO and molar ratio of methanol to GTO is 3:1, and the methanol was added in three equal amounts with 12-h interval. A high conversion (>95%) for esterification was achieved at the end of 84 h. As shown in Fig. 44, the whole cell biocatalysts are able to convert the high amount of FFA in grease to FAME and can be used as an efficient pretreatment step. If desired, the remaining triglycerides in grease can also be easily converted to FAME via conventional base-catalysis.

As another representative example, the *E. coli* whole cells expressing lipases CALB and TLL in one plasmid was also used for esterification of FFA in GTO containing 21.4% of FFA with methanol. The reaction was conducted at 30°C and 500 rpm, 4 wt% of catalyst loading based on GTO and molar ratio of methanol to GTO is 3:1, and the methanol was added in three equal amounts with 6-h intervals. As shown in Fig. 46, 87% FAME yield was achieved in 72 h. These cells can further be recycled up to five times while retaining 75% of the original productivity, shown in Fig. 47.

Accordingly, the cell catalyst may be used in a one-pot reaction for esterification of free fatty acids to FAME and the transesterification of triglycerides to FAME as shown in Fig. 35 or as a pre-treatment step esterification of free fatty acids to FAME in the two-step reaction shown in Fig. 36.

As a further illustrative example, Fig. 49 provides an exemplary graphical representation of a cell catalyzing particle that may be adopted by some of the particles described in Examples 27-38. Referring to Fig. 49, the particle comprises a cell body 432 that produces catalysing entities 436 for the catalysis of FFA to fatty acid esters. The dotted lines 438 represent that the catalyzing entities 436 are produced and/or secreted by the cell body 432.

APPLICATIONS

Embodiments of the present disclosure advantageously allow free fatty acids (FFA) to be converted to fatty acid esters, in particular fatty acid methyl esters (FAME), at a high conversion rate. More specifically, at least 80% of the FFA may be converted to FAME from a composition comprising more than 10% by weight of FFAs, for example between 15% to 40% wt of FFAs.

Embodiments of the present disclosure can allow the production of FAME from grease in a one-pot reaction at high conversion efficiency, without having to undergo the conventional two-step esterification/transesterification reaction which may incur additional cost and reduce efficiency. Alternatively, embodiments of the present disclosures also have the flexibility to be adopted in the conventional two-step esterification/transesterification reaction, where the embodiments of the catalysts disclosed herein can be used for the esterification process of the two-step reaction.

Advantageously, embodiments of the method disclosed herein do not require high heat or pressure to carry out the reaction. For example, in some embodiments, the conversion of FFA to FAME in grease may be carried out at substantially ambient conditions. In addition, embodiments of the method disclosed herein may be performed under mild reaction conditions with the use of non-toxic and non-corrosive catalyst, and thus does not adversely affect the environment. Corrosive acid catalysts used in conventional esterification/transesterification reaction processes may not be necessary for some embodiments of the method disclosed herein. Furthermore, in some embodiments, the methods disclosed herein do not require the use of a toxic solvent such as *tert*-butanol as compared with conventional processes. Accordingly, in some embodiments, the methods and catalysts disclosed herein can be regarded as being environment friendly.

Even more advantageously, in some embodiments, the catalysts disclosed herein can be recycled and reused in a fast and simple manner. The recycled catalysts can beneficially be used for a plurality of conversion cycles of free fatty acids to fatty acid esters while still maintaining a high conversion rate, that is, at least 80% of the FFA may be converted to FAME from a composition comprising more than 10% by weight of FFAs. The recyclability and reusability of some embodiments of the catalysts can potentially reduce the cost of biodiesel/biofuel production as the cost of catalyst replacement after each cycle is minimized.

Advantageously, embodiments of the catalysts disclosed herein can be produced in a fast, efficient manner and at a low cost. This can potentially decrease the overall production cost biodiesel/biofuel production.

SOME EMBODIMENTS (SEs)

The following recites some embodiments of the present disclosure.

SE 1: A method of producing fatty acid esters from a composition comprising at least 10% by weight of free fatty acids (FFA), the method comprising:

- 5 incubating a plurality of micro- or nano-sized catalysing particles with the composition, to catalyse the conversion of FFA to fatty acid esters, such that at least 80% of the FFA are converted to fatty acid esters.

SE 2: The method of SE 1, wherein the fatty acid esters comprises fatty acid alky esters.

- 10 **SE 3:** The method of any one of the preceding SEs, wherein the incubation step comprises incubating the plurality of micro- or nano-sized catalysing particles with the composition in the presence of an alcohol.

SE 4: The method of any one of the preceding SEs, wherein the incubation step is carried out at temperature of less than 150°C.

- 15 **SE 5:** The method of any one of the preceding SEs, wherein the incubation step is carried out at a pressure of less than 121 KPa.

SE 6: The method of any one of the preceding SEs, wherein the incubation step is carried out from between 30 to 5760 minutes.

- 20 **SE 7:** The method of any one of the preceding SEs, wherein the micro- or nano-sized catalysing particles have an average particle size or diameter of no more than 800 µm.

SE 8: The method of any one of the preceding SEs, wherein the composition further comprises glycerides, preferably triglycerides.

- 25 **SE 9:** The method of any one of the preceding SEs, wherein the composition comprises grease, preferably brown grease.

SE 10: The method of SE 8, wherein the micro- or nano-sized catalysing particles is capable of additionally catalysing the conversion of the glycerides to fatty acid esters.

- 30 **SE 11:** The method of SE 10, wherein the conversion of FFA to fatty acid esters and the conversion of glycerides to fatty acid esters is carried out simultaneously.

SE 12: The method of SE 11, wherein the conversion of FFA to fatty acid esters and the conversion of glycerides to fatty acid esters is a one-step process.

SE 13: The method of SE 10, further comprises exposing the glyceride to a base to catalyse the conversion of the glycerides to fatty acid esters.

SE 14: The method of any one of SEs 3 to 13, wherein the alcohol is selected from the group consisting of methanol, glycerol, ethanol, propanol, butanol, amyl alcohol, and mixtures thereof.

SE 15: The method of any one of SEs 3 to 14, wherein molar ratio of the alcohol to the composition is from 2:1 to 40:1.

SE 16: The method of any one of the preceding SEs, wherein the micro- or nano-sized catalysing particles are selected from a group consisting of polymer particles, silica particles, micro-organism cells and mixtures thereof.

SE 17: The method of SE 16, wherein the polymer particles or silica particles are magnetic particles.

SE 18: The method of SE 17, wherein the magnetic particles are paramagnetic particles.

SE 19: The method of SE 17, wherein the magnetic particle comprises:

an outer shell;

a magnetic core at least partially encapsulated by the outer shell; and

a catalyzing entity immobilized on the outer shell, for catalyzing the conversion of FFA to fatty acid esters.

SE 20: The method of SE 17, wherein the magnetic property of the magnetic core is provided by a metal oxide, preferably an iron oxide.

SE 21: The method of any one of SEs 19 to 20, wherein the magnetic core comprises a plurality of magnetic nano-sized particles.

SE 22: The method of SE 21, wherein the magnetic nano-sized particles are coated with a capping agent, preferably oleic acid.

SE 23: The method of any one of SEs 21 to 22, wherein the magnetic nano-sized particles have an average diameter of 5-20 nm.

SE 24: The method of any one of SE 18 to 23, wherein the outer shell is at least one of a polymer shell or a silica shell.

SE 25: The method of SE 24, wherein the polymer shell comprises a polymer selected from the group consisting of poly(glycidyl methacrylate) (PGMA), polystyrene (PS) and poly(methyl methacrylate) (PMMA).

SE 26: The method of any one of SE 19 to 25, wherein the catalyzing entity is selected from at least one of an enzyme and an acid group.

SE 27: The method of SE 26, wherein the enzyme comprises a hydrolase.

SE 28: The method of SE 26, wherein the acid group comprises sulfonic acid.

SE 29: The method of SE 27, wherein the hydrolase is selected from the group consisting of *Candida antarctica* Lipase B (CALB) and *Thermomyces lanuginose* Lipase.

5 **SE 30:** The method of any one of SE 19 to 29, wherein the hydrolase is immobilized on the polymer shell via a linker selected from the group comprising epoxide functional group containing linker, amine functional group containing linker, aldehyde functional group containing linker, benzene functional group containing linker, ester functional group containing linker and mixtures thereof.

10 **SE 31:** The method of SE 30, wherein the linker is 4,7,10-trioxa-1,13-tridecanediamine.

SE 32: The method of SE 16, wherein the micro-organism is a wild type strain of micro-organism expressing hydrolase.

15 **SE 33:** The method of SE 15, wherein the micro-organism is a recombinant micro-organism expressing hydrolase.

SE 34: The method of any one of SE 32 to 33, wherein the hydrolase comprises lipase.

SE 35: The method of SE 32 wherein the wild type strain of micro-organism is SM YXJ-1002.

20 **SE 36:** The method of SE 33, wherein the recombinant micro-organism is *Escherichia coli* expressing lipase.

SE 37: The method of SE 32, wherein the wild type strain of micro-organism expressing hydrolase is isolated from soil using oil and/or fatty acid ester as a carbon source, preferably a sole carbon source.

25 **SE 38:** The method of SE 37, wherein the oil comprises olive oil and the fatty acid ester comprises methyl palmitate.

SE 39: A micro- or nano-sized catalysing particle comprising:

a body having dimensions in the micrometer or nanometer range; and

30 catalytic means associated with the body for catalysing the conversion of free fatty acids (FFA) to fatty acid esters,

wherein the catalysing particle is adapted to catalyse the conversion of at least 80% of the FFA in a composition comprising at least 10% by weight of FFA, to fatty acid esters.

SE 40: The micro- or nano-sized catalysing particle of SE 39, wherein the particle is a magnetic particle and the body comprises:

an outer shell; and

a magnetic core at least partially encapsulated by the outer shell,

- 5 and wherein the catalytic means associated with the body is a catalyzing entity immobilized on the outer shell, for catalyzing the conversion of FFA to fatty acid esters.

SE 41: The micro- or nano-sized catalysing particle of SE 40, wherein the magnetic property of the magnetic core is provided by a metal oxide.

- 10 **SE 42:** The micro- or nano-sized catalysing particle of any one of SEs 40 to 41, wherein the magnetic core comprises a plurality of magnetic nano-sized particles.

SE 43: The micro- or nano-sized catalysing particle of SE 42, wherein the magnetic nano-sized particles are coated with a capping agent, preferably oleic acid.

- 15 **SE 44:** The micro- or nano-sized catalysing particle of any one of SEs 40 to 43, wherein the outer shell is at least one of a polymer shell or a silica shell.

SE 45: The micro- or nano-sized catalysing particle of SE 44, wherein the polymer shell comprises a polymer selected from the group consisting of poly(glycidyl methacrylate) (PGMA), polystyrene (PS) and poly(methyl methacrylate) (PMMA).

SE 46: The micro- or nano-sized catalysing particle of any one of SEs 40 to 45, wherein the catalyzing entity is selected from the group consisting of an enzyme and an acid group.

- 25 **SE 47:** The micro- or nano-sized catalysing particle of SE 46, wherein the enzyme comprises a hydrolase.

SE 48: The micro- or nano-sized catalysing particle of SE 46, wherein the acid group comprises sulfonic acid.

- 30 **SE 49:** The micro- or nano-sized catalysing particle of SE 47, wherein the hydrolase is selected from the group consisting of *Candida antartica* Lipase B (CALB) and *Thermomyces lanuginose* Lipase.

SE 50: The micro- or nano-sized catalysing particle of any one of SE 40 to 49, wherein the hydrolase is immobilized on the outer shell *via* a linker selected from the group comprising epoxide functional group containing linker, amine functional group containing linker, aldehyde functional group containing linker, benzene functional group containing linker, ester functional group containing linker and mixtures thereof.

SE 51: The micro- or nano-sized catalysing particle of any one of SE 40 to 50, wherein the particle has a specific loading of 10-100 mg enzyme per particle.

SE 52: The micro- or nano-sized catalysing particle of SE 50, wherein the linker is 4,7,10-trioxa-1,13-tridecanediamine.

SE 53: The micro- or nano-sized catalysing particle of SE 39, wherein the micro- or nano-sized catalysing particle is a cell of a micro-organism, the body of the particle is the body of the cell, and the catalytic means associated with the body is an enzyme produced in or from the body of the cell for catalyzing the conversion of FFA to fatty acid esters.

SE 54: The micro- or nano-sized catalysing particle of SE 53, wherein the enzyme is hydrolase.

SE 55: The micro- or nano-sized catalysing particle of any one of SEs 53 to 54, wherein the micro-organism is a recombinant micro-organism expressing the hydrolase.

SE 56: The micro- or nano-sized catalysing particle of any one of SEs 53 to 55, wherein the hydrolase comprises lipase.

SE 57: The micro- or nano-sized catalysing particle of SE 55, wherein the recombinant micro-organism comprises one or more nucleic acid sequence that encodes for the hydrolase, the nucleic acid sequence having at least about 80% homology/identity, at least about 90% homology/identity, at least about 95% homology/identity, at least about 96% homology/identity, at least about 97% homology/identity, at least about 98% homology/identity, at least about 99% homology/identity or about 100% homology/identity to SEQ ID No. 1 and/or SEQ ID No. 3 and/or SEQ ID No. 5.

SEQ ID No. 1:

atgaagctac tctctctgac cgggtgtggct ggtgtgcttg cgacttgcgt tgcagccact ctttgggtga
agcgtctacc ttccggttcg gacctgcct ttctgcagcc caagtcggtg ctctgatcgg gtctgacctg
ccagggtgct tcgccatcct cggcttccaa acccatcctt ctctgtcccc gaaccggcac cacagggtcca
5 cagtcgttcg actcgaactg gatccccctc tcaacgcagt tgggttacac accctgctgg atctcacccc
cgccgttcat gctcaacgac acccagggtca acacggagta catgggtcaac gccatcacccg
cgctctacgc tgggtcgggc aacaacaagc ttcccgctgt tacctgggtcc cagggtgggc tgggtgcaca
gtgggggtctg accttcttcc ccagtatcag gtccaaggct gatcgactta tggcctttgc gcccgactac
aaggggcaccg tctctgccgg ccctctcgat gactcgcgg ttagtgcacc ctccgtatgg cagcaaacca
10 ccggttcggc actcaccacc gactccgaa acgcagggtg tctgaccag atcgtgcccc
ccaccaacct ctactcggcg accgacgaga tcttcagcc tcagggtgtcc aactcgccac tctactcatc
ctaccttctc aacggaaaga acgtccaggc acaggccgtg tgtgggccgc tgttctcat cgaccatgca
ggctcgtcctc cctcgcagtt ctctacgtc gtccgtcgat ccgccctgcg ctccaccacg ggccagggtc
gtagtgcaga ctatggcatt acggactgca acctcttcc cgccaatgat ctgactcccg agcaaaagggt
15 cgccgcggct ggcctcctgg cgccggcagc tgcagccatc gtggcgggtc caaagcagaa
ctgcgagccc gacctcatgc cctacgcccg cccctttgca gtaggcaaaa ggacctgtc cgcatcgtc
acccctga

SEQ ID No. 3:

20 ggatccacca tgaggagctc ccttgtctg ttcttgtct ctgcgtggac ggccttggcc agtctattc
gtcgagaggt ctgcaggat ctgttaacc agttcaatct cttgcacag tattctgcag ccgcatactg
cggaaaaaac aatgatgcc cagctggtac aaacattacg tgcacgggaa atgcctgcc cgaggtagag
aaggcggatg caacgtttct ctactcgtt gaagactctg gagtgggcga tgcaccggc ttcttctc
25 tgcacaacac gaacaaattg atcgtcctct cttccgtgg ctctcgttcc atagagaact ggatcgggaa
tcttaacttc gacttgaaag aaataaatga catttgctcc ggctgcaggg gacatgacgg ctctactctg
tcttgagggt ctgtagccga tacgttaagg cagaagggtg aggatgtgtg gagggagcat cccgactatc
gcgtggtgtt taccggacat agcttgggtg gtgcattggc aactgttgc ggagcagacc tgcgtggaaa
tgggtatgat atcgacgtgt ttcatatgg cgcccccgca gtcggaaaca gggcttttgc agaattcctg
30 accgtacaga ccggcggaac actctaccgc attaccaca ccaatgatat tgcctctaga ctccgcccg
gcgaattcgg ttacagccat tctagcccag agtactggat caaatctgga acctgttcc ccgtcacccg
aaacgatatc gtgaagatag aaggcatcga tgccaccggc ggcaataacc agcctaacat tccggatatc
cctgcgcacc tatgttactt cgggttaatt gggacatgtc ttagtggcc ggcgcggctg ggtcgactct
agcgagctcg agatctaga

SEQ ID No 5:

ccaagcgccg cataccaata acgtttcatc aatcagtctc cttaatgtct atgcagagct atcagtatag
gagagccagc gccggcactg ttaaccaacg cacaatctcg ccaattgat tcgcacgcct aatatttagg
5 gctaatacta ttctaccga tgttggtcct ctgaccagct gtcgttcggc taacgttgtt tccctgtttc caccgcccagc
gcatgagagt tcaactccccg gccaggcggc ataattcata aggaactgat atgggcatct ttagctataa
ggatttggac gaaaacgcgt cgaaagcgct gttttccgac gccttggcca tctccaccta cgcttaccac
aatatcgata acggcttoga cgaaggctac caccagaccg gtttcggtct tggcctgccg ctgacgctga
tcaccgcgct gatcggcagc acccaatcgc agggcggcct gccccgatt ccttgaacc ccgactccga
10 acaggccgcg caggagacgg tgaacaatgc cggctggtcg gtcacagcg ccgcgcagct gggttacgcc
ggcaaaaccg atgcacgcgg cacctattac ggcgagaccg ccggttacac caccgcgcag gccgaggtgc
tgggcaaata tgacagcgaa ggcaatctca ccgccattgg tatctcattt cgcggtacca gcggcccgcg
cgagtcgctg atcggcgata ccatcggcga tgtgattaac gatctgctgg ccggttcgg gccgaaaggc
tacgtgacg gctacacgct gaacgccttc ggcaatctgc tgggcgacgt ggcgaaattc gcgcaggcgc
15 acgggctgag cggcgaggac gtagtggtca gcggccacag cctcggcggg ctggcggtca acagcatggc
ggcgcagagc gacgccaact ggggcggctt ctacgcgcag tccaactatg tcgccttcgc ctgcgccacc
cagtacgaag ccggcggcaa ggtgatcaac atcggctacg agaacgaccg ggtgttcgc gcgctgcagc
gcacctcgt aacctgccg tcaactggcg tacacgatgc gccgcacgcc tccgccacca acaatatcgt
caacttcaac gaccactacg cgtcggacgc ctggaacctg ctgccgtttt ccatttcaa cattccgacc
20 tggctgtgc acctgccgtt ctctatcag gacgggctga tgcgggtgct gaactccgag tttattcgc
tgaccgacaa ggactcgacc atcatcgtct ccaacctgtc gaacgtgacg cgcggcaata cctgggtgga
agacctgaac cgcaacgcgg aaacgcacag cggaccgacg ttatcatcg gcagcgacgg caatgattg
atcaagggcg gcaaaggcaa cgactatctc gagggccgcg acggcgacga tatctccgc gacgccggcg
gctataacct gatcgccggc ggcaaaggcc acaatatctt cgatacccaa caggcggtga aaaacaccga
25 ggtgcctac gacggcaata cgctttacct gcgcgacgcc aaaggcggtta ttacgtggc agacgacatc
agcacctgc gcagcaaaga aacctcctgg ctgatttca gcaaagaggt ggatcatcag gtgaccgctg
cgggattgaa atcggactcg ggcctcaaag cctatgccgc cgccaccacc ggcggcgacg gcgatgacgt
cctgcaggct cgcagccacg acgcctggct gttcggcaac gccggcaacg acacgctgat cggccatgcc
ggcggcaacc tgaccttctg cggcggcagc ggcgatgaca tctgaaggc cgccggcaac ggtaatacct
30 tctgttcag cggcgatttc ggccgcgacc agctgtatgg tttaacgcc accgataaac tgggtttat
cggtagcgaa ggcgccagcg ggaatatccg cgactatgcc acacagcaaa acgacgatct ggtgctggcc
ttcggccacg gccaggtcac gctgatcggc gtctcgtcg atcaactcaa caccgatcgg gtggtgttgg
cctaagggtc ggcgtaaaaa aagccgggcg ctttcgccgc ccggcttcc tctttttt gctccgcctt
acggcacgtc ataccccagc gccgccttcg gaatgcggaa ccaactgctg cggttcagca ccagttctg

cgccctcagc gccgagcgca cccgctcaat ttgccggag ccgataatcg gcagcggcga tgacggcagg
cgcatcacc aggcgtagac cacctgctcg atggctcgg cgccgatctc ttgcgccacc cgttcagct
cgtcgcgag cggctggaac tcggcgctgt taaacaggcg cccgcccccc aggcaggacc aggccag

5

SE 58: The micro- or nano-sized catalysing particle of SE 55, wherein the recombinant micro-organism expresses the enzyme that is capable of catalysing the conversion of FFA to fatty acid esters, the enzyme comprising an amino acid sequence that at least about 80% homology/identity, at least about 90% homology/identity, at least about 95% homology/identity, at least about 96% homology/identity, at least about 97% homology/identity, at least about 98% homology/identity, at least about 99% homology/identity or about 100% homology/identity to SEQ ID No. 2 or SEQ ID No. 4 or SEQ ID No. 6.

15 SEQ ID No 2:

MKLLSLTGVAGVLATCVAATPLVKRLPSGSDPAFSQPKSVLDAGLTCQGASPS
SVSKPILLVPGTGTTPQSFDSNWIPSTQLGYTPCWISPPPFMLNDTQVNT
MVNAITALYAGSGNNKLPVLTWSQGGGLVAQWGLTFFPSIRSKVDRLMAFAPDY
KGTVLGAGPLDALAVSAPSVWQQTTGSALTALRNAGGLTQIVPTTNLYSATDEI
20 VQPQVSNSPLDSSYLFNGKNVQAQAVCGPLFVIDHAGSLTSQFSYVVG
STTGQARSADYGITDCNPLPANDLTPEQKVAAAALLAPAAAAIVAGPKQNC
LMPYARPFVAGKRTCSGIVTP

SEQ ID No. 4:

25 MRSSLVLFFVSAWTALASPIRREVVSQDLFNQFNLFQAQYSAAYCGKNNDAPAGTNI
TCTGNACPEVEKADATFLYSFEDSGVGDTVGFALDNTNKLIVLSFRGSR
SIENWIG
NLNFDLKEINDICSGCRGHDGFTSSWRSVADTLRQKVEDAVREHPDYRVVFTGHS
LGGALATVAGADLRGNGYDIDVFSYGAPRVGNRAFAEFLTVQTGGTLYRITH
TNDIV
PRLPPREFGYSHSSPEYWIKSGTLVPVTRNDIVKIEGIDATGGNNQPNIPDIP
30 YFGLIGTCL

SEQ ID No 6:

MGIFSYKDLDENASKALFSDALAISTYAYHNIDNGFDEGYHQTGFGLGLPLTLITALI
 GSTQSQGGLPRIPWNPDSEQAAQETVNNAGWSVISAAQLGYAGKTDARGTTYGE
 5 TAGYTТАQAEVLGKYDSEGNLTAIGISFRGTSGPRESLIGDTIGDVINDLLAGFGPKG
 YADGYTLNAFGNLLGDVAKFAQAHGLSGEDVVVSGHSLGGLAVNSMAAQSDANW
 GGFYAQSΝYVAFASPTQYEAGGKVINIGYENDPVFRALDGTSLTLP SLGVHDAPHA
 SATNNIVNFNDHYASDAWNLLPFSILNIPTWLSHLPFFYQDGLMRVLNSEFYSLTDK
 DSTIIVSNLSNVTRGNTWVEDLNRNAETHSGPTFIIGSDGNDLIKGGKGN DYLEGRD
 10 GDDIFRDAGGYNLIAGGKGHNIFDTQQALKNTEVAYDGNTLYLRDAKGGITLADDIS
 TLRSKETSWLIFSKEVDHQVTAAGLKSDSGLKAYAAATTGGDGDDVLQARSHDAW
 LFGNAGNDTLIGHAGGNLTFVGGSGDDILKGAGNGNTFLFSGDFGRDQLYGFNAT
 DKLVFIGTEGASGNIRDYATQQNDDLVLAFGHGQVTLIGVSLDHFNTDRVVLA

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SE 59: The micro- or nano-sized catalysing particle of SE 55, wherein the recombinant micro-organism is *Escherichia coli* expressing lipase.

SE 60: A method of producing a micro- or nano-sized catalysing particle, the method comprising:

forming a magnetic core;
 encapsulating at least part of the magnetic core with an outer shell; and
 immobilizing a catalyzing entity on the outer shell,
 wherein the catalysing entity is adapted to catalyse the conversion of at least 80%
 25 of the FFA in a composition comprising at least 10% by weight of FFA, to fatty acid esters.

SE 61: The method as claimed of SE 60, wherein the step of forming a magnetic core comprises forming a plurality of magnetic nano-sized particles.

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SE 62: The method of SE 61, wherein the step of forming a plurality of magnetic nano-sized particles comprises coprecipitation to obtain the magnetic nano-sized particles.

SE 63: The method of any one of SEs 61 to 62, wherein the magnetic nano-sized particles have an average diameter of 5-20 nm.

SE 64: The method of any one of SEs 60 to 63, wherein the outer shell is at least one of a polymer shell or a silica shell.

SE 65: The method of SE 64, wherein the step of encapsulating the magnetic core with the polymer shell comprises:

mixing the magnetic core with one or more monomer precursor of the polymer shell and an initiator in water; and
polymerizing the monomer precursors to form a polymer shell that encapsulates the magnetic core.

SE 66: The method of SE 65, wherein the step of encapsulating the magnetic core with the silica shell comprises:

mixing the magnetic core with tetraethyl orthosilicate in water containing an alcohol and ammonia to obtain a mixture; and
precipitating silica from the mixture to form a silica shell that encapsulates the magnetic core.

SE 67: The method of any one of SEs 60 to 66, wherein the step of immobilizing a catalyzing entity to the outer shell comprising at least one of chemical coupling catalyzing entity to the outer shell or physically adsorbing the catalyzing entity to the outer shell.

SE 68: The method of any one of SEs 60 to 66, wherein the step of immobilizing a catalyzing entity to the outer shell comprising:

- (i) covalently coupling the catalyzing entity with the functional group of the outer shell which is chemically reactive to the catalyzing entity, to immobilize the catalyzing entity to the outer shell; or
- (ii) functionalizing the surface of the outer shell with a functional group chemically reactive to the catalyzing entity; and chemically coupling

the catalyzing entity with the functional group to immobilize the catalyzing entity to the outer shell.

5 **SE 69:** A method of a obtaining a cell catalyst comprising:

10 a) identifying a strain of micro-organism from a repertoire of hydrolase producing micro-organisms, the identified strain of micro-organism capable of catalysing the conversion of more FFA to fatty acid esters than at least 50% of the other strains of micro-organisms in the repertoire;

15 b) identifying a gene sequence of the hydrolase that is capable of catalysing the conversion of FFA to fatty acid esters from the identified strain in step a);

20 c) introducing the gene sequence in a host cell to obtain a recombinant host cell that is capable of expressing the hydrolase in an amount that is more than the strain of micro-organism identified in step a) under substantially similar conditions,

25 wherein the recombinant host cell is capable of catalysing the conversion of at least 80% of the FFA in a composition comprising at least 10% by weight of FFA, to fatty acid esters.

SE 70: A method of separating the magnetic catalysing particles of any one of SE 40 to 52 from a mixture, the method comprising:

applying an external magnetic field or a centrifugal force to consolidate the magnetic catalysing particles together; and

30 removing the rest of the mixture from the consolidated particles magnetic catalysing particles.

SE 71: A method of producing fatty acid esters from a composition comprising at least 10% by weight of free fatty acids (FFA), the method comprising:

incubating a plurality of micro- or nano-sized catalysing particles obtained from the method of SE 68 with the composition, to catalyse the conversion of FFA to fatty acid esters, such that at least 80% of the FFA are converted to fatty acid esters.

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In one embodiment, there is provided a process for producing FAME from grease trap oil (GTO) containing high concentration of FFA by using nano- or micro-biocatalysts.

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In one embodiment, the disclosed process producing FAME from grease trap oil (GTO) containing high concentration of FFA is achieved by both esterification and transesterification in one-pot with nano- or micro-biocatalysts.

15 In one embodiment, the disclosed process for producing FAME from grease trap oil (GTO) containing high concentration of FFA involves two steps in which high yielding of transformation of FFA in grease to the FAME is achieved by using nano- or micro-biocatalysts in the first step, and the conversion of the remaining triglyceride to FAME is achieved in the second step.

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In one embodiment, the disclosed the grease trap oil (GTO) is brown grease containing 14-22 wt% of FFA.

25 In one embodiment, the disclosed nano- and micro-biocatalysts are enzymes immobilized on magnetic nanoparticles.

In one embodiment, the disclosed magnetic nanoparticles are nanoparticles containing magnetic core, polymer shell, and functional groups on the surface. In one embodiment, the disclosed magnetic core comprises multiple sub-
30 nanoparticles with superparamagnetic properties and size or diameter of 5-20 nm.

In one embodiment, the disclosed sub-nanoparticle is iron oxide nanoparticle. In one embodiment, the disclosed sub-nanoparticle is iron oxide nanoparticle with oleic acid coating.

- 5 In one embodiment, the disclosed the polymer shell is poly(glycidyl methacrylate) (PGMA).

In one embodiment, the disclosed polymer shell is polystyrene (PS).

- 10 In one embodiment, the disclosed polymer shell is poly(methyl methacrylate) (PMMA).

In one embodiment, the disclosed functional group on the surface is epoxide. In one embodiment, the disclosed functional group on the surface is amine.

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In one embodiment, the disclosed functional group on the surface is aldehyde.

In one embodiment, the disclosed functional group on the surface in is benzene.

- 20 In one embodiment, the disclosed functional group on the surface is ester.

In one embodiment, the disclosed amine group is introduced *via* the reaction of epoxide surface group with a diamine.

- 25 In one embodiment, the disclosed diamine is ethylenediamine. In one embodiment, the disclosed diamine is 4,7,10-trioxa-1,13-tridecanediamine.

In one embodiment, the disclosed aldehyde group is introduced *via* the reaction of amine surface group with glutaraldehyde.

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In one embodiment, the disclosed magnetic nanoparticles have a uniform size distribution with size or diameter at 40-800 nm.

In one embodiment, the disclosed enzyme is a hydrolase. In one embodiment, the disclosed hydrolase is *Candida antartica* Lipase B (CALB).

5 In one embodiment, the disclosed hydrolase is *Thermomyces Lanuginosus* Lipase (TLL).

In one embodiment, the disclosed enzyme is immobilized on magnetic nanoparticles by covalent binding.

10 In one embodiment, the disclosed enzyme is immobilized on magnetic nanoparticles by physical interaction.

In one embodiment, the disclosed microbiocatalysts are clusters of individual nanobiocatalyst.

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In one embodiment, the disclosed clusters are non-crosslinked clusters.

In one embodiment, the disclosed clusters are reversible clusters.

20 In one embodiment, the disclosed microbiocatalysts are MNA TL which is the non-crosslinked cluster of nano-biocatalysts containing *Thermomyces Lanuginosus* Lipase (TLL) on the magnetic nanoparticles (CHO-MNPs) consisting of iron oxide core, poly(glycidyl methacrylate) shell, and aldehyde surface group.

25

In one embodiment, the disclosed microbiocatalysts MNA TL is prepared by shaking of TLL and CHO-MNPs in phosphate buffer (7mM, pH 5-8) at 4-30°C for 0.5-12h.

30 In one embodiment, the disclosed microbiocatalysts is MNA CA which is the non-crosslinked cluster of nano-biocatalysts containing *Candida Antarctica* Lipase B (CALB) on the magnetic nanoparticles (CHO- MNPs) consisting of iron oxide core, poly(glycidyl methacrylate) shell, and aldehyde surface group. In one

embodiment, the disclosed microbiocatalysts MNA CA is prepared by shaking CALB and CHO-MNPs in phosphate buffer (7mM, pH 5-8) at 4-30°C for 0.5-12h.

5 In one embodiment, the disclosed nanobiocatalysts in is GAP CA which contains CALB on the magnetic nanoparticles (GA-MNPs) consisting of iron oxide core, poly(glycidyl methacrylate) shell, and aldehyde surface group.

10 In one embodiment, the disclosed nanobiocatalysts GAP CA is prepared by shaking CALB and GA-MNPs in phosphate salt buffer at pH of 7 and 4-30°C for 4-12h.

15 In one embodiment, the disclosed nanobiocatalysts is EDAP CA which contains CALB on the magnetic nanoparticles (EDA-MNPs) consisting of iron oxide core, poly(glycidyl methacrylate) shell, and amine surface group.

In one embodiment, the disclosed nanobiocatalysts EDAP CA is prepared by shaking CALB, glutaraldehyde, and EDA-MNPs in phosphate salt buffer at pH of 7 and 4-30°C for 4-12h.

20 In one embodiment, the disclosed nanobiocatalysts is PSP CA which contains CALB on the magnetic nanoparticles (PS-MNPs) consisting of iron oxide core, polystyrene shell, and benzene surface group.

25 In one embodiment, the disclosed nanobiocatalysts PSAP CA is prepared *via* physical adsorption by shaking CALB and PS-MNPs in phosphate salt buffer at pH of 7 and 4-30°C for 4-12h.

30 In one embodiment, the disclosed nanobiocatalysts is PMMAP CA which contains CALB on the magnetic nanoparticles (PMMA-MNPs) consisting of iron oxide core, poly(methyl methacrylate) shell, and ester surface group.

In one embodiment, the disclosed nanobiocatalysts PMMAP CA is prepared *via* physical adsorption by shaking CALB and PMMA-MNPs in phosphate salt buffer at pH of 7 and 4-30°C for 4-12h.

In one embodiment, the disclosed micro-biocatalysts have a regular shape with size or diameter smaller than 100µm. In one embodiment, the disclosed micro-biocatalysts have a specific loading of 10-100 mg enzyme per gram particles.

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In one embodiment, the disclosed nano-biocatalysts have a specific loading of 10-300 mg enzyme per gram particles.

10 In one embodiment, the disclosed separation of the nano- or micro-biocatalysts is achieved by using external magnetic field.

In one embodiment, the disclosed separation of the nano- or micro-biocatalysts is achieved by centrifugation followed by magnetic separation.

15 In one embodiment, the disclosed high yielding production of FAME from grease trap oil (GTO) containing high concentration of FFA is achieved by the reaction of grease trap oil (GTO) with MeOH in the presence of silica gel microbead with nano- or micro-biocatalysts.

20 In one embodiment, the disclosed biocatalysts are recyclable, with high yielding of FAME in each cycle of biotransformation.

In one embodiment, the disclosed biocatalysts are separated from the reaction mixture under external magnetic field after each cycle of reaction, washed,
25 freeze-dried, and then used for next cycles of reactions.

In one embodiment, the disclosed high-yielding transformation of FFA in GTO to FAME is achieved by the reaction of grease trap oil (GTO) with MeOH with nano- or micro-biocatalysts, leaving the product with <1 wt% FFA which is
30 suitable for further transformation of the remaining triglyceride to FAME by conventional base catalysis.

In one embodiment, the disclosed biocatalysts are recyclable, with effectively reduction of FFA in GTO to 1-2 wt% in each cycle of biotransformation.

In one embodiment, the disclosed biocatalysts are separated from the reaction mixture under external magnetic field after each cycle of reaction, washed, freeze-dried, and then used for next cycles of reactions.

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In one embodiment, there is provided a process for reducing free fatty acid in grease *via* esterification with an alcohol by using a recyclable nano- or micro-size paramagnetic solid acid catalyst.

10 In one embodiment, the disclosed alcohol is methanol.

In one embodiment, the disclosed alcohol is glycerol.

15 In one embodiment, the disclosed recyclable nano-size paramagnetic solid acid catalyst is core-shell nanoparticles which consist of magnetic cores, polymer or inorganic shell, and acid groups on the surface.

20 In one embodiment, the disclosed magnetic cores comprises of multiple sub-nanoparticles with superparamagnetic properties and size or diameter of 5-20 nm.

In one embodiment, the disclosed sub-nanoparticles are iron oxide nanoparticles.

25 In one embodiment, the disclosed sub-nanoparticles are iron oxide coated with oleic acid.

In one embodiment, the disclosed polymer shell is poly(glycidyl methacrylate).

In one embodiment, the disclosed polymer shell is polystyrene.

30

In one embodiment, the disclosed inorganic shell is silica.

In one embodiment, the disclosed acid group is sulfonic acid.

In one embodiment, the disclosed recyclable micro-size paramagnetic solid acid catalyst is the aggregated recyclable nano-size paramagnetic solid acid catalysts.

5 In one embodiment, the disclosed recyclable nano-size paramagnetic solid acid catalyst is prepared by mixing poly(glycidyl methacrylate)-coated magnetic nanoparticles with sodium sulfite, followed by mixing with hydrochloric acid.

10 In one embodiment, the disclosed recyclable nano-size paramagnetic solid acid catalyst is prepared by shaking polystyrene-coated magnetic nanoparticles with sulfuric acid.

15 In one embodiment, the disclosed recyclable nano-size paramagnetic solid acid catalyst is prepared by mixing silica-coated magnetic nanoparticles with 3-mercaptopropyl trimethoxysilane in ethanol and deionized water, followed by mixing with hydrogen peroxide in methanol and deionized water, and followed by mixing with sulfuric acid solution.

20 In one embodiment, the disclosed recyclable micro-size paramagnetic solid acid catalyst is prepared by drying the polystyrene-coated magnetic nanoparticles under vacuum and shaking the resultant microparticles with sulfuric acid.

In one embodiment, the disclosed recyclable nano-size paramagnetic solid acid catalyst is separated by using external magnetic field.

25 In one embodiment, the disclosed recyclable micro-size paramagnetic solid acid catalyst is separated by centrifugation.

30 In one embodiment, the disclosed process for reducing free fatty acid in grease is achieved by the reaction of grease with methanol by using recyclable nano- or micro-size paramagnetic solid acid catalysts, leaving the product with low value of free fatty acid which is subjected to base-catalyzed transesterification with methanol to produce biodiesel.

In one embodiment, the disclosed recyclable nano- or micro-size paramagnetic solid acid catalysts are separated from reaction mixture by centrifugation or under magnetic field, washed, dried, and subsequently used in the next reaction round.

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In one embodiment, the disclosed recyclable nano- or micro-size paramagnetic solid acid catalysts are reused without any regeneration needed, leaving the product with low value of free fatty acid after each reaction round.

10 In one embodiment, the disclosed process for reducing free fatty acid in grease is achieved by the reaction of grease with glycerol by using recyclable nano- or micro-size paramagnetic solid acid catalyst, leaving the product with low value of free fatty acid which is subjected to base-catalyzed transesterification with methanol to produce biodiesel.

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In one embodiment, the disclosed recyclable nano- or micro-size paramagnetic solid acid catalysts are separated from reaction mixture by centrifugation or under magnetic field, washed, dried, and subsequently used in the next reaction round.

20

In one embodiment, the disclosed recyclable nano- or micro-size paramagnetic solid acid catalysts are reused without any regeneration needed, leaving the product with low value of free fatty acid after each reaction round.

25 In one embodiment, there is provided a process for production of FAME with high yield from grease trap oil containing high concentration of FFA by using whole cell biocatalysts.

In one embodiment, the disclosed process for producing FAME from grease trap
30 oil (GTO) containing high concentration of FFA is achieved by both esterification and transesterification in one-pot reaction with whole cell biocatalysts.

In one embodiment, the disclosed process for producing FAME from grease trap oil (GTO) containing high concentration of FFA involves two steps in which high

yielding of transformation of FFA in grease to the FAME is achieved by using whole cell biocatalyst in the first step, and the conversion of the remaining triglyceride to FAME is achieved in the second step with base catalysts.

- 5 In one embodiment, the disclosed grease trap oil (GTO) is brown grease containing 15-40 wt% of FFA.

In one embodiment, the disclosed whole cell biocatalysts are cells of a microorganism.

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In one embodiment, the disclosed microorganism is a wild type strain.

In one embodiment, the disclosed microorganism is a recombinant microorganism.

- 15 In one embodiment, the disclosed recombinant microorganism is recombinant *Escherichia coli*.

In one embodiment, the disclosed recombinant *Escherichia coli* is used for expressing enzyme.

20

In one embodiment, the disclosed enzyme is a hydrolase.

In one embodiment, the disclosed hydrolase is lipase SML from a wild type strain.

- 25 In one embodiment, the disclosed hydrolase is *Candida antarctica* Lipase B (CALB).

In one embodiment, the disclosed wild type strain is *Serratia marcescens* YXJ-1002.

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In one embodiment, the disclosed *Serratia marcescens* YXJ-1002 was isolated from soil using oil or fatty acid ester as sole carbon source.

In one embodiment, the disclosed oil is olive oil.

In one embodiment, the disclosed oil is grease trap oil (GTO).

In one embodiment, the disclosed fatty acid ester is methyl palmitate.

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In one embodiment, the disclosed soil samples were collected in Singapore.

In one embodiment, the disclosed recombinant *Escherichia coli* is *Escherichia coli* T7 express.

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In one embodiment, the disclosed recombinant *Escherichia coli* is *Escherichia coli* BL 21(DE3).

In one embodiment, the disclosed whole cell biocatalysts are the lyophilized cells.

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In one embodiment, the disclosed lyophilized cells are lyophilized *Escherichia coli* T7 express cells expressing lipase SML.

In one embodiment, the disclosed lyophilized cells are lyophilized *Escherichia coli* BL 21(DE3) cells expressing lipase CALB.

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In one embodiment, the disclosed preparation of lyophilized whole cell biocatalysts comprising 1) Inoculating the *Escherichia coli* T7 or *Escherichia coli* BL 21(DE3) cells to LB medium for seed cultivation; 2) Inoculating seed culture to LB or TB medium containing kanamycin for cultivation; 3) Inducing the enzyme expression by addition of isopropylthiogalactoside (IPTG); 4) Harvesting the cells by centrifugation and washing using de-ionized water; 5) Lyophilizing the wet whole cells using vacuum freeze to obtain the lyophilized cells.

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In one embodiment, the disclosed seed cultivation is conducted at 20-40°C and 150-400 rpm for 6-18 h.

In one embodiment, the amount of seed culture used for inoculation is 0.5-5% (v/v).

In one embodiment, the OD600 value for enzyme induction is 0.6-0.8.

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In one embodiment, the final concentration of IPTG used for induction is 0.05-5 mM.

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In one embodiment, the enzyme expression is conducted at 15-30°C and 150-400 rpm for 6-18 h.

In one embodiment, the cells harvested are washed with de-ionized water for 1-3 times.

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In one embodiment, the lyophilization is done for 24-72 h.

In one embodiment, the high yielding production of FAME from grease trap oil (GTO) containing high concentration of FFA is achieved by reaction of grease trap oil (GTO) with methanol using the whole cell biocatalysts.

20

In one embodiment, the catalyst loading of the whole cell biocatalysts is 1-10 wt% based on the grease trap oil (GTO).

25

In one embodiment, the total molar ratio of methanol to grease trap oil (GTO) was 3:1-8:1.

In one embodiment, the reaction was conducted at 20-50°C.

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In one embodiment, the high-yielding transformation of FFA in GTO to FAME is achieved by the reaction of grease trap oil (GTO) with methanol with whole cell biocatalyst, leaving the product with <1 wt% FFA which is suitable for further transformation of the remaining triglyceride to FAME by conventional base catalysis.

In one embodiment, the catalyst loading of the whole cell biocatalysts is 1-10 wt% based on the grease trap oil (GTO).

In one embodiment, the total molar ratio of methanol to grease trap oil (GTO) was 2:1-4:1.

5

In one embodiment, the reaction was conducted at 20-50°C.

10 It will be appreciated by a person skilled in the art that other variations and/or modifications may be made to the embodiments disclosed herein without departing from the spirit or scope of the disclosure as broadly described. The present embodiments are, therefore, to be considered in all respects to be illustrative and not restrictive.

CLAIMS

1. A method of producing fatty acid esters from a composition comprising glycerides and at least 10% by weight of free fatty acids (FFA), the method comprising:
 - incubating a plurality of micro- or nano-sized catalysing particles with the composition, to catalyse the conversion of FFA to fatty acid esters, such that at least 80% of the FFA are converted to fatty acid esters.
2. The method as claimed in claim 1, wherein the incubation step is carried out in the presence of an alcohol.
3. The method as claimed in claim 1, wherein the micro- or nano-sized catalysing particles have an average particle size or diameter of no more than 800 μm .
4. The method as claimed in claim 1, wherein the micro- or nano-sized catalysing particles is capable of additionally catalysing the conversion of the glycerides to fatty acid esters.
5. The method as claimed in claim 1, wherein the micro- or nano-sized catalysing particles are selected from a group consisting of polymer particles, silica particles, micro-organism cells and mixtures thereof.
6. The method as claimed in claim 5, wherein at least one of the polymer particles and silica particles are magnetic particles, the magnetic particle comprising:
 - an outer shell;
 - a magnetic core at least partially encapsulated by the outer shell;
 - and
 - a catalyzing entity selected from at least one of an inorganic acid group or an enzyme, the catalyzing entity being immobilized on the outer shell, for catalyzing the conversion of FFA to fatty acid esters, optionally for additionally catalysing the conversion of glycerides to fatty acid esters.

7. The method as claimed in claim 5, wherein the micro-organism is a wild type strain of micro-organism expressing hydrolase or a recombinant micro-organism expressing hydrolase.

5 8. A micro- or nano-sized catalysing particle for producing fatty acid esters from a composition comprising glycerides and at least 10% by weight of free fatty acids (FFA), the particle comprising:

a body having dimensions in the micrometer or nanometer range;

10 and

catalytic means associated with the body for catalysing the conversion of free fatty acids (FFA) to fatty acid esters, and optionally for additionally catalysing the conversion of glycerides to fatty acid esters,

15 wherein the catalysing particle is adapted to catalyse the conversion of at least 80% of the FFA in a composition comprising at least 10% by weight of FFA, to fatty acid esters.

9. The micro- or nano-sized catalysing particle as claimed in claim 8, wherein the particle is a magnetic particle and the body comprises:

20 an outer shell; and

a magnetic core at least partially encapsulated by the outer shell,

25 wherein the catalytic means associated with the body is a catalyzing entity immobilized on the outer shell, for catalyzing the conversion of FFA to fatty acid esters, and optionally for additionally catalysing the conversion of glycerides to fatty acid esters.

10. The micro- or nano-sized catalysing particle as claimed in claim 9, wherein the magnetic core comprises a plurality of magnetic nano-sized particles having an average size or diameter of from 5 to 20 nm.

11. The micro- or nano-sized catalysing particle as claimed in claim 9, wherein the outer shell is at least one of a silica shell or polymer shell

made from the group of polymers consisting of poly(glycidyl methacrylate) (PGMA), polystyrene (PS) and poly(methyl methacrylate) (PMMA).

5 12. The micro- or nano-sized catalysing particle as claimed in claim 9, wherein the catalyzing entity is selected from at least one of an inorganic acid group or an enzyme.

10 13. The micro- or nano-sized catalysing particle as claimed in claim 11, wherein the catalyzing entity is immobilized on the polymer shell via a linker selected from the group comprising epoxide functional group containing linker, amine functional group containing linker, aldehyde functional group containing linker, benzene functional group containing linker, ester functional group containing linker and mixtures thereof.

15 14. The micro- or nano-sized catalysing particle as claimed in claim 12, wherein the particle has a specific loading of from 10 to 500 mg enzyme per particle or a specific loading of from 0.1 to 3 mmol H⁺ per gram of particle.

20 15. The micro- or nano-sized catalysing particle as claimed in claim 8, wherein the micro- or nano-sized catalysing particle is a cell of a micro-organism, the body of the particle is the body of the cell, and the catalytic means associated with the body is an enzyme produced in or from the body of the cell for catalyzing the conversion of FFA to fatty acid esters and optionally for additionally catalysing the conversion of glycerides to fatty acid esters.

25 30 16. The micro- or nano-sized catalysing particle as claimed in claim 15, wherein the recombinant micro-organism comprises a nucleic acid sequence that encodes for the enzyme, the nucleic acid sequence having at least 80% homology/identity to SEQ ID No. 1 or SEQ ID No. 3 or SEQ ID No. 5.

SEQ ID No. 1:

atgaagctac tctctctgac cgggtgtggct ggtgtgcttg cgacttgctg tgcagccact
 ccttggtga agcgtctacc ttccggttcg gaccctgcct ttctgcagcc caagtgcgtg
 ctcgatgcgg gtctgacctg ccaggggtgt tcgccatcct cgggtctcaa acccatcctt
 5 ctctgccccg gaaccggcac cacaggtcca cagtcgttcg actcgaactg gatccccctc
 tcaacgcagt tgggttacac accctgctgg atctcacccc cgccgttcac gctcaacgac
 acccaggtca acacggagta catggtcaac gccatcaccc cgctctacgc tgggtcgggc
 aacaacaagc ttccgtgct tacctggctc caggggtggc tgggtgcaca gtgggggtctg
 accttcttc ccagtatcag gtccaaggct gatcgactta tggcctttgc gcccgactac
 10 aagggcacccg tctcgcgccg ccctctcgat gcactcgcgg ttagtgacc ctccgtatgg
 cagcaaacca ccggttcggc actcaccacc gcactccgaa acgcaggtgg tctgaccag
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 aactcgccac tcgactcatc ctaccttctc aacggaaaga acgtccaggc acaggccgtg
 tgtggggccgc tgttcgtcat cgaccatgca ggctcgtca cctcgcagtt ctctacgtc
 15 gtcggtcgat ccgccctgcg ctccaccacg ggccaggctc gtagtgacaga ctatggcatt
 acggactgca acctcttcc cgccaatgat ctgactcccg agcaaaaggt cgccgcgggt
 gcgtctctgg cgccggcagc tgcagccatc gtggcgggtc caaagcagaa ctgcgagccc
 gacctcatgc cctacgcccg ccccttgca gtaggcaaaa ggacctgtc cggcacgtc
 accccctga

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SEQ ID No. 3:

ggatccacca tgaggagctc ccttgtgctg ttctttgtct ctgcgtggac ggcottggcc
 agtcctattc gtcgagaggt ctgcaggat ctgtttaacc agttcaatct cttgcacag
 25 tattctgcag ccgcatactg cggaaaaaac aatgatgcc cagctggtac aaacattacg
 tgcacgggaa atgcctgccc cgaggtagag aaggcggatg caacgtttct ctactcgtt
 gaagactctg gagtgggcga tgcaccggc ttcttgctc tcgacaacac gaacaaattg
 atcgtctctt cttccgtgg ctctcgttc atagagaact ggatcgggaa tcttaacttc
 gactgaaag aaataaatga catttgctc ggctgcaggg gacatgacgg ctctacttcg
 30 tcttgagggt ctgtagccga tacgttaagg cagaagggtg aggatgctgt gagggagcat
 cccgactatc gcgtggtgtt taccggacat agcttgggtg gtgcattggc aactgttgcc
 ggagcagacc tgcgtggaaa tgggtatgat atcgacgtgt ttcatatgg cggccccga
 gtcggaaaca gggcttttgc agaattcctg accgtacaga ccggcggaac actctaccgc
 attaccaca ccaatgatat tgtccctaga ctcccgccgc gcgaattcgg ttacagccat
 35 tctagcccag agtactggat caaatctgga accttgctc ccgtcaccgg aaacgatatc
 gtgaagatag aaggcatcga tgccaccggc ggcaataacc agcctaacat tccggatata

cctgcgcacc tatggtactt cgggtaatt gggacatgtc ttagtggcc ggcgcggctg
ggtcgactct agcgagctcg agatctaga

SEQ ID No 5:

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ccaagcgccg cataccaata acgtttcatc aatcagtctc cttaatgtct atgcagagct
atcagtatag gagagccagc gccggcactg ttaaccaacg cacaatctcg ccaatttgat
tcgcacgcct aatatttagg gctaatacta tttctaccga tgttggtcct ctgaccagct
gtcgttcggc taacgttggt tccctgtttc caccgccgac gcatgagagt tcaactccccg
gccaggcggc ataattcata aggaactgat atgggcatct ttagctataa ggatttggac
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17.A method of producing a micro- or nano-sized catalysing particle, the method comprising:

forming a magnetic core;

encapsulating at least part of the magnetic core with an outer shell;

and

immobilizing a catalyzing entity on the outer shell,

wherein the catalysing entity is adapted to catalyse the conversion of at
 least 80% of the FFA in a composition comprising at least 10% by weight
 of FFA, to fatty acid esters, and optionally for additionally catalysing the
 conversion of glycerides to fatty acid esters.

18.The method as claimed in claim 17, wherein the step of forming a
 magnetic core comprises forming a plurality of magnetic nano-sized
 particles having an average size or diameter of from 5 to 20 nm.

19.The method as claimed in claim 18, wherein the step of forming a plurality
 of magnetic nano-sized particles comprises co-precipitation to obtain the
 magnetic nano-sized particles.

20. The method as claimed in claim 17, wherein the outer shell comprises a polymer shell and the step of encapsulating at least part of the magnetic core with the polymer shell comprises:

5 mixing the magnetic core with one or more monomer precursor of the polymer shell; and

 polymerizing the monomer precursors to form a polymer shell the encapsulates at least part of the magnetic core.

10 21. The method as claimed in claim 17, wherein the outer shell comprises a silica shell and the step of encapsulating at least part of the magnetic core with the silica shell comprises:

 mixing the magnetic core with alkyl silicate, an alkali and an alcohol to obtain a mixture; and

15 forming a silica shell the encapsulates at least part of the magnetic core from precipitation of the mixture.

22. The method as claimed in claim 17, wherein the step of immobilizing a catalyzing entity to the outer shell comprising:

20 (i) covalently coupling the catalyzing entity with the functional group of the outer shell which is chemically reactive to the catalyzing entity, to immobilize the catalyzing entity to the outer shell; or

25 (ii) functionalizing the surface of the outer shell with a functional group chemically reactive to the catalyzing entity; and covalently or non covalently coupling the catalyzing entity with the functional group to immobilize the catalyzing entity to the outer shell.

30 23. A method of obtaining a cell catalyst for producing fatty acid esters from a composition comprising glycerides and at least 10% by weight of free fatty acids (FFA), the method comprising:

 a. identifying a strain of micro-organism from a repertoire of hydrolase producing micro-organisms, the identified strain of micro-organism capable of catalysing the conversion of more FFA to fatty acid

esters than at least 50% of the other strains of micro-organisms in the repertoire, and optionally capable of additionally catalysing the conversion of glycerides to fatty acid esters;

b. identifying a gene sequence of the hydrolase that is capable of catalysing the conversion of FFA to fatty acid esters and optionally capable of additionally catalysing the conversion of glycerides to fatty acid esters, from the identified strain in step a); and

c. introducing the gene sequence in a host cell to obtain a recombinant host cell that is capable of expressing the hydrolase in an amount that is more than the strain of micro-organism identified in step a) under substantially similar conditions,

wherein the recombinant host cell is capable of catalysing the conversion of at least 80% of the FFA in a composition comprising at least 10% by weight of FFA, to fatty acid esters, and optionally capable of additionally catalysing the conversion of glycerides to fatty acid esters.

24. A method of separating a plurality of micro- or nano-sized catalysing particles as claimed in claim 1 from a mixture, the method comprising:

applying an external magnetic field or a centrifugal force to consolidate the catalysing particles together; and

removing the rest of the mixture from the consolidated catalysing particles.

25. A method of producing fatty acid esters from a composition comprising glycerides and at least 10% by weight of free fatty acids (FFA), the method comprising:

incubating a plurality of micro- or nano-sized catalysing particles obtained from the method of claim 24 with the composition, to catalyse the conversion of FFA to fatty acid esters, such that at least 80% of the FFA are converted to fatty acid esters, and optionally to additionally catalyse the conversion of glycerides to fatty acid esters.