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(54) Title: METHOD FOR DETECTING HYDROGEN PEROXIDE CONVERSION ACTIVITY OF AN ENZYME

(57) Abstract: The present invention relates to the detection of enzyme activity, in particular hydrogen conversion activity of an enzyme such as peroxygenase or peroxidase activity. More particularly, the invention provides a microplate-scale absorbance/fluorescence-based method for the detection of hydrogen conversion activity of an enzyme based on the measurement of hydrogen peroxide changes. The invention further provides screening methods for enzymes with improved hydrogen conversion activity, and a kit and combination of reagents suitable for use in the methods of the invention.



METHOD FOR DETECTING HYDROGEN PEROXIDE CONVERSION ACTIVITY OF AN ENZYME

TECHNICAL FIELD

5 The present invention generally relates to the detection of enzyme activity. In particular, the present invention relates to an improved method of detecting hydrogen peroxide conversion activity, such as peroxygenase or peroxidase activity, of an enzyme and screening methods for enzymes with improved hydrogen peroxide conversion activity. The present invention also relates to a kit and combination of reagents for the detection
10 of hydrogen peroxide conversion activity of an enzyme, in particular peroxygenase and/or peroxidase activity.

BACKGROUND

The interest in using enzymes as biocatalysts continues to grow rapidly. Enzymes can
15 be employed e.g. in whole-cell biotransformations or fermentation-based processes for the production of a wide variety of chemical and pharmaceutical products.

Thanks to advances in recombinant DNA technology, new enzymes can now be isolated directly from microorganisms that are difficult to cultivate by high-throughput screening of expressed libraries of environmental DNA. However, enzymes isolated
20 from natural resources usually have moderate activities. Directed evolution is a promising approach to generate enzymes with improved catalytic activity and/or substrate specificity. Identifying new or improved enzymes by these routes requires the development of high-throughput screening methods that are accurate, sensitive, efficient, and simple to implement.

25 Conventional screening methods for enzymatic activity, albeit accurate, may be laborious and/or costly and therefore not suitable for high-throughput screening of large libraries. Examples of such screening methods are gas chromatographic (GC)-based or high performance liquid chromatography (HPLC)-based screening methods e.g. for evaluating oxidative fatty acid decarboxylation activity. In these methods, a test
30 enzyme is reacted with a substrate and eventually co-substrate, and the reaction product formed, e.g. α -alkenes when screening for fatty acid decarboxylase activity, or

the remaining substrate or co-substrate is detected by gas chromatography-mass spectroscopy analysis of the sample. Another problem encountered with these GC-based or HPLC-based screening methods is that they are time-consuming with regard to samples preparation and analysis, and therefore not suitable for high-throughput screening applications (Wang et al. (2016) ChemComm 52:8131-8133).

High-throughput screening methods for enzymatic activities do already exist, but may be specific for one particular enzymatic activity and not applicable to the screening for related enzymatic activities. For instance, Cirino and Arnold (2003. Angew. Chem. Int. Ed. 42:3299-3301) developed an assay for evaluating fatty acid hydroxylation activity of cytochrome P450 mutants wherein p-nitrophenoxycarboxylic acids (pNCAs) were used as fatty acid surrogate substrates, and enzymatic activity was evaluated by spectrophotometrically monitoring the formation of the colorimetric product p-nitrophenolate (pNP). This assay thus requires the synthesis of particular substrates and is therefore difficult to adapt for evaluating other substrates or other catalytic enzyme activities.

SUMMARY OF THE INVENTION

The present invention solves one or more of the above described problems of the prior art. In particular, methods are provided for the detection of hydrogen peroxide converting activity, in particular peroxygenase and/or peroxidase activity, of an enzyme, which are accurate, sensitive, fast and cost-efficient and thus suitable for high-throughput screening. Moreover, the methods are broadly applicable for evaluating a variety of catalytic enzyme activities that require H_2O_2 as substrate or co-substrate such as peroxygenase and peroxidase activities. More particularly, the methods described herein utilize a catalase-deficient host cell for expression of a (test) enzyme and hydrogen peroxide conversion activity is detected by measuring H_2O_2 changes via sensitive absorbance/fluorescence-based assays at microplate scale directly from the whole cell lysates.

The present invention is in particular captured by any one or any combination of one or more of the below numbered aspects and embodiments (i) to (xv):

(i) A method for detecting hydrogen peroxide conversion activity of an enzyme, the method comprising the steps of:

- providing a catalase-deficient host cell;
- expressing said enzyme in said catalase-deficient host cell;
- preparing a cell lysate of said catalase-deficient host cell expressing said enzyme;
- contacting said cell lysate with H_2O_2 and optionally a further substrate for said
- 5 enzyme for a given time period; and thereafter
- determining the remaining H_2O_2 level in said cell lysate.

In particular embodiments, said enzyme is a catalytic enzyme requiring H_2O_2 as a co-substrate; in further particular embodiments, said enzyme is a peroxygenase.

- (ii) The method according to embodiment (i), wherein said method comprises
- 10 comparing said remaining H_2O_2 level in said cell lysate to the remaining H_2O_2 level in a cell lysate of a catalase-deficient host cell not expressing said enzyme and wherein a reduced H_2O_2 level in said cell lysate of said catalase-deficient host cell expressing said enzyme compared to the H_2O_2 level in said cell lysate of said catalase-deficient host cell not expressing said enzyme indicates that the enzyme has hydrogen peroxide
- 15 conversion activity.

(iii) The method according to embodiment (i) or (ii), wherein the remaining H_2O_2 level determined in said cell lysate of said catalase-deficient host cell expressing said enzyme inversely correlates with the hydrogen peroxide conversion activity of said enzyme.

- (iv) The method according to any one of embodiments (i) to (iii), wherein the catalase-deficient host cell is a host cell wherein endogenous catalase-encoding genes have been deleted or inactivated.

- (v) The method according to embodiment (iv), wherein the catalase-deficient host cell is an *E. coli* cell, preferably an BL21(DE3) *E. coli* cell, wherein the *katE* and *katG* genes
- 25 have been deleted.

(vi) The method according to any one of embodiments (i) to (v), wherein the cell lysate is prepared using a lysis buffer that comprises a nonionic surfactant.

- (vii) The method according to any one of embodiments (i) to (vi), wherein determining the remaining H_2O_2 level in said cell lysate comprises reacting said cell lysate
- 30 comprising enzyme, after the catalytic reaction has been stopped, with a peroxidase and a substrate for said peroxidase, and detecting the reaction product formed.

(viii) The method according to embodiment (vii), wherein said substrate for said peroxidase is a chromogenic, fluorogenic or luminescent substrate.

(ix) The method according to any one of embodiments (i) to (ix), wherein said hydrogen peroxide conversion activity is selected from peroxygenase activity and/or peroxidase activity.

(x) The method according to any one of embodiments (i) to (ix), wherein said enzyme is an enzyme involved in the H_2O_2 -assisted conversion of fatty acids and said further substrate for said enzyme is a free fatty acid, preferably a C_8 - C_{14} free fatty acid, more preferably a C_{12} free fatty acid.

(xi) The method according to any one of embodiments (i) to (x), wherein a library of enzymes is expressed in said catalase-deficient host cells.

(xii) The method according to any one of embodiments (i) to (xi), which is a method for screening variants of an enzyme for improved hydrogen peroxide conversion activity compared to the corresponding wild-type enzyme, the method comprising the steps of:

- expressing a library of variants of said wild-type enzyme in a catalase-deficient host cell;

- preparing cell lysates of said host cells expressing said variants;

- contacting said cell lysates with H_2O_2 and optionally a further substrate for said enzyme for a given time period; and thereafter

- determining the remaining H_2O_2 level in said cell lysates, wherein a reduced H_2O_2 level in a cell lysate of a host cell expressing a variant enzyme compared to the remaining H_2O_2 level in a cell lysate of a host cell expressing the wild-type enzyme indicates that the variant has improved hydrogen peroxide conversion activity.

(xiii) The method according to embodiment (xi), further comprising the step of recovering the variants with improved hydrogen peroxide conversion activity from the cell lysates.

(xiv) A combination of reagents for detecting hydrogen peroxide conversion activity of an enzyme comprising:

- a catalase-deficient host cell;

- a lysis buffer comprising a nonionic surfactant;

- hydrogen peroxide;

- optionally a further substrate for said enzyme; and

- an assay for determining H_2O_2 levels.

(xv) The combination according to embodiment (xiv), wherein said assay for determining H_2O_2 levels comprises a peroxidase and a chromogenic, fluorogenic or luminescent substrate for said peroxidase.

5 BRIEF DESCRIPTION OF THE FIGURES

The teaching of the application is illustrated by the following Figures which are to be considered as illustrative only and do not in any way limit the scope of the claims.

Figure 1: Schematic representation of a screening method for an enzyme with hydrogen peroxide converting activity according to an embodiment of the invention.

10 **Figure 2:** Substantial soluble enzyme release into the supernatant (SN) fraction of cell lysates after addition of 0.1% Triton X-100 (TX-100) or 0.5% Tween-20 (TN-20) to the cell lysis buffer.

Figure 3: Levels of soluble cytochrome P450 fatty acid decarboxylase OleT_{JE} (P1-7 to P1-9) and CYP-Sm46 Δ 29 (P1-10 to P1-12) in the cleared cell lysate supernatants from
15 a 96-deepwell plate with HX Δ EG as host cells. Lane P1-2 represents negative controls of the host cell lysate. P1-3 and P1-5 are respectively the two enzymes expressed in wild-type BL21(DE3) host cells.

Figure 4: Results of the Amplex-Red H_2O_2 assay to measure the remaining H_2O_2 levels following the catalytic conversion of C12 fatty acid to 1-undecene by the OleT_{JE}⁻ and
20 CYP-Sm46 Δ 29-containing cell lysates shown in Figure 3. Results from 2 different plates are shown. BL21 and HX Δ EG are cell lysates of respectively, wild-type and catalase-deficient host cells.

Figure 5: Percentage H_2O_2 consumption by purified CYP-Sm46 Δ 29 enzyme exogenously added to wild-type (BL21) or catalase-deficient (HX Δ EG) host cell lysate.
25 Graph shows that H_2O_2 consumption positively correlates with the amount (and therefore the activity) of purified enzyme exogenously added to the catalase-deficient host cell lysate. Wild-type BL21(DE3) cell lysate itself completely consumed the H_2O_2 in the reaction system due to its endogenous catalase activity, therefore no CYP-Sm46 Δ 29-specific consumption of H_2O_2 was detected.

Figure 6: Parallel GC-analysis of C12 fatty acid consumption and 1-undecene production from the catalytic reaction samples shown in Figure 3. Concentration of C12 fatty acid (light gray) and C11 alkene (dark grey) in the reaction mixtures are shown. P1-1 and P1-2 are BL21(DE3) and HXΔEG cells, respectively. P1-3 and P1-5 are
5 respectively, OleT_{JE} and CYP-Sm46Δ29 expressed in wild-type BL21(DE3) cells. P1-7 to P1-9 and P2-7 to P2-9 are cell lysates of catalase-deficient HXΔEG cells expressing OleT_{JE}, and P1-10 to P1-12 and P2-10 to P2-12 are catalase-deficient HXΔEG cells expressing CYP-Sm46Δ29.

Figure 7: Representative data of mutant library screening for enzyme variants with
10 improved hydrogen peroxide conversion activities. Shown are data from one of the 96-well plates. Several mutant variants exhibited significantly decreased residual H₂O₂, i.e. improved H₂O₂ conversion activity, compared with the wild-type enzymes OleT_{JE} and CYP-Sm46Δ29.

Figure 8: Parallel GC-analysis of substrate conversion rate of C12 fatty acid by some
15 of the variants from Figure 7.

DETAILED DESCRIPTION OF THE INVENTION

Unless otherwise defined, all terms used in disclosing the invention, including technical and scientific terms, have the meaning as commonly understood by one of ordinary
20 skill in the art to which this invention belongs. By means of further guidance, term definitions are included to better appreciate the teaching of the present invention.

As used herein, the singular forms "a", "an", and "the" include both singular and plural referents unless the context clearly dictates otherwise.

The terms "comprising", "comprises" and "comprised of" as used herein are
25 synonymous with "including", "includes" or "containing", "contains", and are inclusive or open-ended and do not exclude additional, non-recited members, elements or method steps. Where reference is made to embodiments as comprising certain elements or steps, this encompasses also embodiments which consist essentially of the recited elements or steps.

The recitation of numerical ranges by endpoints includes all numbers and fractions
30 subsumed within the respective ranges, as well as the recited endpoints.

The term "about" as used herein when referring to a measurable value such as a parameter, an amount, a temporal duration, and the like, is meant to encompass variations of +/-10% or less, preferably +/-5% or less, more preferably +/-1% or less, and still more preferably +/-0.1% or less of and from the specified value, insofar such variations are appropriate to perform in the disclosed invention. It is to be understood that the value to which the modifier "about" refers is itself also specifically, and preferably, disclosed.

All documents cited in the present specification are hereby incorporated by reference in their entirety.

An "enzyme" denotes herein a biological molecule that catalyzes a biochemical reaction. The "activity" of an enzyme refers to its ability to catalyze a biochemical reaction.

As used herein, "hydrogen peroxide conversion activity" or "hydrogen peroxide converting activity" or "hydrogen consumption activity" refer to the ability of an enzyme to catalyse a biochemical reaction wherein hydrogen peroxide is a substrate or co-substrate.

An "oxygenase" is an enzyme that catalyses certain oxidations by adding oxygen from an oxygen donor to a substrate compound, and/or by reducing the relative amount of hydrogen in the substrate compound. The terms "oxygen donor", "oxidizing agent" and "oxidant" are used as synonyms and refer to a substance, molecule or compound which donates oxygen to a substrate compound in an oxidation reaction, or which removes hydrogen from a substrate compound. Typically, the oxygen donor is thereby "reduced" and the substrate compound becomes "oxygenated" or "oxidised". Non-limiting examples of oxygen donors include molecular oxygen or dioxygen (O₂) and peroxides such as hydrogen peroxide (H₂O₂).

As used herein, an "enzyme having peroxygenase activity" refers to enzyme that utilizes hydrogen peroxide as oxidizing agent in an oxidation reaction.

As used herein, a "peroxidase" refers to an enzyme that is capable of oxidizing a hydrogen donor such as hydrogen peroxide at the expense of a peroxide. While the methods of the invention can be used to detect catalase activity, in particular

embodiments, the enzyme is not a peroxidase. In particular embodiments, the enzyme is not a catalase.

“Alpha-olefins”, “ α -olefins”, “1-alkenes” or “terminal olefins” are used as synonyms herein and denote olefins or alkenes having a double bond at the primary or alpha (α) position.

As used herein, the term “fatty acid” or “free fatty acid” means a carboxylic acid having the formula RCOOH , or a salt (RCOO^-) thereof. R represents an aliphatic group, preferably an alkyl group. Fatty acids can be saturated, mono-unsaturated, or poly-unsaturated. The term “medium-chain fatty acid” or “medium-chain free fatty acid” as used herein denotes a fatty acid or free fatty acid having 8 to 14 carbon atoms.

The term “host cell” means any cell type that is susceptible to transformation, transfection, transduction, or the like with a suitable nucleic acid construct or expression vector to express an enzyme as taught herein. The term “host cell” encompasses any progeny of a parent cell, including progeny that is not identical to the parent cell due to random mutations that occur during replication.

The terms “genetically engineered” or “genetically modified” or “recombinant” as used herein with reference to a host cell denote a non-naturally occurring host cell, as well as its recombinant progeny, that has at least one genetic alteration not found in a naturally occurring strain of the referenced species or wild-type strain of the referenced species. Such genetic modification is typically achieved by technical means (i.e. non-naturally) through human intervention and may include, e.g., the introduction of an exogenous nucleic acid and/or the modification, over-expression, inactivation or deletion of an endogenous nucleic acid.

The term “exogenous” or “foreign” as used herein is intended to mean that the referenced molecule, in particular nucleic acid, is not naturally present in the host cell.

The term “endogenous” or “native” as used herein denotes that the referenced molecule, in particular nucleic acid, is present in the host cell.

By “encoding” is meant that a nucleic acid sequence or part(s) thereof corresponds, by virtue of the genetic code of an organism in question, to a particular amino acid sequence, e.g., the amino acid sequence of a desired polypeptide or protein. By means of example, nucleic acids “encoding” a particular polypeptide or protein, e.g. an

enzyme, may encompass genomic, hnRNA, pre-mRNA, mRNA, cDNA, recombinant or synthetic nucleic acids.

Preferably, a nucleic acid encoding a particular polypeptide or protein may comprise an open reading frame (ORF) encoding said polypeptide or protein. An “open reading
5 frame” or “ORF” refers to a succession of coding nucleotide triplets (codons) starting with a translation initiation codon and closing with a translation termination codon known per se, and not containing any internal in-frame translation termination codon, and potentially capable of encoding a polypeptide or protein. Hence, the term may be synonymous with “coding sequence” as used in the art.

10

The present application generally relates to the detection of enzyme activity, in particular enzyme activity that consumes or converts hydrogen peroxide such as peroxygenase and/or peroxidase activity.

15

More particularly, the application relates to methods for detecting hydrogen peroxide conversion activity of an enzyme and screening methods for enzymes with improved hydrogen peroxide conversion activity, and host cells and kits suitable for use in these methods. The methods make use of a catalase-deficient host cell. Indeed, it has been found that the use of catalase-deficient host cells allows for efficient evaluation of hydrogen peroxide converting enzyme activity, which can then be very sensitively
20 detected.

In an aspect, methods are provided for detecting hydrogen peroxide conversion activity of an enzyme, which involve the use of a catalase-deficient host cell. More particularly, a catalase-deficient host cell is transformed with a nucleic acid comprising a sequence encoding the enzyme of interest, and, upon expression of the enzyme, the hydrogen
25 peroxide conversion activity of the enzyme is determined. In particular embodiments, the methods comprise the steps of:

- providing a catalase-deficient host cell;
- expressing the enzyme of interest in the catalase-deficient host cell;
- preparing a cell lysate of the host cell expressing the enzyme of interest;
- 30 - contacting the cell lysate with H₂O₂ and optionally a further substrate for the enzyme of interest for a given time period; and thereafter
- determining the remaining H₂O₂ level in the cell lysate.

The methods provided herein are particularly well suited for screening large numbers of enzymes or enzyme variants and in particular to determine relative hydrogen peroxide converting activity, in particular peroxygenase and/or peroxidase activity, of these enzymes or enzyme variants. The methods thus allow the identification of enzymes with improved activity to convert or consume hydrogen peroxide, in particular enzymes with improved peroxygenase and/or peroxidase activity, e.g. compared to a reference enzyme. Accordingly, also provided herein are screening methods for an enzyme with improved activity for converting or consuming hydrogen peroxide such as improved peroxygenase and/or peroxidase activity, more particularly as compared to a corresponding wild-type enzyme, the method comprising the steps of:

- expressing a library of enzymes such as variants of an enzyme in a catalase-deficient host cell;
- preparing cell lysates of the host cells expressing the enzymes or enzyme variants;
- contacting the cell lysates with H_2O_2 and optionally a further substrate for the enzymes for a given time period; and thereafter
- determining the remaining H_2O_2 level in the cell lysates.

In particular embodiments, the remaining H_2O_2 level of the cell lysates is compared to a control. In particular embodiments, the control is the remaining H_2O_2 level in a cell lysate of the host cell expressing a control enzyme to which H_2O_2 and optionally the further substrate have been added. More particularly, where the enzymes expressed are variants of a wild-type enzyme, the control is the cell lysate of the host cell expressing the wild-type enzyme to which the H_2O_2 and optionally the further substrate have been added. In these methods, a reduced H_2O_2 level compared to the H_2O_2 level in the control indicates a higher activity to consume or convert hydrogen peroxide by the variant than the control. More particularly, a reduced H_2O_2 level in the cell lysate of the host cell expressing the variant compared to the H_2O_2 level of a cell lysate of the host cell expressing the wild-type enzyme indicates that the variant has improved hydrogen peroxide converting activity compared to the wild-type enzyme.

Hydrogen peroxide conversion activity

Advantageously, the methods described herein are suitable for evaluating any enzyme activity that consumes or converts H_2O_2 . These activities can be one of varying types of enzyme activities which require H_2O_2 as a substrate or co-substrate whereby the enzyme activity will vary with the nature of the enzyme and its substrate/co-substrate.

Non-limiting examples of H₂O₂-consuming or -converting enzyme activity that can be detected using the methods described herein include oxidative fatty acid decarboxylase activity, hydroxylase activity, cytochrome P450 peroxidase/peroxygenase activity, chloroperoxidase activity, aromatase activity, epoxidase activity, sulfoxidase activity, laccase activity, etc.

In particular embodiments, the methods described herein are for the detection of H₂O₂-consuming or -converting enzyme activity selected from peroxygenase activity and peroxidase activity. In further embodiments, the methods described herein are for the detection of peroxygenase activity of an enzyme. In other embodiments, the methods are for the detection of peroxidase activity of an enzyme.

Enzymes with a primary peroxygenase activity are summarized under EC 1.11.2, which comprises unspecific peroxygenase (UPO, EC 1.11.2.1, formerly aromatic peroxygenase), plant seed peroxygenase (EC 1.11.2.3), fatty-acid peroxygenase (EC 1.11.2.4), and myeloperoxidase (EC 1.11.2.2)

However, many enzymes use hydrogen peroxide as a substrate/co-substrate. Non-limiting examples of such enzymes with peroxygenase activity include, without limitation, cytochrome P450 enzymes (P450), such as cytochrome P450 OleT_{JE} from *Jeotgalicoccus* sp. ATCC 8456, P450_{BSβ} (CYP152A1) from *Bacillus subtilis*, P450_{SPα} (CYP152B1) from *Sphingomonas paucimobilis* and other CYP152 family members, chloroperoxidase (CPO), *Agrocybe aegerita* peroxidase (AaP), methane monooxygenase (MMO), toluene monooxygenase, toluene dioxygenase (TDO), biphenyl dioxygenase, naphthalene dioxygenase (NDO), etc.

Enzymes with a primary peroxidase activity are summarized under EC 1.11.1, which comprises NADH peroxidase (EC 1.11.1.1), NADPH peroxidase (EC 1.11.1.2), fatty-acid peroxidase (EC 1.11.1.3), tryptophan peroxidase (EC 1.11.1.4), cytochrome-c peroxidase (EC 1.11.1.5), catalase (EC 1.11.1.6), peroxidase (EC 1.11.1.7), iodide peroxidase (EC 1.11.1.8), glutathione peroxidase (EC 1.11.1.9), chloride peroxidase (EC 1.11.1.10), L-ascorbate peroxidase (EC 1.11.1.11), phospholipid-hydroperoxide glutathione peroxidase (EC 1.11.1.12), manganese peroxidase (EC 1.11.1.13), lignin peroxidase (EC 1.11.1.14), peroxiredoxin (EC 1.11.1.15), versatile peroxidase (EC 1.11.1.16), glutathione amide-dependent peroxidase (EC 1.11.1.17), bromide peroxidase (EC 1.11.1.18), dye decolorizing peroxidase (EC 1.11.1.19),

prostamide/prostaglandin F(2- α) synthase (EC 1.11.1.20), catalase peroxidase (EC 1.11.1.21), hydroperoxy fatty acid reductase (EC 1.11.1.22), (S)-2-hydroxypropylphosphonic acid epoxidase (EC 1.11.1.23).

- In particular embodiments, the enzyme tested is an oxidative fatty acid decarboxylase enzyme. The term “fatty acid decarboxylases” as envisaged herein refers to enzymes which catalyze the decarboxylation of fatty acids to form α -alkenes using H_2O_2 as the sole electron and oxygen donor. Non-limiting examples of enzymes having fatty acid decarboxylase activity include cytochrome P450 OleT_{JE} from *Jeotgalicoccus sp.* ATCC 8456 (OleT_{JE}, SEQ ID NO:3; Genbank Accession No. ADW41779.1), P450_{BS β} fatty acid hydroxylase from *Bacillus subtilis* subsp. *subtilis* str. 168 (Bs168, SEQ ID NO:4; Genbank Accession No. WP_003246284), and the olefin-producing enzymes termed Aa162 (SEQ ID NO:5 ; Genbank Accession No. WP_008340313) and Sm46 (SEQ ID NO:6 ; Genbank Accession No. WP_039990689) from *Alicyclobacillus acidocaldarius* and *Staphilococcus massiliensis* described in WO 2017/001606.
- In particular embodiments, the methods of the present invention are screening methods for enzymes with improved fatty acid conversion activity such as fatty acid decarboxylase activity and/or fatty acid hydroxylase activity. Such methods may involve the screening of a library of variants of OleT_{JE}, Bs168, Aa162 or Sm46 for hydrogen peroxide consuming activity, in particular peroxygenase activity, whereby the hydrogen peroxide consuming activity, in particular the peroxygenase activity, of the enzyme corresponds to its fatty acid conversion activity. In these methods for detecting hydrogen peroxide conversion activity or fatty acid conversion activity and screening methods for enzymes with improved hydrogen peroxide conversion/fatty acid conversion activity as taught herein, the lysate of host cells expressing an enzyme, such as a variant of OleT_{JE}, Bs168, Aa162 or Sm46, is reacted with medium-chain free fatty acids, in particular C₈-C₁₄ free fatty acids, more particularly C₁₂ free fatty acids, and hydrogen peroxide, and residual hydrogen peroxide level in the cell lysate is determined to evaluate hydrogen peroxide conversion activity, in particular peroxygenase activity, and thus fatty acid conversion activity of the enzyme.
- The present inventors found a good correlation between the methods of the present invention and gas chromatographic (GC)-based fatty acid consumption and hydrocarbon production methods for detecting fatty acid decarboxylase activity. The

herein described methods thus accurately detect the P450 peroxygenase activity, and advantageously are less labor intensive and cheaper, accelerate the turnover time and improve the throughput compared to (GC)-based substrate/product detection methods.

Catalase-deficient host cell

5 The present invention provides catalase-deficient host cells, i.e. host cells that do not express functional catalases, for use in the methods described herein. The use of catalase-deficient host cells advantageously eliminates interference of these enzymes for reacting with the H₂O₂ that is added to the cell lysates. As a result, hydrogen peroxide conversion activity can be evaluated directly from the whole cell lysates,
10 without the need of enzyme purification.

Catalase-deficient host cells can be generated through inactivation or deletion of endogenous catalase-encoding genes, such as the *katE* and *katG* genes in *E. coli*. Accordingly, in particular embodiments, the present invention provides host cells wherein endogenous catalase-encoding genes have been inactivated or deleted, more
15 particularly *E. coli* cells wherein the *katE* and *katG* genes have been inactivated or deleted.

The inactivation or deletion of endogenous catalase-encoding genes results in a reduction or loss of endogenous catalase activity and consequently a reduction of H₂O₂ consumption compared to the corresponding wild-type host cell. Preferably the
20 reduction is a reduction of more than 50%, more preferably of more than 60%, even more preferably of more than 70% or more than 80%, still more preferably a reduction of more than 90%. H₂O₂ consumption can be determined by methods well known to the skilled person, e.g. using an H₂O₂ assay, as described elsewhere herein. Accordingly, in embodiments, the catalase-deficient host cells are host cells wherein endogenous
25 catalase-encoding genes have been deleted or inactivated, preferably deleted (or knocked out), so that H₂O₂ consumption is reduced by more than 50%, preferably by more than 60% or 70%, more preferably by more than 80%, still more preferably by more than 90% compared to the corresponding wild-type host cell.

The inactivation or deletions envisaged herein can be accomplished by genetic
30 engineering methods, forced evolution or mutagenesis and/or selection or screening. Indeed, the state of the art provides a wide variety of techniques that can be used for

the inactivation or deletion of genes in cell population. Such molecular techniques include but are not limited to:

- (i) gene inactivation techniques based on natural gene silencing methods including antisense RNA, ribozymes and triplex DNA formation,
- 5 (ii) techniques for single gene mutation such as gene inactivation by single crossing over with non-replicative plasmid and gene inactivation with a non-replicative plasmid or a linearized DNA fragment capable of double-crossover chromosomal integration (Finchham, 1989, Microbiological Reviews, 53: 148-170; Archer et al., 2006, Basic Biotechnology: 95-126), and
- 10 (iii) techniques for multiple unmarked mutations in the same strain, such as but not limited to:
 - (a) deletion and replacement of the target gene by an antibiotic resistance gene by a double-crossover integration through homologous recombination of an integrative plasmid, giving segregationally highly stable mutants;
 - 15 (b) removing of the antibiotic resistance gene with the Fip recombinase system from *Saccharomyces cerevisiae* allowing the repeated use of the method for construction of multiple, unmarked mutations in the same strain and
 - (c) generating a strain deleted for the *upp* gene, encoding uracil phosphoribosyl transferase, thus allowing the use of 5-fluorouracyl as a counter selectable marker and
 - 20 a positive selection of the double-crossover integrants.

In particular embodiments, the inactivation or deletion of endogenous catalase-encoding genes is performed using a recombinase system such as the phage lambda Red recombination system as described in Datsenko and Wanner (2000 Proc Natl Acad Sci U S A 97(12): 6640-6645).

- 25 The types of host cells provided herein and suitable for use in the methods, kits and combination of reagents provided herein are those that are suitable for transformation and expression of enzymes. Non-limiting examples of host cells envisaged herein include bacterial cells such as *E. coli*, *Bacillus* species, *Pseudomonas* species, yeast cells, such as *S. cerevisiae*, insect cells and filamentous fungi, such as *Aspergillus*
- 30 species. Preferred host cells are *E. coli* cells, such as BL21 cells or BL21(DE3) cells.

Expression of enzymes

According to the present invention, enzymes are expressed in a catalase-deficient host cell as described herein. This can be accomplished in one or more steps via the design and construction of appropriate vectors comprising the coding sequence for the enzyme and transformation of the catalase-deficient host cells, such as catalase-deficient *E. coli* cells, with those vectors.

Electroporation and/or chemical (such as calcium chloride- or lithium acetate-based) transformation methods or *Agrobacterium tumefaciens*-mediated transformation methods as known in the art can be used.

Numerous vectors are known to practitioners skilled in the art, and selection of an appropriate vector is a matter of choice. Non-limiting examples of expression vectors which be used include viral particles, baculovirus, phage, plasmids, phagemids, cosmids, phosmids, bacterial artificial chromosomes, viral DNA, P1-based artificial chromosomes, yeast plasmids, yeast artificial chromosomes. The vectors can either be cut with particular restriction enzymes or used as circular DNA.

The vectors that are used for transformation of the catalase-deficient host cells in the context of the present invention typically comprises a coding sequence for an enzyme placed under the transcriptional control of one or more promoters and one or more terminators, both of which are functional in the host cell.

Promoter and terminator sequences may be native to the host cell or exogenous to the host cell. Useful promoter and terminator sequences include those that are highly identical (i.e. having an identities score of 90% or more, preferably 95% or more, most preferably 99% or more) in their functional portions compared to the functional portions of promoter and terminator sequences, respectively, that are native to the host cell.

The use of native (to the host cell) promoters and terminators, together with their respective upstream and downstream flanking regions, can permit the targeted integration of the coding sequence into specific loci of the host cell genome.

The promoter may be an inducible promoter, the activation of which is regulated by a specific substrate (inducer).

The vectors disclosed herein preferably comprise the coding sequence of an enzyme and associated promoter and terminator sequences. The vector may contain restriction sites of various types for linearization or fragmentation. Vectors may further contain a

backbone portion (such as for propagation in *E. coli*) many of which are conveniently obtained from commercially available yeast or bacterial vectors. The vector preferably comprises one or more selection marker gene cassettes. A "selection marker gene" is one that encodes a protein needed for the survival and/or growth of the transformed cell in a selective culture medium. Typical selection marker genes encode proteins that (a) confer resistance to antibiotics or other toxins such as chloramphenicol, zeocin (sh *ble* gene from *Streptoalloteichus hindustanus*), genetecin, melibiase (MEL5), hygromycin (aminoglycoside antibiotic resistance gene from *E. coli*), ampicillin, tetracycline, or kanamycin (kanamycin resistance gene of Tn903), (b) complement auxotrophic deficiencies of the cell. Two prominent examples of auxotrophic deficiencies are the amino acid leucine deficiency (e.g. LEU2 gene) or uracil deficiency (e.g. URA3 gene). Cells that are orotidine-5'-phosphate decarboxylase negative (*ura3*-) cannot grow on media lacking uracil. Thus a functional URA3 gene can be used as a marker on a cell having a uracil deficiency, and successful transformants can be selected on a medium lacking uracil. Only cells transformed with the functional URA3 gene are able to synthesize uracil and grow on such medium. If the wild-type strain does not have a uracil deficiency (as is the case with *I. orientalis*, for example), an auxotrophic mutant having the deficiency must be made in order to use URA3 as a selection marker for the strain. Methods for accomplishing this are well known in the art.

Successful transformants can be selected for in known manner, by taking advantage of the attributes contributed by the marker gene, or by other characteristics contributed by the inserted recombinant nucleic acids. Screening can also be performed by PCR or Southern analysis to confirm that the desired insertions, and optionally deletions have taken place, to confirm copy number and to identify the point of integration of coding sequences into the host genome.

Once the host cell has been transformed with a vector as taught herein, the host cell is cultured under conditions with promote expression of the enzyme. Said culturing of the host cells may take place in one or more phases. For example, the transformed host cells may first be cultured under conditions which promote exponential logarithmic growth of the cells, prior to entering into the production phase. The term "production phase" as used herein refers to the period during which production of the enzyme is primary. Production of the enzyme may be induced e.g. through changing the culturing

conditions, e.g. by lowering the temperature, or through addition of an appropriate inducer when the coding sequence for the enzyme is under control of an inducible promoter as known in the art.

Accordingly, the invention also provides recombinant catalase-deficient host cells which comprise at least one transgene encoding an enzyme of interest, i.e. an enzyme having or suspected to have hydrogen peroxide conversion activity such as peroxygenase or peroxidase activity. As detailed above, in particular embodiments, the catalase-deficient host cell is characterized by one or more dysfunctional catalase genes. As also detailed herein, in particular embodiments, the enzyme having hydrogen peroxide conversion activity, e.g. peroxygenase and/or peroxidase activity, can be an enzyme having oxidative fatty acid decarboxylase activity, hydroxylase activity, aromatase activity, epoxidase activity, sulfoxidase activity requiring H₂O₂ as a (co-)substrate. In particular embodiments, the enzyme is a cytochrome P450 enzyme.

Library of (test) enzymes

The methods described herein are well suited for screening large number of different enzymes or potential enzymes (also referred to herein as test enzymes) for hydrogen peroxide conversion activity, such as peroxygenase and/or peroxidase activity. Accordingly, in certain embodiments, catalase-deficient host cells as taught herein are transformed with a number of vectors expressing different (test) enzymes e.g. for comparing relative hydrogen peroxide conversion activity of the different (test) enzymes and to screen for an enzyme with improved hydrogen peroxide conversion activity.

In certain embodiments, the different enzymes are mutants of a given enzyme. The mutants can be prepared by subjecting the enzyme or a polynucleotide encoding the enzyme to mutagenesis techniques as known in the art. Non-limiting examples of mutagenesis techniques include DNA shuffling, as shown for example in US Patent No. 5,605,793, error-prone PCR and oligonucleotide directed mutagenesis.

The term "error-prone PCR" refers to a process for performing PCR under conditions where the copying fidelity of the DNA polymerase is low, such that a high rate of point mutations is obtained along the entire length of the PCR product (Leung, D. W., et al., Technique, 1:11-15 (1989) and Caldwell, R. C. & Joyce G. F., PCR Methods Applic., 2:28-33 (1992)).

The term "oligonucleotide directed mutagenesis" refers to a process which allows for the generation of site-specific mutations in any cloned DNA segment of interest. Reidhaar-Olson, J. F. & Sauer, R. T., et al., Science, 241:53-57 (1988).

A library of cells may be prepared by transforming catalase-deficient host cells as taught herein with vectors as taught herein comprising the mutagenized polynucleotides encoding the enzyme. Accordingly, the invention also provides a library of catalase-deficient host cells which comprise a library of enzyme-encoding sequences which are variants of a given enzyme sequence, wherein the enzyme is an enzyme having hydrogen peroxide conversion activity. In particular embodiments, the host cells comprise variants of a given enzyme-encoding sequence obtained by random or directed mutagenesis.

The resultant libraries of transformed clones can then be screened for clones which express an enzyme having hydrogen peroxide conversion activity using the methods as taught herein. In particular, the clones are cultured under conditions to promote protein (or enzyme) expression and cell lysates are prepared. In further steps, the whole cell lysates are reacted with a substrate for the enzyme and H_2O_2 , and residual H_2O_2 level is determined. Clones of which the residual H_2O_2 level is lower than the H_2O_2 level in cell lysates of a clone expressing the wild-type enzyme are considered to comprise mutant enzymes with improved hydrogen peroxide conversion activity. The clone with the lowest residual H_2O_2 is considered to comprise a mutant with the highest hydrogen peroxide conversion activity.

Accordingly, also provided herein are methods for providing an enzyme having improved hydrogen peroxide conversion activity, e.g. peroxygenase and/or peroxidase activity, as compared to a corresponding wild-type enzyme, comprising:

- subjecting at least one polynucleotide encoding the wild-type enzyme to mutagenesis;
- transforming catalase-deficient host cells with the at least one mutagenized polynucleotide encoding a variant or mutated enzyme;
- culturing the catalase-deficient host cells under conditions to produce the mutated enzymes;
- preparing cell lysates of the host cells expressing the mutated enzymes;
- providing H_2O_2 and optionally a further substrate for the enzymes to the cell lysates and allowing the cell lysates to react with H_2O_2 and optionally the further substrate;

- determining the remaining H_2O_2 level in the cell lysates and selecting a host cell expressing an enzyme with improved hydrogen peroxide conversion activity based thereon; and

- optionally recovering the variant enzyme from the cell lysate of the host cell expressing the enzyme with improved hydrogen peroxide conversion activity or expressing said variant enzyme in a host cell.

In particular embodiments, the residual or remaining H_2O_2 level of the cell lysates is compared e.g. with the H_2O_2 level of cell lysates comprising the wild-type enzyme (as well as the added substrate and H_2O_2). In the methods of the invention, cell lysates having a lower residual H_2O_2 level compared to the H_2O_2 level in cell lysates comprising the wild-type enzyme comprise an enzyme with improved hydrogen peroxide conversion activity.

A library of enzymes can also be generated from environmental DNA, such as DNA recovered from an environmental sample containing microorganisms which are not or cannot be cultured. Sources of microorganism DNA as a starting material library from which DNA is obtained are particularly contemplated to include environmental samples, such as microbial samples obtained from Arctic and Antarctic ice, water or permafrost sources, materials of volcanic origin, materials from soil or plant sources in tropical areas, etc. Thus, for example, genomic DNA may be recovered from either uncultured or non-culturable microorganism and employed to produce an appropriate library of clones for subsequent determination of enzyme activity, in particular hydrogen peroxide conversion activity, e.g. peroxygenase and/or peroxidase activity. Such libraries of environmental DNA can be screened for new enzymes having hydrogen peroxide conversion activity using the herein described methods.

Cell lysis

A variety of techniques can be used to lyse cells, including mechanical or physical disruption of cell membranes, such as, for example, sonication, and enzymatic or chemical cell lysis. Mechanical or physical lysis may require specialized and expensive equipment and may generate heat that may denature the target polypeptide or protein. Enzymatic or chemical lysis method may result in too low yields of soluble enzyme in the cell lysate.

The present inventors have found that supplementation of a cell lysis buffer with a nonionic surfactant substantially increases the release of soluble enzyme in the cell lysate. Accordingly, in particular embodiments, a cell lysis buffer comprising nonionic surfactant is used for disruption of the host cells in the methods described herein. The surfactant may be present in the cell lysis buffer in an amount ranging from about 0.005 to about 10% (w/w), preferably from about 0.01 to about 5%(w/w), even more preferably from about 0.05 to about 1% (w/w), such as about 0.05, 0.10, 0.20, 0.30, 0.40, 0.50, 0.60, 0.70, 0.80, 0.90 or 1.00% (w/w). Non-limiting examples of nonionic surfactants include Triton X-100, Tween 20, Tween 80 and NP-40. In preferred embodiments, the nonionic surfactant is Triton X-100 or Tween 20.

Contacting with H₂O₂ and optionally with a further substrate

Detection of a desired enzymatic activity as described herein is achieved through contacting the enzyme with its substrate and/or hydrogen peroxide. Where the enzyme is a catalase, H₂O₂ will act as a substrate. Where the enzyme is a peroxygenase, H₂O₂ will act as a co-substrate. Accordingly, the methods involve providing hydrogen peroxide and optionally an appropriate further substrate for the enzyme to the host cell lysates comprising the enzyme. For instance, for the detection of a fatty acid decarboxylase peroxygenase activity, the cell lysates are supplemented with free fatty acids and hydrogen peroxide. For the detection of rabbit CYP2B4 activity, the cell lysates can be supplemented with aniline and H₂O₂. For the detection of human CYP2S1 activity, the cell lysates can be supplemented with 7,12-Dimethylbenz[a]anthracene (DMBA) and H₂O₂. For the detection of bacterial P450_{BSP} activity, the cell lysates can be supplemented with free fatty acids, ethylbenzene or styrene and H₂O₂. For the detection of bacterial CYP101A1 activity, the cell lysates can be supplemented with dehydrocamphor or naphthalene and H₂O₂. For the detection of fungal lignin peroxidase activity, the cell lysates can be supplemented with 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol and H₂O₂. For the detection of CPO activity, the cell lysates can be supplemented with guaiacol, pyrogallol or thiourea as substrate and hydrogen peroxide as co-substrate. Other examples of combinations of enzymes with hydrogen peroxide consuming activity and appropriate substrates therefor are easily recognized by one skilled in the art. Preferably, the substrate is a naturally occurring molecule. In particular embodiments, the substrate is

a naturally occurring peroxidase or peroxygenase substrate. It will be understood to the skilled person that as the methods of the present invention rely on indirect detection of residual H_2O_2 , the substrate is preferably not a chromogenic, fluorogenic or luminescent substrate.

- 5 The methods of the invention encompass contacting the cell lysates with hydrogen peroxide. The hydrogen peroxide may be provided as an aqueous solution of hydrogen peroxide or a hydrogen peroxide precursor for *in situ* production of hydrogen peroxide. Any solid entity which liberates upon dissolution hydrogen peroxide which is useable by an enzyme having H_2O_2 conversion activity can serve as a source of hydrogen
- 10 peroxide. Compounds which yield hydrogen peroxide upon dissolution in water or an appropriate aqueous based medium include but are not limited to metal peroxides, percarbonates, persulphates, perphosphates, peroxyacids, alkylperoxides, acylperoxides, peroxyesters, urea peroxide, perborates and peroxycarboxylic acids or salts thereof.
- 15 The amount of H_2O_2 and, where applicable, substrate added to the cell lysate will depend on a variety of factors including the concentration of the enzyme in the cell lysate, the activity of the enzyme etc., and can easily be determined by the skilled person. In particular embodiments, the amount of H_2O_2 and/or substrate added to the cell lysate is between 10 μM and 500 μM , such as about 200 μM . In particular
- 20 embodiments, the methods comprise contacting the cell lysate with different concentrations of H_2O_2 and/or substrate. In further particular embodiments, the methods comprise contacting various amounts of cell lysate with a given amount of substrate and/or H_2O_2 .

- In the methods provided herein the cell lysate comprising the crude enzyme is
- 25 contacted with H_2O_2 and optionally a further substrate of said enzyme during a given time period. In particular embodiments, the time period is between 5 min and 12 h, more particularly between 10 minutes and 5 hours such as for 30-90 minutes, more particularly 1 hour. In further particular embodiments, the reaction is stopped by addition of a strong acid. In particular embodiments, the reaction is stopped by adding
- 30 1M HCl.

H_2O_2 assay

The methods described herein are indirect in that they do not detect the presence of the product that is produced by action of the (test) enzyme on its substrate(s). Instead the residual H_2O_2 level following the catalytic reaction of the enzyme on its substrate(s) is determined and thus gives an indication of the H_2O_2 consumption by the enzyme and thus of the hydrogen peroxide conversion activity of the enzyme. Accordingly, in the methods of the invention the residual or remaining H_2O_2 level inversely correlates with the hydrogen peroxide conversion activity of the enzyme. Thus, hydrogen peroxide conversion activity of an enzyme is detected in the methods described herein when the residual H_2O_2 level decreases or is determined to be lower compared to e.g. the residual H_2O_2 level in a cell lysate of a non-transformed host cell (i.e. a catalase-deficient host cell that does not express a (test) enzyme) to which H_2O_2 has been added. When comparing relative hydrogen peroxide conversion activity of enzymes, such as when screening a library of (test) enzymes, the cell lysate wherein the lowest remaining H_2O_2 level is determined, is considered to comprise the enzyme with highest hydrogen peroxide conversion activity.

As used herein, the term “determining” the remaining H_2O_2 level in the cell lysate may denote a qualitative and/or quantitative assessment of the remaining H_2O_2 level.

Methods for qualitative and/or quantitative assessment of the presence of H_2O_2 are known in the art. Suitable methods can be based on the detection of a reaction product of a substrate with H_2O_2 using an enzyme that catalyzes the conversion of the respectively selected substrate into a detectable reaction product. Preferably, said substrate is a chromogenic, fluorogenic or luminescent substrate that undergoes a color change in the presence of hydrogen peroxide and the enzyme. In particular, chromogenic substrates produce a colored reaction product, fluorogenic substrates produce a fluorescent product, and chemiluminescent substrates produce light as the detectable product. The identification and quantification of the detectable signals described herein (e.g., the formation of colored products or the detection of color or the absorption of light) may be performed by any suitable means known. The simplest is visual observation of color development or color change. Alternatively, embodiments of the herein described methods requiring quantitative measurement will best be performed by spectrophotometry. The use of these hydrogen peroxide assays advantageously reduces labor and cost, and accelerates the assay turnover time and improves the throughput of the methods described herein for instance in comparison to

methods based on the detection of a particular enzyme substrate-product. Accordingly, in particular embodiments of the methods of the invention, the residual H_2O_2 level is measured using a chromogenic substrate. The skilled person will understand that this also encompasses substrates which indirectly generate a chromogenic reaction, e.g. by conversion of another substrate upon activation by a peroxidase enzyme. In particular embodiments, the cell lysate is supplemented with a peroxidase, such as horseradish peroxidase (HRP), and a substrate for said peroxidase, and the presence of oxidation product is detected to determine residual H_2O_2 level in the cell lysate. Preferably, the substrate changes color upon reacting with hydrogen peroxide, which color change can be detected. The color varies in intensity, depending upon the amount of hydrogen peroxide present and thus provides a quantitative measure of the hydrogen peroxide that is present in the cell lysate. Upon measuring color intensity of the oxidation product, H_2O_2 level in the cell lysate can be quantitatively determined. Non-limiting examples of chromogenic substrate for peroxidases, in particular horseradish peroxidase (HRP), include tetramethylbenzidine (TMB), o-phenylenediamine (ABTS) and guacajol. A non-limiting example of fluorogenic substrate for peroxidases, in particular horseradish peroxidase (HRP), includes Amplex Red. A non-limiting example of luminescent substrate for peroxidases, in particular horseradish peroxidase (HRP), includes luminol.

Kits and combinations of reagents

As noted before, the use of catalase-deficient host cells as taught herein for expressing a test enzyme eliminates interference of these catalases for reacting with hydrogen peroxide and thus allows to determine hydrogen peroxide levels directly from whole cell lysates. These catalase-deficient host cells are thus suitable for use in the assays and methods described herein, but also e.g., in assays wherein hydrogen peroxide is generated through enzymatic activity and convenient assays for peroxidase activity. Accordingly, also disclosed herein are kits for the detection of enzyme activity, wherein the enzyme consumes or generates hydrogen peroxide, said kit comprising a catalase-deficient host cell as taught herein, preferably an *E. coli* cell, such as an *E. coli* BL21(DE3) cell, wherein the *katE* and *katG* genes have been inactivated or deleted.

Preferably, the kits and combination of reagents for use in the methods described herein further comprise reagents necessary to initiate an enzymatic reaction, in

particular the reaction catalyzed by the enzyme of interest, and reagents that facilitate the determination of enzyme activity. Accordingly, in preferred embodiments, the kits or combination of reagents further comprise:

- hydrogen peroxide;

- 5 - optionally a substrate for the enzyme; and- an assay for determining H₂O₂ levels as taught herein.

In particular embodiments, the hydrogen peroxide and optionally the substrate are provided in a specified concentration. In preferred embodiments, said assay for determining H₂O₂ levels comprises a peroxidase, such as horseradish peroxidase
10 (HRP), and a chromogenic, fluorogenic or luminescent substrate for said peroxidase.

The kit may further comprise a lysis buffer comprising a nonionic surfactant as taught herein.

15 The present invention will now be further illustrated by means of the following non-limiting examples.

EXAMPLES

Example 1: Assay for peroxygenase activity in cell lysates in 96-well plates

Material and methods

20 *Generation of catalase-deficient E. coli cells*

The *katE* (SEQ ID NO:1) and *katG* (SEQ ID NO:2) genes in BL21(DE3) *E. coli* cells (Novagen) were knocked-out using a phage λ RED recombination system as described in Datsenko and Wanner (2000 Proc Natl Acad Sci U S A 97(12): 6640-6645). The catalase-deficient *E. coli* cells are referred to herein as "HX Δ EG".

25 *Expression of test enzyme in the HX Δ EG cells*

HX Δ EG cells were transformed via electrotransformation with vector pET28b/*oleT* or pET28b/*sm46-29* comprising the coding sequence for the cytochrome P450 fatty acid decarboxylases OleT_{JE} (Genbank Accession No: ADW41779) or CYP-Sm46 Δ 29 (Genbank Accession No: WP_039990689), respectively.

30 *Colony culture and protein expression*

The transformed HX Δ EG cells were grown overnight on LB agar plates supplemented with 50 μ g/ml kanamycin at 37°C. The individual colonies were picked and then transferred into 96-deep well plates containing 400 μ l/well of LB medium supplemented with kanamycin and grown at 37°C for 3 h with shaking at 500 rpm. To induce protein
5 expression, temperature was lowered to 16°C and colonies were further cultured at 16°C for 24 hours.

Cell lysis

Following protein expression, cells were harvested by centrifugation and disrupted by addition of 1 mg/ml lysozyme in a 50 mM Na₃PO₄, 10% glycerol, 300 mM NaCl, pH 7.4
10 buffer supplemented with 0.1% Triton X-100 or 0.5% Tween-20, or not.

Catalytic reaction

The lysed cells were centrifuged and the cleared cell lysates containing the soluble proteins were used as crude (i.e. not purified) enzyme samples for catalytic reactions of converting C₁₂ fatty acid to C₁₁ alkene in a 96-well microplate with H₂O₂ as co-factor/co-
15 substrate. Samples were prepared by mixing cleared cell lysate (100 μ l), C₁₂ fatty acid (200 μ M) and H₂O₂ (200 μ M) in a 50 mM Na₃PO₄, 10% glycerol, 300 mM NaCl, pH 7.4 buffer to a total volume of 200 μ l. The catalytic reactions were stopped by adding 1/10 volume of 1.0 M HCl after 1 hour of incubation at 30°C.

Peroxidase/H₂O₂ assay

20 Following the catalytic reactions, 10 μ l of the reaction mixture was diluted into 50 ml of 50 mM Na₃PO₄, pH 7.4 buffer followed by mixing with equal volume of the Amplex-Red H₂O₂ assay reagent (Life Technologies) and incubating for 30 min at room temperature in the dark. Residual H₂O₂ levels were measured by reading the absorbance of resorufin at 560 nm.

25 *GC-analysis*

Following the Amplex-Red H₂O₂ assay, the reaction mixture was subjected to ethyl acetate extraction to recover the fatty acid substrate and olefin product in the reaction. Quantitative analysis of the C12 fatty acid conversion and C11 undecene production were performed using a gas chromatographic (GC) method.

30 Results

Supplementation of the cell lysis buffer with 0.1% Triton X-100 or 0.5% Tween-20 significantly increased cell disruption and resulted in substantial enzyme release into the soluble fraction (Fig. 2). Significant amount of soluble protein was also detected in the cleared cell lysates (Fig. 3).

5 Figure 4 shows that samples comprising the high activity OleT_{JE} enzyme contained significantly decreased residual H₂O₂ level, whereas the CYP-Sm46Δ29 enzyme barely consumed any H₂O₂ indicating very low activity. Figures 4 and 5 further show that in comparison with the wild-type BL21(DE3) strain showing high consumption of H₂O₂ due to its endogenous catalase activity, the catalase-deficient host cells HXΔEG
10 retained most of the H₂O₂ in the reaction system. Moreover, H₂O₂ consumption positively correlated with increasing amount of P450 enzyme exogenously added to HXΔEG cell lysate (Fig. 5), indicative of increased catalytic activity, whereas fatty acid decarboxylase activity of CYP-Sm46Δ29 enzyme could not be detected using the present method when the enzyme was added to wild-type BL21(DE3) cell lysates (Fig.
15 5) due to interference of the endogenous catalases for reaction with the added H₂O₂.

The results obtained using the method described above according to an embodiment of the present invention correlate well with the results obtained by GC-analysis of the same samples for C₁₂ fatty acid substrate consumption (correlation coefficient: 0.9942) and 1-undecene production (correlation coefficient: 0.9866) (Fig. 4&6). This shows that
20 the present method is thus an accurate method for the detection of fatty acid decarboxylase activity.

Example 2: Screening assay for improved activity of P450 fatty acid decarboxylase towards C12 fatty acid substrate

25 Materials and methods

Construction of a library of mutants of P450 fatty acid decarboxylases in the HXΔEG cells

A mutant library was generated by DNA shuffling of OleT_{JE} and CYP-Sm46Δ29 genes. Briefly, the encoding DNA sequences of OleT_{JE} and CYP-Sm46Δ29 were subjected to
30 DNase I digestion or fragmentation. The 100-150 bp DNA fragments were gel purified and reassembled by a primerless PCR, thereby creating a pool of chimeric sequences

containing crossovers between the two parent sequences. Then a final step of PCR with primers flanking the original parent sequence was performed using the above reassembled fragments as template to obtain the full-length library of the shuffled genes. These shuffled products were then cloned into pET28b vector and used to electrotransform the catalase-deficient HXΔEG cells. The resultant library of transformed clones were plated on LB plus kanamycin plates.

Colony culture and protein expression, cell lysis, catalytic reaction and peroxidase/H₂O₂ assay were performed as described in Example 1.

For certain variants GC-analysis of the wells was performed as described in Example 1 in parallel with the peroxidase/H₂O₂ assay.

Results

A screening assay for an enzyme with improved fatty acid decarboxylase activity was conducted as schematically shown in Figure 1. More particularly, a library of gene variants of an enzyme of interest was generated and expressed in the catalase-deficient HXΔEG *E. coli* cells. The resultant mutant library of colonies was transferred into 96-deepwell plates for individual colony culture and protein expression. The cells were then harvested by centrifugation and disrupted using a lysis buffer supplemented with a nonionic surfactant such as Triton X-100. The cleared cell lysates containing soluble protein were used as crude enzyme solutions for a catalytic reaction, in particular for the conversion of C₁₂ fatty acid into 1-undecene, wherein H₂O₂ is co-substrate. By measuring the remaining H₂O₂ level (or H₂O₂ consumption) after the catalytic reaction, e.g. using a fluorometric H₂O₂ assay and reading absorbance of the reaction product using a spectrophotometer, enzyme activities among the different enzyme variants can be compared and variants with improved enzymatic activity can be selected.

Figure 7 shows representative data of the Amplex-Red H₂O₂ assay of one of the 96-well plates. Several mutants exhibited significantly improved enzymatic activity compared with the wild-type enzymes OleT_{JE} and CYP-Sm46Δ29 as can be deduced from the decreased residual H₂O₂ levels measured. GC-analysis of selected wells showed good correlations between the present method and the GC-analysis-based method (Fig. 7 and 8). For example, wells A6 and F3 had low residual H₂O₂ levels and

the variants showed extensive C₁₂ fatty acid consumption, whereas the mutants in wells like A3, B9, H6 and H8 that consumed almost no H₂O₂ (high residual H₂O₂ levels) exhibited substantial C12 fatty acid substrate retrieval in GC-analysis. These data demonstrate that the present screening method provides good accuracy for detecting

5 fatty acid decarboxylase activity.

CLAIMS

1. A method for detecting hydrogen peroxide conversion activity of an enzyme, the method comprising the steps of:
 - 5 - providing a catalase-deficient host cell;
 - expressing said enzyme in said catalase-deficient host cell;
 - preparing a cell lysate of said catalase-deficient host cell expressing said enzyme;
 - contacting said cell lysate with H_2O_2 and optionally a further substrate for said enzyme for a given time period; and thereafter
 - 10 - determining the remaining H_2O_2 level in said cell lysate.
2. The method according to claim 1, wherein said method comprises comparing said remaining H_2O_2 level in said cell lysate to the remaining H_2O_2 level in a cell lysate of a catalase-deficient host cell not expressing said enzyme and wherein a reduced H_2O_2
 - 15 level in said cell lysate of said catalase-deficient host cell expressing said enzyme compared to the H_2O_2 level in said cell lysate of said catalase-deficient host cell not expressing said enzyme indicates that the enzyme has hydrogen peroxide conversion activity.
- 20 3. The method according to claim 1 or 2, wherein the remaining H_2O_2 level determined in said cell lysate of said catalase-deficient host cell expressing said enzyme inversely correlates with the hydrogen peroxide conversion activity of said enzyme.
4. The method according to any one of claims 1 to 3, wherein the catalase-deficient
 - 25 host cell is a host cell wherein endogenous catalase-encoding genes have been deleted or inactivated.
5. The method according to claim 4, wherein the catalase-deficient host cell is an *E. coli* cell, preferably an BL21(DE3) *E. coli* cell, wherein the *katE* and *katG* genes have
 - 30 been deleted.
6. The method according to any one of claims 1 to 5, wherein the cell lysate is prepared using a lysis buffer that comprises a nonionic surfactant.

7. The method according to any one of claims 1 to 6, wherein determining the remaining H_2O_2 level in said cell lysate comprises reacting said cell lysate comprising enzyme, after the catalytic reaction has been stopped, with a peroxidase and a substrate for said peroxidase and detecting the reaction product formed.

8. The method according to claim 7, wherein said substrate for said peroxidase is a chromogenic, fluorogenic or luminescent substrate.

9. The method according to any one of claims 1 to 8, wherein said hydrogen peroxide conversion activity is selected from peroxygenase activity and/or peroxidase activity.

10. The method according to any one of claims 1 to 9, wherein said enzyme is an enzyme involved in the H_2O_2 -assisted conversion of fatty acids and said further substrate for said enzyme is a free fatty acid, preferably a C_8 - C_{14} free fatty acid, more preferably a C_{12} free fatty acid.

11. The method according to any one of claims 1 to 10, wherein a library of enzymes is expressed in said catalase-deficient host cells.

12. The method according to any one of claims 1 to 11, which is a method for screening variants of an enzyme for improved hydrogen peroxide conversion activity compared to the corresponding wild-type enzyme, the method comprising the steps of:

- expressing a library of variants of said wild-type enzyme in a catalase-deficient host cell;

- preparing cell lysates of said host cells expressing said variants;

- contacting said cell lysates with H_2O_2 and optionally a further substrate for said enzyme for a given time period; and thereafter

- determining the remaining H_2O_2 level in said cell lysates, wherein a reduced H_2O_2

level in a cell lysate of a host cell expressing a variant enzyme compared to the remaining H_2O_2 level in a cell lysate of a host cell expressing the wild-type enzyme indicates that the variant has improved hydrogen peroxide conversion activity.

13. The method according to claim 11, further comprising the step of recovering the variants with improved hydrogen peroxide conversion activity from the cell lysates.

14. A combination of reagents for detecting hydrogen peroxide conversion activity of an enzyme comprising:

- a catalase-deficient host cell;
- a lysis buffer comprising a nonionic surfactant
- optionally a further substrate for said enzyme;
- hydrogen peroxide; and
- an assay for determining H₂O₂ levels.

15. The combination according to claim 14, wherein said assay for determining remaining H₂O₂ levels comprises a peroxidase and a chromogenic, fluorogenic or luminescent substrate for said peroxidase.

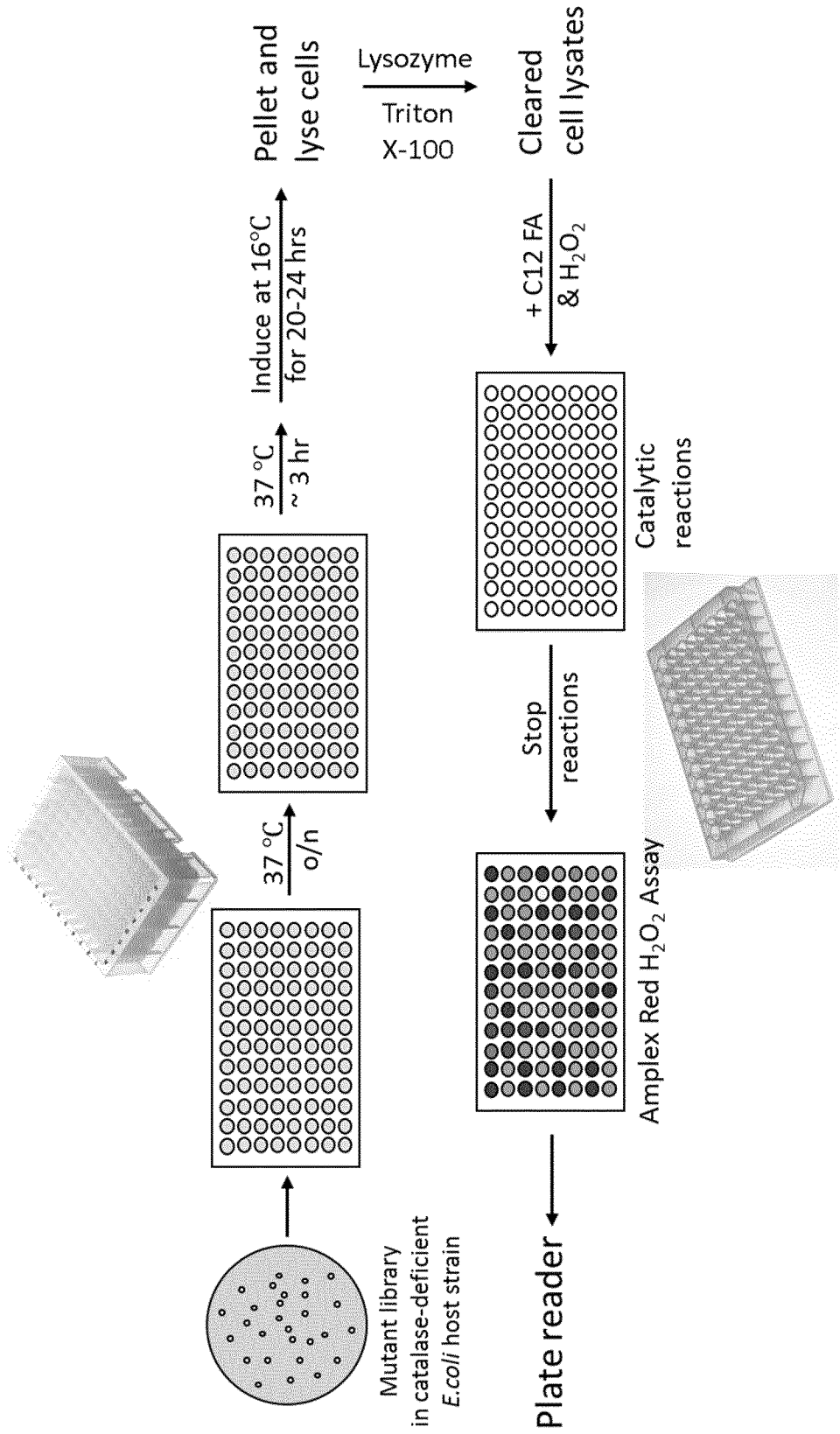


FIG. 1

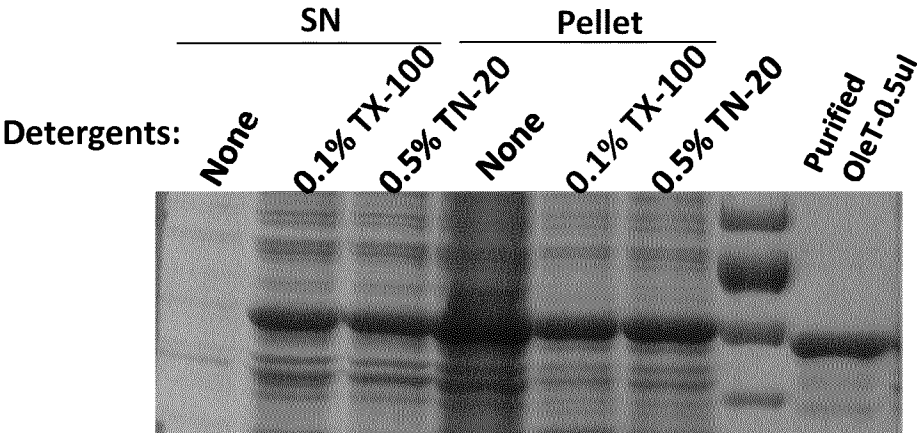


FIG. 2

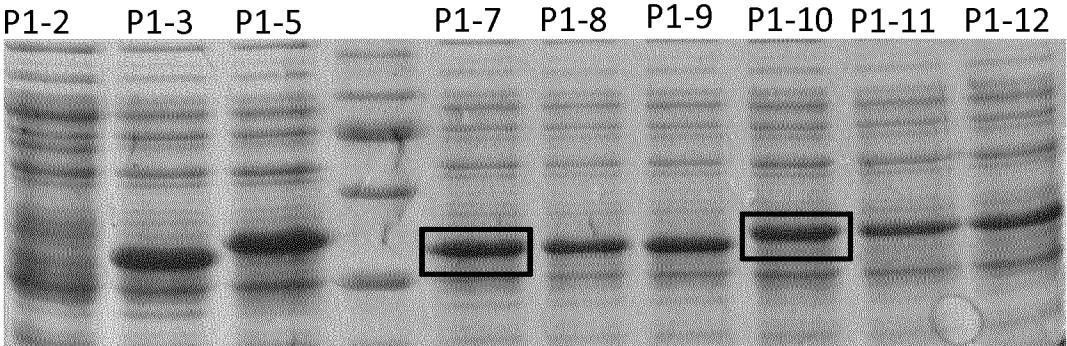


FIG. 3

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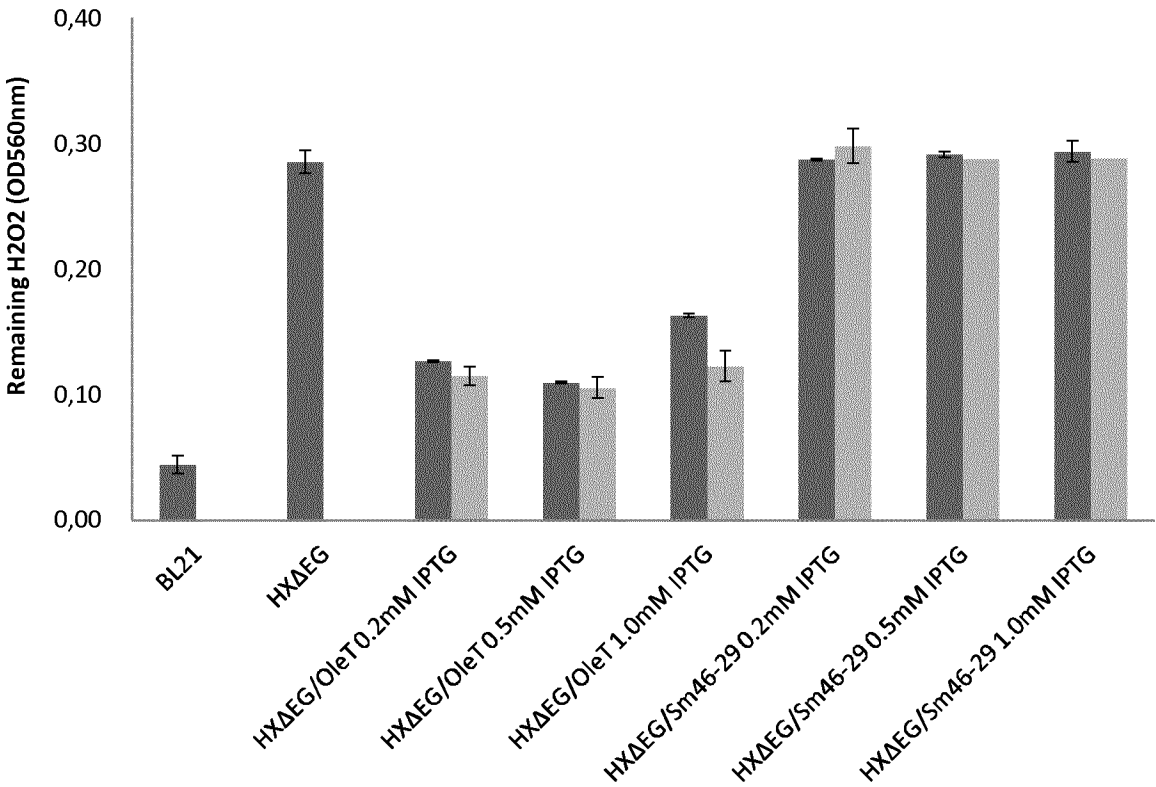


FIG. 4

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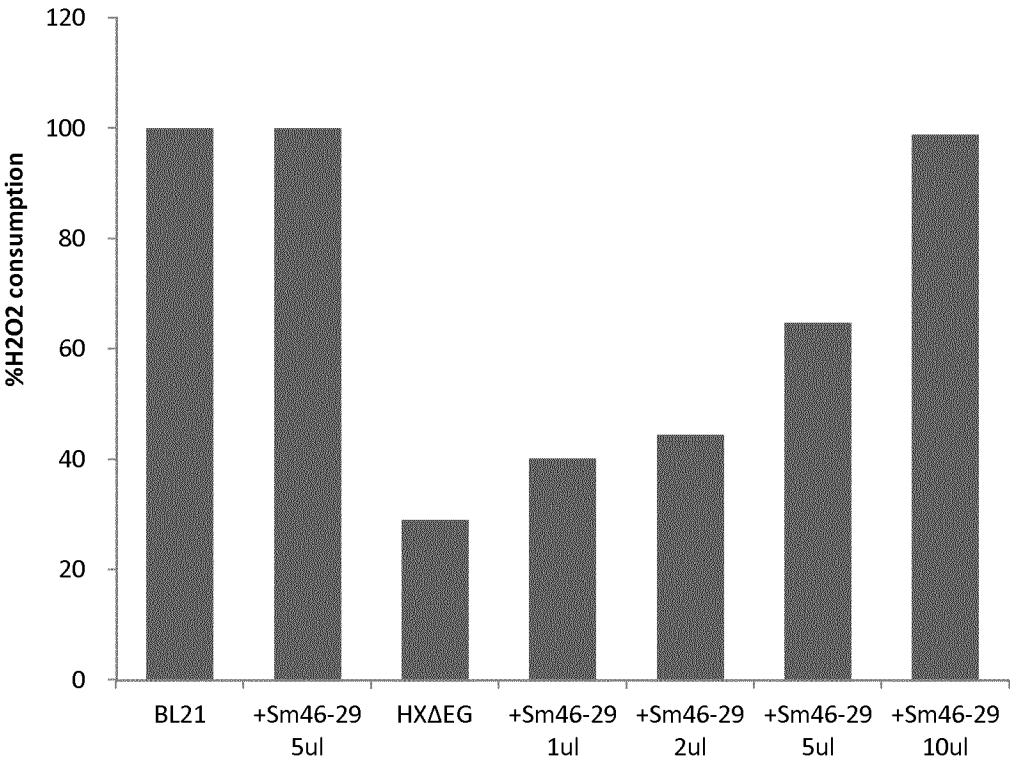


FIG. 5

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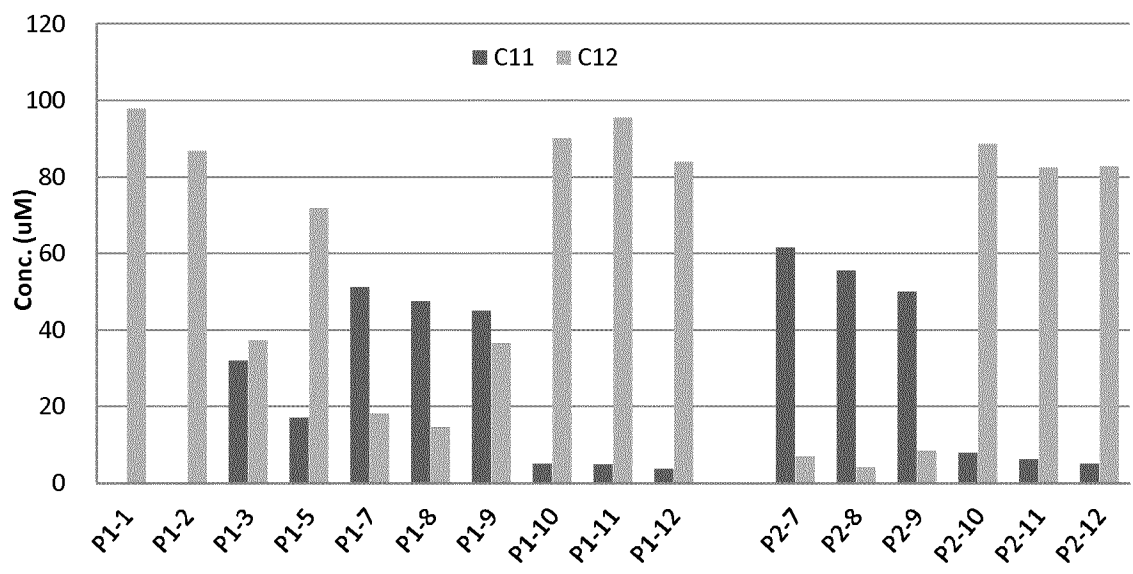


FIG. 6

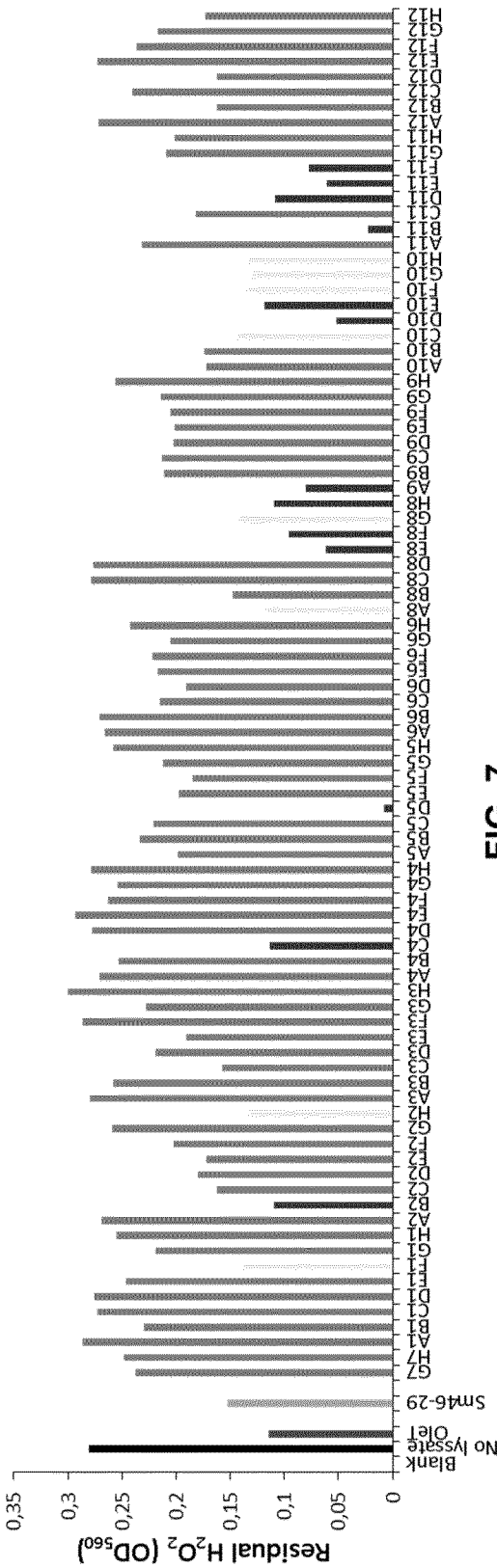


FIG. 7

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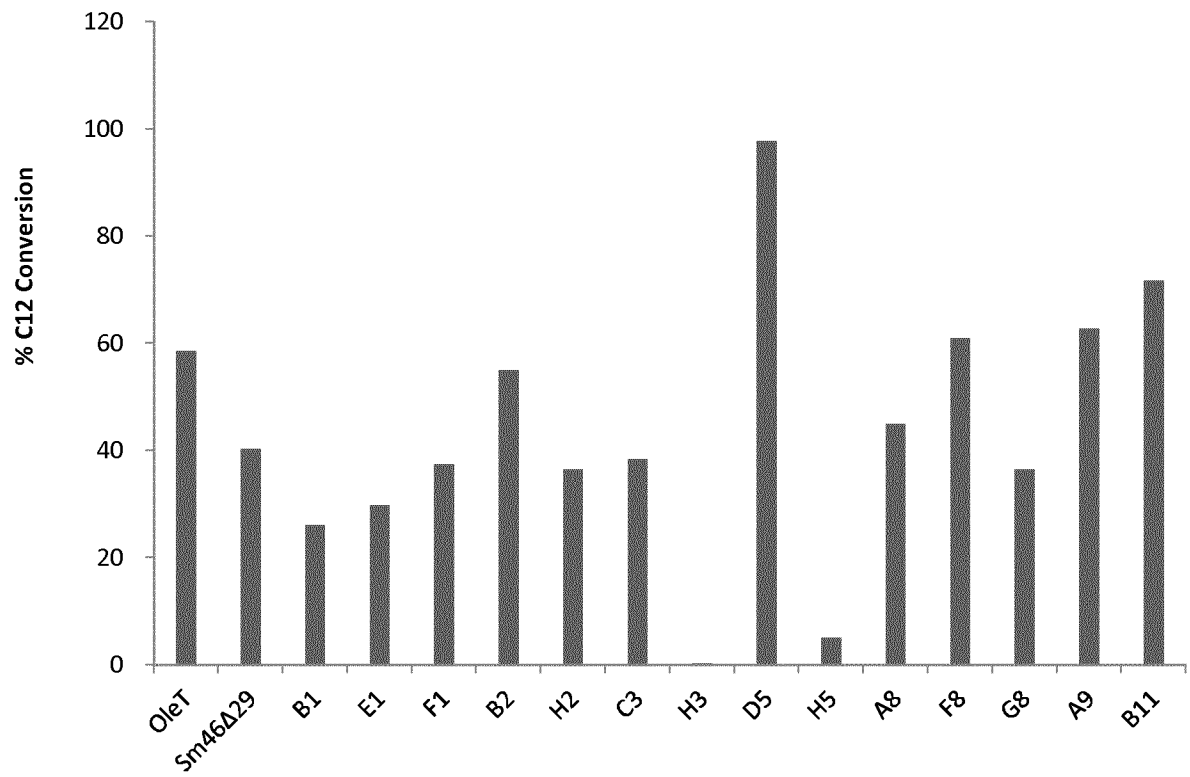


FIG. 8