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(54) **Title:** METHIONINE PRODUCTION

(57) **Abstract:** The present invention relates to a microbial cell capable of producing at least one acetylhomoserine from 4-acetyl-butyric acid and/or ester thereof, wherein the cell is genetically modified to comprise -at least a first genetic mutation that increases the expression relative to the wild type cell of an enzyme E₁ capable of catalysing the hydroxylation of 4-acetyl-butyric acid and/or ester thereof to an alpha-hydroxy acetyl butanone, and -at least a second genetic mutation that increases the expression relative to the wild type cell of enzyme E₂ capable of catalysing the amination of the alpha-hydroxy acetyl butanone to the acetylhomoserine.



WO 2016/184663 A1

METHIONINE PRODUCTION

FIELD OF THE INVENTION

The present invention relates to a biotechnological method for producing methionine. In particular, the method may use carbon as the starting material.

BACKGROUND OF THE INVENTION

Amino acids are especially useful as additives in animal feed and as nutritional supplements for human beings. They can also be used in infusion solutions and may function as synthetic intermediates for the manufacture of pharmaceuticals and agricultural chemicals. Compounds such as cysteine, homocysteine, methionine and S-adenosylmethionine are usually industrially produced to be used as food or feed additives and also in pharmaceuticals. In particular, methionine, an essential amino acid, which cannot be synthesized by animals, plays an important role in many body functions. D,L-methionine is presently being produced by chemical synthesis from hydrogen cyanide, acrolein and methyl mercaptan. These petroleum based starting materials such as acrolein and methyl mercaptan are obtained by cracking gasoline or petroleum which is bad for the environment. Also, since the costs for these starting materials will be linked to the price of petroleum, with the expected increase in petroleum prices in the future, prices of methionine will also increase relative to the increase in the petroleum prices.

There are several chemical means of producing methionine. In one example, 3-methylthiopropional is used as a raw material with hydrocyanic acid in the presence of a base. The reaction results in ammonium carbonate, which is then hydrolysed. In this method, carbon dioxide is introduced into the reaction liquid after hydrolysis, whereby crystallization occurs and methionine is separated as a crystal. Carbon dioxide and hydrogen are used as raw materials for producing methionine using this method. However, a large amount of hydrogen is left over, making this method inefficient.

With the increasing methionine demand, thus microbial production of methionine is always an attractive alternative.

The pathway for L-methionine synthesis is well known in many microorganisms (Figge RM (2006)). *E. coli* and *C. glutamicum* methionine producer strains have also been described in patent applications WO2005/111202, WO2007/077041, WO2007/012078 and WO2007/135188.

Methionine produced by fermentation needs to be purified from the fermentation broth. Cost-efficient purification of methionine relies on producer strains and production processes that minimize the amount of by-products in the fermentation broth. Further, most of these biotechnological methods of producing methionine use nutrients including, but not limited to, carbohydrate sources, e.g., sugars, such as glucose, fructose, or sucrose, hydrolyzed starch, nitrogen sources, e.g., ammonia, and sulfur sources e.g., sulfate and/or thiosulfate, together with other necessary or supplemental media

components as a starting material. This process would yield L-methionine and biomass as a by-product with no toxic dangerous, flammable, unstable, noxious starting materials. However, this is an expensive raw material and the yield too low to consider this method commercially viable.

Accordingly, there is a need in the art for a cheaper and more efficient biotechnological means of producing methionine.

DESCRIPTON OF THE INVENTION

The present invention provides a cell that may be capable of producing homoserines that are essential in the production of methionine. In particular, the cell may be genetically modified to express at least an enzyme E₁ capable of catalysing the hydroxylation of 4-acetyl-butyric acid and/or ester thereof to an alpha-hydroxy acetyl butanoate. The cell may also be used in a method of converting 4-acetyl-butyric acid and/or ester thereof to an alpha-hydroxy acetyl butanoate. More in particular, the cell may be genetically modified to comprise other specific enzymes that are capable of catalysing the conversion of a simple carbon source to acetyl-butyric acid and/or ester and also the conversion of alpha-hydroxy acetyl butanoate to a homoserine. The ability of enzyme E₁ to catalyse the hydroxylation of 4-acetyl-butyric acid and/or ester thereof to an alpha-hydroxy acetyl butanoate, allows for the biotechnological production of homoserine to be more efficient and cost effective. This is because, the carbon source may include CO and/or CO₂ allowing the feedstock to be cheap and carbon yield to be close to 100%.

According to one aspect of the present invention, there is provided a microbial cell capable of producing at least one acetylhomoserine from 4-acetyl-butyric acid and/or ester thereof, wherein the cell is genetically modified to comprise

- at least a first genetic mutation that increases the expression relative to the wild type cell of an enzyme E₁ capable of catalysing the hydroxylation of 4-acetyl-butyric acid and/or ester thereof to an alpha-hydroxy acetyl butanone, and
- at least a second genetic mutation that increases the expression relative to the wild type cell of enzyme E₂ capable of catalysing the amination of the alpha-hydroxy acetyl butanone to the acetylhomoserine.

The starting material for the hydroxylation may be 4-acetyl-butyric acid and/or ester thereof. In particular, the ester of 4-acetyl-butyric acid may be produced in the presence of at least one WS/DGAT enzyme found in the cell according to any aspect of the present invention and/or other esterification processes known in the art.

In one example, the method of esterification may be a chemical process. Any chemical reaction known in the art may be used to convert 4-acetyl-butyric acid to 4- acetoxybutanoate. More in particular, the method involves reacting 4-acetyl-butyric acid with an alcohol. 4-acetyl-butyric acid may be contacted with at least one alcohol to produce a 4- acetoxybutanoate. In particular, the alcohol may be methanol,

ethanol, isopropyl alcohol, and/or butyl alcohol. In particular, the alcohol may be selected from the group consisting of methanol, ethanol, isopropyl alcohol, and/or butyl alcohol to produce methyl, ethyl, propyl or butyl butanoate respectively. This reaction may take place in the presence of at least one catalyst. The catalyst is usually an acidic catalyst such as a sulphonic acid, a base such as an alkali hydroxide or an alkali alcoholate, a metal oxide or a metal alkylate. In particular, the catalyst may be $\text{ZrOCl}_2 \cdot 8\text{H}_2\text{O}$.

In one example, the 4-acetyl-butyric acid reacts with ethanol in the presence of $\text{ZrOCl}_2 \cdot 8\text{H}_2\text{O}$ to produce ethyl 4-acetoxybutanoate.

In another example, the method of esterification may be a biochemical process where at least one enzyme may be involved in catalysing the esterification process. The enzyme for esterification may be selected from the group consisting of a thioesterase enzyme, an acyl-CoA synthetase enzyme, an ester synthase enzyme and a lipase. At least one of these enzymes may be expressed in the cell according to any aspect of the present invention. In one example, the acetogenic bacteria and/or hydrogen oxidising bacteria may overexpress an esterification enzyme that may be capable of esterification of the 4-acetyl-butyric acid. In another example, a further cell expressing an esterification enzyme may be included to esterify the 4-acetyl-butyric acid to 4-acetoxybutanoate.

In particular, the enzymes in the cell according to any aspect of the present invention may comprise at least one of the following enzymes: cytochrome P450 BM3 (CYP102A1) (E_1) and a leucine dehydrogenase or alpha transaminase (E_2). More in particular, the cell according to any aspect of the present invention may be capable of increased expression relative to the wild type cell of cytochrome P450 BM3 (CYP102A1) (E_1) and a leucine dehydrogenase (E_2).

The cell according to any aspect of the present invention, allows for a two-step process to be carried out in a single pot with no product purification/separation of the polar substrate intermediates (α -hydroxy acid, α -keto acid) required between the stages.

The cell according to any aspect of the present invention further comprises at least a third genetic mutation that increases the expression relative to the wild type cell of at least one enzyme (E_5) capable of cofactor regeneration. In particular, E_5 may be capable of NAD(P)H regeneration. E_5 may be any enzyme that may be capable of NAD(P)H regeneration. In particular, E_5 may be a dehydrogenase/ oxidoreductase which uses NAD(P) as electron acceptor (EC 1.1.1.X). More in particular, E_5 may be any enzyme with KEGG no. EC 1.1.1.X in the Brenda database as of 24th February 2014. For example, E_5 may be selected from the group consisting of alcohol dehydrogenase, glycerol phosphate dehydrogenase, histidinol dehydrogenase, shikimate dehydrogenase, lactate dehydrogenase, 3-hydroxyaryl-CoA dehydrogenase, malate dehydrogenase, isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, formate dehydrogenase, horse liver alcohol dehydrogenase, glucose dehydrogenase, amino acid dehydrogenase, sorbitol dehydrogenase, 20- β -hydroxysteroid dehydrogenase and formaldehyde dehydrogenase. Even more in particular, enzyme

(E₅) may be selected from the group consisting of glucose dehydrogenase (E_{5a}) (EC 1.1.99.10), phosphite dehydrogenase (E_{5b}) (EC 1.20.1.1) and formate dehydrogenase (E_{5c}) (EC 1.2.1.43) where glucose, phosphite and formate are used as reducing agents respectively. The presence of enzyme (E₅) allows for cofactor regeneration that enables the process of producing alpha-hydroxy acetyl butanone from 4-acetyl-butyric acid and/or ester thereof to be self-sustaining. No external energy would thus have to be introduced into the system of producing alpha-hydroxy acetyl butanone. Accordingly, the cell according to any aspect of the present invention may be able to generate at least one alpha-hydroxy acetyl butanone from a 4-acetyl-butyric acid and/or ester thereof in the presence of at least enzymes E₁, and/or E₅ without any external energy source needed.

In one example, the glucose dehydrogenase (E_{5a}) may be NADP⁺-specific glucose dehydrogenase. The organism that serves as the source of glucose dehydrogenase (E_{5a}) is not subject to limitation, and may be a microorganism or a higher organism, and microorganisms such as bacteria, fungi, and yeast are suitable. For example, a microorganism of the genus *Bacillus*, in particular *Bacillus megaterium*, may be the source. In another example, the source may be a microorganism belonging to the genus *Cryptococcus*, the genus *Gluconobacter*, or the genus *Saccharomyces*. In particular, a microorganism belonging to the genus *Cryptococcus* may be selected, more in particular, the microorganism may be selected from the group consisting of *Cryptococcus albidus*, *Cryptococcus humicola*, *Cryptococcus terreus*, and *Cryptococcus uniguttulatus*.

In another example, enzyme E₅ may be phosphite dehydrogenase (E_{5b}) or formate dehydrogenase (E_{5c}). The organism that serves as the source of phosphite dehydrogenase (E_{5b}) or formate dehydrogenase (E_{5c}) may not be subject to limitation, and may be a microorganism or a higher organism, and microorganisms such as bacteria, fungi, and yeast are suitable.

In one example, the glucose dehydrogenase (E_{5a}) may be NADP⁺-specific glucose dehydrogenase. The organism that serves as the source of glucose dehydrogenase (E_{5a}) is not subject to limitation, and may be a microorganism or a higher organism, and microorganisms such as bacteria, fungi, and yeast are suitable. For example, a microorganism of the genus *Bacillus*, in particular *Bacillus megaterium*, may be the source. In another example, the source may be a microorganism belonging to the genus *Cryptococcus*, the genus *Gluconobacter*, or the genus *Saccharomyces*. In particular, a microorganism belonging to the genus *Cryptococcus* may be selected, more in particular, the microorganism may be selected from the group consisting of *Cryptococcus albidus*, *Cryptococcus humicola*, *Cryptococcus terreus*, and *Cryptococcus uniguttulatus*.

In another example, enzyme E₅ may be phosphite dehydrogenase (E_{5b}) or formate dehydrogenase (E_{5c}). The organism that serves as the source of phosphite dehydrogenase (E_{5b}) or formate dehydrogenase (E_{5c}) may not be subject to limitation, and may be a microorganism or a higher organism, and microorganisms such as bacteria, fungi, and yeast are suitable.

Another advantage of the cell according to any aspect of the present invention lies in the activity of enzymes E₁ and E₅ being redox-neutral, which saves significant cost of redox equivalents and the reaction equilibrium may be completely shifted towards amino acid production. In particular, enzymes E₁ and E₅ allow for recycling of NAD(P)H co-factor.

The cell according to any aspect of the present invention may refer to a wide range of microbial cells. In particular, the cell may be a prokaryotic or a lower eukaryotic cell selected from the group consisting of *Pseudomonas*, *Corynebacterium*, *Bacillus* and *Escherichia*. In one example, the cell may be *Escherichia coli*. In another example, the cell may be a lower eukaryote, such as a fungus from the group comprising *Saccharomyces*, *Candida*, *Pichia*, *Schizosaccharomyces* and *Yarrowia*, particularly, *Saccharomyces cerevisiae*. The cell may be an isolated cell, in other words a pure culture of a single strain, or may comprise a mixture of at least two strains. Biotechnologically relevant cells are commercially available, for example from the American Type Culture Collection (ATCC) or the German Collection of Microorganisms and Cell Cultures (DSMZ). Particles for keeping and modifying cells are available from the prior art, for example Sambrook/Fritsch/Maniatis (1989).

The cell may be genetically modified to increase the expression relative to the wild type cell of enzyme E₁ capable of catalysing the hydroxylation of the 4-acetyl-butyric acid and/or ester thereof to an alpha-hydroxy acetyl butanone, and/or enzyme E₂ capable of catalysing the oxidation of the alpha-hydroxy acetyl butanone to the acetylhomoserine. E₁ may be cytochrome P450 BM3 (CYP102A1) (E₁), and/or E₂ may be an amino acid dehydrogenase.

The phrase "wild type" as used herein in conjunction with a cell or microorganism may denote a cell with a genome make-up that is in a form as seen naturally in the wild. The term may be applicable for both the whole cell and for individual genes. The term 'wild type' may thus also include cells which have been genetically modified in other aspects (i.e. with regard to one or more genes) but not in relation to the genes of interest. The term "wild type" therefore does not include such cells or such genes where the gene sequences have been altered at least partially by man using recombinant methods. A wild type cell according to any aspect of the present invention thus refers to a cell that has no genetic mutation with respect to the whole genome and/or a particular gene. Therefore, in one example, a wild type cell with respect to enzyme E₁ may refer to a cell that has the natural/ non-altered expression of the enzyme E₁ in the cell. The wild type cell with respect to enzyme E₂, E₃, E₄, E₅, E₆, E₇, E₈, E₉, E₁₀, E₁₁, E₁₂, E₁₃, E₁₄, E₁₅, E₁₆, E₁₇, E₁₈, E₁₉, E₂₀, E₂₁, etc. may be interpreted the same way and may refer to a cell that has the natural/ non-altered expression of the enzyme E₂, E₃, E₄, E₅, E₆, E₇, E₈, E₉, E₁₀, E₁₁, E₁₂, E₁₃, E₁₄, E₁₅, E₁₆, E₁₇, E₁₈, E₁₉, E₂₀, E₂₁, etc. respectively in the cell.

Any of the enzymes used according to any aspect of the present invention, may be an isolated enzyme. In particular, the enzymes used according to any aspect of the present invention may be used in an active state and in the presence of all cofactors, substrates, auxiliary and/or activating polypeptides or factors essential for its activity. The term "isolated", as used herein, means that the enzyme of interest is enriched compared to the cell in which it occurs naturally. The enzyme may be

enriched by SDS polyacrylamide electrophoresis and/or activity assays. For example, the enzyme of interest may constitute more than 5, 10, 20, 50, 75, 80, 85, 90, 95 or 99 percent of all the polypeptides present in the preparation as judged by visual inspection of a polyacrylamide gel following staining with Coomassie blue dye.

The cell and/or enzyme used according to any aspect of the present invention may be recombinant. The term "recombinant" as used herein, refers to a molecule or is encoded by such a molecule, particularly a polypeptide or nucleic acid that, as such, does not occur naturally but is the result of genetic engineering or refers to a cell that comprises a recombinant molecule. For example, a nucleic acid molecule is recombinant if it comprises a promoter functionally linked to a sequence encoding a catalytically active polypeptide and the promoter has been engineered such that the catalytically active polypeptide is overexpressed relative to the level of the polypeptide in the corresponding wild type cell that comprises the original unaltered nucleic acid molecule.

The phrase "increased activity of an enzyme", as used herein is to be understood as increased intracellular activity. Basically, an increase in enzymatic activity can be achieved by increasing the copy number of the gene sequence or gene sequences that code for the enzyme, using a strong promoter or employing a gene or allele that codes for a corresponding enzyme with increased activity, altering the codon utilization of the gene, increasing the half-life of the mRNA or of the enzyme in various ways, modifying the regulation of the expression of the gene and optionally by combining these measures. Genetically modified cells used according to any aspect of the present invention are for example produced by transformation, transduction, conjugation or a combination of these methods with a vector that contains the desired gene, an allele of this gene or parts thereof and a vector that makes expression of the gene possible. Heterologous expression is in particular achieved by integration of the gene or of the alleles in the chromosome of the cell or an extrachromosomally replicating vector. An increase in expression of an enzyme is synonymous with overexpression of the respective enzyme.

In the same context, the phrase "decreased activity of an enzyme E_x " used with reference to any aspect of the present invention may be understood as meaning an activity decreased by a factor of at least 0.5, particularly of at least 0.1, more particularly of at least 0.01, even more particularly of at least 0.001 and most particularly of at least 0.0001. The phrase "decreased activity" also comprises no detectable activity ("activity of zero"). The decrease in the activity of a certain enzyme can be effected, for example, by selective mutation or by other measures known to the person skilled in the art for decreasing the activity of a certain enzyme. In particular, the person skilled in the art finds instructions for the modification and decrease of protein expression and concomitant lowering of enzyme activity by means of interrupting specific genes, for example at least in Dubeau *et al.* 2009. Singh & Röhm. 2008., Lee *et al.*, 2009 and the like. The decrease in the enzymatic activity in a cell according to any aspect of the present invention may be achieved by modification of a gene comprising one of the nucleic acid sequences, wherein the modification is selected from the group comprising, consisting of, insertion of foreign DNA in the gene, deletion of at least parts of the gene, point mutations in the gene

sequence, RNA interference (siRNA), *antisense* RNA or modification (insertion, deletion or point mutations) of regulatory sequences, such as, for example, promoters and terminators or of ribosome binding sites, which flank the gene.

Whether or not a nucleic acid molecule, polypeptide, more specifically an enzyme used according to any aspect of the present invention, is recombinant or not has not necessarily implications for the level of its expression. However, in one example one or more recombinant nucleic acid molecules, polypeptides or enzymes used according to any aspect of the present invention may be overexpressed. The term "overexpressed", as used herein, means that the respective polypeptide encoded or expressed is expressed at a level higher or at higher activity than would normally be found in the cell under identical conditions in the absence of genetic modifications carried out to increase the expression, for example in the respective wild type cell. The person skilled in the art is familiar with numerous ways to bring about overexpression. For example, the nucleic acid molecule to be overexpressed or encoding the polypeptide or enzyme to be overexpressed may be placed under the control of a strong inducible promoter such as the lac promoter. The state of the art describes standard plasmids that may be used for this purpose, for example the pET system of vectors exemplified by pET-3a (commercially available from Novagen). Whether or not a nucleic acid or polypeptide is overexpressed may be determined by way of quantitative PCR reaction in the case of a nucleic acid molecule, SDS polyacrylamide electrophoreses, Western blotting or comparative activity assays in the case of a polypeptide. Genetic modifications may be directed to transcriptional, translational, and/or post-translational modifications that result in a change of enzyme activity and/or selectivity under selected and/or identified culture conditions. Thus, in various examples of the present invention, to function more efficiently, a microorganism may comprise one or more gene deletions. Gene deletions may be accomplished by mutational gene deletion approaches, and/or starting with a mutant strain having reduced or no expression of one or more of these enzymes, and/or other methods known to those skilled in the art.

E₁, the cytochrome P450 BM3 (CYP102A1), may lead to double oxidation of 4-acetyl-butyric acid and/or ester thereof to form the equivalent ketone, alpha-hydroxy acetyl butanone. In one example, E₁ may comprise an amino acid sequence that has 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100% sequence identity to SEQ ID NO:1. More in particular, E₁ may comprise mutations in at least one amino acid. In one example, E₁ may comprise point mutations at least on amino acids at positions 47, 51 and/or 401. In particular, the sequence of E₁ may comprise point mutations R47S, Y51W, I401M.

In one example, O₂ may be used as an oxidant.

In another example, the 4-acetyl-butyric acid may first be esterified by simple methods known in the art to form 4-acetyl-butanoate. 4-acetyl-butanoate (also known as 4-acetyl-butyrate) may be used as the substrate for hydroxylation to form the respective ketone, alpha-hydroxy acetyl butanone.

In one example, the oxidation of 4-acetyl-butyric acid and/or ester thereof to form the alpha-hydroxy acetyl butanone is carried out in 2 steps and catalysed by two different enzymes. In this example, E₃ and E₄ carry out the activity of E₁. In particular, E₃ may catalyse the hydroxylation of 4-acetyl-butyric acid and/or ester thereof to an alpha-hydroxy acetyl butanoate, and E₄ may catalyse the oxidation of the alpha-hydroxy acetyl butanoate and/or ester to the alpha-hydroxy acetyl butanone. In this example, therefore the cell according to any aspect of the present invention may be genetically modified to only increase the expression relative to the wild type cell of two enzymes: E₃ and E₄.

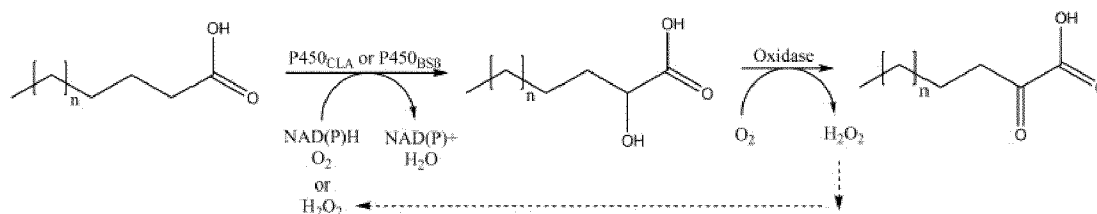
According to another aspect of the present invention, there is provided a microbial cell capable of producing at least one acetylhomoserine from 4-acetyl-butyric acid and/or ester thereof, wherein the cell is genetically modified to comprise

- at least a first genetic mutation that increases the expression relative to the wild type cell of an enzyme E₃ capable of catalysing the hydroxylation of 4-acetyl-butyric acid and/or ester thereof to an alpha-hydroxy acetyl butanoate,
- at least a second genetic mutation that increases the expression relative to the wild type cell of an enzyme E₄ capable of catalysing the oxidation of the alpha-hydroxy acetyl butanoate and/or ester to an alpha-hydroxy acetyl butanone, and
- at least a further genetic mutation that increases the expression relative to the wild type cell of enzyme E₂ capable of catalysing the amination of the alpha-hydroxy acetyl butanone to the acetylhomoserine.

E₃ may be any alpha monooxygenase used according to any aspect of the present invention may be an enzyme capable of catalyzing the alpha-hydroxylation of 4-acetyl-butyric acid and/or ester thereof. In particular, E₃ may be an oxidase selected from the group consisting of rubredoxin-dependent alkane oxidases (EC 1.14.15.3), cytochrome P450 alkane oxidases (EC 1.14.14.1), xylene monooxygenases (EC 1.14.15.-), methane monooxygenases (EC 1.14.13.25), lipooxygenase (EC 1.13.11.-), hydratase (EC 4.2.1.-), 12-hydroxylase (EC 1.14.13.-), diol synthase (EC 1.13.11.- and EC 1.13.12.-), and variants thereof. In particular, the monooxygenase may be capable of oxidising the 4-acetyl-butyric acid and/or ester thereof to an alpha-hydroxy acetyl butanoate. In particular, the monooxygenase may be a cytochrome P450 enzyme. More in particular, the cytochrome P450 enzyme may be P450_{Cla} or P450_{BSB} (EC 1.11.2.4).

In one example, E₁ and/or E₃ may be a cytochrome P450 enzyme that comes in contact with H₂O₂ according to any aspect of the present invention. The application of H₂O₂ and stability of the P450 enzyme in presence of peroxide may be improved with use of an in-situ H₂O₂ producing system. An example of the in-situ H₂O₂ producing system may involve the controlled formation of peroxide in the reaction vessel. In particular, controlled formation of peroxide in the reaction vessel may be carried out using a sensor-controlled electrochemical generation of H₂O₂ (Getrey et al., 2014 and Kreig et al., 2011). In another example, E₁ and/or E₃ may be capable of catalysing O₂-dependent hydroxylation where O₂ may be the sole oxidant.

According to any aspect of the present invention, the dehydrogenase (E_4) may be capable of catalysing the conversion/ oxidation of α -hydroxy acetyl butanoate and/or ester to an α -hydroxy acetyl butanone. In particular, E_4 may be an α -hydroxyacid dehydrogenase (EC 1.1.3.15). More in particular, E_4 may be 2-hydroxyisocaproate dehydrogenase (EC 1.1.1.-) and/or lactate dehydrogenase (EC 1.1.1.28). Even more in particular, the dehydrogenase used according to any aspect of the present invention may comprise an amino acid sequence that has 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100% sequence identity to SEQ ID NO: 3 and/or SEQ ID NO: 5. In another example, E_4 may be an α -hydroxy acid oxidase (EC 1.1.3.15), which can catalyse the reaction using O_2 as oxidant (producing H_2O_2). This would be advantageous for the oxidation of α -hydroxyacids 1a-d (scheme 1), as it is irreversible and produces H_2O_2 (Malca et al., 2011) using P_{450} catalysts (Scheme 1). In particular, E_4 may be an FMN-dependent 2-hydroxyacid oxidase (EC 1.1.3.15). More in particular, E_4 may be selected from the group consisting of lactate oxidase (EC 1.1.3.2), (S)-2-hydroxy-acid oxidase (EC 1.1.3.15) and D- or L-mandelate oxidase [EC 1.1.3.X].



Scheme 1: Irreversible double oxidation of fatty acids employing a P_{450} monooxygenase and a hydroxyacid oxidase to produce α -keto acids in a one pot reaction. H_2O_2 produced by the 2-hydroxy-acid oxidase can be used by the P_{450} catalyst.

In another example, E_4 may be a monooxygenase where O_2 may be used as an oxidant. In another example, E_4 may be a lactate monooxygenase (EC 1.13.12.4) or a P_{450} monooxygenase which may be capable of oxidising α -hydroxy acetyl butanoate and/or ester to an α -hydroxy acetyl butanone.

The teachings of the present invention may not only be carried out using biological macromolecules having the exact amino acid or nucleic acid sequences referred to in this application explicitly, for example by name or accession number, or implicitly, but also using variants of such sequences. The term "variant", as used herein, comprises amino acid or nucleic acid sequences, respectively, that are at least 70, 75, 80, 85, 90, 92, 94, 95, 96, 97, 98 or 99 % identical to the reference amino acid or nucleic acid sequence, wherein preferably amino acids other than those essential for the function, for example the catalytic activity of a protein, or the fold or structure of a molecule are deleted, substituted or replaced by insertions or essential amino acids are replaced in a conservative manner to the effect that the biological activity of the reference sequence or a molecule derived therefrom is preserved. The state of the art comprises algorithms that may be used to align two given nucleic acid or amino acid sequences and to calculate the degree of identity, see Arthur Lesk (2008), Thompson *et al.*, 1994, and Katoh *et al.*, 2005. The term "variant" is used synonymously and interchangeably with the term "homologue". Such variants may be prepared by introducing deletions, insertions or substitutions

in amino acid or nucleic acid sequences as well as fusions comprising such macromolecules or variants thereof. In one example, the term “variant”, with regard to amino acid sequence, comprises, in addition to the above sequence identity, amino acid sequences that comprise one or more conservative amino acid changes with respect to the respective reference or wild type sequence or comprises nucleic acid sequences encoding amino acid sequences that comprise one or more conservative amino acid changes. In one example, the term “variant” of an amino acid sequence or nucleic acid sequence comprises, in addition to the above degree of sequence identity, any active portion and/or fragment of the amino acid sequence or nucleic acid sequence, respectively, or any nucleic acid sequence encoding an active portion and/or fragment of an amino acid sequence. The term “active portion”, as used herein, refers to an amino acid sequence or a nucleic acid sequence, which is less than the full length amino acid sequence or codes for less than the full length amino acid sequence, respectively, wherein the amino acid sequence or the amino acid sequence encoded, respectively retains at least some of its essential biological activity. For example an active portion and/or fragment of a protease may be capable of hydrolysing peptide bonds in polypeptides. The phrase “retains at least some of its essential biological activity”, as used herein, means that the amino acid sequence in question has a biological activity exceeding and distinct from the background activity and the kinetic parameters characterising said activity, more specifically k_{cat} and K_M , are preferably within 3, 2, or 1 order of magnitude of the values displayed by the reference molecule with respect to a specific substrate. Similarly, the term “variant” of a nucleic acid comprises nucleic acids the complementary strand of which hybridises, preferably under stringent conditions, to the reference or wild type nucleic acid. A skilled person would be able to easily determine the monooxygenases that will be capable of making hydroxyl fatty acids.

Stringency of hybridisation reactions is readily determinable by one ordinary skilled in the art, and generally is an empirical calculation dependent on probe length, washing temperature and salt concentration. In general, longer probes require higher temperatures for proper annealing, while shorter probes need lower temperatures. Hybridisation generally depends on the ability of denatured DNA to reanneal to complementary strands when present in an environment below their melting temperature. The higher the degree of desired homology between the probe and hybridisable sequence, the higher the relative temperature which may be used. As a result it follows that higher relative temperatures would tend to make the reaction conditions more stringent, while lower temperature less so. For additional details and explanation of stringency of hybridisation reactions, see F. M. Ausubel (1995). The person skilled in the art may follow the instructions given in the manual “The DIG System Users Guide for Filter Hybridization”, Boehringer Mannheim GmbH, Mannheim, Germany, 1993 and in Liebl *et al.*, 1991 on how to identify DNA sequences by means of hybridisation. In one example, stringent conditions are applied for any hybridisation, i.e. hybridisation occurs only if the probe is 70 % or more identical to the target sequence. Probes having a lower degree of identity with respect to the target sequence may hybridise, but such hybrids are unstable and will be removed in a washing step under stringent conditions, for example by lowering the concentration of salt to 2 x SSC or, optionally and subsequently, to 0,5 x SSC, while the temperature is, in order of increasing preference, approximately 50 °C – 68 °C, approximately 52 °C – 68 °C, approximately 54 °C – 68 °C,

approximately 56 °C – 68 °C, approximately 58 °C – 68 °C, approximately 60 °C – 68 °C, approximately 62 °C – 68 °C, approximately 64 °C – 68 °C, approximately 66 °C – 68 °C. In a particularly preferred embodiment, the temperature is approximately 64 °C – 68 °C or approximately 66 °C – 68 °C. It is possible to adjust the concentration of salt to 0.2 x SSC or even 0.1 x SSC. Polynucleotide fragments having a degree of identity with respect to the reference or wild type sequence of at least 70, 80, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 % may be isolated. The term “homologue” of a nucleic acid sequence, as used herein, refers to any nucleic acid sequence that encodes the same amino acid sequence as the reference nucleic acid sequence, in line with the degeneracy of the genetic code.

E₂ used according to any aspect of the present invention may be an amino acid dehydrogenase. In particular, the amino acid dehydrogenase may be capable of catalysing the conversion of alpha-hydroxy acetyl butanone, ammonia and NADH to acetylhomoserine, water and NAD⁺. The amino acid dehydrogenase may be an alanine dehydrogenase, i.e. an enzyme capable of catalysing the conversion of pyruvate, ammonia and NADH to L-alanine, water and NAD⁺. Examples of suitable amino acid dehydrogenases comprise alanine dehydrogenases from *Bacillus subtilis* (accession number: L20916), *Rhizobium leguminosarum* (accession number: CP001622), *Vibrio proteolyticus* (accession number: AF070716), *Mycobacterium tuberculosis* (accession number: X63069) and *Enterobacter aerogenes* (accession number AB013821). In another example, E₂ may be leucine dehydrogenase (EC 1.4.1.9) capable of deamination of L-norleucine and L-leucine. More in particular, the leucine dehydrogenase used according to any aspect of the present invention may comprise an amino acid sequence that has 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100% sequence identity to SEQ ID NO:4. In another example, E₂ may be an ω -transaminase. The ω -transaminase may be selected from any of them as described in Hwang and Kim (2004), Kaulmann et al. (2007), and Yun et al. (2005).

A skilled person would be capable of easily measuring the activity of each of the enzymes E₁, E₂, E₃ and E₄. For example, to determine if the expression of E₁ and/or E₃ is increased in a cell, a skilled person may use the assay disclosed in Malca S.H, 2011. For example, to determine if the expression of E₄ is increased in a cell, a skilled person may use the assay disclosed in H. Schütte et al, 1984 and Hummel W., et al., 1985. The expression of E₂ in a cell, whether it is increased or decreased, may be measured using the assay disclosed at least in Abrahamson M.J., 2012. A skilled person would easily be able to identify other well-known methods in the art that may be used for measuring the expression of the enzymes used in the cell of the present invention.

In one example, a source of amine may be added to the cell according to any aspect of the present invention to produce acetylhomoserine from 4-acetyl-butyric acid and/or ester thereof. In particular, the source of amines may be any ammonium salt known in the art. More in particular, the amine may be selected from the group consisting of ammonium formate, ammonium chloride, ammonium sulphate and the like. In one example, when E₂ may be an alpha transaminase, the source of amine may be alanine.

The cell according to any aspect of the present invention may have reduced capacity of fatty acid degradation by beta-oxidation relative to the wild type cell. In particular, the reduced fatty acid degradation activity compared to the wild type cell may be a result of decreased expression relative to the wild type cell of at least one enzyme selected from the group consisting of acyl-CoA dehydrogenase (FadE) (E₆) (EC:1.3.99.-), enoyl-CoA hydratase (FadB) (E₇) (EC 4.2.1.17), (*R*)-3-hydroxyacyl-CoA dehydrogenase (FadB) (E₈) (EC 1.1.1.35) and 3-ketoacyl-CoA thiolase (FadA) (E₉) (EC:2.3.1.16).

The term “having a reduced fatty acid degradation capacity”, as used herein, means that the respective cell degrades fatty acids, in particular those taken up from the environment, at a lower rate than a comparable cell or wild type cell having normal fatty acid degradation capacity would under identical conditions. In one example, the fatty acid degradation of such a cell is lower on account of deletion, inhibition or inactivation of at least one gene encoding an enzyme involved in the β -oxidation pathway. In one example, at least one enzyme involved in the β -oxidation pathway has lost, in order of increasing preference, 5, 10, 20, 40, 50, 75, 90 or 99 % activity relative to the activity of the same enzyme under comparable conditions in the respective wild type microorganism. The person skilled in the art may be familiar with various techniques that may be used to delete a gene encoding an enzyme or reduce the activity of such an enzyme in a cell, for example by exposition of cells to radioactivity followed by accumulation or screening of the resulting mutants, site-directed introduction of point mutations or knock out of a chromosomally integrated gene encoding for an active enzyme, as described in Sambrook/Fritsch/Maniatis (1989). In addition, the transcriptional repressor FadR may be over expressed to the effect that expression of enzymes involved in the β -oxidation pathway is repressed (Fujita, Y., et al, 2007). The phrase “deletion of a gene”, as used herein, means that the nucleic acid sequence encoding said gene is modified such that the expression of active polypeptide encoded by said gene is reduced. For example, the gene may be deleted by removing in-frame a part of the sequence comprising the sequence encoding for the catalytic active centre of the polypeptide. Alternatively, the ribosome binding site may be altered such that the ribosomes no longer translate the corresponding RNA. It would be within the routine skills of the person skilled in the art to measure the activity of enzymes expressed by living cells using standard essays as described in enzymology text books, for example Cornish-Bowden, 1995.

Degradation of fatty acids is accomplished by a sequence of enzymatically catalysed reactions. First of all, fatty acids are taken up and translocated across the cell membrane *via* a transport/acyl-activation mechanism involving at least one outer membrane protein and one inner membrane-associated protein which has fatty acid-CoA ligase activity, referred to in the case of *E. coli* as FadL and FadD / FadK, respectively. Inside the cell, the fatty acid to be degraded is subjected to enzymes catalysing other reactions of the β -oxidation pathway. The first intracellular step involves the conversion of acyl-CoA to enoyl-CoA through acyl-CoA dehydrogenase, the latter referred to as FadE in the case of *E. coli*. The activity of an acyl-CoA dehydrogenase may be assayed as described in the state of art, for example by monitoring the concentration of NADH spectrophotometrically at 340 nm in 100 mM

MOPS, pH 7.4, 0.2 mM Enoyl-CoA, 0.4 mM NAD⁺. The resulting enoyl-CoA is converted to 3-ketoacyl-CoA via 3-hydroxyacyl-CoA through hydration and oxidation, catalysed by enoyl-CoA hydratase/(*R*)-3-hydroxyacyl-CoA dehydrogenase, referred to as FadB and FadJ in *E. coli*. Enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase activity, more specifically formation of the product NADH may be assayed spectrophotometrically as described in the state of the art, for example as outlined for FadE. Finally, 3-ketoacyl-CoA thiolase, FadA and FadI in *E. coli*, catalyses the cleavage of 3-ketoacyl-CoA, to give acetyl-CoA and the input acyl-CoA shortened by two carbon atoms. The activity of ketoacyl-CoA thiolase may be assayed as described in the state of the art, for example in Antonenkov, V., et al, 1997.

The phrase “a cell having a reduced fatty acid degradation capacity”, as used herein, refers to a cell having a reduced capability of taking up and/or degrading fatty acids, particularly those having at least eight carbon chains. The fatty acid degradation capacity of a cell may be reduced in various ways. In particular, the cell according to any aspect of the present invention has, compared to its wild type, a reduced activity of an enzyme involved in the β -oxidation pathway. The term “enzyme involved in the β -oxidation pathway”, as used herein, refers to an enzyme that interacts directly with a fatty acid or a derivative thereof formed as part of the degradation of the fatty acid via the β -oxidation pathway. The β -oxidation pathway comprises a sequence of reactions effecting the conversion of a fatty acid to acetyl-CoA and the CoA ester of the shortened fatty acid. The enzyme involved in the β -oxidation pathway may by recognizing the fatty acid or derivative thereof as a substrate, converts it to a metabolite formed as a part of the β -oxidation pathway. For example, the acyl-CoA dehydrogenase (EC 1.3.99.-) is an enzyme involved in the β -oxidation pathway as it interacts with fatty acid-CoA and converts fatty acid-CoA ester to enoyl-CoA, which is a metabolite formed as part of the β -oxidation. In another example, the term “enzyme involved in the β -oxidation pathway”, as used herein, comprises any polypeptide from the group comprising acyl-CoA dehydrogenase (EC 1.3.99.-), enoyl-CoA hydratase (EC 4.2.1.17), 3-hydroxyacyl-CoA dehydrogenase EC 1.1.1.35) and 3-keto-acyl-CoA thiolase (EC 2.3.1.16). The acyl-CoA synthetase (EC 6.2.1.1) may catalyse the conversion of a fatty acid to the CoA ester of a fatty acid, *i.e.* a molecule, wherein the functional group –OH of the carboxy group is replaced with –S-CoA and introducing the fatty acid into the β -oxidation pathway. For example, the polypeptides FadD and FadK in *E. coli* (accession number: BAA15609.1 and NP_416216.4, respectively) are acyl-CoA dehydrogenases. In one example, the term “acyl-CoA dehydrogenase”, as used herein, may be a polypeptide capable of catalysing the conversion of an acyl-CoA to enoyl-CoA, as part of the β -oxidation pathway. For example, the polypeptide FadE in *E. coli* (accession number: BAA77891.2) may be an acyl-CoA dehydrogenase. The term “enoyl-CoA hydratase”, as used herein, also referred to as 3-hydroxyacyl-CoA dehydrogenase, refers to a polypeptide capable of catalysing the conversion of enoyl-CoA to 3-ketoacyl-CoA through hydration and oxidation, as part of the β -oxidation pathway. For example, the polypeptides FadB and FadJ in *E. coli* (accession numbers: BAE77457.1 and P77399.1, respectively) are enoyl-CoA hydratases. The term “ketoacyl-CoA thiolase”, as used herein, may refer to a polypeptide capable of catalysing the cleaving of 3-ketoacyl-CoA, resulting in an acyl-CoA shortened by two carbon atoms and acetyl-CoA,

as the final step of the β -oxidation pathway. For example, the polypeptides FadA and FadI in *E. coli* (accession number: YP_491599.1 and P76503.1, respectively) are ketoacyl-CoA thiolases.

According to any aspect of the present invention, the cell may comprise a further genetic mutation that increases the expression relative to the wild type cell of an enzyme E₁₀, an alkane oxidase capable of catalysing the oxidation of butylacetate to 4-acetyl-butyric acid and/or ester thereof.

Various alkane oxidases have been described in the literature, for example basidiomycete heme-thiolate peroxidases (Gutierrez, A., 2011, Lo Piccolo, L., 2011, Grant, C., 2011, Koch, D., 2009). In one example, the alkane oxidase may be an alkB type alkane oxidase. AlkB is an oxidoreductase from the *Pseudomonas putida* AlkBGT system, dependent on two auxiliary polypeptides, AlkG and AlkT. AlkT is a FAD-dependent rubredoxin reductase transferring electrons from NADH to AlkG. AlkG is a rubredoxin, an iron-containing redox protein functioning as a direct electron donor to AlkB. In one example, the term "alkB type alkane oxidase", as used herein, refers to AlkB from *Pseudomonas putida* Gpo1 (Access code: CAB54050.1, any access code used in the application refers to the respective sequence from the Genbank database run by the NCBI, wherein the release referred to is the one online on the 6th May, 2015) or a variant thereof.

In particular, the enzyme E₁₀ may be selected from the group consisting of rubredoxin-dependent alkane oxidase (EC 1.14.15.3), cytochrome P450 enzyme (EC 1.14.x.x), xylene monooxygenase (EC:1.14.13.-; EC:1.18.1.3) and methane monooxygenase (EC 1.14.13.25; EC 1.14.18.3).

The term "rubredoxin-dependent alkane oxidase", as used herein refers to an oxidoreductase that recognises as its substrate an alkane receiving electrons *via* a rubredoxin, the latter being, an iron-sulphur protein having an $\alpha+\beta$ class fold with 2 α helices and 2 to 3 β -strands transferring electrons to the alkane oxidase and AlkG from *Pseudomonas putida* or a variant thereof. Examples include AlkG from *Pseudomonas putida*. The term "cytochrome P450 enzyme", as used herein, refers to an oxidoreductase having a P450-type cytochrome having, in its CO-bound form an absorption band at 450 nm and may be capable of oxidizing an alkane. Examples include cytochrome P450 BM-3 from *Bacillus megaterium* (Koch, D. J., 2009.) The term "xylene monooxygenase", as used herein, refers to a membrane-spanning, non-heme diiron enzyme oxidoreductase with a histidine-rich active site and preferably capable of oxidizing an alkane. Examples include XylM from *Pseudomonas putida* (Austin, R. N., 2003). The term "methane monooxygenase", as used herein, refers to an oxidoreductase that is either a soluble methane monooxygenase comprising di-iron center bridged by an oxygen atom (Fe-O-Fe) and comprising three protein components, a hydroxylase, a β unit, and a reductase, from a methanotropic bacterium or is a particulate methane monooxygenase, a membrane-protein in a methanotrophic bacterium, comprising a copper-containing active site. Examples of soluble and particulate methane monooxygenases comprises the soluble methane monooxygenase from *Methylosinus trichosporium* OB3b (A C Rosenzweig, 1993) and the methane monooxygenase from *Methylococcus capsulatus* (Bath) (Nguyen, H. H. T., 1998).

The cell according to any aspect of the present invention may also have increased expression relative to a wild type cell of at least one enzyme that may allow the cell according to any aspect of the present invention to convert carbon sources containing CO and/or CO₂ to butanol and/or acetic acid. In particular, the cell may comprise enzymes that are able to catalyse the acetyl-CoA or Wood-Ljungdahl pathway and the ethanol-carboxylate fermentation pathway to produce butanol and/or acetic acid. The butanol and/or acetic acid may then be esterified by state of the art methods like acid catalysts and/or lipases as known in the art to produce butylacetate.

The phrase "wild type" as used herein in conjunction with a cell or microorganism may denote a cell with a genome make-up that is in a form as seen naturally in the wild. The term may be applicable for both the whole cell and for individual genes. The term "wild type" therefore does not include such cells or such genes where the gene sequences have been altered at least partially by man using recombinant methods.

A skilled person would be able to use any method known in the art to genetically modify a cell or microorganism. According to any aspect of the present invention, the genetically modified cell may be genetically modified so that in a defined time interval, within 2 hours, in particular within 8 hours or 24 hours, it forms at least twice, especially at least 10 times, at least 100 times, at least 1000 times or at least 10000 times more carboxylic acid and/or the respective carboxylic acid ester than the wild-type cell. The increase in product formation can be determined for example by cultivating the cell according to any aspect of the present invention and the wild-type cell each separately under the same conditions (same cell density, same nutrient medium, same culture conditions) for a specified time interval in a suitable nutrient medium and then determining the amount of target product (carboxylic acid) in the nutrient medium.

In another aspect of the present invention, there is provided a method of producing acetylhomoserine from at least one carbon source, the method comprising a step of contacting an enzyme E₁ with 4-acetyl-butyric acid and/or ester thereof, wherein the enzyme E₁ is capable of catalysing the hydroxylation of 4-acetyl-butyric acid and/or ester thereof to an alpha-hydroxy acetyl butanone. In particular, the enzyme E₁ may be cytochrome P450 BM3 (CYP102A1). This step may be a step in any biotechnological method of producing acetylhomoserine, where 4-acetyl-butyric acid and/or ester thereof may be found as a substrate and/or raw material to be hydroxylated.

According to another aspect of the present invention, there is provided a method of producing acetylhomoserine from 4-acetyl-butyric acid and/or ester thereof, the method comprising the step of contacting the cell according to any aspect of the present invention with a medium comprising 4-acetyl-butyric acid and/or ester thereof.

The term "contacting", as used herein, means bringing about direct contact between the of 4-acetyl-butyric acid and/or ester thereof with the enzyme E₁ and/or cell according to any aspect of the present invention in an aqueous solution. For example, the cell and 4-acetyl-butyric acid and/or ester thereof

may be in the same container allowing the enzyme to work on the 4-acetyl-butyric acid and/or ester thereof. If the 4-acetyl-butyric acid and/or ester thereof is soluble and may be taken up by the cell or can diffuse across biological membranes, it may simply be added to the cell according to any aspect of the present invention in an aqueous solution. In case it is insufficiently soluble, it may be solved in a suitable organic solvent prior to addition to the aqueous solution. The person skilled in the art is able to prepare aqueous solutions of 4-acetyl-butyric acid and/or ester thereof having insufficient solubility by adding suitable organic and/or polar solvents. Such solvents may be provided in the form of an organic phase comprising liquid organic solvent. In one example, the organic solvent or phase may be considered liquid when liquid at 25 °C and standard atmospheric pressure.

The term "an aqueous solution" or "medium" comprises any solution comprising water, mainly water as solvent that may be used to keep the cell according to any aspect of the present invention, at least temporarily, in a metabolically active and/or viable state and comprises, if such is necessary, any additional substrates. The person skilled in the art is familiar with the preparation of numerous aqueous solutions, usually referred to as media that may be used to keep inventive cells, for example LB medium in the case of *E. coli*. It is advantageous to use as an aqueous solution a minimal medium, *i.e.* a medium of reasonably simple composition that comprises only the minimal set of salts and nutrients indispensable for keeping the cell in a metabolically active and/or viable state, by contrast to complex mediums, to avoid dispensable contamination of the products with unwanted side products. For example, M9 medium may be used as a minimal medium.

According to a further aspect of the present invention, there is provided a method comprising a step of contacting the cell according to any aspect of the present invention with a medium comprising butylacetate.

According to another aspect of the present invention, there is provided a method of producing acetylhomoserine from a carbon source, the method comprising the steps of:

- (i) contacting a reaction mixture comprising a first and a second microorganism with the carbon source, wherein
 - the first microorganism is an acetogenic microorganism capable of converting the carbon source to acetate and/or ethanol;
 - the second microorganism is capable of carrying out the ethanol carboxylate fermentation pathway and convert the acetate and/or ethanol to form butyric acid; and
 - the first microorganism is further capable of converting the butyric acid to butanol;
- (ii) esterifying the butanol to form butylacetate;
- (iii) contacting the butylacetate with at least one enzyme E₃, an alkane oxidase that is capable of oxidising butylacetate to form 4-acetyl butyric acid and/or ester thereof; and
- (iv) contacting the 4-acetyl butyric acid and/or ester thereof with a cell according to any aspect of the present invention.

In one example, the first and second microorganisms are added simultaneously. In another example, the first and second microorganisms are added consequently. The concentration of the first and second microorganisms may be constantly maintained to keep the reaction progressing. In one example, before the second microorganism is added, the concentration of ethanol and/or acetate is measured to ensure an optimum concentration is reached for the second microorganism. In particular, the acetate and/or ethanol concentration may be at an optimum level for there to be sufficient substrate in the aqueous medium for the second microorganism to form at least butyric acid. A skilled person would be able to easily determine the suitable time to include the second organism into the aqueous medium.

In another example, the first and second microorganisms are added simultaneously. In this example, the second microorganism may not be active (i.e. may remain latent) until a sufficient amount of acetate and/or ethanol is made available by the activity of the first microorganism. The presence of either microorganism in the aqueous medium does not disrupt or hinder the activity and/or efficiency of the other.

An advantage of the present invention may be that much more favorable CO₂/CO mixtures of raw materials can be used. These various sources include natural gas, biogas, coal, oil, plant residues and the like. Another advantage of the method may be the high carbon yield. This is made possible by the return of formed CO₂. Namely, the CO₂ can be reacted in the first stage back to acetic acid. Another advantage may lie in greater flexibility with regard to the fermentation conditions used, as any acetogenic and any microorganism capable of carrying out the ethanol-carboxylate fermentation pathway may be used in combination for the actual production of higher alcohols. Another advantage of the present invention may be that since the second microorganism may function and/or produce an acid from the acetate and/or ethanol in the presence of CO, both the first and second microorganisms may be present in a homogenous mixture for the production of higher alcohols from a carbon source comprising CO. This feature of the second microorganism enables the production of higher alcohols from a carbon source like CO to be a one step process making the process more efficient and the yield greater. Surprisingly, because of this advantage of the second microorganism, the one-step procedure for making higher alcohols may be carried out in a single fermenter without an intermediate separation step. There may also be an increased concentration of the final product using this one step procedure. This is surprising as Baffert C., 2011 and Thauer, R.K., 1973 both teach that hydrogenases were inhibited in the presence of CO.

The term "acetogenic bacteria" as used herein refers to a microorganism which is able to perform the Wood-Ljungdahl pathway and thus is able to convert CO, CO₂ and/or hydrogen to acetate. These microorganisms include microorganisms which in their wild-type form do not have a Wood-Ljungdahl pathway, but have acquired this trait as a result of genetic modification. Such microorganisms include but are not limited to *E. coli* cells. These microorganisms may be also known as carboxydophilic bacteria. Currently, 21 different genera of the acetogenic bacteria are known in the art (Drake et al., 2006), and these may also include some *clostridia* (Drake & Kusel, 2005). These bacteria are able to

use carbon dioxide or carbon monoxide as a carbon source with hydrogen as an energy source (Wood, 1991). Further, alcohols, aldehydes, carboxylic acids as well as numerous hexoses may also be used as a carbon source (Drake et al., 2004). The reductive pathway that leads to the formation of acetate is referred to as acetyl-CoA or Wood-Ljungdahl pathway.

In particular, the acetogenic bacteria may be selected from the group consisting of *Acetoanaerobium notera* (ATCC 35199), *Acetonema longum* (DSM 6540), *Acetobacterium carbinolicum* (DSM 2925), *Acetobacterium malicum* (DSM 4132), *Acetobacterium species no. 446* (Morinaga et al., 1990, J. Biotechnol., Vol. 14, p. 187-194), *Acetobacterium wieringae* (DSM 1911), *Acetobacterium woodii* (DSM 1030), *Alkalibaculum bacchi* (DSM 22112), *Archaeoglobus fulgidus* (DSM 4304), *Blautia producta* (DSM 2950, formerly *Ruminococcus productus*, formerly *Peptostreptococcus productus*), *Butyribacterium methylotrophicum* (DSM 3468), *Clostridium aceticum* (DSM 1496), *Clostridium autoethanogenum* (DSM 10061, DSM 19630 and DSM 23693), *Clostridium carboxidivorans* (DSM 15243), *Clostridium coskatii* (ATCC no. PTA-10522), *Clostridium drakei* (ATCC BA-623), *Clostridium formicoaceticum* (DSM 92), *Clostridium glycolicum* (DSM 1288), *Clostridium ljungdahlii* (DSM 13528), *Clostridium ljungdahlii C-01* (ATCC 55988), *Clostridium ljungdahlii ERI-2* (ATCC 55380), *Clostridium ljungdahlii O-52* (ATCC 55989), *Clostridium mayombeii* (DSM 6539), *Clostridium methoxybenzovorans* (DSM 12182), *Clostridium ragsdalei* (DSM 15248), *Clostridium scatologenes* (DSM 757), *Clostridium species ATCC 29797* (Schmidt et al., 1986, Chem. Eng. Commun., Vol. 45, p. 61-73), *Desulfotomaculum kuznetsovii* (DSM 6115), *Desulfotomaculum thermobezoicum* subsp. *thermosyntrophicum* (DSM 14055), *Eubacterium limosum* (DSM 20543), *Methanosarcina acetivorans C2A* (DSM 2834), *Moorella sp. HUC22-1* (Sakai et al., 2004, Biotechnol. Let., Vol. 29, p. 1607-1612), *Moorella thermoacetica* (DSM 521, formerly *Clostridium thermoaceticum*), *Moorella thermoautotrophica* (DSM 1974), *Oxobacter pfennigii* (DSM 322), *Sporomusa aerivorans* (DSM 13326), *Sporomusa ovata* (DSM 2662), *Sporomusa silvacetica* (DSM 10669), *Sporomusa sphaeroides* (DSM 2875), *Sporomusa termitida* (DSM 4440) and *Thermoanaerobacter kivui* (DSM 2030, formerly *Acetogenium kivui*). More in particular, the strain ATCC BAA-624 of *Clostridium carboxidivorans* may be used. Even more in particular, the bacterial strain labelled "P7" and "P11" of *Clostridium carboxidivorans* as described for example in U.S. 2007/0275447 and U.S. 2008/0057554 may be used.

Another particularly suitable bacterium may be *Clostridium ljungdahlii*. In particular, strains selected from the group consisting of *Clostridium ljungdahlii* PETC, *Clostridium ljungdahlii* ERI2, *Clostridium ljungdahlii* COL and *Clostridium ljungdahlii* O-52 may be used in the conversion of synthesis gas to hexanoic acid. These strains for example are described in WO 98/00558, WO 00/68407, ATCC 49587, ATCC 55988 and ATCC 55989.

The acetogenic bacteria may be used in conjunction with a second microorganism that may be capable of carrying out the ethanol-carboxylate fermentation pathway. In one example, both an acetogenic bacteria and a second microorganism that may be capable of carrying out the ethanol-carboxylate fermentation pathway may be used to produce a butyric acid from the carbon source. The

butyric acid may then be converted to butanol. In one example, a single microorganism may be able to carry out the activity of an acetogenic bacterium and the ethanol-carboxylate fermentation pathway. For example, the bacterium may be *C. Carboxidivorans*.

In one example, the ethanol and/or acetate may be converted to butyric acid in the presence of the second microorganism capable of carrying out the ethanol-carboxylate fermentation pathway. The ethanol-carboxylate fermentation pathway is described in detail at least in Seedorf, H., et al., 2008. In particular, the second organism may be selected from the group consisting of *Clostridium kluyveri*, *C. Carboxidivorans* and the like. These second microorganisms include microorganisms which in their wild-type form do not have an ethanol-carboxylate fermentation pathway, but have acquired this trait as a result of genetic modification. In particular, the second microorganism may be *Clostridium kluyveri*.

In another example, the second microorganism may be a wild type organism that expresses at least one enzyme selected from the group consisting of E₁₁ to E₂₁, wherein E₁₁ is an alcohol dehydrogenase (adh), E₁₂ is an acetaldehyde dehydrogenase (ald), E₁₃ is an acetoacetyl- CoA thiolase (thl), E₁₄ is a 3-hydroxybutyryl-CoA dehydrogenase (hbd), E₁₅ is a 3-hydroxybutyryl-CoA dehydratase (crt), E₁₆ is a butyryl-CoA dehydrogenase (bcd), E₁₇ is an electron transfer flavoprotein subunit (etf), E₁₈ is a coenzyme A transferase (cat), E₁₉ is an acetate kinase (ack), E₂₀ is phosphotransacetylase (pta) and E₂₁ is a transhydrogenase. In particular, the wild type second microorganism according to any aspect of the present invention may express at least E₁₂, E₁₃ and E₁₄. Even more in particular, the wild type second microorganism according to any aspect of the present invention may express at least E₁₄.

In another example, the second microorganism according to any aspect of the present invention may be a genetically modified organism that has increased expression relative to the wild type microorganism of at least one enzyme selected E₁₁ to E₂₁ wherein E₁₁ is an alcohol dehydrogenase (adh), E₁₂ is an acetaldehyde dehydrogenase (ald), E₁₃ is an acetoacetyl-CoA thiolase (thl), E₁₄ is a 3-hydroxybutyryl-CoA dehydrogenase (hbd), E₁₅ is a 3-hydroxybutyryl-CoA dehydratase (crt), E₁₆ is a butyryl-CoA dehydrogenase (bcd), E₁₇ is an electron transfer flavoprotein subunit (etf), E₁₈ is a coenzyme A transferase (cat), E₁₉ is an acetate kinase (ack) E₂₀ is phosphotransacetylase (pta) and E₂₁ is a transhydrogenase. In particular, the genetically modified second microorganism according to any aspect of the present invention may express at least enzymes E₁₂, E₁₃ and E₁₄. Even more in particular, the genetically modified second microorganism according to any aspect of the present invention may express at least E₁₄. The enzymes E₁₁ to E₂₁ may be isolated from *Clostridium kluyveri*. A skilled person may be capable of measuring the activity of each of these enzymes using methods known in the art. In particular, the activity of enzymes E₁₁ and E₁₂ may be measured using the assays taught at least in Hillmer P., 1972, Lurz R., 1979; the activity of enzyme E₁₂ may also be measured using the assay taught in Smith L.T., 1980; the activity of enzymes E₁₃ and E₁₄ may be measured using the assays taught at least in Sliwowski M.X., 1984; the activity of E₁₄ may also be measured using the assay taught in Madan, V.K., 1972; the activity of E₁₅ may also be measured using the assay taught in Bartsch, R.G., 1961; the activity of enzymes E₁₆ and E₁₇ may be measured using the assay

taught in Li, F., 2008; the activity of E₁₇ may also be measured using the assay taught in Chowdhury, 2013; the activity of E₁₈ may be measured using the assay taught in Stadman, 1953; the activity of E₁₉ may be measured using the assay taught in Winzer, K., 1997; the activity of E₂₀ may be measured using the assay taught in Smith L.T., 1976; and the activity of E₂₁ may be measured using the assay taught in Wang S, 2010.

According to any aspect of the present invention, the first and/or second microorganism may be a genetically modified microorganism. The genetically modified cell or microorganism may be genetically different from the wild type cell or microorganism. The genetic difference between the genetically modified microorganism according to any aspect of the present invention and the wild type microorganism may be in the presence of a complete gene, amino acid, nucleotide etc. in the genetically modified microorganism that may be absent in the wild type microorganism. In one example, the genetically modified microorganism according to any aspect of the present invention may comprise enzymes that enable the microorganism to produce at least one carboxylic acid. The wild type microorganism relative to the genetically modified microorganism according to any aspect of the present invention may have none or no detectable activity of the enzymes that enable the genetically modified microorganism to produce at least one carboxylic acid. As used herein, the term 'genetically modified microorganism' may be used interchangeably with the term 'genetically modified cell'. The genetic modification according to any aspect of the present invention may be carried out on the cell of the microorganism.

Even more in particular, according to any aspect of the present invention, the acid is produced in the presence of at least one acetogenic microorganism and *Clostridium kluyveri*. In one example, the acetogenic microorganism may be *Clostridium ljungdahlii* or *Clostridium ragsdalei*.

The term "acetate" as used herein, refers to both acetic acid and salts thereof, which results inevitably, because as known in the art, since the microorganisms work in an aqueous environment, and there is always a balance between salt and acid present.

According to any aspect of the present invention, the carbon source may be synthesis gas. Synthesis gas can for example be produced as a by-product of coal gasification. Accordingly, the microorganism of the mixed culture according to any aspect of the present invention may be capable of converting a substance which is a waste product into a valuable resource. In another example, synthesis gas may be a by-product of gasification of widely available, low-cost agricultural raw materials for use with the mixed culture of the present invention to produce at least one higher alcohol.

There are numerous examples of raw materials that can be converted into synthesis gas, as almost all forms of vegetation can be used for this purpose. In particular, raw materials are selected from the group consisting of perennial grasses such as miscanthus, corn residues, processing waste such as sawdust and the like.

In general, synthesis gas may be obtained in a gasification apparatus of dried biomass, mainly through pyrolysis, partial oxidation and steam reforming, wherein the primary products of the synthesis gas are CO, H₂ and CO₂.

Usually, a portion of the synthesis gas obtained from the gasification process is first processed in order to optimize product yields, and to avoid formation of tar. Cracking of the undesired tar and CO in the synthesis gas may be carried out using lime and/or dolomite. These processes are described in detail in for example, Reed, 1981.

Mixtures of sources can be used as a carbon source.

A skilled person would understand the other conditions necessary to carry out the method according to any aspect of the present invention. In particular, the conditions in the container (e.g. fermenter) may be varied depending on the first and second microorganisms used. The varying of the conditions to be suitable for the optimal functioning of the microorganisms is within the knowledge of a skilled person.

In one example, the method according to any aspect of the present invention may be carried out in an aqueous medium with a pH between 5 and 8, 5.5 and 7. The pressure may be between 1 and 10 bar.

In particular, the aqueous medium may comprise a carbon source comprising CO and/or CO₂. More in particular, the carbon source comprising CO and/or CO₂ is provided to the aqueous medium in a continuous gas flow. Even more in particular, the continuous gas flow comprises synthesis gas. In one example, the gases are part of the same flow/stream. In another example, each gas is a separate flow/stream provided to the aqueous medium. These gases may be divided for example using separate nozzles that open up into the aqueous medium, frits, membranes within the pipe supplying the gas into the aqueous medium and the like.

In particular, the reaction mixture according to any aspect of the present invention (i.e. mixture of the first microorganism- the acetogenic organism, the second microorganism, and the carbon source can be employed in any known bioreactor or fermenter to carry out any aspect of the present invention.

Acetylhomoserine is one of the substrates used in the production of methionine. In particular, the reaction is:



Methanethiol can be chemically synthesised from H₂S and MeOH. O-acetyl-L-homoserine may be produced according to any aspect of the present invention. By combining homoserine with methanethiol, L-methionine may then be produced.

BRIEF DESCRIPTION OF FIGURES

The inventions are further illustrated by the following figures and non-limiting examples from which further embodiments, aspects and advantages of the present invention may be taken.

Figure 1 is LC-MS scan for mass 190.08+1 after conversion of EAB with P450 BM3 variant M2.
a = product peak 1 with mass 191. b = product peak 2 with mass 191.

EXAMPLES

The foregoing describes preferred embodiments, which, as will be understood by those skilled in the art, may be subject to variations or modifications in design, construction or operation without departing from the scope of the claims. These variations, for instance, are intended to be covered by the scope of the claims.

Example 1

Conversion of ethyl 4-acetoxybutanoate with P450 BM3

Ethyl acetoxy-butanoate (EAB) was converted with P450 BM3 variant M2 employing O₂ as oxidant.

General methodology

The 3.1 kb P450 BM3 gene from *Bacillus megaterium* (CYP102A1) was cloned into the 2 kb pET22b-derived pALXtreme-1 a plasmid (Blanusa et al., 2011). For expression of the P450 BM3 monooxygenase (EC 1.14.14.1) the pALXtreme-1, a plasmid, was transformed into chemical competent *E. coli* BL21 Gold (DE3)-derived *E. coli* laqI^{Q1} Gold (DE3) cells. Preparation of chemical competent cells was achieved following a standard protocol generating 108 clones per µξ pUC19 (Inoue et al., 1990). All plasmid preparations were performed using the QIAGEN QIAprep Spin Miniprep Kit (Hilden, Germany). All chemicals used were of analytical grade and purchased from Sigma Aldrich (Steinheim, Germany), abcr (Karlsruhe, Germany) or AppliChem (Darmstadt, Germany). Oligonucleotides used for mutagenesis were obtained from Eurofins MWG Operon (Ebersberg, Germany) and produced at HPLC purity. Enzymes were received from New England Biolabs (Frankfurt, Germany) and Sigma Aldrich (Steinheim, Germany). dNTPs were purchased from Fermentas (St. Leon-Rot, Germany).

For protein activity measurement of the P450 BM3 mutant libraries, frozen pellets of grown expression cultures stored for 4h at -20° C in 96-deep-well plates were resuspended by retro-pipetting in 150 µl 50 mM potassium phosphate buffer (KPi) pH 7.5. Additional 150 µl 50 mM KPi pH 7.5 was added containing 5 mg/ml lysozyme. Plates were incubated for 1 h at 900 rpm and 37°C in an Infors HT Microtron shaker to ensure entire cell lysis. Plates with lysed cells were centrifuged for 15 min at 3200 g and 4° C in an Eppendorf 581 OR centrifuge to spin down cell debris. The clear supernatant was used for assaying monooxygenase activity by measuring NADPH depletion at 340 nm in a Tecan Sunrise MTP reader (Groeding, Austria; Glieder & Meinhld, 2003). 96-well quartz glass MTPs (Hellma, Muellheim, Germany) were used during all measurements with p-xylene since the substrate reacts with most poly-propylene and poly-styrene based materials. A reaction mixture in MTP format

contained 1.5 % DMSO, 200 mM p-xylene, 10 to 50 µl cell lysate and 0.16 mM NADPH in a final volume of 250 µl 50 mM KPi buffer pH 7.5. The amount of lysate had to be adjusted in a way that linear depletion of NADPH could be monitored reliably for 2 min. The reaction was stopped by pipetting 25 µl quenching buffer (4 M urea in 0.1 M NaOH) in the reaction mixture, followed by addition of 20 µl 4-aminoantipyrine (4-AAP) (5 mg/ml) and 20 µl potassium peroxodisulphate (5 mg/ml) for phenolic product quantification (Wong et al., 2005). After 30 min incubation at 750 rpm on an Eppendorf MixMate MTP shaker (Hamburg, Germany) the amount of 2,5-DMP was quantified at 509 nm absorbance in a Tecan Sunrise MTP reader (Wong et al., supra). Mutants for next rounds of saturation were selected based upon best performance for NADPH depletion as well as highest formation of 2,5-DMP. Improved variants of P450 BM3 were sequenced at MWG Eurofins DNA (Ebersberg, Germany) and analyzed using Clone Manager 9 Professional Edition software (Scientific & Educational Software, Cary, NC, USA).

Generation of P450 BM3 mutants

Three amino acid residues were chosen for iterative NNK saturation (R47, Y51, and I401). Amino acid residues R47 and Y51 were simultaneously saturated meanwhile site I401 was saturated as a single position. Oligonucleotides were designed for optimal PCR product yields according to recommendations of user's manual from Stratagene's QuikChange Site-directed mutagenesis Kit (Stratagene, La Jolla, CA, USA). A standard PCR set-up contained 20 ng plasmid DNA, 5 U Phusion DNA polymerase, 1 x Phusion DNA polymerase buffer, 0.2 mM dNTP mix and 0.4 µM of forward and reverse primer (Table 4). All PCRs were performed in thin wall PCR tubes (Saarstedt, Nuembrecht, Germany) and using an Eppendorf Mastercycler proS (Hamburg, Germany). The cycling protocol was performed according to Stratagene's QuikChange Site-directed mutagenesis Kit manual instruction (Stratagene, La Jolla, CA, USA). Temperatures and incubation times during each PCR cycle were adjusted to provider's recommendation for Phusion DNA polymerase (New England Biolabs, Frankfurt, Germany). Annealing temperature during each PCR cycle was adjusted to 55 °C for 30 s. Successful PCR amplifications were verified on 0.8% agarose gel according to a standard protocol (Maniatis et al. 1982). Digestion of template DNA was achieved by addition of 20 U DpnI to 50 µl PCR mixture followed by incubation at 37°C for 3 h. The PCR products were transformed without further purification into chemically competent *E. coli* Gold (DE3) λ q1^{Q1} cells using a heat shock transformation protocol (Hanahan et al., 1991). Recovered cells were plated and incubated overnight at 37° C on LB agar plates containing 100 Mg/ml kanamycin. Obtained colonies were transferred with sterile toothpicks into Greiner BioOne 96-well microtiterplates (MTP) (Frickenhausen, Germany) pre-filled with 100 µl LB media and 100 Mg/ml kanamycin. The plates were incubated for 14 h at 900 rpm and 37° C in an Infors HT Microtron shaker (Bottmingen, Switzerland) followed by addition of 100 µl of 50% glycerol (W/V). All MTPs were stored at -80° C prior to expression of mutant libraries.

Position	Primer name	SEQ ID No:	Primer sequence 5' – 3'

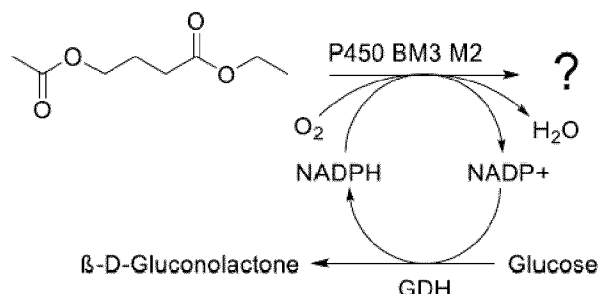
R47/Y51	R47/Y51	6	CGAGGCGCCTGGTNNKGTAACGCGCNNKTTATCAAGTCAGCG
	Fw	7	CGCTGACTTGATAAMNNGCGCGTTACMNNACCAGGCGCCTCG
	R47/Y51		
	Rv		
I401	I401 Fw	8	CAGCGTGCGTGTNNKGGTCAGCAGTTC
	I401 Rw	9	GAACTGCTGACCMNNACACGCACGCTG

Table 4. Oligonucleotides used for NNK saturation of targeted amino acid residues.

Expression and purification of P450 BM3 mutants

The grown culture of the P450 BM3 mutant were centrifuged for 15 min at 2900 g and 4° C in an Eppendorf 5804R centrifuge (Hamburg, Germany) for removal of residual expression media. Obtained pellets were washed in 50 mM KPi pH 7.8 and spin down again applying the same centrifugation program (15 min; 2900 g; 4° C). The supernatant was removed and pellets were stored at -20° C for 12 h. For purification, frozen cell pellets were resuspended in 10 ml 50mM KPi buffer pH 7.8 prior to disruption in an Avestin EmulsiFlex-C3 high-pressure homogenizer (Ottawa, ON, Canada) by applying three cycles of 1500 bar pressure. Cell debris was removed by centrifuging homogenized samples for 30 min at 16000 g and 4° C in a Sorvall RC-6 Plus ultracentrifuge (Thermo Scientific, Rockford, IL, USA). Removal of small insoluble particles was achieved by pressing the supernatant through a 0.45 µm membrane filter. The filtered cell lysate was loaded on a Kronlab TAC15/ 125PE5-AB-2 column (YMC Europe GmbH, Dinslaken, Germany) prefilled with Toyopearl DEAE 650S anion exchange matrix (Tosoh Bioscience, Stuttgart, Germany). An AKTAprime Plus chromatography system with UV-detection (GE Healthcare, Muenchen, Germany) was used for purification and collection of eluted protein samples. A standard protocol was applied to purify P450 BM3 wild-type as well as the variant M2 (R47S/Y51W/I401M) (Schwaneberg et al.1999). Eluted protein samples were collected and purity was estimated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions containing active and approximately 90% pure protein, were pooled in an Amicon Ultra-4 centrifugation tube (30 kDa cutoff; Millipore, Schwalbach, Germany) and concentrated to a final volume of 2 ml. Concentrated protein samples were desalted and equilibrated in 50 mM KPi pH 7.5 buffer using a PD-10 gel-filtration column (GE Healthcare, Muenchen, Germany). Finally enzyme samples were shock-frozen for long time storage in liquid N2 and lyophilized in an Alpha 1 -2 LD plus freeze-dryer (Christ, Osterode am Harz, Germany) for 48 h at -54° C.

The essential NADPH cofactor was regenerated using a glucose dehydrogenase (GDH) and glucose as shown in Scheme 1.



Scheme 1: Conversion of EAB with P450 BM3 variant M2 in presence of a cofactor regeneration system. GDH = glucose dehydrogenase.

Reactions were done in 1 mL scale (KPi-buffer, pH 7.5, 0.1 M) containing following components:

12 U GDH

100 mM glucose

400 μ M NADP+

50 mM EBA (substrate) in DMSO (5 % V/V DMSO final)

50 mg cell free freeze dried lysate of P450 BM3 variant M2 (SEQ ID NO:2)

Control reactions:

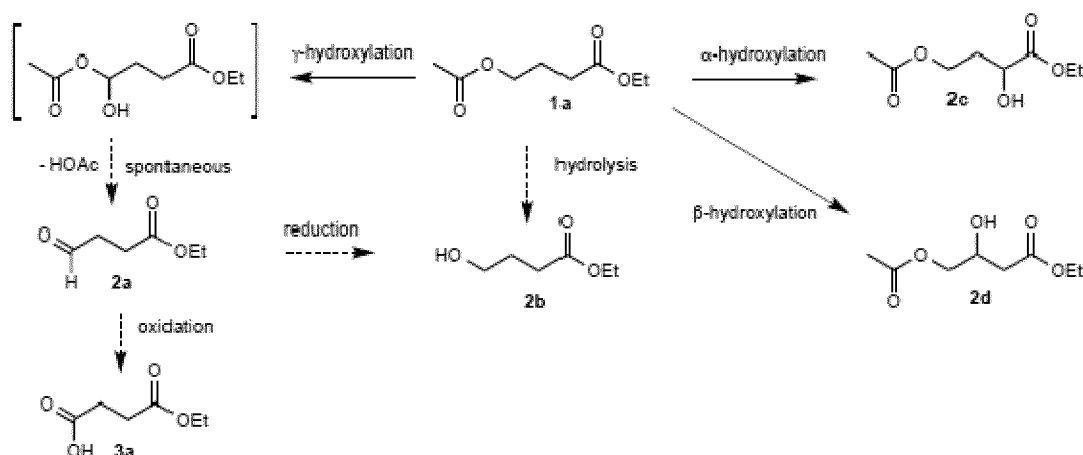
- Empty vector; 50 mg of freeze dried *E. coli* BL21 (DE3) cells containing no P450 enzyme
- Reaction containing no substrate (5 % V/V DMSO supplemented instead)

GC-MS analysis

The product was recovered by liquid-liquid extraction using 500 μ L EtOAc as organic phase. No product could be identified by GC-MS.

LC-MS analysis

LC-MS analysis as formation of unstable (polar) intermediates/products occurred (e.g. hemiacetals), depending on the regioselectivity of the P450 monooxygenase catalyst (Scheme 2).



Scheme 2: Potential products formed during conversion of EAB with P450 BM3.

For analysis, 100 µl of the reaction volume were mixed with 100 µl acetonitrile followed by centrifugation to remove insoluble particles. The supernatant was diluted 1:50 in H₂O/acetonitrile (50:50; V/V) followed by injection into the LC-MS.

To identify potentially formed side-products, LC-MS scans were done for following masses:

- a) 130.06+1 = Product 2a
- b) 146.06+1 = Product 3a
- c) 132.08+1 = Product 2b
- d) 190.08+1 = Product 2c and 2d
- e) 174.09+1 = Substrate (EAB) 1a

Mass-scanning of reaction samples

A mass-scanning was done to identify potential products formed during conversion of EAB with P450 BM3. At 2.96 min retention time a peak was detected that elutes before the substrate (retention time 3.699) indicating formation of a more polar compound. The same product peak was not detected in the control reaction which contains *E. coli* cells without P450 BM3.

Analysis of reaction samples for mass 190.08+1 (target product 2c and 2d)

Detection of mass 190.08+1 revealed formation of two product peaks at 2.9 and 3.0 min that were only present in the reaction samples containing P450 BM3 variant M2 and EAB as substrate (Figure 1). Both peaks were not detectable in the control reactions nor in the substrate as possible contamination. In addition, the product peaks elute earlier than the substrate (3.699 min) indicating a higher polarity possibly due to a hydroxylation of EAB.

Analysis of reaction samples for mass 132.08+1 (Product 2b) – potential hydrolysis of the acetoxylgroup

A large peak signal was obtained for mass 132.06+1 (~20 % area) most likely corresponding to the formation of compound 2b (Scheme 2).

Formation of an unstable hemiacetal by P450 BM3 variant M2 is very unlikely since the same compound was also detected in the EV control reaction and no aldehyde formation occurred (2a, Scheme 1). Accordingly, intrinsic *E. coli* hydrolases were most likely responsible for the catalysing the formation of 2b.

P450 BM3 variant M2 possibly catalyses the formation of target 2c and unwanted side product 2d in rather equal amount.

NMR Analysis

NMR was also carried out on the reaction mixtures. The results also showed that 450 BM3 variant M2 possibly catalyses the formation of target 2c and unwanted side product 2d.

Example 2

For in vivo studies cytochrome P450 BM3 (CYP102A1) of SEQ ID NO:1 was used as E₁, rubredoxin-dependent alkane oxidase, in particular AlkG from *Pseudomonas putida* was used as E₃ and alpha-hydroxyacid dehydrogenase, in particular 2-hydroxyisocaproate dehydrogenase of SEQ ID NO: 3 and SEQ ID NO: 5 were both used as E₄. These enzymes were transferred into *E. coli* W3110. In a first step the E₁ or E₃ and E₄ of SEQ ID: 3 and of SEQ ID NO:5 are cloned into a standard expression vector under the control of a lac promotor (e.g. pUC19 vector). The name of the plasmids are:

- Containing E1 pUC_E1
- Containing E3 and E4_pUC_E3_3_E4
- Containing E3 and E4_5_pUC_E3_5_E4

These plasmids are transferred into *E. coli* by using standard procedures.

The *E. coli* W3110 strain harbouring the plasmid pUC_E1 is named *E. coli* W3110 pUC_E1. The *E. coli* W3110 strain harbouring the plasmid pUC_E3_3_E4 is named *E. coli* W3110 pUC_E3_3_E4. The *E. coli* W3110 strain harbouring the plasmid pUC_E3_5_E4 is named *E. coli* W3110 pUC_E3_5_E4.

The recombinant *E. coli* W3110 strains are cultivated on plate count agar (Merck, Germany) with 100 mg/l ampicillin. For a first preculture 25 mL of LB medium (Merck, Germany) with 100 mg/l ampicillin in a 250 mL shaking flask are inoculated with a single colony from a fresh incubated agar plate and is cultivated at 37°C and 200 rpm for 16 h. For a second preculture 100 mL of HZD medium (1,8 g/L (NH₄)₂SO₄, 19,1 g/L K₂HPO₄, 12,5 g/L KH₂PO₄, 6,7 g/L yeast extract, 2,3 g/L Na₃-Citrat * 2 H₂O, 170 mg/L NH₄Fe-Citrat, 5 mL/L trace elements US3 (80 mL/L 37 %HCl, 1,9 g/L MnCl₂*4H₂O, 1,9 g/L ZnSO₄*7H₂O, 0,9 g/L Na-EDTA*2H₂O, 0,3 g/L H₃BO₃, 0,3 g/L Na₂MoO₄*2H₂O, 4,7 g/L CaCl₂*2H₂O,

17,8 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0,2 g/L $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 30 mL/L HZD-feed (50 g/kg Glucose x H_2O , 10 g/kg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 22 g/kg NH_4Cl)) with 100 mg/l ampicillin in a 1000 mL shaking flask are inoculated with $\text{OD}_{600\text{nm}}$ of 0,1 from the first preculture and cultivated at 37°C and 200 rpm for 8 h.

For the main culture 15 L of fresh HZD medium (pH 6.8) with 15 g/L glucose in a 20 L stirred tank bioreactor are inoculated with cells from the second preculture to an $\text{OD}_{600\text{nm}}$ of 0.1. The fermentation is carried out at 37°C and 30% pO_2 (250 to 1200 rpm with an airflow of 4.2-30 L/min). The pH is held at 6.8 with 25% NH_4OH . When the pO_2 reaches 45%, a feed with 5 g/L *h glucose is started. 4 h before harvest the culture is induced with 1 mM IPTG. After harvest at high $\text{OD}_{600\text{nm}}$ the cells are centrifuged and stored at -20°C.

For the conversion reaction 15 mL assay buffer (pH 7.4, 1.347 g/L KH_2PO_4 , 6.98 g/L K_2HPO_4 , 0.5 g/L NH_4Cl) with 1 g/L EAB as substrate in a 50 mL reaction tube are inoculated with 1.6 g/L washed cells from the frozen stock of the main culture and is incubated at 30°C and 300 rpm in a water bath shaker for 30 h.

At the start and during the incubation period, samples are taken. These are tested for optical density, pH and the different analytes. The determination of the product concentrations is performed by semi quantitative ^1H -NMR spectroscopy. As an internal quantification standard sodium trimethylsilylpropionate (T(M)SP) is used.

During the incubation period of 30 h the concentration of product 2a, 2b, 2c and 2d increases.

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CLAIMS

1. A microbial cell capable of producing at least one acetylhomoserine from 4-acetyl-butyric acid and/or ester thereof, wherein the cell is genetically modified to comprise
 - at least a first genetic mutation that increases the expression relative to the wild type cell of an enzyme E₁ capable of catalysing the hydroxylation of 4-acetyl-butyric acid and/or ester thereof to an alpha-hydroxy acetyl butanone, and
 - at least a second genetic mutation that increases the expression relative to the wild type cell of enzyme E₂ capable of catalysing the amination of the alpha-hydroxy acetyl butanone to the acetylhomoserinewherein enzyme E₁ is cytochrome P450 BM3 (CYP102A1) or the activity of enzyme E₁ is carried out by enzymes E₃ and E₄; and
E₃ is selected from the group consisting of rubredoxin-dependent alkane oxidase (EC 1.14.15.3), cytochrome P450 alkane oxidase (EC 1.14.14.1), xylene monooxygenase (EC 1.14.15.-), methane monooxygenase (EC 1.14.13.25), lipoxygenase (EC 1.13.11.-), hydratase (EC 4.2.1.-), 12-hydroxylase (EC 1.14.13.-), and diol synthase (EC 1.13.11.- and EC 1.13.12.-); and
E₄ is selected from the group consisting of alpha-hydroxyacid dehydrogenase (EC 1.1.3.15) and a monooxygenase; and
E₂ is selected from the group consisting of leucine dehydrogenase and alpha transaminase.
2. The cell according to claim 1, wherein E₁ comprises 60% sequence identity to SEQ ID NO: 1.
3. The cell according to either claim 1 or 2, wherein the cell further comprises at least a third genetic mutation that increases the expression relative to the wild type cell of at least one enzyme (E₅) capable of NAD(P)H regeneration.
4. The cell according to claim 3, wherein the enzyme (E₅) is selected from the group consisting of glucose dehydrogenase, phosphite dehydrogenase and formate dehydrogenase.
5. The cell according to any one of the preceding claims, wherein the cell is genetically modified to comprise at least a further genetic mutation that increases the expression relative to the wild type cell of an enzyme E₁₀, an alkane oxidase capable of catalysing the oxidation of butylacetate to 4-acetyl-butyric acid and/or ester thereof.
6. The cell according to claim 5, wherein enzyme E₁₀ is selected from the group consisting of rubredoxin-dependent alkane oxidase (EC 1.14.15.3), cytochrome P450 enzyme (EC 1.14.x.x), xylene monooxygenase (EC:1.14.13.-; EC:1.18.1.3) and methane monooxygenase (EC 1.14.13.25; EC 1.14.18.3).
7. A method of producing acetylhomoserine from at least one carbon source, the method comprising:

- contacting an enzyme E₁ with 4-acetyl-butyric acid and/or ester thereof, wherein the enzyme E₁ is capable of catalysing the hydroxylation of 4-acetyl-butyric acid and/or ester thereof to an alpha-hydroxy acetyl butanone; and
 - contacting an enzyme E₂ with the alpha-hydroxy acetyl butanone, wherein the enzyme E₂ is capable of catalysing the conversion of alpha-hydroxy acetyl butanone to acetylhomoserine.
8. The method according to claim 7, wherein the enzyme E₁ is cytochrome P450 BM3 (CYP102A1).
9. The method according to claim 7 or 8, wherein the enzyme E₂ is leucine dehydrogenase or alpha transaminase
10. A method of producing acetylhomoserine from 4-acetyl-butyric acid and/or ester thereof, the method comprising:
- contacting the cell according to any one of claims 1 to 4 with a medium comprising 4-acetyl-butyric acid and/or ester thereof.
11. A method of producing acetylhomoserine from butylacetate, the method comprising:
- contacting the cell according to either one of claims 5 or 6 with a medium comprising butylacetate.
12. A method of producing acetylhomoserine from a carbon source, the method comprising the steps of:
- (i) contacting a reaction mixture comprising a first and a second microorganism with the carbon source, wherein
 - the first microorganism is an acetogenic microorganism capable of converting the carbon source to acetate and/or ethanol;
 - the second microorganism is capable of carrying out the ethanol carboxylate fermentation pathway and convert the acetate and/or ethanol to form butyric acid; and
 - the first microorganism is further capable of converting the butyric acid to butanol;
 - (ii) esterifying the butanol to form butylacetate;
 - (iii) contacting the butylacetate with at least one enzyme E₁₀, an alkane oxidase that is capable of oxidising butylacetate to form 4-acetyl butyric acid and/or ester thereof; and
 - (iv) contacting the 4-acetyl butyric acid and/or ester thereof with a cell according to any of the claims 1 to 6.
13. The method according to claim 12, wherein the carbon source comprises CO and/or CO₂.
14. The method according to either claim 12 or 13, wherein wherein
- the first microorganism is selected from the group consisting of *Clostridium autothenogenum* DSMZ 19630, *Clostridium ragsdalei* ATCC no. BAA-622, *Clostridium autoethanogenum*, *Moorella* sp HUC22-1, *Moorella thermoaceticum*, *Moorella thermoautotrophica*, *Rumicoccus*

productus, *Acetoanaerobium*, *Oxobacter pfennigii*, *Methanosarcina barkeri*, *Methanosarcina acetivorans*, *Carboxydotherrmus*, *Desulfotomaculum kutznetsovii*, *Pyrococcus*, *Peptostreptococcus*, *Butyribacterium methylotrophicum* ATCC 33266, *Clostridium formicoaceticum*, *Clostridium butyricum*, *Lactobacillus delbrukii*, *Propionibacterium acidopropionici*, *Propionispora arboris*, *Anaerobierspirillum succiniproducens*, *Bacterioides amylophilus*, *Bacterioides ruminicola*, *Thermoanaerobacter kivui*, *Acetobacterium woodii*, *Acetoanaerobium notera*, *Clostridium aceticum*, *Butyribacterium methylotrophicum*, *Moorella thermoacetica*, *Eubacterium limosum*, *Peptostreptococcus productus*, *Clostridium ljungdahlii*, *Clostridium* ATCC 29797 and *Clostridium carboxidivorans*; and/or

- the second microorganism is selected from the group consisting of *Clostridium kluyveri*, and *C. Carboxidivorans*.

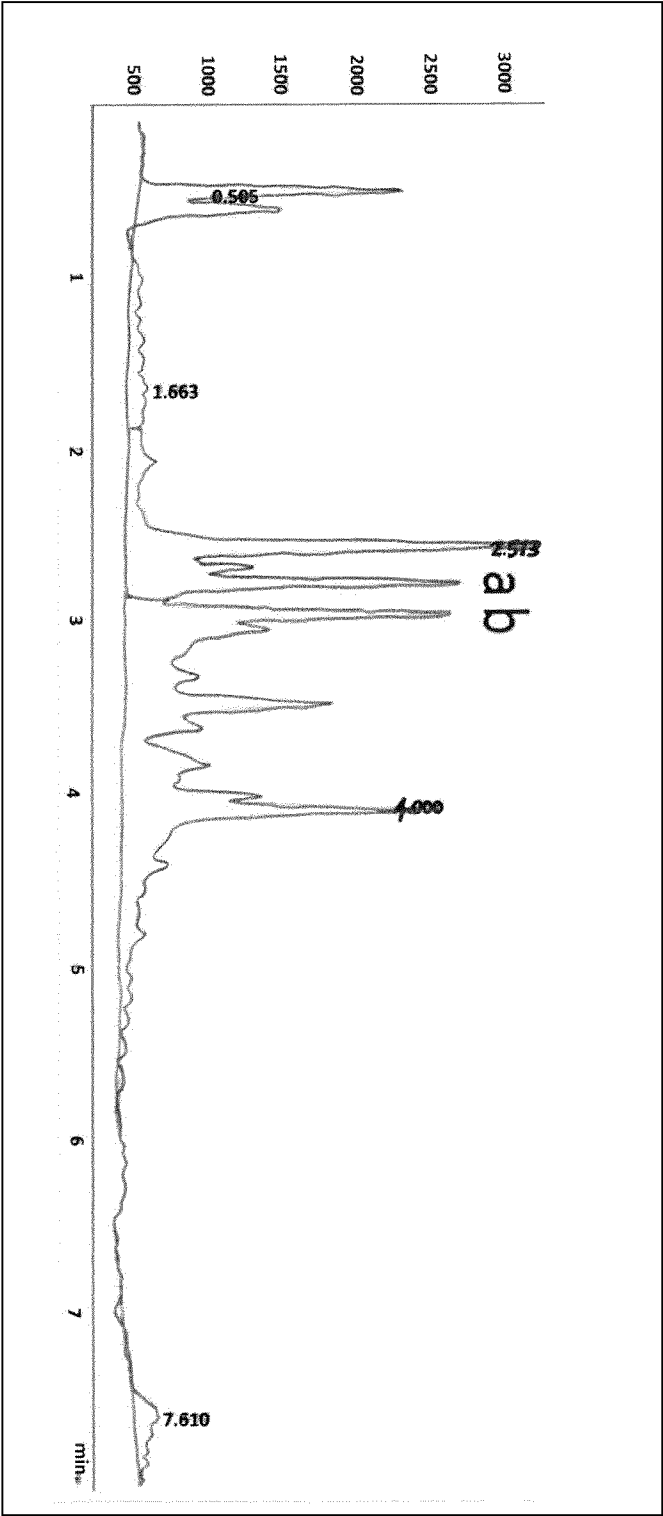


FIGURE 1

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2016/059619

A. CLASSIFICATION OF SUBJECT MATTER
 INV. C12N15/52 C12R1/00
 ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 C12N C12R

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/012078 A1 (BASF AG [DE]; ZELDER OSKAR [DE]; HAEFNER STEFAN [DE]; KLOPPROGGE CORIN) 25 January 2007 (2007-01-25) cited in the application example 1 tables III-VIII	1-14
A	WO 2014/100461 A2 (VERDEZYNE INC [US]) 26 June 2014 (2014-06-26) page 263 SEQ ID NO: 66 -& DATABASE Geneseq Nucleic Acids [Online] 14 August 2014 (2014-08-14), XP002753433, Database accession no. BBJ02596	1-14



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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

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

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

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

6 July 2016

Date of mailing of the international search report

20/07/2016

Name and mailing address of the ISA/

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Barnas, Christoph

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2016/059619

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 1, 3, 5, 7, 10-14(all partially)
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.2

Claims Nos.: 1, 3, 5, 7, 10-14(all partially)

Claims 1, 3, 5, 7 and 12 refer to enzymes capable of catalysing enzymatic reactions as indicated. The wording of said claims covers all kind of enzymes having these capabilities whereas the description provides support and disclosure in the sense of Article 6 and 5 PCT for a limited number of such enzymes. In the present case, the claims so lack support (Art. 6 PCT), and the application so lacks disclosure (Art. 5 PCT). Independent of the above reasoning, the claims also lack clarity (Art. 6 PCT): an attempt is made to define the enzymes by reference to a result to be achieved. Further, from said claims the skilled reader does not know which enzymes are embraced. Said claims are therefore also because of this reason not clear (Art. 6 PCT).

The enzyme E1 was searched and examined as far as relating to cytochrome P450 BM3 (CYP102A1).

The enzyme E2 was searched and examined as far as relating to leucine dehydrogenase or alpha transaminase.

The enzyme E4 was searched and examined as far as relating to alpha-hydroxyacid dehydrogenase (EC 1.1.3.15), a lactate monooxygenase (EC 1.13.12.4) or a P450 monooxygenase.

The enzyme E5 was searched and examined as far as relating to glucose dehydrogenase, phosphite dehydrogenase and formate dehydrogenase.

The enzyme E10 was searched and examined as far as relating to rubredoxin-dependent alkane oxidase (EC 1.14.15.3), cytochrome P450 enzyme (EC 1.14.x.x), xylene monooxygenase (EC: 1.14.13.-; EC: 1.18.1.3) and methane monooxygenase (EC 1.14.13.25; EC 1.14.18.3).

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guidelines C-IV, 7.2), should the problems which led to the Article 17(2) declaration be overcome.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2016/059619

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		JP 2016501040 A	18-01-2016
		SG 11201504837W A	30-07-2015
		WO 2014100461 A2	26-06-2014
