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(54) Title: METHOD FOR PRODUCING ISOBUTENE FROM 3-METHYLCROTONYL-CoA

(57) Abstract: Described is a method for the production of isobutene from 3-methylcrotonyl-CoA comprising the steps of: (a) enzymatically converting 3-methylcrotonyl-CoA into 3-methylbutyric acid; and (b) further enzymatically converting the thus produced 3-methylbutyric acid into isobutene. The conversion of 3-methylcrotonyl-CoA into 3-methylbutyric acid can be achieved by first enzymatically converting 3-methylcrotonyl-CoA into 3-methylbutyryl-CoA and further enzymatically converting the thus produced 3-methylbutyryl-CoA into 3-methylbutyric acid. Alternatively, the conversion of 3-methylcrotonyl-CoA into 3-methylbutyric acid can be achieved by first enzymatically converting 3-methylcrotonyl-CoA into 3-methylcrotonic acid and then further enzymatically converting the thus produced 3-methylcrotonic acid into 3-methylbutyric acid.



Method for producing isobutene from 3-methylcrotonyl-CoA

The present invention relates to a method for the production of isobutene from 3-methylcrotonyl-CoA comprising the steps of:

- (a) enzymatically converting 3-methylcrotonyl-CoA into 3-methylbutyric acid; and
- (b) further enzymatically converting the thus produced 3-methylbutyric acid into isobutene.

The conversion of 3-methylcrotonyl-CoA into 3-methylbutyric acid can be achieved by first enzymatically converting 3-methylcrotonyl-CoA into 3-methylbutyryl-CoA and further enzymatically converting the thus produced 3-methylbutyryl-CoA into 3-methylbutyric acid. Alternatively, the conversion of 3-methylcrotonyl-CoA into 3-methylbutyric acid can be achieved by first enzymatically converting 3-methylcrotonyl-CoA into 3-methylcrotonic acid and then further enzymatically converting the thus produced 3-methylcrotonic acid into 3-methylbutyric acid.

A large number of chemical compounds are currently derived from petrochemicals. Alkenes (such as ethylene, propylene, the different butenes, or else the pentenes, for example) are used in the plastics industry, for example for producing polypropylene or polyethylene, and in other areas of the chemical industry and that of fuels.

Butylene exists in four forms, one of which, isobutene (also referred to as isobutylene), enters into the composition of methyl-tert-butyl-ether (MTBE), an anti-knock additive for automobile fuel. Isobutene can also be used to produce isooctene, which in turn can be reduced to isooctane (2,2,4-trimethylpentane); the very high octane rating of isooctane makes it the best fuel for so-called "gasoline" engines. Alkenes such as isobutene are currently produced by catalytic cracking of petroleum products (or by a derivative of the Fischer-Tropsch process in the case of hexene, from coal or gas). The production costs are therefore tightly linked to the price of oil. Moreover, catalytic cracking is sometimes associated with considerable technical

difficulties which increase process complexity and production costs.

The production by a biological pathway of alkenes such as isobutene is called for in the context of a sustainable industrial operation in harmony with geochemical cycles. The first generation of biofuels consisted in the fermentative production of ethanol, as fermentation and distillation processes already existed in the food processing industry. The production of second generation biofuels is in an exploratory phase, encompassing in particular the production of long chain alcohols (butanol and pentanol), terpenes, linear alkanes and fatty acids. Two recent reviews provide a general overview of research in this field: Ladygina et al. (Process Biochemistry 41 (2006), 1001) and Wackett (Current Opinions in Chemical Biology 21 (2008), 187).

The conversion of isovalerate to isobutene by the yeast *Rhodotorula minuta* has been described (Fujii et al. (Appl. Environ. Microbiol. 54 (1988), 583)), but the efficiency of this reaction, less than 1 millionth per minute, or about 1 for 1000 per day, is far from permitting an industrial application. The reaction mechanism was elucidated by Fukuda et al. (BBRC 201 (1994), 516) and involves a cytochrome P450 enzyme which decarboxylates isovalerate by reduction of an oxoferryl group $\text{Fe}^{\text{V}}=\text{O}$. Large-scale biosynthesis of isobutene by this pathway seems highly unfavorable, since it would require the synthesis and degradation of one molecule of leucine to form one molecule of isobutene and the cytochrome P450 from *Rhodotorula minuta* is a membrane bound protein, poorly lending itself to a recombinant expression in bacteria in a soluble form. For all these reasons, it appears very unlikely that this pathway can serve as a basis for industrial exploitation. Other microorganisms have been described as being marginally capable of naturally producing isobutene from isovalerate; the yields obtained are even lower than those obtained with *Rhodotorula minuta* (Fukuda et al. (Agric. Biol. Chem. 48 (1984), 1679)).

Gogerty et al. (Appl. Environm. Microbiol. 76 (2010), 8004-8010) and van Leeuwen et al. (Appl. Microbiol. Biotechnol. 93 (2012), 1377-1387) describe the production of isobutene from acetoacetyl-CoA by enzymatic conversions wherein the last step of the proposed pathway is the conversion of 3-hydroxy-3-methylbutyric acid (also referred to as 3-hydroxyisovalerate (HIV)) by making use of a mevalonate diphosphate decarboxylase. This reaction for the production of isobutene from 3-hydroxy-3-methylbutyric acid is also described in WO2010/001078. In Gogerty et al. (loc. cit.) and in van Leeuwen et al. (loc. cit.) the production of 3-hydroxy-3-

methylbutyric acid is proposed to be achieved by the conversion of 3-methylcrotonyl-CoA via 3-hydroxy-3-methylbutyryl-CoA. Van Leeuwen et al. (loc cit.) also describe alternative routes for producing isobutene from renewable resources, one of which comprises the conversion of isovalerate (3-methylbutyrate) into isobutene wherein the isovalerate is provided by a hydrolysis reaction from isovaleryl-CoA. The isovaleryl-CoA itself is proposed to be produced from 2-oxoisocaproate and CoA by making use of a 4-methyl-2-oxopentanoate:NAD⁺ oxidoreductase. In order to further improve the efficiency and variability of methods for producing isobutene from renewable resources, there is a need for alternative routes for the provision of precursors which can be used as a substrate for the enzymatic conversion into isobutene.

The present invention meets this demand by providing a method for the production of isobutene from 3-methylcrotonyl-CoA comprising the steps of:

- (a) enzymatically converting 3-methylcrotonyl-CoA into 3-methylbutyric acid; and
- (b) further enzymatically converting the thus produced 3-methylbutyric acid into isobutene.

The conversion of 3-methylcrotonyl-CoA into 3-methylbutyric acid (isovalerate) according to step (a) provides an alternative route to that proposed in the prior art which converts 2-oxoisocaproate via 3-methylbutyryl-CoA (isovaleryl-CoA) into 3-methylbutyric acid (isovalerate).

According to the present invention the conversion of 3-methylcrotonyl-CoA into 3-methylbutyric acid can be achieved via different enzymatic routes. One possibility is to first convert 3-methylcrotonyl-CoA into 3-methylbutyryl-CoA and then to further convert 3-methylbutyryl-CoA into 3-methylbutyric acid. Another possibility is to first convert 3-methylcrotonyl-CoA into 3-methylcrotonic acid and then to further convert 3-methylcrotonic acid into 3-methylbutyric acid.

Thus, in one preferred embodiment the conversion of 3-methylcrotonyl-CoA into 3-methylbutyric acid is achieved by first converting 3-methylcrotonyl-CoA into 3-methylbutyryl-CoA and then to further convert 3-methylbutyryl-CoA into 3-methylbutyric acid. Accordingly, the present invention relates to a method for the production of isobutene from 3-methylcrotonyl-CoA comprising the steps of:

- (a) enzymatically converting 3-methylcrotonyl-CoA into 3-methylbutyric acid; and
- (b) further enzymatically converting the thus produced 3-methylbutyric acid into isobutene,

wherein the enzymatic conversion of 3-methylcrotonyl-CoA into 3-methylbutyric acid according to step (a) is achieved by a method comprising the following steps:

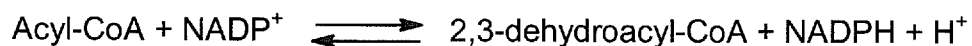
- (a1) enzymatically converting 3-methylcrotonyl-CoA into 3-methylbutyryl-CoA; and
- (a2) further enzymatically converting the thus produced 3-methylbutyryl-CoA into 3-methylbutyric acid.

The enzymatic conversion of 3-methylcrotonyl-CoA into 3-methylbutyryl-CoA according to step (a1), i.e. the reduction of the double bond in 3-methylcrotonyl-CoA, can, for example, be achieved by employing an enzyme classified as EC 1.3.____. Enzymes classified as EC 1.3.____ are oxidoreductases acting on the CH-CH group of a donor molecule. This subclass contains enzymes that reversibly catalyze the conversion of a carbon-carbon single bond to a carbon-carbon double bond between two carbon atoms. Sub-classes of EC 1.3 are classified depending on the acceptor. In one preferred embodiment the enzyme is an enzyme which is classified as EC 1.3.____ and which uses NADH or NADPH as co-factor.

In one particularly preferred embodiment the enzyme is an enzyme which uses NADPH as a co-factor. The conversion using such an enzyme is schematically shown in Figure 1. In a preferred embodiment the enzyme is selected from the group consisting of:

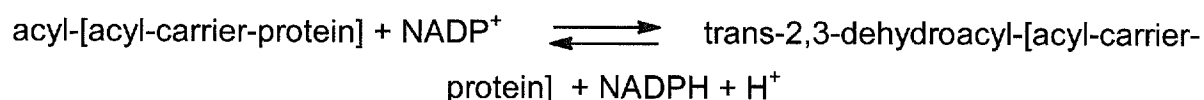
- acyl-CoA dehydrogenase (NADP+) (EC 1.3.1.8);
- enoyl-[acyl-carrier-protein] reductase (NADPH, Si-specific) (EC 1.3.1.10);
- cis-2-enoyl-CoA reductase (NADPH) (EC 1.3.1.37);
- trans-2-enoyl-CoA reductase (NADPH) (EC 1.3.1.38);
- enoyl-[acyl-carrier-protein] reductase (NADPH, Re-specific) (EC 1.3.1.39); and
- crotonyl-CoA reductase (EC 1.3.1.86).

Thus, in one preferred embodiment the conversion of 3-methylcrotonyl-CoA into 3-methylbutyryl-CoA is achieved by making use of an acyl-CoA dehydrogenase (NADP+) (EC 1.3.1.8). Acyl-CoA dehydrogenases are enzymes which catalyze the following reaction:



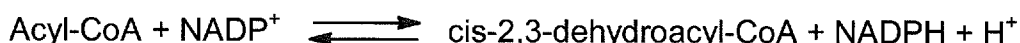
This enzyme occurs in a variety of organism, including eukaryotic and prokaryotic organisms, such as plants, animals, fungi and bacteria. The enzyme has, e.g., been described in *Bos taurus*, *Rattus norvegicus*, *Mus musculus*, *Columba sp.*, *Arabidopsis thaliana*, *Nicotiana benthamiana*, *Allium ampeloprasum*, *Euglena gracilis*, *Candida albicans*, *Streptococcus collinus*, *Rhodobacter sphaeroides* and *Mycobacterium smegmatis*.

In a further preferred embodiment the conversion of 3-methylcrotonyl-CoA into 3-methylbutyryl-CoA is achieved by making use of an enoyl-[acyl-carrier-protein] reductase (NADPH, Si-specific) (EC 1.3.1.10). Enoyl-[acyl-carrier-protein] reductases (NADPH, Si-specific) are enzymes which catalyze the following reaction:



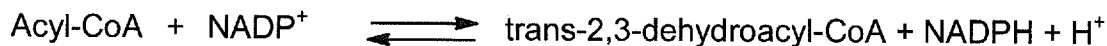
This enzyme occurs in a variety of organism, including eukaryotic and prokaryotic organisms, such as plants, fungi and bacteria. The enzyme has, e.g., been described in *Carthamus tinctorius*, *Candida tropicalis*, *Saccharomyces cerevisiae*, *Streptococcus collinus*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Porphyromonas gingivalis*, *Escherichia coli* and *Salmonella enterica*.

In a further preferred embodiment the conversion of 3-methylcrotonyl-CoA into 3-methylbutyryl-CoA is achieved by making use of a cis-2-enoyl-CoA reductase (NADPH) (EC 1.3.1.37). Cis-2-enoyl-CoA reductases (NADPH) are enzymes which catalyze the following reaction:



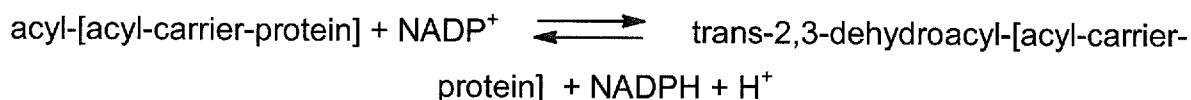
This enzyme has been described to occur in *Escherichia coli*.

In a further preferred embodiment the conversion of 3-methylcrotonyl-CoA into 3-methylbutyryl-CoA is achieved by making use of a trans-2-enoyl-CoA reductase (NADPH) (EC 1.3.1.38). Trans-2-enoyl-CoA reductases (NADPH) are enzymes which catalyze the following reaction:



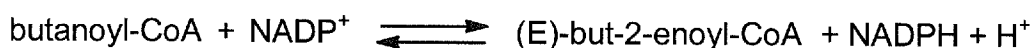
This enzyme occurs in a variety of organism, including eukaryotic and prokaryotic organisms, such as plants, animals and bacteria. The enzyme has, e.g., been described in *Homo sapien*, *Rattus norvegicus*, *Mus musculus*, *Cavia porcellus*, *Caenorhabditis elegans*, *Phalaenopsis amabilis*, *Gossypium hirsutum*, *Mycobacterium tuberculosis*, *Streptococcus collinu* and *Escherichia coli*.

In a further preferred embodiment the conversion of 3-methylcrotonyl-CoA into 3-methylbutyryl-CoA is achieved by making use of an enoyl-[acyl-carrier-protein] reductase (NADPH, Re-specific) (EC 1.3.1.39). Enoyl-[acyl-carrier-protein] reductases (NADPH, Re-specific) are enzymes which catalyze the following reaction:



This enzyme occurs in a variety of organism, including eukaryotic and prokaryotic organisms, such as animals and bacteria. The enzyme has, e.g., been described in *Gallus gallus*, *Pigeon*, *Rattus norvegicus*, *Cavia porcellus*, *Staphylococcus aureus*, *Bacillus subtilis* and *Porphyromonas gingivalis*.

In a further preferred embodiment the conversion of 3-methylcrotonyl-CoA into 3-methylbutyryl-CoA is achieved by making use of a crotonyl-CoA reductase (EC 1.3.1.86). Crotonyl-CoA reductases are enzymes which catalyze the following reaction:

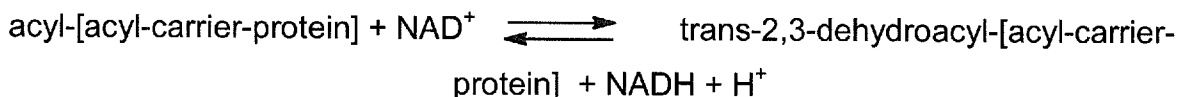


This enzyme occurs in a variety of organism, including eukaryotic and prokaryotic organisms, such as animals, fungi and bacteria. The enzyme has, e.g., been described in *Bos taurus*, *Salinospora tropica*, *Clostridium difficile*, *Streptomyces collinus*, *Streptomyces cinnamomensis* and *Streptomyces hygroscopicus*.

In another particularly preferred embodiment the enzyme is an enzyme which uses NADH as a co-factor. The conversion using such an enzyme is schematically shown in Figure 2. In a preferred embodiment the enzyme is selected from the group consisting of:

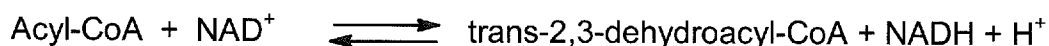
- enoyl-[acyl-carrier-protein] reductase (NADH) (EC 1.3.1.9); and
- trans-2-enoyl-CoA reductase (NAD⁺) (EC 1.3.1.44).

Thus, in one preferred embodiment the conversion of 3-methylcrotonyl-CoA into 3-methylbutyryl-CoA is achieved by making use of an enoyl-[acyl-carrier-protein] reductase (NADH) (EC 1.3.1.9). Enoyl-[acyl-carrier-protein] reductases (NADH) are enzymes which catalyze the following reaction:



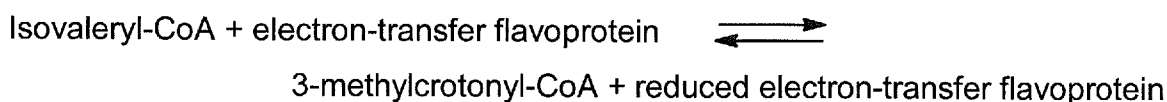
This enzyme occurs in a variety of organism, including eukaryotic and prokaryotic organisms, such as plants and bacteria. The enzyme has, e.g., been described in *Arabidopsis thaliana*, *Plasmodium falciparum*, *Eimeria tenella*, *Toxoplasma gondii*, *Mycobacterium tuberculosis*, *Streptococcus pneumoniae*, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus anthracis*, *Birkholderia mallei*, *Pseudomonas aeruginosa*, *Helicobacter pylori*, *Yersinia pestis* and many others.

In a further preferred embodiment the conversion of 3-methylcrotonyl-CoA into 3-methylbutyryl-CoA is achieved by making use of a trans-2-enoyl-CoA reductase (NAD⁺) (EC 1.3.1.44). Trans-2-enoyl-CoA reductases (NAD⁺) are enzymes which catalyze the following reaction:



This enzyme occurs in a variety of organism, including eukaryotic and prokaryotic organisms, such as plants, animals and bacteria. The enzyme has, e.g., been described in *Ratus norvegicus*, *Euglena gracilis*, *Mycobacterium smegmatis*, *Pseudomonas fluorescens*, *Clostridium acetobutylicum*, *Butyrivibrio fibrisolvens*, *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis* and *Treponema denticola*.

In another preferred embodiment the enzyme is an enzyme which is classified as EC 1.3.8 and which uses a flavin prosthetic group as acceptor. The conversion using such an enzyme is schematically shown in Figure 3. In a preferred embodiment the enzyme is an isovaleryl-CoA dehydrogenase (EC 1.3.8.4). Isovaleryl-CoA dehydrogenases are enzymes which catalyze the following reaction:



This enzyme occurs in a variety of organism, including eukaryotic and prokaryotic organisms, such as plants, animals, fungi and bacteria. The enzyme has, e.g., been described in *Homo sapiens*, *Bos taurus*, *Rattus norvegicus*, *Mus musculus*, *Cavia porcellus*, *Bombix mori*, *Caenorhabditis elegans*, *Solanum tuberosum*, *Arabidopsis thaliana*, *Pisum sativum*, *Aspergillus oryzae*, *Pseudomonas aeruginosa* and *Halobacterium salinarum*.

In another embodiment the conversion of 3-methylcrotonyl-CoA into 3-methylbutyryl-CoA may, for example, be achieved by making use of the enzyme from *Myxococcus* sp. which is encoded by the *liuA* gene (Li et al., *Angew. Chem. Int. Ed.* 52 (2013), 1304-1308). This enzyme (AibC, LiuA) was annotated as an oxidoreductase and as belonging to the zinc-binding dehydrogenase family. The enzyme was shown to reduce 3-methylcrotonyl-CoA into 3-methylbutyryl-CoA using NADH as co-factor. The amino acid sequence of said protein is available under Uniprot Accession Number Q1D4I2.

In a preferred embodiment such an enzyme has an amino acid sequence as shown in SEQ ID NO: 3 or shows an amino acid sequence which is at least x% homologous

to SEQ ID NO: 3 and has the activity of an oxidoreductase with x being an integer between 30 and 100, preferably 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99 wherein such an enzyme is capable of converting 3-methylcrotonyl-CoA into 3-methylbutyryl-CoA as set forth herein above.

Preferably, the degree of identity is determined by comparing the respective sequence with the amino acid sequence of any one of the above-mentioned SEQ ID NOs. When the sequences which are compared do not have the same length, the degree of identity preferably either refers to the percentage of amino acid residues in the shorter sequence which are identical to amino acid residues in the longer sequence or to the percentage of amino acid residues in the longer sequence which are identical to amino acid residues in the shorter sequence. The degree of sequence identity can be determined according to methods well known in the art using preferably suitable computer algorithms such as CLUSTAL.

When using the Clustal analysis method to determine whether a particular sequence is, for instance, 80% identical to a reference sequence default settings may be used or the settings are preferably as follows: Matrix: blosum 30; Open gap penalty: 10.0; Extend gap penalty: 0.05; Delay divergent: 40; Gap separation distance: 8 for comparisons of amino acid sequences. For nucleotide sequence comparisons, the Extend gap penalty is preferably set to 5.0.

Preferably, the degree of identity is calculated over the complete length of the sequence.

The conversion of 3-methylbutyryl-CoA into 3-methylbutyric acid according to step (a2) can be achieved by different enzymatic conversions. One possibility is the direct conversion via a hydrolysis reaction. Another possibility is the direct conversion via a reaction catalyzed by a CoA-transferase and a third possibility is a two-step conversion via 3-methylbutyryl phosphate. These three options will be further discussed in the following.

In one embodiment the conversion of 3-methylbutyryl-CoA into 3-methylbutyric acid according to step (a2) can be achieved by a method comprising the step of enzymatically converting 3-methylbutyryl-CoA into 3-methylbutyric acid in a hydrolysis reaction by making use of a thioester hydrolase (EC 3.1.2; in the following referred to as thioesterase).

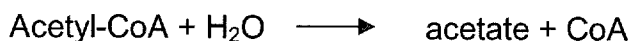
Thioesterases (TEs; also referred to as thioester hydrolases) are enzymes which are classified as EC 3.1.2. Presently thioesterases are classified as EC 3.1.2.1 through EC 3.1.2.27 and EC 3.1.2.- for unclassified TEs. Cantu et al. (Protein Science 19 (2010), 1281-1295) describe that there are 23 families of thioesterases which are unrelated to each other as regards the primary structure. However, it is assumed that all members of the same family have essentially the same tertiary structure. Thioesterases hydrolyze the thioester bond between a carbonyl group and a sulfur atom.

The conversion of 3-methylbutyryl-CoA into 3-methylbutyric acid catalyzed by a thioesterase is schematically shown in Figure 4.

In a preferred embodiment, a thioesterase employed in a method according to the present invention for converting 3-methylbutyryl-CoA into 3-methylbutyric acid is selected from the group consisting of:

- acetyl-CoA hydrolase (EC 3.1.2.1);
- palmitoyl-CoA hydrolase (EC 3.1.2.2);
- 3-hydroxyisobutyryl-CoA hydrolase (EC 3.1.2.4);
- oleoyl-[acyl-carrier-protein] hydrolase (EC 3.1.2.14);
- ADP-dependent short-chain-acyl-CoA hydrolase (EC 3.1.2.18);
- ADP-dependent medium-chain-acyl-CoA hydrolase (EC 3.1.2.19); and
- acyl-CoA hydrolase (EC 3.1.2.20).

Thus, in one preferred embodiment the direct conversion of 3-methylbutyryl-CoA into 3-methylbutyric acid is achieved by making use of an acetyl-CoA hydrolase (EC 3.1.2.1). Acetyl-CoA hydrolases are enzymes which catalyze the following reaction:



This enzyme occurs in a variety of organism, including eukaryotic and prokaryotic organisms, such as plants, animals, fungi and bacteria. The enzyme has, e.g., been described in *Rattus norvegicus* (Uniprot Accession number: Q99NB7), *Mus musculus*, *Sus scrofa*, *Bos taurus*, *Gallus gallus*, *Platyrrhini*, *Ovis aries*, *Mesocricetus auratus*, *Ascaris suum*, *Homo sapiens* (Uniprot Accession number: Q8WYK0), *Pisum sativum*, *Cucumis sativus*, *Panicus sp.*, *Ricinus communis*, *Solanum tuberosum*,

Spinacia oleracea, *Zea mays*, *Glycine max*, *Saccharomyces cerevisiae*, *Neurospora crassa*, *Candida albicans*, *Trypanosoma brucei brucei*, *Trypanosoma cruzi*, *Trypanosoma dionisii*, *Trypanosoma vespertilionis*, *Crithidia fasciculata*, *Clostridium aminovalericum*, *Acidaminococcus fermentans*, *Bradyrhizobium japonicum* and *Methanosarcina barkeri*.

In another preferred embodiment the direct conversion of 3-methylbutyryl-CoA into 3-methylbutyric acid is achieved by making use of a palmitoyl-CoA hydrolase (EC 3.1.2.2). Palmitoyl-CoA hydrolases are enzymes which catalyze the following reaction:



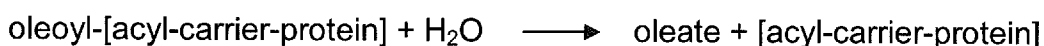
This enzyme occurs in a variety of organism, including eukaryotic and prokaryotic organisms, such as plants, animals, fungi and bacteria. The enzyme has, e.g., been described in *Arabidopsis thaliana* (Uniprot Accession number: Q8GYW7), *Pisum sativum*, *Spinacia oleracea*, *Bumilleriopsis filiformis*, *Eremosphaera viridis*, *Mougeotia scalaris*, *Euglena gracilis*, *Rhodotorula aurantiaca*, *Saccharomyces cerevisiae*, *Candida rugosa*, *Caenorhabditis elegans*, *Mus musculus* (Uniprot Accession number: P58137), *Homo sapiens*, *Platyrrhini*, *Bos taurus*, *Canis lupus familiaris*, *Sus scrofa*, *Cavia procellus*, *Columba sp.*, *Cricetulus griseus*, *Mesocricetus auratus*, *Drosophila melanogaster*, *Rattus norvegicus*, *Oryctolagus cuniculus*, *Gallus gallus*, *Anas platyrhynchos*, *Mycobacterium tuberculosis*, *Mycobacterium phlei*, *Mycobacterium smegmatis*, *Acinetobacter colaceticus*, *Haemophilus influenza*, *Helicobacter pylori*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Rhodobacter sphaeroides*, *Streptomyces coelicolor*, *Streptomyces venezuelae* and *E. coli*.

In a further preferred embodiment the direct conversion of 3-methylbutyryl-CoA into 3-methylbutyric acid is achieved by making use of a 3-hydroxyisobutyryl-CoA hydrolase (EC 3.1.2.4). 3-hydroxyisobutyryl-CoA hydrolases are enzymes which catalyze the following reaction:



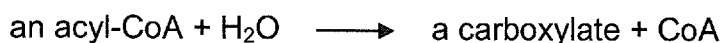
This enzyme occurs in a variety of organism, including eukaryotic and prokaryotic organisms, such as plants, animals, fungi and bacteria. The enzyme has, e.g., been described in *Arabidopsis thaliana*, *Homo sapiens*, *Canis lupus familiaris*, *Rattus norvegicus*, *Bacillus cereus*, *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*.

In yet another preferred embodiment the direct conversion of 3-methylbutyryl-CoA into 3-methylbutyric acid is achieved by making use of an oleoyl-[acyl-carrier-protein] hydrolase (EC 3.1.2.14). Oleoyl-[acyl-carrier-protein] hydrolases are enzymes which catalyze the following reaction:



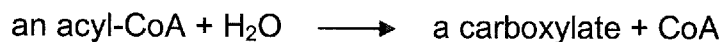
This enzyme occurs in a variety of plants and bacteria. The enzyme has, e.g., been described in *Arabidopsis thaliana*, *Allium ampeloprasum*, *Curcubita moschata*, *Cuphea calophylla*, *Cuphea hookeriana*, *Cuphea lanceolata*, *Cuphea wrightii*, *Umbellularia californica*, *Coriandrum sativum*, *Spinacia oleracea*, *Elaeis* sp., *Elaeis guineensis*, *Glycine max*, *Persea americana*, *Pisum sativum*, *Sinapis alba*, *Ulmus americana*, *Zea mays*, *Brassica juncea*, *Brassica napus*, *Brassica rapa* subsp. *campestris*, *Jatropha curcas*, *Ricinus communis*, *Cinnamomum camphorum*, *Macadamia tetraphylla*, *Magnifera indica*, *Madhuca longifolia*, *Populus tomentosa*, *Chimonanthus praecox*, *Gossypium hirsutum*, *Diploknema butyracea*, *Helianthus annuus* and *Streptococcus pyogenes*.

In yet another preferred embodiment the direct conversion of 3-methylbutyryl-CoA into 3-methylbutyric acid is achieved by making use of an ADP-dependent short-chain-acyl-CoA hydrolase (EC 3.1.2.18). ADP-dependent short-chain-acyl-CoA hydrolases are enzymes which catalyze the following reaction:



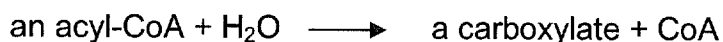
This enzyme occurs in a variety of animals and has, e.g., been described in *Mus musculus*, *Rattus norvegicus* and *Mesocricetus auratus*.

In yet another preferred embodiment the direct conversion of 3-methylbutyryl-CoA into 3-methylbutyric acid is achieved by making use of an ADP-dependent medium-chain-acyl-CoA hydrolase (EC 3.1.2.19). ADP-dependent medium-chain-acyl-CoA hydrolases are enzymes which catalyze the following reaction:



This enzyme occurs in a variety of animals and has, e.g., been described in *Rattus norvegicus* and *Mesocricetus auratus*.

In a further preferred embodiment the direct conversion of 3-methylbutyryl-CoA into 3-methylbutyric acid is achieved by making use of an acyl-CoA hydrolase (EC 3.1.2.20). Acyl-CoA hydrolases are enzymes which catalyze the following reaction:



This enzyme occurs in a variety of organism, including eukaryotic and prokaryotic organisms, such as plants, animals, fungi and bacteria. The enzyme has, e.g., been described in *Arabidopsis thaliana*, *Rhodotorula aurantiaca*, *Bumilleriopsis filiformis*, *Eremosphaera viridis*, *Euglena gracilis*, *Mus musculus*, *Rattus norvegicus*, *Homo sapiens*, *Sus scrofa*, *Bos taurus*, *Canis lupus familiaris*, *Cavia porcellus*, *Cricetus griseus*, *Drosophila melanogaster*, *Anas platyrhynchos*, *Gallus gallus*, *Caenorhabditis elegans*, *Saccharomyces cerevisiae*, *Candida rugosa*, *Escherichia coli*, *Haemophilus influenzae*, *Xanthomonas campestris*, *Streptomyces* sp., *Streptomyces coelicolor*, *Alcaligenes faecalis*, *Pseudomonas aeruginosa*, *Amycolatopsis mediterranei*, *Acinetobacter calcoaceticus*, *Helicobacter pylori*, *Rhodobacter sphaeroides* and *Mycobacterium phlei*.

In a preferred embodiment the acyl-CoA hydrolase is an enzyme from *Escherichia coli* or from *Haemophilus influenza*, more preferably the YciA enzyme from *E. coli* or its closely related homolog HI0827 from *Haemophilus influenza* (Zhuang et al., *Biochemistry* 47 (2008), 2789-2796). In another preferred embodiment the acetyl-CoA hydrolase is an enzyme from *Homo sapiens* (Cao et al., *Biochemistry* 48 (2009), 1293-1304). The enzyme from *Haemophilus influenza* has been reported to

be able to catalyze the conversion of 3-methylbutyryl-CoA into 3-methylbutyric acid (Zhuang et al., loc.cit.).

In another embodiment the conversion of 3-methylbutyryl-CoA into 3-methylbutyric acid according to step (a2) can be achieved by a method comprising the step of enzymatically converting 3-methylbutyryl-CoA into 3-methylbutyric acid in a transferase reaction by making use of a CoA-transferase (EC 2.8.3).

The conversion of 3-methylbutyryl-CoA into 3-methylbutyric acid catalyzed by a CoA-transferase is schematically shown in Figure 5.

CoA-transferases are found in organisms from all lines of descent. Most of the CoA-transferase belong to two well-known enzyme families (referred to in the following as families I and II) and there exists a third family which had been identified in anaerobic metabolic pathways of bacteria. A review describing the different families can be found in Heider (FEBS Letters 509 (2001), 345-349).

Family I contains, e.g., the following CoA-transferases:

For 3-oxo acids: enzymes classified in EC 2.8.3.5 or EC 2.8.3.6;

for short chain fatty acids: enzymes classified in EC 2.8.3.8 or EC 2.8.3.9;

for glutaconate: enzymes classified in EC 2.8.3.12;

for succinate: succinyl-CoA:acetate CoA-transferases, i.e. enzymes classified in EC 2.8.3.18 (see also Mullins et al., Biochemistry 51(2012), 8422-34; Mullins et al., J. Bacteriol. 190 (2006), 4933-4940).

Most enzymes of family I naturally use succinyl-CoA or acetyl-CoA as CoA donors. These enzymes contain two dissimilar subunits in different aggregation states. Two conserved amino acid sequence motives have been identified:

Prosites entry PS01273 (<http://prosite.expasy.org/cgi-bin/prosite/prosite-search-ac?PDOC00980>)

COA_TRANSF_1, <http://prosite.expasy.org/PS01273>; Coenzyme A transferases signature 1 (PATTERN)

Consensus pattern:

[DN]-[GN]-x(2)-[LIVMFA](3)-G-G-F-x(3)-G-x-P

and

Prosites entries PS01273 (<http://prosite.expasy.org/cgi-bin/prosite/prosite-search-ac?PDOC00980>)

COA_TRANSF_2, PS01274; Coenzyme A transferases signature 2 (PATTERN)

Consensus pattern:

[LF]-[HQ]-S-E-N-G-[LIVF](2)-[GA]

E (glutamic acid) is an active site residue.

The family II of CoA-transferases consists of the homodimeric α -subunits of citrate lyase (EC 2.8.3.10) and citramalate lyase (EC 2.8.3.11). These enzymes catalyse the transfer of acyl carrier protein (ACP) which contains a covalently bound CoA-derivative. It was shown that such enzymes also accept free CoA-thioester in vitro, such as acetyl-CoA, propionyl-CoA, butyryl-CoA in the case of citrate lyase (Dimroth et al., Eur. J. Biochem. 80 (1977), 479-488) and acetyl-CoA and succinyl-CoA in the case of citramalate lyase (Dimroth et al., Eur. J. Biochem. 80 (1977), 469-477).

According to Heider (loc. cit.) family III of CoA-transferases consists of formyl-CoA:oxalate CoA-transferase, succinyl-CoA:(*R*)-benzylsuccinate CoA-transferase, (*E*)-cinnamoyl-CoA:(*R*)-phenyllactate CoA-transferase and butyrobetainyl-CoA:(*R*)-carnitine CoA-transferase. A further member of family III is succinyl-CoA:L-malate CoA-transferase whose function in autotrophic CO₂ fixation of *Chloroflexus aurantiacus* is to activate L-malate to its CoA thioester with succinyl-CoA as the CoA donor (Friedman S. et al. J. Bacteriol. 188 (2006), 2646-2655). The amino acid sequences of the CoA-transferase of this family show only a low degree of sequence identity to those of families I and II. These CoA-transferases occur in prokaryotes and eukaryotes,

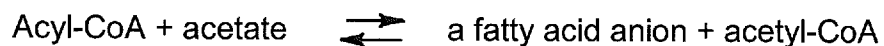
In a preferred embodiment the CoA-transferase employed in a method according to the present invention is a CoA-transferase which belongs to family I or II as described herein-above.

Preferably, the CoA-transferase employed in a method according to the present invention for the direct conversion of 3-methylbutyryl-CoA into 3-methylbutyric acid is selected from the group consisting of:

- acetate CoA-transferase (EC 2.8.3.8); and
- butyrate-acetoacetate CoA-transferase (EC 2.8.3.9).

Thus, in one preferred embodiment the direct conversion of 3-methylbutyryl-CoA into 3-methylbutyric acid is achieved by making use of an acetate CoA-transferase (EC

2.8.3.8). Acetate CoA-transferases are enzymes which catalyze the following reaction:



This enzyme occurs in a variety of bacteria and has, e.g., been described in *Anaerostipes caccae*, *Eubacterium hallii*, *Faecalibacterium prausnitzii*, *Roseburia hominis*, *Roseburia intestinalis*, *Coprococcus* sp. and *Escherichia coli*,

In another preferred embodiment the direct conversion of 3-methylbutyryl-CoA into 3-methylbutyric acid is achieved by making use of a butyrate-acetoacetate CoA-transferase (EC 2.8.3.9). Butyrate-acetoacetate CoA-transferase are enzymes which catalyze the following reaction:



This enzyme occurs in a variety of organism, including eukaryotic and prokaryotic organisms, such as animals and bacteria. The enzyme has, e.g., been described in *Bos taurus*, *Clostridium* sp. and *Escherichia coli*.

As mentioned above, the conversion of 3-methylbutyryl-CoA into 3-methylbutyric acid can in the alternative also be achieved by a conversion which first encompasses the conversion of 3-methylbutyryl-CoA into 3-methylbutyryl phosphate and the subsequent conversion of 3-methylbutyryl phosphate into 3-methylbutyric acid. The corresponding reaction is schematically shown in Figure 6. This pathway has the advantage of generating one molecule of ATP and is, thus, energetically more favourable for a cell if carried out in vivo.

The conversion of 3-methylbutyryl-CoA into 3-methylbutyryl phosphate can, e.g., be achieved by the use of a phosphate butyryltransferase (EC 2.3.1.19) or a phosphate acetyltransferase (EC 2.3.1.8).

Phosphate butyryltransferase (EC 2.3.1.19) naturally catalyzes the following reaction



It has been described by Wiesenborn et al. (Appl. Environ. Microbiol. 55 (1989), 317-322) and by Ward et al. (J. Bacteriol. 181 (1999), 5433-5442) that phosphate butyryltransferases (EC 2.3.1.19) can use a number of substrates in addition to butyryl coenzyme A (butyryl-CoA), in particular acetyl-CoA, propionyl-CoA, isobutyryl-CoA, valeryl-CoA and isovaleryl-CoA.

The enzyme has been described to occur in a number of organism, in particular in bacteria and in protozoae. In one embodiment the enzyme is from the protozoae *Dasytricha ruminantium*. In a preferred embodiment the phosphate butyryltransferase is a phosphate butyryltransferase from a bacterium, preferably from a bacterium of the genus *Bacillus*, *Butyrivibrio*, *Enterococcus* or *Clostridium*, more preferably *Enterococcus* or *Clostridium*, and even more preferably from *Bacillus megaterium*, *Bacillus subtilis*, *Butyrivibrio fibrisolvens*, *Clostridium acetobutylicum*, *Clostridium beijerinckii*, *Clostridium butyricum*, *Clostridium kluyveri*, *Clostridium saccharoacetobutylicum*, *Clostridium sporogenes* or *Enterococcus faecalis*. Most preferably, the enzyme is from *Bacillus subtilis* (strain 168) (Uniprot Accession number P54530), *Clostridium acetobutylicum*, in particular the enzyme encoded by the *ptb* gene (Uniprot Accession number F0K6W0; Wiesenborn et al. (Appl. Environ. Microbiol. 55 (1989), 317-322)) or from *Enterococcus faecalis*, in particular *Enterococcus faecalis* MTUP9 (Uniprot Accession number K4YRE8 or Uniprot Accession number A0A038BNC2; Ward et al. (J. Bacteriol. 181 (1999), 5433-5442)). The sequences available for the phosphate butyryltransferase from *Enterococcus faecalis* under Uniprot Accession number K4YRE8 and Uniprot Accession number A0A038BNC2 have a sequence homology of 99.3%. The sequences available for the phosphate butyryltransferase from *Enterococcus faecalis* under Uniprot Accession number A0A038BNC2 is the more preferred one.

As mentioned, in a preferred embodiment, the enzyme is a phosphate butyryltransferase (EC 2.3.1.19) from *Bacillus subtilis* (strain 168) (Uniprot Accession number P54530). In a particularly preferred embodiment, the phosphate butyryltransferase (EC 2.3.1.19) employed in the method of the invention has an amino acid sequence as shown in SEQ ID NO: 8 or shows an amino acid sequence which is at least x% homologous to SEQ ID NO: 8 and has the activity of a phosphate butyryltransferase with x being an integer between 30 and 100, preferably 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99

wherein such an enzyme is capable of converting 3-methylcrotonyl-CoA into 3-methylcrotonyl-phosphate as set forth herein above.

In another preferred embodiment, as mentioned, the enzyme is a phosphate butyryltransferase (EC 2.3.1.19) from *Enterococcus faecalis* MTUP9 (Uniprot Accession number K4YRE8 or Uniprot Accession number A0A038BNC2). In a particularly preferred embodiment, the phosphate butyryltransferase (EC 2.3.1.19) employed in the method of the invention has an amino acid sequence as shown in SEQ ID NO: 9 or shows an amino acid sequence which is at least x% homologous to SEQ ID NO: 9 and has the activity of a phosphate butyryltransferase with x being an integer between 30 and 100, preferably 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99 wherein such an enzyme is capable of converting 3-methylcrotonyl-CoA into 3-methylcrotonyl-phosphate as set forth herein above.

As regards the determination of the degree of identity, the same applies as has been set forth herein above.

Phosphate acetyltransferase (EC 2.3.1.8) naturally catalyzes the following reaction



It has been described by Veit et al. (J. Biotechnol. 140 (2009), 75-83) that phosphate acetyltransferase can also use as a substrate butyryl-CoA or propionyl-CoA.

The accession numbers for this enzyme family in InterPro database are IPR012147 and IPR002505, "http://www.ebi.ac.uk/interpro/entry/IPR002505"

(<http://www.ebi.ac.uk/interpro/entry/IPR012147>

<http://www.ebi.ac.uk/interpro/entry/IPR002505>)

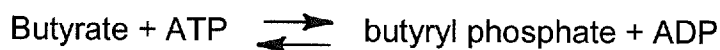
See also <http://pfam.sanger.ac.uk/family/PF01515>

The enzyme has been described in a variety of organisms, in particular bacteria and fungi. Thus, in one preferred embodiment the enzyme is an enzyme from a bacterium, preferably of the genus *Escherichia*, *Chlorogonium*, *Clostridium*, *Veillonella*, *Methanosarcina*, *Corynebacterium*, *Ruegeria*, *Salmonella*, *Azotobacter*, *Bradorhizobium*, *Lactobacillus*, *Moorella*, *Rhodopseudomonas*, *Sinorhizobium*, *Streptococcus*, *Thermotoga* or *Bacillus*, more preferably of the species *Escherichia coli*, *Chlorogonium elongatum*, *Clostridium kluyveri*, *Clostridium acetobutylicum*,

Clostridium acidurici, *Veillonella parvula*, *Methanosarcina thermophila*, *Corynebacterium glutamicum*, *Ruegeria pomeroyi*, *Salmonella enterica*, *Azotobacter vinelandii*, *Bradyrhizobium japonicum*, *Lactobacillus fermentum*, *Lactobacillus sanfranciscensis*, *Moorella thermoacetica*, *Rhodopseudomonas palustris*, *Sinorhizobium meliloti*, *Streptococcus pyogenes*, *Thermotoga maritima* or *Bacillus subtilis*. In another preferred embodiment the enzyme is an enzyme from a fungus, preferably from the genus *Saccharomyces*, more preferably of the species *Saccharomyces cerevisiae*.

The conversion of 3-methylbutyryl phosphate into 3-methylbutyric acid can, e.g., be achieved by the use of a butyrate kinase (EC 2.7.2.7), a branched-chain-fatty-acid kinase (EC 2.7.2.14), a propionate kinase (EC 2.7.2.15) or an acetate kinase (EC 2.7.2.1).

Butyrate kinases (EC 2.7.2.7) naturally catalyze the following reaction



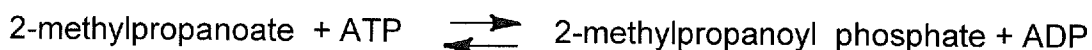
It has been described, e.g. by Hartmanis (J. Biol. Chem. 262 (1987), 617-621) that butyrate kinase can use a number of substrates in addition to butyrate, e.g. valerate, isobutyrate, isovalerate and vinyl acetate. The enzyme has been described in a variety of organisms, in particular bacteria. In one preferred embodiment the enzyme is from a bacterium, preferably from a bacterium of the genus *Clostridium*, *Butyrivibrio*, *Thermotoga*, *Enterococcus*, *Lactobacillus* or *Geobacillus*. Preferred is *Clostridium*, *Lactobacillus* or *Geobacillus*. More preferably the enzyme is from a bacterium of the species *Clostridium acetobutylicum*, *Clostridium proteoclasticum*, *Clostridium tyrobutyricum*, *Clostridium butyricum*, *Clostridium pasteurianum*, *Clostridium tetanomorphum*, *Butyrivibrio fibrosolvens*, *Butyrivibrio hungatei*, *Thermotoga maritima*, *Enterococcus durans*, *Enterococcus faecalis*, *Lactobacillus casei* (Uniprot Accession number K0N529) or *Geobacillus* sp. (Uniprot Accession number L8A0E1). Preferred is *Clostridium acetobutylicum*, *Lactobacillus casei* W56 or *Geobacillus* sp. GHH01. For *Clostridium acetobutylicum* two butyrate kinases have been described: butyrate kinase 1 (Uniprot Accession number: Q45829) and butyrate kinase II (Uniprot Accession number: Q97II19). Preferably, the enzyme is an enzyme encoded by the buk gene from *Clostridium acetobutylicum* (Hartmanis (J. Biol. Chem.

262 (1987), 617-621)) or the homologue of this enzyme which has been found in *Enterococcus faecalis* (Ward et al. (J. Bacteriol. 181 (1999), 5433-5442)).

As mentioned, in a preferred embodiment, the enzyme is a butyrate kinase (EC 2.7.2.7) from *Lactobacillus casei* W56 (Uniprot Accession number K0N529). In a particularly preferred embodiment, the butyrate kinase (EC 2.7.2.7) employed in the method of the invention has an amino acid sequence as shown in SEQ ID NO: 10 or shows an amino acid sequence which is at least x% homologous to SEQ ID NO: 10 and has the activity of a butyrate kinase with x being an integer between 30 and 100, preferably 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99 wherein such an enzyme is capable of converting 3-methylcrotonyl-phosphate into 3-methylcrotonic acid as set forth herein above.

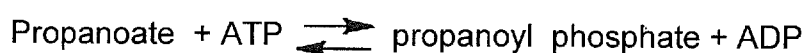
In another preferred embodiment, the enzyme is a butyrate kinase (EC 2.7.2.7) from *Geobacillus* sp. GHH01 (Uniprot Accession number L8A0E1). In a particularly preferred embodiment, the butyrate kinase (EC 2.7.2.7) employed in the method of the invention has an amino acid sequence as shown in SEQ ID NO: 11 or shows an amino acid sequence which is at least x% homologous to SEQ ID NO: 11 and has the activity of a butyrate kinase with x being an integer between 30 and 100, preferably 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99 wherein such an enzyme is capable of converting 3-methylcrotonyl-phosphate into 3-methylcrotonic acid as set forth herein above.

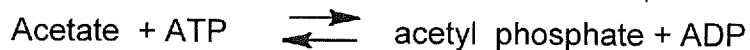
Branched-chain-fatty-acid kinases (EC 2.7.2.14) naturally catalyze the following reaction



This enzyme has been described to occur in a number of bacteria. Thus, in one preferred embodiment the enzyme is an enzyme from a bacterium, preferably of the genus *Spirochaeta* or *Thermotoga*, more preferably *Thermotoga maritima*.

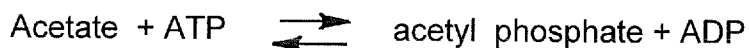
Propionate kinases (EC 2.7.2.15) naturally catalyze the following reactions





This enzyme has been described to occur in a number of bacteria, in particular Enterobacteriaceae. Thus, in one preferred embodiment the enzyme is an enzyme from a bacterium, preferably of the genus *Salmonella* or *Escherichia*, more preferably of the species *Salmonella enterica* or *Escherichia coli*.

Acetate kinases (EC 2.7.2.1) naturally catalyze the following reaction



This enzyme has been described to occur in a number of organisms, in particular bacteria and eukaryotes. In one preferred embodiment the enzyme is from a bacterium, preferably from a bacterium of the genus *Methanosarcina*, *Cryptococcus*, *Ethanoligenens*, *Propionibacterium*, *Roseovarius*, *Streptococcus*, *Salmonella*, *Acholeplasma*, *Acinetobacter*, *Ajellomyces*, *Bacillus*, *Borrelia*, *Chaetomium*, *Clostridium*, *Coccidioides*, *Coprinopsis*, *Cryptococcus*, *Cupriavidus*, *Desulfovibrio*, *Enterococcus*, *Escherichia*, *Ethanoligenes*, *Geobacillus*, *Helicobacter*, *Lactobacillus*, *Lactococcus*, *Listeria*, *Mesoplasma*, *Moorella*, *Mycoplasma*, *Oceanobacillus*, *Propionibacterium*, *Rhodospseudomonas*, *Roseovarius*, *Salmonella*, *Staphylococcus*, *Thermotoga* or *Veillonella*, more preferably from a bacterium of the species *Methanosarcina thermophila*, *Cryptococcus neoformans*, *Ethanoligenens harbinense*, *Propionibacterium acidipropionici*, *Streptococcus pneumoniae*, *Streptococcus enterica*, *Streptococcus pyogenes*, *Acholeplasma laidlawii*, *Acinetobacter calcoaceticus*, *Ajellomyces capsulatus*, *Bacillus subtilis*, *Borrelia burgdorferi*, *Chaetomium globosum*, *Clostridium acetobutylicum*, *Clostridium thermocellum*, *Coccidioides immitis*, *Coprinopsis cinerea*, *Cryptococcus neoformans*, *Cupriavidus necator*, *Desulfovibrio vulgaris*, *Enterococcus faecalis*, *Escherichia coli*, *Ethanoligenes harbinense*, *Geobacillus stearothermophilus*, *Helicobacter pylori*, *Lactobacillus delbrueckii*, *Lactobacillus acidophilus*, *Lactobacillus sanfranciscensis*, *Lactococcus lactis*, *Listeria monocytogenes*, *Mesoplasma florum*, *Methanosarcina acetivorans*, *Methanosarcina mazei*, *Moorella thermoacetica*, *Mycoplasma pneumoniae*, *Oceanobacillus iheyensis*, *Propionibacterium freudenreichii*,

Propionibacterium acidipropionici, *Rhodospseudomonas palustris*, *Salmonella enteric*, *Staphylococcus aureus*, *Thermotoga maritime* or *Veillonella parvula*.

In another preferred embodiment the enzyme is an enzyme from a fungus, preferably from a fungus of the genus *Aspergillus*, *Gibberella*, *Hypocrea*, *Magnaporthe*, *Phaeosphaeria*, *Phanerochaete*, *Phytophthora*, *Sclerotinia*, *Uncinocarpus*, *Ustilago* or *Neurospora* even more preferably from a fungus of the species *Aspergillus fumigates*, *Aspergillus nidulans*, *Gibberella zeae*, *Hypocrea jecorina*, *Magnaporthe grisea*, *Phaeosphaeria nodorum*, *Phanerochaete chrysosporium*, *Phytophthora ramorum*, *Phytophthora sojae*, *Sclerotinia sclerotiorum*, *Uncinocarpus reesii*, *Ustilago maydis* or *Neurospora crassa*.

In a further preferred embodiment the enzyme is an enzyme from a plant or an algae, preferably from the genus *Chlamydomonas*, even more preferably from the species *Chlamydomonas reinhardtii*.

In another embodiment the enzyme is from an organism of the genus *Entamoeba*, more preferably of the species *Entamoeba histolytica*.

The above mentioned enzyme families suitable for the conversion of 3-methylbutyryl phosphate into 3-methylbutyric acid have been shown to be evolutionary related and contain common sequence signatures. These signatures are referenced and described in Prosite database:

<http://prosite.expasy.org/cgi-bin/prosite/nicedoc.pl?PS01075>

Gao et al. (FEMS Microbiol. Lett. 213 (2002), 59-65) already described genetically modified *E. coli* cells which have been transformed, inter alia, with the *ptb* gene and the *buk* gene from *Clostridium acetobutylicum* encoding a phosphate butyryltransferase (EC 2.3.1.19) and a butyrate kinase (EC 2.7.2.7), respectively. These *E. coli* cells have been shown to be able to D-(-)-3-hydroxybutyric acid (3HB).

As described above the conversion of 3-methylcrotonyl-CoA into 3-methylbutyric acid can also be achieved by first converting 3-methylcrotonyl-CoA into 3-methylcrotonic acid and then further converting 3-methylcrotonic acid into 3-methylbutyric acid, i.e. by a method wherein the enzymatic conversion of 3-methylcrotonyl-CoA into 3-methylbutyric acid according to step (a) is achieved by a method comprising the following steps:

(a1) enzymatically converting 3-methylcrotonyl-CoA into 3-methylcrotonic acid; and

- (all) further enzymatically converting the thus produced 3-methylcrotonic acid into 3-methylbutyric acid.

The conversion of 3-methylcrotonyl-CoA into 3-methylcrotonic acid according to step (a1) can be achieved by different enzymatic conversions. One possibility is the direct conversion via a hydrolysis reaction. Another possibility is the direct conversion via a reaction catalyzed by a CoA-transferase and a third possibility is a two-step conversion via 3-methylcrotonyl phosphate. These three options will be further discussed in the following.

In one embodiment the conversion of 3-methylcrotonyl-CoA into 3-methylcrotonic acid according to step (a1) can be achieved by a direct conversion, i.e. by a method comprising the step of enzymatically converting 3-methylcrotonyl-CoA into 3-methylcrotonic acid in a hydrolysis reaction by making use of a thioesterase (EC 3.1.2). The conversion of 3-methylcrotonyl-CoA into 3-methylcrotonic acid catalyzed by a thioesterase is schematically shown in Figure 7.

Thioesterases have been described herein above and the same as has been set forth above also applies in connection with this embodiment of a method according to the invention. In this context it may be noteworthy that the acyl-CoA hydrolases (EC 3.1.2.20) from *E. coli*, *Haemophilus influenza* and *Homo sapiens* have been reported to be able to accept 3-methylcrotonyl-CoA (*aka* beta-methylcrotonyl-CoA) as a substrate.

Particularly preferred are the enzymes acyl-CoA thioesterase 2 from *E. coli* (Uniprot P0AGG2; SEQ ID NO: 12) and acyl-CoA thioesterase II from *Pseudomonas putida* (Uniprot Q88DR1; SEQ ID NO: 13).

In another embodiment the conversion of 3-methylcrotonyl-CoA into 3-methylcrotonic acid according to step (a1) can be achieved by a direct conversion, i.e. by a method comprising the step of enzymatically converting 3-methylcrotonyl-CoA into 3-methylcrotonic acid in a transferase reaction by making use of a CoA-transferase (EC 2.8.3).

The conversion of 3-methylcrotonyl-CoA into 3-methylcrotonic acid catalyzed by a CoA-transferase is schematically shown in Figure 8.

CoA-transferases have been described herein above and the same as has been set forth above also applies in connection with this embodiment of a method according to the invention.

As mentioned above, the conversion of 3-methylcrotonyl-CoA into 3-methylcrotonic acid can in the alternative also be achieved by a conversion which first encompasses the conversion of 3-methylcrotonyl-CoA into 3-methylcrotonyl phosphate and the subsequent conversion of 3-methylcrotonyl phosphate into 3-methylcrotonic acid. The corresponding reaction is schematically shown in Figure 9.

The conversion of 3-methylcrotonyl-CoA into 3-methylcrotonyl phosphate can, e.g., be achieved by the use of a phosphate butyryltransferase (EC 2.3.1.19) or a phosphate acetyltransferase (EC 2.3.1.8). These enzymes have been described above in connection with the conversion of 3-methylbutyryl-CoA into 3-methylbutyryl phosphate and the same which has been set forth above also applies in connection with the present embodiment.

The conversion of 3-methylcrotonyl phosphate into 3-methylcrotonic acid can, e.g., be achieved by the use of enzymes which are classified in EC 2.7.2, i.e. enzymes which are classified as phosphotransferases with a carboxy group as acceptor. In a preferred embodiment the conversion of 3-methylcrotonyl phosphate into 3-methylcrotonic acid can, e.g., be achieved by the use of a butyrate kinase (EC 2.7.2.7), a branched-chain-fatty-acid kinase (EC 2.7.2.14), a propionate kinase (EC 2.7.2.15) or an acetate kinase (EC 2.7.2.1). These enzymes have been described above in connection with the conversion of 3-methylbutyryl phosphate into 3-methylbutyric acid and the same which has been set forth above also applies in connection with the present embodiment.

The 3-methylcrotonic acid which is obtained in step (aI) of the above described method is enzymatically further converted into 3-methylbutyric acid according to step (aII) of the above described method. This conversion can, e.g., be achieved by making use of an enzyme which is classified as a 2-enoate reductase (EC 1.3.1.31). The reaction is schematically shown in Figure 10. 2-enoate reductases are enzymes which naturally catalyze the following reaction:



This enzyme occurs in a variety of organism, including eukaryotic and prokaryotic organisms, such as animals, fungi and bacteria. The enzyme has, e.g., been described in *Cichorium intybus*, *Marchantia polymorpha*, *Solanum lycopersicum*, *Absidia glauca*, *Kluyveromyces lactis*, *Penicillium citrinum*; *Rhodosporidium*, *Saccharomyces cerevisiae*, *Clostridium kluyveri*, *Clostridium bifermentans*, *Clostridium botulinum*, *Clostridium difficile*, *Clostridium ghonii*, *Clostridium manganotii*, *Clostridium oceanicum*, *Clostridium sordellii*, *Clostridium sporogenes*, *Clostridium sticklandii*, *Clostridium tyrobutyricum*, *Achromobacter* sp., *Burkholderia* sp., *Gluconobacter oxydans*, *Lactobacillus casei*, *Pseudomonas putida*, *Shewanella* sp., *Yersinia bercovieri*, *Bacillus subtilis*, *Moorella thermoacetica* and *Peptostreptococcus anaerobius*. The enoate reductase of *Clostridia* has been described, e.g., in Tischler et al. (*Eur. J. Biochem.* 97 (1979), 103-112).

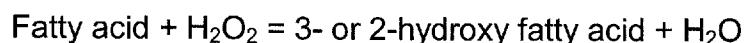
The 3-methylbutyric acid produced according to a method of the present invention can be further enzymatically converted into isobutene, preferably by an oxidative decarboxylation. The reaction is schematically shown in Figure 11a.

This conversion can be achieved by means and methods known in the art or by new means as described herein further below.

One possibility which has been described in van Leeuwen et al. (*Appl. Microbiol. Biotechnol.* 93 (2012), 1377-1387) involves the use of a cytochrome P450 from *Rhodotorula minuta* which has been reported to be able to catalyze the conversion of 3-methylbutyric acid (isovalerate) into isobutene (Fukuda et al., *Biochem. Biophys. Res. Commun.* 21(1994), 516-522 and Fukuda et al., *J. Biochem.* 119 (1996), 314-318). This cytochrome P450 is referred to as P450_{rm}. It is a membrane protein, in particular a microsomal protein and has been annotated as an "isobutene-forming enzyme and benzoate 4-hydroxylase". Thus, in one preferred embodiment the conversion of 3-methylbutyric acid into isobutene is achieved by making use of a cytochrome P450, more preferably of the cytochrome P450 of *R. minuta*. The sequence of this enzyme is available under UniProt Accession number Q12668. In a preferred embodiment such an enzyme has an amino acid sequence as shown in SEQ ID NO: 4 or shows an amino acid sequence which is at least x% homologous to

SEQ ID NO: 4 and has the activity of an cytochrome P450 with x being an integer between 30 and 100, preferably 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99 wherein such an enzyme is capable catalyze the conversion of 3-methylbutyric acid (isovalerate) into isobutene.

According to one particularly preferred embodiment of the present invention 3-methylbutyric acid is enzymatically converted into isobutene by an enzymatically catalyzed oxidative decarboxylation catalyzed by a cytochrome P450 olefin forming fatty acid decarboxylase. The term "cytochrome P450 olefin forming fatty acid decarboxylase" refers to a cytochrome P450 which belongs to the cyp152 family and which has the ability to decarboxylate fatty acids to the terminal olefins. In general, P450s form a large superfamily of multifunctional proteins and are divided into different CYP families according to their sequence similarity (Ortiz de Montellano, P. R. (ed.), 2005, Cytochrome P450: structure, mechanism and biochemistry, 3rd ed. Kluwer Academics, New York, NY). Belcher et al. (J. Biol. Chem. 289 (2014), 6535-6550; Figure 2) shows an overview of members of the cyp152 family and their phylogenetic relationship. The family members of the cyp152 family are characterized in that they are cytosolic hemoproteins with sequence homology to P450 monooxygenases. Enzymes of this type are produced by bacteria (e.g. *Sphingomonas paucimobilis*, *Bacillus subtilis* and those mentioned herein). Catalytic turnover rates are high compared with those of monooxygenation reactions as well as peroxide shunt reactions catalysed by the common P450s. The catalyzed reaction is hydroxylation of fatty acids in α - and/or β -position:



As reported by Rude et al. (Appl. Environ. Microbiol. 77 (2011), 1718-1727) some cyp152 P450 enzymes have the ability to decarboxylate and to hydroxylate fatty acids (in α - and/or β -position), suggesting a common reaction intermediate in their catalytic mechanism and specific structural determinants that favor one reaction over the other.

More preferably the term "cytochrome P450 olefin forming fatty acid decarboxylase" refers to the CYP450 olefin forming fatty acid decarboxylase of the bacterium *Jeotgalicoccus* sp. ATCC 8456 or a highly related enzyme which has the ability to decarboxylate fatty acids. The CYP450 olefin forming fatty acid decarboxylase of the

bacterium *Jeotgalicoccus* sp. 8456 is in the following referred to as "Ole T JE". In the literature this enzyme is also referred to as "CYP152L1" (Belcher et al., J. Biol. Chem. 289 (2014), 6535-6550).

Ole T JE had been identified in the bacterium *Jeotgalicoccus* sp. ATCC 8456 as a terminal olefin-forming fatty acid decarboxylase (Rude et al., Appl. Environm. Microbiol. 77 (2011), 1718-1727). The nucleotide sequence of the gene encoding the protein has been deposited in GenBank under accession number HQ709266 and due to sequence homologies it has been assigned to the cyp152 enzyme family of P450 peroxygenases (Rude et al., loc. cit.). The protein sequence is available at Uniprot accession number: E9NSU2. As reported in Rude et al. (loc. cit.), *Jeotgalicoccus* sp. ATCC 8456 was able to produce terminal olefins with 18 to 20 C atoms. It has been reported by Belcher et al. (loc. cit.) that Ole T JE binds avidly to a range of long chain fatty acids and produces terminal alkenes from a range of saturated fatty acids (C12 – C20).

Although it has been described in the literature that Ole T JE uses as substrates long chain fatty acids, the inventors surprisingly found that Ole T JE can actually accept 3-methylbutyric acid as a substrate and convert it into isobutene.

In a preferred embodiment the Ole T JE is an enzyme which

- (a) comprises the sequence as shown in SEQ ID NO:1 or a sequence which is at least 60% identical to the amino acid sequence shown in SEQ ID NO: 1; and
- (b) shows the activity of converting 3-methylbutyric acid via oxidative decarboxylation into isobutene.

As mentioned above in (a), the enzyme comprises the sequence as shown in SEQ ID NO:1 or a sequence which is at least 60% identical to the amino acid sequence shown in SEQ ID NO: 1. SEQ ID NO: 1 represents the amino acid sequence of the Ole T JE protein. In one preferred embodiment the Ole T JE enzyme employed in a method according to the present invention is the Ole T JE protein comprising the amino acid sequence as shown in SEQ ID NO: 1. In another preferred embodiment the Ole T JE enzyme employed in the method according to the present invention is an enzyme which is structurally related to the Ole T JE protein and which also shows the property of being able to convert 3-methylbutyric acid via oxidative decarboxylation into isobutene. The term "structurally related" means that the amino

acid sequence of the enzyme shows at least 60% sequence identity to the amino acid sequence shown in SEQ ID NO:1. Preferably, the sequence identity is at least 70%, more preferably at least 80%, 85% or 90%, even more preferably 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% and particularly preferred at least 99% to SEQ ID NO:1.

Structure/function analyses of the Ole T JE protein have shown that the residue in position 85 of SEQ ID NO:1 may play a role in the decarboxylation activity of the Ole T JE protein. In one preferred embodiment the enzyme employed in the method according to the present invention is an enzyme which has an amino acid sequence which is at least 60% identical to the amino acid sequence as shown in SEQ ID NO: 1 and in which the amino acid residue corresponding to position 85 of SEQ ID NO: 1 is not glutamine. In another preferred embodiment the enzyme employed in the method according to the present invention is an enzyme which has an amino acid sequence which is at least 60% identical to the amino acid sequence as shown in SEQ ID NO: 1 and in which the amino acid residue corresponding to position 85 of SEQ ID NO: 1 is histidine.

As regards the determination of sequence identity, the following should apply: When the sequences which are compared do not have the same length, the degree of identity either refers to the percentage of amino acid residues in the shorter sequence which are identical to amino acid residues in the longer sequence or to the percentage of amino acid residues in the longer sequence which are identical to amino acid residues in the shorter sequence. Preferably, it refers to the percentage of amino acid residues in the shorter sequence which are identical to amino acid residues in the longer sequence. The degree of sequence identity can be determined according to methods well known in the art using preferably suitable computer algorithms such as CLUSTAL.

When using the Clustal analysis method to determine whether a particular sequence is, for instance, at least 60% identical to a reference sequence default settings may be used or the settings are preferably as follows: Matrix: blosum 30; Open gap penalty: 10.0; Extend gap penalty: 0.05; Delay divergent: 40; Gap separation distance: 8 for comparisons of amino acid sequences. For nucleotide sequence comparisons, the Extend gap penalty is preferably set to 5.0.

In a preferred embodiment ClustalW2 is used for the comparison of amino acid sequences. In the case of pairwise comparisons/alignments, the following settings are preferably chosen: Protein weight matrix: BLOSUM 62; gap open: 10; gap extension: 0.1. In the case of multiple comparisons/alignments, the following settings are preferably chosen: Protein weight matrix: BLOSUM 62; gap open: 10; gap extension: 0.2; gap distance: 5; no end gap.

Preferably, the degree of identity is calculated over the complete length of the sequence.

Amino acid residues located at a position corresponding to a position as indicated herein-below in the amino acid sequence shown in SEQ ID NO:1 can be identified by the skilled person by methods known in the art. For example, such amino acid residues can be identified by aligning the sequence in question with the sequence shown in SEQ ID NO:1 and by identifying the positions which correspond to the above indicated positions of SEQ ID NO:1. The alignment can be done with means and methods known to the skilled person, e.g. by using a known computer algorithm such as the Lipman-Pearson method (Science 227 (1985), 1435) or the CLUSTAL algorithm. It is preferred that in such an alignment maximum homology is assigned to conserved amino acid residues present in the amino acid sequences.

In a preferred embodiment ClustalW2 is used for the comparison of amino acid sequences. In the case of pairwise comparisons/alignments, the following settings are preferably chosen: Protein weight matrix: BLOSUM 62; gap open: 10; gap extension: 0.1. In the case of multiple comparisons/alignments, the following settings are preferably chosen: Protein weight matrix: BLOSUM 62; gap open: 10; gap extension: 0.2; gap distance: 5; no end gap.

As mentioned above in (b), the enzyme shows the activity of converting 3-methylbutyric acid via oxidative decarboxylation into isobutene. This activity can be assayed as described in the appended Examples.

In another embodiment the conversion of 3-methylbutyric acid into isobutene is achieved by making use a cytochrome P450 fatty acid decarboxylase from *Macrococcus caseolyticus*, preferably from strain JCSC5402. The amino acid sequence of said protein is shown in SEQ ID NO: 2 (Uniprot Accession number:

B9EBA0). It is of course not only possible to use an enzyme exactly showing this amino acid. It is also possible to use an enzyme which comprises a sequence which is at least 60% identical to the amino acid sequence shown in SEQ ID NO: 2. Preferably, the sequence identity is at least 70%, more preferably at least 80%, 85% or 90%, even more preferably 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% and particularly preferred at least 99% to SEQ ID NO:2 and the enzyme has the enzymatic activity of converting 3-methylbutyric acid into isobutene. As regards the determination of the sequence identity, the same applies as has been set forth above.

In a preferred embodiment the conversion of 3-methylbutyric acid into isobutene is achieved by making use of a cytochrome P450 in combination with a cytochrome P450 reductase.

The reductase can be directly fused to the cytochrome P450 or it can be present as a separate enzyme.

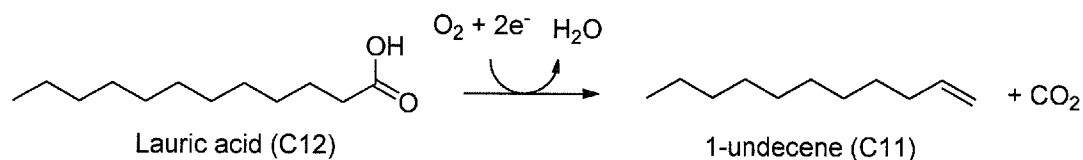
In one preferred embodiment the reaction uses NADPH as a reducing agent. In such an embodiment it is preferred that the P450 reductase is NADPH dependent. An example and preferred embodiment is the Rhodococcus fusion reductase (RhFRED) domain from Rhodococcus, e.g. Rhodococcus sp. NCIMB 9784 (Roberts et al., J. Bacteriol. 184 (2002), 3898-3908). When used in connection with a cytochrome P450 enzyme or fused to a cytochrome P450 enzyme the resultant catalytic activity of the fusion CYP450 enzyme can be driven by NADPH.

In a further preferred embodiment the reaction employs a flavoprotein/flavodoxin reductase as redox mediator protein. The corresponding reaction scheme is shown in Figure 11b. Examples are the flavodoxin (Fld) and flavodoxin reductase (FdR) proteins from E. coli (Liu et al., Biotechnology for Biofuels 7 (2014), 28).

In another preferred embodiment the reaction employs ferredoxin/ferredoxin reductase as redox partner.

According to another possibility, the enzymatic conversion of 3-methylbutyric acid into isobutene can preferably be achieved by making use a non-heme iron oxygenase. Non-heme iron oxygenases have been reported to be able to catalyze the oxidative decarboxylation of a C_n carboxylic acid into the respective C_n-1 terminal alkene. Rui et al. describes the biosynthesis of medium-chain 1-alkenes by a non-heme iron oxygenase (Rui et al., "Microbial biosynthesis of medium-chain 1-

alkenes by non-heme oxidase"; Proc. Natl. Acad. Sci., published ahead of print on December 8, 2014; doi:10.1073/pnas.1419701112) which is found in several *Pseudomonas* species. The x-ray structure of these non-heme iron oxygenases show a typical non-heme iron oxygenase active site having a coordination triade of His/His/Glu. These non-heme iron oxygenases have been shown to catalyze the following reaction:



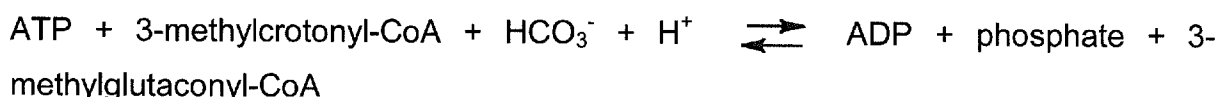
Rui et al. describe that the described non-heme iron oxygenases have a specificity for a chain length of the Cn carboxylic acid from C10 to C14. Yet, in the context of the present invention, these non-heme iron oxygenases which are capable of catalyzing the oxidative decarboxylation of a Cn carboxylic acid into the respective Cn-1 terminal alkene can be used to catalyze the conversion of 3-methylbutyric acid into isobutene according to the present invention.

Accordingly, in a preferred embodiment, the conversion of 3-methylbutyric acid into isobutene is achieved by making use a non-heme iron oxygenases capable of catalyzing the oxidative decarboxylation of a Cn carboxylic acid into the respective Cn-1 terminal alkene from *Pseudomonas* sp., preferably from *Pseudomonas aeruginosa*, more preferably *Pseudomonas aeruginosa* strain UCBPP-PA14, *Pseudomonas syringae* pv. Tomato, more preferably *Pseudomonas syringae* pv. Tomato strain DC3000, and *Pseudomonas putida*, more preferably *Pseudomonas putida* strain F1. The amino acid sequences of said proteins are shown in SEQ ID NOs: 5 to 7, respectively.

It is, of course, not only possible to use an enzyme exactly showing any of these amino acid sequences of SEQ ID NOs: 5 to 7. It is also possible to use an enzyme which comprises a sequence which is at least 60% identical to any one of the amino acid sequences shown in SEQ ID NOs: 5 to 7. Preferably, the sequence identity is at least 70%, more preferably at least 80%, 85% or 90%, even more preferably 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% and particularly preferred at least 99% to any one of SEQ ID NOs: 5 to 7 and the enzyme has the enzymatic activity of converting

3-methylbutyric acid into isobutene. As regards the determination of the sequence identity, the same applies as has been set forth above.

The 3-methylcrotonyl-CoA which is converted according to the method of the present invention into 3-methylbutyric acid according to any of the above described methods may itself be provided by an enzymatic reaction, e.g. by the conversion of 3-methylglutaconyl-CoA via decarboxylation into 3-methylcrotonyl-CoA. This reaction is schematically shown in Figure 12. It may be catalyzed by different enzymes. In one preferred embodiment, the conversion of 3-methylglutaconyl-CoA via decarboxylation into 3-methylcrotonyl-CoA is catalyzed by a methylcrotonyl-CoA carboxylase (EC 6.4.1.4). Methylcrotonyl-CoA carboxylases have been described to catalyze the following reaction:



, i.e. the carboxylation, but they can be used to catalyze the reaction of decarboxylation. Methylcrotonyl-CoA carboxylases occur in a variety of organism, including eukaryotic and prokaryotic organisms, such as plants, animals, fungi and bacteria. The enzyme has, e.g., been described in *Daucus carota*, *Glycine max*, *Hordeum vulgare*, *Pisum sativum*, *Solanum lycopersicum*, *Solanum tuberosum*, *Zea mays*, *Arabidopsis* sp., *Lens culinaris*, *Homo sapiens*, *Bos taurus*, *Rattus norvegicus*, *Mus musculus*, *Pagrus major*, *Emericella nidulans*, *Pseudomonas aeruginosa*, *Pseudomonas citronellolis*, *Acidaminococcus fermentans*, *Escherichia coli*, *Mycobacterium* sp. and *Achromobacter* sp.

In another preferred embodiment the conversion of 3-methylglutaconyl-CoA via decarboxylation into 3-methylcrotonyl-CoA is catalyzed by a geranoyl-CoA carboxylase (EC 6.4.1.5). Geranoyl-CoA carboxylases naturally catalyze the following reaction:



The enzymes occurs in eukaryotes and prokaryotes, such as plants and bacteria. The enzyme has, e.g., been described in *Daucus carota*, *Glycine max*, *Zea mays*, *Pseudomonas* sp., *Pseudomonas aeruginosa*, *Pseudomonas citronellolis* and *Pseudomonas mendocina*.

In another preferred embodiment the conversion of 3-methylglutaconyl-CoA via decarboxylation into 3-methylcrotonyl-CoA is catalyzed by a 3-methylglutaconyl-CoA decarboxylase, e.g. a 3-methylglutaconyl-CoA decarboxylase of *Myxococcus xanthus* encoded by the *liuB* gene. This gene codes for an enzyme having the two subunits AibA and AibB (Li et al., *Angew. Chem. Int. Ed.* 52 (2013), 1304-1308).

Thus, the present invention also relates to a method for producing isobutene from 3-methylglutaconyl-CoA in which 3-methylglutaconyl-CoA is first converted by a decarboxylation reaction into 3-methylcrotonyl-CoA which is then further enzymatically converted into 3-methylbutyric acid as described herein above which is then further converted into isobutene.

The 3-methylglutaconyl-CoA to be converted into 3-methylcrotonyl-CoA can itself be provided by an enzymatic reaction which occurs naturally, which involves the conversion of 3-hydroxy-3-methylglutaryl-CoA into 3-methylglutaconyl-CoA and which is catalyzed, e.g., by enzymes classified as 3-methylglutaconyl-coenzyme A hydratase (EC 4.2.1.18). This reaction is schematically shown in Figure 13. 3-methylglutaconyl-coenzyme A hydratase are enzymes which catalyze the following reaction:



This enzyme occurs in a variety of organism, including eukaryotic and prokaryotic organisms, such as plants, animals and bacteria. The enzyme has, e.g., been described in *Catharantus roseus*, *Homo sapiens*, *Bos taurus*, *Ovis aries*, *Acinetobacter* sp. and *Pseudomonas putida*.

The conversion of 3-hydroxy-3-methylglutaryl-CoA into 3-methylglutaconyl-CoA can also be achieved by making use of a 3-hydroxy-3-methylglutaryl-coenzyme A dehydratase activity which has been identified, e.g., in *Myxococcus xanthus* and

which is encoded by the *liuC* gene (Li et al., *Angew. Chem. Int. Ed.* 52 (2013), 1304-1308).

Thus, the present invention also relates to a method for producing isobutene from 3-hydroxy-3-methylglutaryl-CoA in which 3-hydroxy-3-methylglutaryl-CoA is first converted into 3-methylglutaconyl-CoA which is then converted by a decarboxylation reaction into 3-methylcrotonyl-CoA, which is then further enzymatically converted into 3-methylbutyric acid and isobutene as described herein above.

The 3-hydroxy-3-methylglutaryl-CoA which is converted into 3-methylglutaconyl-CoA can itself be provided enzymatically, e.g. by the condensation of acetyl-CoA and acetoacetyl-CoA, a reaction which is naturally catalyzed by the enzyme 3-hydroxy-3-methylglutaryl-CoA synthase (also referred to as HMG-CoA synthase). HMG-CoA synthases are classified in EC 2.3.3.10 (formerly, HMG-CoA synthase has been classified as EC 4.1.3.5 but has been transferred to EC 2.3.3.10). The term "HMG-CoA synthase" refers to any enzyme which is able to catalyze the reaction where acetyl-CoA condenses with acetoacetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) (see Figure 14). HMG-CoA synthase is part of the mevalonate pathway. Two pathways have been identified for the synthesis of isopentenyl pyrophosphate (IPP), i.e. the mevalonate pathway and the glyceraldehyde 3-phosphate-pyruvate pathway. HMG-CoA synthase catalyzes the biological Claisen condensation of acetyl-CoA with acetoacetyl-CoA and is a member of a superfamily of acyl-condensing enzymes that includes beta-ketothiolases, fatty acid synthases (beta-ketoacyl carrier protein synthase) and polyketide synthases.

HMG-CoA synthase has been described for various organisms. Also amino acid and nucleic acid sequences encoding HMG-CoA synthases from numerous sources are available. Generally, the sequences only share a low degree of overall sequence identity. For example, the enzymes from *Staphylococcus* or *Streptococcus* show only about 20% identity to those of human and avian HMG-CoA synthase. In some sources it is reported that the bacterial HMG-CoA synthases and their animal counterparts exhibit only about 10% overall sequence identity (Sutherland et al., *J. Bacteriol.* 184 (2002), 4065-4070). However, the amino acid residues involved in the acetylation and condensation reactions are conserved among bacterial and eukaryotic HMG-CoA synthases (Campobasso et al., *J. Biol. Chem.* 279 (2004), 44883-44888). The three-dimensional structure of three HMG-CoA synthase

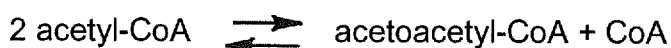
enzymes has been determined and the amino acids crucial for the enzymatic reaction are in principle well characterized (Campobasso et al., loc. cit.; Chun et al., J. Biol.Chem. 275 (2000), 17946-17953; Nagegowda et al., Biochem. J. 383 (2004), 517-527; Hegardt, Biochem. J. 338 (1999), 569-582). In eukaryotes there exist two forms of the HMG-CoA synthase, i.e. a cytosolic and a mitochondrial form. The cytosolic form plays a key role in the production of cholesterol and other isoprenoids and the mitochondrial form is involved in the production of ketone bodies.

In principle any HMG-CoA synthase enzyme can be used in the context of the present invention, in particular from prokaryotic or eukaryotic organisms.

Prokaryotic HMG-CoA synthases are described, e.g., from *Staphylococcus aureus* (Campobasso et al., loc. cit.; Uniprot accession number Q9FD87), *Staphylococcus epidermidis* (Uniprot accession number Q9FD76), *Staphylococcus haemolyticus* (Uniprot accession number Q9FD82), *Enterococcus faecalis* (Sutherland et al., loc. cit.; Uniprot accession number Q9FD7), *Enterococcus faecium* (Uniprot accession number Q9FD66), *Streptococcus pneumonia* (Uniprot accession number Q9FD56), *Streptococcus pyogenes* (Uniprot accession number Q9FD61) and *Methanobacterium thermoautotrophicum* (accession number AE000857), *Borrelia burgdorferi* (NCBI accession number BB0683). Further HMG-CoA synthases are, e.g., described in WO 2011/032934.

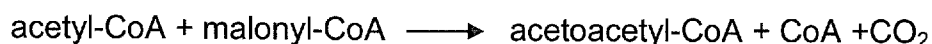
Thus, the present invention also relates to a method for producing isobutene from acetyl-CoA and acetoacetyl-CoA in which acetyl-CoA and acetoacetyl-CoA are first condensed so as to form 3-hydroxy-3-methylglutaryl-CoA, in which 3-hydroxy-3-methylglutaryl-CoA is then converted into 3-methylglutaconyl-CoA, which is then converted by a decarboxylation reaction into 3-methylcrotonyl-CoA, which is then further enzymatically converted into 3-methylbutyric acid, which is then further converted into isobutene as described herein above.

The acetoacetyl-CoA which is used in the production of 3-hydroxy-3-methylglutaryl-CoA can itself be provided by enzymatic reactions. For example, acetoacetyl-CoA can be produced from acetyl-CoA as, e.g., described in WO 2013/057194. Thus, according to the present invention, acetyl-CoA can, for example, be converted into acetoacetyl-CoA by the following reaction:



This reaction is catalyzed by enzymes called acetyl-CoA C-acetyltransferases which are classified as EC 2.3.1.9. Enzymes belonging to this class and catalyzing the above shown conversion of two molecules of acetyl-CoA into acetoacetyl-CoA and CoA occur in organisms of all kingdoms, i.e. plants, animals, fungi, bacteria etc. and have extensively been described in the literature. Nucleotide and/or amino acid sequences for such enzymes have been determined for a variety of organisms, like *Homo sapiens*, *Arabidopsis thaliana*, *E. coli*, *Bacillus subtilis* and *Candida*, to name just some examples. In principle, any acetyl-CoA C-acetyltransferase (EC 2.3.1.9) can be used in the context of the present invention.

Alternatively, the provision of acetoacetyl-CoA may also be achieved by the enzymatic conversion of acetyl-CoA and malonyl-CoA into acetoacetyl-CoA according to the following reaction.



This reaction is catalyzed by an enzyme called acetoacetyl-CoA synthase (EC 2.3.1.194). The gene encoding this enzyme was identified in the mevalonate pathway gene cluster for terpenoid production in a soil-isolated Gram-positive *Streptomyces* sp. Strain CL190 (Okamura et al., PNAS USA 107 (2010), 11265-11270, 2010). Moreover a biosynthetic pathway using this enzyme for acetoacetyl-CoA production was recently developed in *E. coli* (Matsumoto K et al., Biosci. Biotechnol. Biochem, 75 (2011), 364-366).

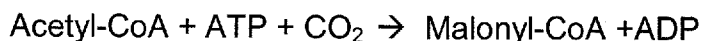
Accordingly, in a preferred embodiment, the enzymatic conversion of acetyl-CoA into said acetoacetyl-CoA consists of a single enzymatic reaction in which acetyl-CoA is directly converted into acetoacetyl-CoA. Preferably, the enzymatic conversion of acetyl-CoA into acetoacetyl-CoA is achieved by making use of an acetyl-CoA acetyltransferase (EC 2.3.1.9) as described above.

Alternatively, the acetoacetyl-CoA can also be provided by an enzymatic conversion which comprises two steps, i.e.;

- (i) the enzymatic conversion of acetyl-CoA into malonyl-CoA; and

- (ii) the enzymatic conversion of malonyl-CoA and acetyl-CoA into acetoacetyl-CoA.

Preferably, the enzymatic conversion of acetyl-CoA into malonyl-CoA is achieved by the use of an acetyl-CoA carboxylase (EC 6.4.1.2). This enzyme catalyzes the following reaction:



Preferably, the enzymatic conversion of malonyl-CoA and acetyl-CoA into acetoacetyl-CoA is achieved by the use of an acetoacetyl-CoA synthase (EC 2.3.1.194). In principle, any acetyl-CoA acetyltransferase (EC 2.3.1.9), acetyl-CoA carboxylase (EC 6.4.1.2) and/or acetoacetyl-CoA synthase (EC 2.3.1.194) can be applied in the method according to the invention.

Figure 15 shows schematically possible ways of producing acetoacetyl-CoA from acetyl-CoA.

Thus, the present invention also relates to a method for producing isobutene from acetyl-CoA, in which acetoacetyl-CoA is produced from acetyl-CoA as described above, in which the thus produced acetoacetyl-CoA is then condensed with acetyl-CoA so as to form 3-hydroxy-3-methylglutaryl-CoA, in which 3-hydroxy-3-methylglutaryl-CoA is then converted into 3-methylglutaconyl-CoA, which is then converted by a decarboxylation reaction into 3-methylcrotonyl-CoA, which is then further enzymatically converted into 3-methylbutyric acid and isobutene as described herein above.

A method according to the present invention may be carried out in vitro or in vivo. An in vitro reaction is understood to be a reaction in which no cells are employed, i.e. an acellular reaction. Thus, in vitro preferably means in a cell-free system. The term "in vitro" in one embodiment means in the presence of isolated enzymes (or enzyme systems optionally comprising possibly required cofactors). In one embodiment, the enzymes employed in the method are used in purified form.

For carrying out the method in vitro the substrates for the reaction and the enzymes are incubated under conditions (buffer, temperature, cosubstrates, cofactors etc.) allowing the enzymes to be active and the enzymatic conversion to occur. The

reaction is allowed to proceed for a time sufficient to produce the respective product. The production of the respective products can be measured by methods known in the art, such as gas chromatography possibly linked to mass spectrometry detection. The enzymes may be in any suitable form allowing the enzymatic reaction to take place. They may be purified or partially purified or in the form of crude cellular extracts or partially purified extracts. It is also possible that the enzymes are immobilized on a suitable carrier.

In another embodiment the method according to the invention is carried out in culture, in the presence of an organism, preferably a microorganism, producing the enzymes described above for the conversions of the method according to the present invention as described herein above. A method which employs a microorganism for carrying out a method according to the invention is referred to as an "in vivo" method. It is possible to use a microorganism which naturally produces the enzymes described above for the conversions of the method according to the present invention or a microorganism which had been genetically modified so that it expresses (including overexpresses) one or more of such enzymes. Thus, the microorganism can be an engineered microorganism which expresses enzymes described above for the conversions of the method according to the present invention, i.e. which has in its genome a nucleotide sequence encoding such enzymes and which has been modified to overexpress them. The expression may occur constitutively or in an induced or regulated manner.

In another embodiment the microorganism can be a microorganism which has been genetically modified by the introduction of one or more nucleic acid molecules containing nucleotide sequences encoding one or more enzymes described above for the conversions of the methods according to the present invention. The nucleic acid molecule can be stably integrated into the genome of the microorganism or may be present in an extrachromosomal manner, e.g. on a plasmid.

Such a genetically modified microorganism can, e.g., be a microorganism that does not naturally express enzymes described above for the conversions of the method according to the present invention and which has been genetically modified to express such enzymes or a microorganism which naturally expresses such enzymes and which has been genetically modified, e.g. transformed with a nucleic acid, e.g. a vector, encoding the respective enzyme(s), and/or insertion of a promoter in front of

the endogenous nucleotide sequence encoding the enzyme in order to increase the respective activity in said microorganism.

However, the invention preferably excludes naturally occurring microorganisms as found in nature expressing an enzyme as described above at levels as they exist in nature. Instead, the microorganism of the present invention and employed in a method of the present invention is preferably a non-naturally occurring microorganism, whether it has been genetically modified to express (including overexpression) an exogenous enzyme of the invention not normally existing in its genome or whether it has been engineered to overexpress an exogenous enzyme.

Thus, the enzymes and (micro)organisms employed in connection with the present invention are preferably non-naturally occurring enzymes or (micro)organisms, i.e. they are enzymes or (micro)organisms which differ significantly from naturally occurring enzymes or microorganism and which do not occur in nature. As regards the enzymes, they are preferably variants of naturally occurring enzymes which do not as such occur in nature. Such variants include, for example, mutants, in particular prepared by molecular biological methods, which show improved properties, such as a higher enzyme activity, higher substrate specificity, higher temperature resistance and the like. As regards the (micro)organisms, they are preferably genetically modified organisms as described herein above which differ from naturally occurring organisms due to a genetic modification. Genetically modified organisms are organisms which do not naturally occur, i.e., which cannot be found in nature, and which differ substantially from naturally occurring organisms due to the introduction of a foreign nucleic acid molecule.

By overexpressing an exogenous or endogenous enzyme as described herein above, the concentration of the enzyme is substantially higher than what is found in nature, which can then unexpectedly force the reaction of the present invention which uses a non-natural for the respective enzyme. Preferably, the concentration of the overexpressed enzyme is at least 5%, 10%, 20%, 30% or 40% of the total host cell protein.

A "non-natural" substrate is understood to be a molecule that is not acted upon by the respective enzyme in nature, even though it may actually coexist in the microorganism along with the endogenous enzyme. This "non-natural" substrate is not converted by the microorganism in nature as other substrates are preferred (e.g. the "natural substrate"). Thus, the present invention contemplates utilizing a non-

natural substrate with the enzymes described above in an environment not found in nature.

Thus, it is also possible in the context of the present invention that the microorganism is a microorganism which naturally does not have the respective enzyme activity but which is genetically modified so as to comprise a nucleotide sequence allowing the expression of a corresponding enzyme. Similarly, the microorganism may also be a microorganism which naturally has the respective enzyme activity but which is genetically modified so as to enhance such an activity, e.g. by the introduction of an exogenous nucleotide sequence encoding a corresponding enzyme or by the introduction of a promoter for the endogenous gene encoding the enzyme to increase endogenous production to overexpressed (non-natural) levels.

If a microorganism is used which naturally expresses a corresponding enzyme, it is possible to modify such a microorganism so that the respective activity is overexpressed in the microorganism. This can, e.g., be achieved by effecting mutations in the promoter region of the corresponding gene or introduction of a high expressing promoter so as to lead to a promoter which ensures a higher expression of the gene. Alternatively, it is also possible to mutate the gene as such so as to lead to an enzyme showing a higher activity.

By using microorganisms which express enzymes described above for the conversions of the methods according to the present invention, it is possible to carry out the methods according to the invention directly in the culture medium, without the need to separate or purify the enzymes.

In one embodiment the organism employed in a method according to the invention is a microorganism which has been genetically modified to contain a foreign nucleic acid molecule encoding at least one enzyme described above for the conversions of the methods according to the present invention. The term "foreign" or "exogenous" in this context means that the nucleic acid molecule does not naturally occur in said microorganism. This means that it does not occur in the same structure or at the same location in the microorganism. In one preferred embodiment, the foreign nucleic acid molecule is a recombinant molecule comprising a promoter and a coding sequence encoding the respective enzyme in which the promoter driving expression of the coding sequence is heterologous with respect to the coding sequence.

“Heterologous” in this context means that the promoter is not the promoter naturally driving the expression of said coding sequence but is a promoter naturally driving expression of a different coding sequence, i.e., it is derived from another gene, or is a synthetic promoter or a chimeric promoter. Preferably, the promoter is a promoter heterologous to the microorganism, i.e. a promoter which does naturally not occur in the respective microorganism. Even more preferably, the promoter is an inducible promoter. Promoters for driving expression in different types of organisms, in particular in microorganisms, are well known to the person skilled in the art.

In a further embodiment the nucleic acid molecule is foreign to the microorganism in that the encoded enzyme is not endogenous to the microorganism, i.e. is naturally not expressed by the microorganism when it is not genetically modified. In other words, the encoded enzyme is heterologous with respect to the microorganism. The foreign nucleic acid molecule may be present in the microorganism in extrachromosomal form, e.g. as a plasmid, or stably integrated in the chromosome. A stable integration is preferred. Thus, the genetic modification can consist, e.g. in integrating the corresponding gene(s) encoding the enzyme(s) into the chromosome, or in expressing the enzyme(s) from a plasmid containing a promoter upstream of the enzyme-coding sequence, the promoter and coding sequence preferably originating from different organisms, or any other method known to one of skill in the art.

The term “microorganism” in the context of the present invention refers to bacteria, as well as to fungi, such as yeasts, and also to algae and archaea. In one preferred embodiment, the microorganism is a bacterium. In principle any bacterium can be used. Preferred bacteria to be employed in the process according to the invention are bacteria of the genus *Bacillus*, *Clostridium*, *Corynebacterium*, *Pseudomonas*, *Zymomonas* or *Escherichia*. In a particularly preferred embodiment the bacterium belongs to the genus *Escherichia* and even more preferred to the species *Escherichia coli*. In another preferred embodiment the bacterium belongs to the species *Pseudomonas putida* or to the species *Zymomonas mobilis* or to the species *Corynebacterium glutamicum* or to the species *Bacillus subtilis*.

It is also possible to employ an extremophilic bacterium such as *Thermus thermophilus*, or anaerobic bacteria from the family *Clostridia*.

In another preferred embodiment the microorganism is a fungus, more preferably a fungus of the genus *Saccharomyces*, *Schizosaccharomyces*, *Aspergillus*,

Trichoderma, Kluyveromyces or Pichia and even more preferably of the species Saccharomyces cerevisiae, Schizosaccharomyces pombe, Aspergillus niger, Trichoderma reesei, Kluyveromyces marxianus, Kluyveromyces lactis, Pichia pastoris, Pichia torula or Pichia utilis.

In another embodiment, the method according to the invention makes use of a photosynthetic microorganism expressing at least one enzyme for the conversion according to the invention as described above. Preferably, the microorganism is a photosynthetic bacterium, or a microalgae. In a further embodiment the microorganism is an algae, more preferably an algae belonging to the diatomeae.

It is also conceivable to use in the method according to the invention a combination of microorganisms wherein different microorganisms express different enzymes as described above. The genetic modification of microorganisms to express an enzyme of interest will also be further described in detail below.

In another embodiment, the method of the invention comprises the step of providing the organism, preferably the microorganism carrying the respective enzyme activity or activities in the form of a (cell) culture, preferably in the form of a liquid cell culture, a subsequent step of cultivating the organism, preferably the microorganism in a fermenter (often also referred to a bioreactor) under suitable conditions allowing the expression of the respective enzyme and further comprising the step of effecting an enzymatic conversion of a method of the invention as described herein above. Suitable fermenter or bioreactor devices and fermentation conditions are known to the person skilled in the art. A bioreactor or a fermenter refers to any manufactured or engineered device or system known in the art that supports a biologically active environment. Thus, a bioreactor or a fermenter may be a vessel in which a chemical/biochemical like the method of the present invention is carried out which involves organisms, preferably microorganisms and/or biochemically active substances, i.e., the enzyme(s) described above derived from such organisms or organisms harboring the above described enzyme(s). In a bioreactor or a fermenter, this process can either be aerobic or anaerobic. These bioreactors are commonly cylindrical, and may range in size from litres to cubic metres, and are often made of stainless steel. In this respect, without being bound by theory, the fermenter or bioreactor may be designed in a way that it is suitable to cultivate the organisms,

preferably microorganisms, in, e.g., a batch-culture, feed-batch-culture, perfusion culture or chemostate-culture, all of which are generally known in the art.

The culture medium can be any culture medium suitable for cultivating the respective organism or microorganism.

In a preferred embodiment the method according to the present invention also comprises the step of recovering the isobutene produced by the method. For example, if the method according to the present invention is carried out *in vivo* by fermenting a corresponding microorganism expressing the necessary enzymes, the isobutene can be recovered from the fermentation off-gas by methods known to the person skilled in the art.

The enzymes used in the method according to the invention can be a naturally occurring enzymes or enzymes which are derived from a naturally occurring enzymes, e.g. by the introduction of mutations or other alterations which, e.g., alter or improve the enzymatic activity, the stability, etc.

Methods for modifying and/or improving the desired enzymatic activities of proteins are well-known to the person skilled in the art and include, e.g., random mutagenesis or site-directed mutagenesis and subsequent selection of enzymes having the desired properties or approaches of the so-called "directed evolution".

For example, for genetic modification in prokaryotic cells, a nucleic acid molecule encoding a corresponding enzyme can be introduced into plasmids which permit mutagenesis or sequence modification by recombination of DNA sequences. Standard methods (see Sambrook and Russell (2001), *Molecular Cloning: A Laboratory Manual*, CSH Press, Cold Spring Harbor, NY, USA) allow base exchanges to be performed or natural or synthetic sequences to be added. DNA fragments can be ligated by using adapters and linkers complementary to the fragments. Moreover, engineering measures which provide suitable restriction sites or remove surplus DNA or restriction sites can be used. In those cases, in which insertions, deletions or substitutions are possible, *in vitro* mutagenesis, "primer repair", restriction or ligation can be used. In general, a sequence analysis, restriction analysis and other methods of biochemistry and molecular biology are carried out as analysis methods. The resulting enzyme variants are then tested for the desired

activity, e.g., enzymatic activity, with an assay as described above and in particular for their increased enzyme activity.

As described above, the microorganism employed in a method of the invention or contained in the composition of the invention may be a microorganism which has been genetically modified by the introduction of a nucleic acid molecule encoding a corresponding enzyme. Thus, in a preferred embodiment, the microorganism is a recombinant microorganism which has been genetically modified to have an increased activity of at least one enzyme described above for the conversions of the method according to the present invention. This can be achieved e.g. by transforming the microorganism with a nucleic acid encoding a corresponding enzyme. A detailed description of genetic modification of microorganisms will be given further below. Preferably, the nucleic acid molecule introduced into the microorganism is a nucleic acid molecule which is heterologous with respect to the microorganism, i.e. it does not naturally occur in said microorganism.

In the context of the present invention, an "increased activity" means that the expression and/or the activity of an enzyme in the genetically modified microorganism is at least 10%, preferably at least 20%, more preferably at least 30% or 50%, even more preferably at least 70% or 80% and particularly preferred at least 90% or 100% higher than in the corresponding non-modified microorganism. In even more preferred embodiments the increase in expression and/or activity may be at least 150%, at least 200% or at least 500%. In particularly preferred embodiments the expression is at least 10-fold, more preferably at least 100-fold and even more preferred at least 1000-fold higher than in the corresponding non-modified microorganism.

The term "increased" expression/activity also covers the situation in which the corresponding non-modified microorganism does not express a corresponding enzyme so that the corresponding expression/activity in the non-modified microorganism is zero. Preferably, the concentration of the overexpressed enzyme is at least 5%, 10%, 20%, 30%, or 40% of the total host cell protein.

Methods for measuring the level of expression of a given protein in a cell are well known to the person skilled in the art. In one embodiment, the measurement of the level of expression is done by measuring the amount of the corresponding protein. Corresponding methods are well known to the person skilled in the art and include Western Blot, ELISA etc. In another embodiment the measurement of the level of

expression is done by measuring the amount of the corresponding RNA. Corresponding methods are well known to the person skilled in the art and include, e.g., Northern Blot.

In the context of the present invention the term "recombinant" means that the microorganism is genetically modified so as to contain a nucleic acid molecule encoding an enzyme as defined above as compared to a wild-type or non-modified microorganism. A nucleic acid molecule encoding an enzyme as defined above can be used alone or as part of a vector.

The nucleic acid molecules can further comprise expression control sequences operably linked to the polynucleotide comprised in the nucleic acid molecule. The term "operatively linked" or "operably linked", as used throughout the present description, refers to a linkage between one or more expression control sequences and the coding region in the polynucleotide to be expressed in such a way that expression is achieved under conditions compatible with the expression control sequence.

Expression comprises transcription of the heterologous DNA sequence, preferably into a translatable mRNA. Regulatory elements ensuring expression in fungi as well as in bacteria, are well known to those skilled in the art. They encompass promoters, enhancers, termination signals, targeting signals and the like. Examples are given further below in connection with explanations concerning vectors.

Promoters for use in connection with the nucleic acid molecule may be homologous or heterologous with regard to its origin and/or with regard to the gene to be expressed. Suitable promoters are for instance promoters which lend themselves to constitutive expression. However, promoters which are only activated at a point in time determined by external influences can also be used. Artificial and/or chemically inducible promoters may be used in this context.

The vectors can further comprise expression control sequences operably linked to said polynucleotides contained in the vectors. These expression control sequences may be suited to ensure transcription and synthesis of a translatable RNA in bacteria or fungi.

In addition, it is possible to insert different mutations into the polynucleotides by methods usual in molecular biology (see for instance Sambrook and Russell (2001), *Molecular Cloning: A Laboratory Manual*, CSH Press, Cold Spring Harbor, NY, USA),

leading to the synthesis of polypeptides possibly having modified biological properties. The introduction of point mutations is conceivable at positions at which a modification of the amino acid sequence for instance influences the biological activity or the regulation of the polypeptide.

Moreover, mutants possessing a modified substrate or product specificity can be prepared. Preferably, such mutants show an increased activity. Alternatively, mutants can be prepared the catalytic activity of which is abolished without losing substrate binding activity.

Furthermore, the introduction of mutations into the polynucleotides encoding an enzyme as defined above allows the gene expression rate and/or the activity of the enzymes encoded by said polynucleotides to be reduced or increased.

For genetically modifying bacteria or fungi, the polynucleotides encoding an enzyme as defined above or parts of these molecules can be introduced into plasmids which permit mutagenesis or sequence modification by recombination of DNA sequences. Standard methods (see Sambrook and Russell (2001), Molecular Cloning: A Laboratory Manual, CSH Press, Cold Spring Harbor, NY, USA) allow base exchanges to be performed or natural or synthetic sequences to be added. DNA fragments can be connected to each other by applying adapters and linkers to the fragments. Moreover, engineering measures which provide suitable restriction sites or remove surplus DNA or restriction sites can be used. In those cases, in which insertions, deletions or substitutions are possible, *in vitro* mutagenesis, "primer repair", restriction or ligation can be used. In general, a sequence analysis, restriction analysis and other methods of biochemistry and molecular biology are carried out as analysis methods.

Thus, in accordance with the present invention a recombinant microorganism can be produced by genetically modifying fungi or bacteria comprising introducing the above-described polynucleotides, nucleic acid molecules or vectors into a fungus or bacterium.

The polynucleotide encoding the respective enzyme is expressed so as to lead to the production of a polypeptide having any of the activities described above. An overview of different expression systems is for instance contained in Methods in Enzymology 153 (1987), 385-516, in Bitter et al. (Methods in Enzymology 153 (1987), 516-544) and in Sawers et al. (Applied Microbiology and Biotechnology 46 (1996), 1-9), Billman-Jacobe (Current Opinion in Biotechnology 7 (1996), 500-4), Hockney (Trends

in Biotechnology 12 (1994), 456-463), Griffiths et al., (Methods in Molecular Biology 75 (1997), 427-440). An overview of yeast expression systems is for instance given by Hensing et al. (Antonie van Leeuwenhoek 67 (1995), 261-279), Bussineau et al. (Developments in Biological Standardization 83 (1994), 13-19), Gellissen et al. (Antonie van Leeuwenhoek 62 (1992), 79-93, Fleer (Current Opinion in Biotechnology 3 (1992), 486-496), Vedvick (Current Opinion in Biotechnology 2 (1991), 742-745) and Buckholz (Bio/Technology 9 (1991), 1067-1072).

Expression vectors have been widely described in the literature. As a rule, they contain not only a selection marker gene and a replication-origin ensuring replication in the host selected, but also a bacterial or viral promoter, and in most cases a termination signal for transcription. Between the promoter and the termination signal there is in general at least one restriction site or a polylinker which enables the insertion of a coding DNA sequence. The DNA sequence naturally controlling the transcription of the corresponding gene can be used as the promoter sequence, if it is active in the selected host organism. However, this sequence can also be exchanged for other promoter sequences. It is possible to use promoters ensuring constitutive expression of the gene and inducible promoters which permit a deliberate control of the expression of the gene. Bacterial and viral promoter sequences possessing these properties are described in detail in the literature. Regulatory sequences for the expression in microorganisms (for instance *E. coli*, *S. cerevisiae*) are sufficiently described in the literature. Promoters permitting a particularly high expression of a downstream sequence are for instance the T7 promoter (Studier et al., Methods in Enzymology 185 (1990), 60-89), lacUV5, trp, trp-lacUV5 (DeBoer et al., in Rodriguez and Chamberlin (Eds), Promoters, Structure and Function; Praeger, New York, (1982), 462-481; DeBoer et al., Proc. Natl. Acad. Sci. USA (1983), 21-25), lp1, rac (Boros et al., Gene 42 (1986), 97-100). Inducible promoters are preferably used for the synthesis of polypeptides. These promoters often lead to higher polypeptide yields than do constitutive promoters. In order to obtain an optimum amount of polypeptide, a two-stage process is often used. First, the host cells are cultured under optimum conditions up to a relatively high cell density. In the second step, transcription is induced depending on the type of promoter used. In this regard, a tac promoter is particularly suitable which can be induced by lactose or IPTG (=isopropyl- β -D-thiogalactopyranoside) (deBoer et al., Proc. Natl. Acad. Sci. USA 80

(1983), 21-25). Termination signals for transcription are also described in the literature.

The transformation of the host cell with a polynucleotide or vector as described above can be carried out by standard methods, as for instance described in Sambrook and Russell (2001), *Molecular Cloning: A Laboratory Manual*, CSH Press, Cold Spring Harbor, NY, USA; *Methods in Yeast Genetics, A Laboratory Course Manual*, Cold Spring Harbor Laboratory Press, 1990. The host cell is cultured in nutrient media meeting the requirements of the particular host cell used, in particular in respect of the pH value, temperature, salt concentration, aeration, antibiotics, vitamins, trace elements etc.

The present invention furthermore relates to the use of

- a phosphate butyryltransferase (EC 2.3.1.19) or an organism expressing a phosphate butyryltransferase (EC 2.3.1.19); or
- a phosphate acetyltransferase (EC 2.3.1.8) or an organism expressing a phosphate acetyltransferase (EC 2.3.1.8)

for the enzymatic conversion of 3-methylcrotonyl-CoA into 3-methylcrotonyl phosphate.

The present invention furthermore relates to the use of a phosphotransferase (EC 2.7.2), e.g. of a butyrate kinase (EC 2.7.2.7), a branched-chain-fatty-acid kinase (EC 2.7.2.14), a propionate kinase (EC 2.7.2.15) and/or an acetate kinase (EC 2.7.2.1), or of a microorganisms expressing such enzymes for the enzymatic conversion of 3-methylcrotonyl-CoA into 3-methylcrotonyl phosphate.

The present invention also relates to the use of a combination comprising

- a phosphate butyryltransferase (EC 2.3.1.19) and/or a phosphate acetyltransferase (EC 2.3.1.8); and
- a phosphotransferase (EC 2.7.2), e.g. a a butyrate kinase (EC 2.7.2.7), a branched-chain-fatty-acid kinase (EC 2.7.2.14), a propionate kinase (EC 2.7.2.15) and/or an acetate kinase (EC 2.7.2.1),

or of a microorganism expressing such a combination of enzymes for the conversion of 3-methylcrotonyl-CoA into 3-methylcrotonic acid.

Moreover, the present invention relates to the use of a combination comprising

- a phosphate butyryltransferase (EC 2.3.1.19) and/or a phosphate acetyltransferase (EC 2.3.1.8); and
- a phosphotransferase (EC 2.7.2), e.g. a butyrate kinase (EC 2.7.2.7), a branched-chain-fatty-acid kinase (EC 2.7.2.14), a propionate kinase (EC 2.7.2.15) and/or an acetate kinase (EC 2.7.2.1); and
- a 2-enoate reductase (EC 1.3.1.31)

or of a microorganism expressing such a combination of enzymes for the conversion of 3-methylcrotonyl-CoA into 3-methylbutyric acid.

Moreover, the present invention relates to the use of a combination comprising

- a phosphate butyryltransferase (EC 2.3.1.19) and/or a phosphate acetyltransferase (EC 2.3.1.8); and
- a phosphotransferase (EC 2.7.2), e.g. a butyrate kinase (EC 2.7.2.7), a branched-chain-fatty-acid kinase (EC 2.7.2.14), a propionate kinase (EC 2.7.2.15) and/or an acetate kinase (EC 2.7.2.1); and
- a 2-enoate reductase (EC 1.3.1.31); and
- a cytochrome P450 or a non-heme iron oxygenase

or of a microorganism expressing such a combination of enzymes for the conversion of 3-methylcrotonyl-CoA into isobutene.

The present invention also relates to the use of a thioesterase (EC 3.1.2) and/or a CoA-transferase (EC 2.8.3) or of a microorganism expressing such an enzyme for the conversion of 3-methylcrotonyl-CoA into 3-methylcrotonic acid.

The present invention also relates to the use of a combination comprising

- a thioesterase (EC 3.1.2) and/or a CoA-transferase (EC 2.8.3); and
- a 2-enoate reductase (EC 1.3.1.31)

or of a microorganism expressing such a combination of enzymes for the conversion of 3-methylcrotonyl-CoA into 3-methylbutyric acid.

Moreover, the present invention relates to the use of a combination comprising

- a thioesterase (EC 3.1.2) and/or a CoA-transferase (EC 2.8.3); and
- a 2-enoate reductase (EC 1.3.1.31); and

- a cytochrome P450 or a non-heme iron oxygenase
- or of a microorganism expressing such a combination of enzymes for the conversion of 3-methylcrotonyl-CoA into isobutene.

The present invention also relates to the use of a combination comprising

- (i) an enzyme classified as EC 1.3._._; and
 - (ii1) a phosphate butyryltransferase (EC 2.3.1.19) and/or a phosphate acetyltransferase (EC 2.3.1.8); and
a phosphotransferase (EC 2.7.2), e.g. a butyrate kinase (EC 2.7.2.7), a branched-chain-fatty-acid kinase (EC 2.7.2.14), a propionate kinase (EC 2.7.2.15) and/or an acetate kinase (EC 2.7.2.1); or
 - (ii2) a thioesterase (EC 3.1.2); or
 - (ii3) a CoA-transferase (EC 2.8.3)

or of a microorganism expressing such a combination of enzymes for the conversion of 3-methylcrotonyl-CoA into 3-methylbutyric acid.

The present invention also relates to the use of a combination comprising

- (i) an enzyme classified as EC 1.3._._; and
 - (ii1) a phosphate butyryltransferase (EC 2.3.1.19) and/or a phosphate acetyltransferase (EC 2.3.1.8); and
a phosphotransferase (EC 2.7.2), e.g. a butyrate kinase (EC 2.7.2.7), a branched-chain-fatty-acid kinase (EC 2.7.2.14), a propionate kinase (EC 2.7.2.15) and/or an acetate kinase (EC 2.7.2.1); or
 - (ii2) a thioesterase (EC 3.1.2); or
 - (ii3) a CoA-transferase (EC 2.8.3)
- and

- (iii) a cytochrome P450 or a non-heme iron oxygenase
- or of a microorganism expressing such a combination of enzymes for the conversion of 3-methylcrotonyl-CoA into isobutene.

The present invention also relates to microorganisms expressing an enzyme combination as described herein above in connection with the uses of the present invention.

As regards the enzymes and the microorganism recited in the above uses, the same applies as has been set forth above in connection with a method according to the invention.

Figure 1 shows schematically the reaction of the conversion of 3-methylcrotonyl-CoA into 3-methylbutyryl-CoA by making use of an enzyme classified as EC 1.3.1._ which uses NADPH as a co-factor.

Figure 2 shows schematically the reaction of the conversion of 3-methylcrotonyl-CoA into 3-methylbutyryl-CoA by making use of an enzyme classified as EC 1.3.1._ which uses NADH as a co-factor.

Figure 3 shows schematically the reaction of the conversion of 3-methylcrotonyl-CoA into 3-methylbutyryl-CoA by making use of an enzyme classified as EC 1.3.8._ which uses FADH as a co-factor.

Figure 4 shows schematically the conversion of 3-methylbutyryl-CoA into 3-methylbutyric acid catalyzed by a thioesterase (EC 3.1.2).

Figure 5 shows schematically the conversion of 3-methylbutyryl-CoA into 3-methylbutyric acid catalyzed by a CoA-transferase (EC 2.8.3).

Figure 6 shows schematically the conversion of 3-methylbutyryl-CoA into 3-methylbutyryl phosphate and the subsequent conversion of 3-methylbutyryl phosphate into 3-methylbutyric acid.

Figure 7 shows schematically the conversion of 3-methylcrotonyl-CoA into 3-methylcrotonic acid catalyzed by a thioesterase (EC 3.1.2).

Figure 8 shows schematically the conversion of 3-methylcrotonyl-CoA into 3-methylcrotonic acid catalyzed by a CoA-transferase (EC 2.8.3).

- Figure 9** shows schematically the conversion of 3-methylcrotonyl-CoA into 3-methylcrotonyl phosphate and the subsequent conversion of 3-methylcrotonyl phosphate into 3-methylcrotonic acid.
- Figure 10** shows schematically the conversion of 3-methylcrotonic acid into 3-methylbutyric acid by making use of an enzyme which is classified as a 2-enoate reductase (EC 1.3.1.31).
- Figure 11a** shows schematically the conversion 3-methylbutyric acid into isobutene.
- Figure 11b** shows schematically the conversion 3-methylbutyric acid into isobutene making use of NADPH as reducing agent and a flavodoxin/flavodoxin reductase as redox mediator protein.
- Figure 12** shows the conversion of 3-methylglutaconyl-CoA into 3-methylcrotonyl-CoA via a decarboxylation reaction.
- Figure 13** shows conversion of 3-hydroxy-3-methylglutaryl-CoA into 3-methylglutaconyl-CoA.
- Figure 14** shows the condensation of acetyl-CoA and acetoacetyl-CoA into 3-hydroxy-3-methylglutaryl-CoA.
- Figure 15** shows possible pathways for producing acetoacetyl-CoA from acetyl-CoA.
- Figure 16** shows a GC chromatogram obtained for the enzyme-catalyzed oxidative decarboxylation of isovalerate with the cytochrome P450 from *Jeotgalicoccus* sp. (Uniprot Accession number : E9NSU2) as outlined in Example 2.
- Figure 17** shows chromatograms of isobutene produced by an *E.coli* strain expressing cytochrome P450 from *Jeotgalicoccus* sp. and an *E. coli* control strain as outlined in Example 3.

Figure 18 shows a GC chromatogram obtained for the enzyme-catalyzed oxidative decarboxylation of isovalerate with the cytochrome P450 from *Macrococcus caseolyticus* (Uniprot Accession number: B9EBA0) as outlined in Example 4.

Figure 19 shows an example of a typical HPLC-chromatogram obtained for the enzymatic assay with acyl-CoA thioesterase II from *Pseudomonas putida*.

Figure 20a shows an overlay of typical HPLC-chromatograms (analysis of 3-methylcrotonyl-CoA, 3-methylcrotonic acid and CoA-SH) obtained for
a) enzymatic assay (assay A, Example 7)
b) enzyme-free assay (assay H, Example 7).

The consumption of 3-methylcrotonyl-CoA with simultaneous production of CoA-SH and 3-methylcrotonic acid was observed in the enzymatic assay combining phosphate butyryltransferase with butyrate kinase.

Figure 20b shows an overlay of typical HPLC-chromatograms obtained for (analysis of ADP and ATP) obtained for
a) enzymatic assay (assay A, Example 7)
b) enzyme-free assay (assay H, Example 7).

The consumption of ADP with simultaneous production of ATP was observed in the enzymatic assay combining phosphate butyryltransferase with butyrate kinase.

Figure 21 shows the results of the production of 3-methylcrotonic acid and ATP in enzymatic assays comprising phosphate butyryltransferase from *Bacillus subtilis* combined with different butyrate kinases, as well as in different control assays.

Figure 22 shows the results of the production of 3-methylcrotonic acid and ATP in enzymatic assays comprising phosphate butyryltransferase from *Enterococcus faecalis* combined with different butyrate kinases, as well as in different control assays.

In this specification, a number of documents including patent applications are cited. The disclosure of these documents, while not considered relevant for the patentability of this invention, is herewith incorporated by reference in its entirety. More specifically, all referenced documents are incorporated by reference to the same extent as if each individual document was specifically and individually indicated to be incorporated by reference.

The invention will now be described by reference to the following examples which are merely illustrative and are not to be construed as a limitation of the scope of the present invention.

EXAMPLES

GENERAL METHODS AND MATERIALS

All reagents and materials used in the experiences were obtained from Sigma-Aldrich Company (St. Luis, MO) unless otherwise specified. Materials and methods suitable for growth of bacterial cultures and protein expression are well known in the art.

Vector pCAN contained gene coding for flavodoxin reductase from E.coli (Uniprot P0A6L0) was purchased from NAIST (Nara Institute of Science and Technology, Japan, ASKA collection). The provided vector contained a stretch of 6 histidine codons after the methionine initiation codon. Flavodoxin reductase thus cloned was overexpressed in E.coli BL21(DE3) strain and purified on PROTINO-2000 Ni-TED column (Macherey-Nagel) allowing adsorption of 6-His tagged proteins. Fractions contained the enzyme of interest were pooled and concentrated on Amicon Ultra-4 10 kDa filter unit (Millipore). Flavodoxin reductase was then resuspended in 100 mM phosphate buffer pH 7.0, containing 100 mM NaCl to be used in subsequent enzymatic assays. Protein concentration was determined by direct UV 280 nm measurement on the NanoDrop 1000 spectrophotometer (Thermo Scientific).

Example 1: Cloning and expression of recombinant cytochrome P450 fatty acid decarboxylases

Gene synthesis, cloning and expression of recombinant proteins

The sequences of the studied enzymes inferred from the genome of prokaryotic organisms were generated by oligonucleotide concatenation to fit the codon usage of *E. coli* (genes were commercially synthesized by GeneArt®). A stretch of 6 histidine codons was inserted after the methionine initiation codon to provide an affinity tag for purification. The genes thus synthesized were cloned in a pET-25b(+) expression vector (vectors were constructed by GeneArt®).

BL21(DE3) competent cells (Novagen) were transformed with these vectors according to standard heat shock procedure and plated out onto LB agar plates supplemented with the appropriate antibiotic. Single transformants were used to inoculate 200 ml of ZYM-5052 auto-induction medium (Studier FW, Prot. Exp. Pur. 41, (2005), 207-234), supplemented with 0.5 mM aminolevulinic acid for cytochrome P450 expression. The cultures were incubated for 6h at 30°C in a shaker incubator and protein expression was continued at 18°C overnight (approximately 16 h).

The cells were collected by centrifugation at 4°C, 10,000 rpm for 20 min and the pellets were stored at -80°C.

Example 2: *In vitro* oxidative decarboxylation of isovaleric acid into isobutene catalyzed by cytochrome P450 fatty acid decarboxylase from *Jeotgalicoccus* sp.

The pellet from 200 ml of cultured cells was resuspended in 50 ml of lysis buffer (100 mM potassium phosphate pH 7, 100 mM KCl) supplemented with 20 µl of lysonase (Merck-Novagen). Cell suspensions were then incubated for 10 minutes at room temperature followed by 20 minutes on ice. The amount of cytochrome P450 fatty acid decarboxylase in the total cell lysate was estimated on SDS-PAGE using gel densitometry.

0.5 M stock solution of 3-methylbutyrate (isovalerate) was prepared in water and adjusted to pH 7.0 with 10 M solution of NaOH.

Enzymatic assays were set up in 2 ml glass vials (Interchim) in the following conditions:

100 mM potassium phosphate buffer pH 7.0

100 mM NaCl

1 mM NADPH

50 mM isovalerate

0.2 mg/ml purified flavodoxin reductase from *E.coli*

Assays were started by adding the 30 μ l of cell lysate containing the recombinant P450 fatty acid decarboxylase from *Jeotgalicoccus* sp. (Uniprot Accession number: E9NSU2; SEQ ID NO: 1) (total volume 300 μ l).

A series of control assays were performed in parallel (Table 1).

The vials were sealed and incubated for 30 minutes at 30°C. The assays were stopped by incubating for 1 minute at 80°C and the isobutene present in the reaction headspace was analysed by Gas Chromatography (GC) equipped with Flame Ionization Detector (FID).

For the GC headspace analysis, one ml of the headspace gas was separated in a Bruker GC-450 system equipped with a GS-alumina column (30 m x 0.53 mm) (Agilent) using isothermal mode at 130°C. Nitrogen was used as carrier gas with a flow rate of 6 ml/min.

The enzymatic reaction product was identified by comparison with an isobutene standard. Under these GC conditions, the retention time of isobutene was 2.23 min.

A significant production of isobutene from isovalerate was observed in the assay, contained cytochrome P450 fatty acid decarboxylase and redox partners (Table 1, Figure 16).

Table 1

Assay	Isobutene peak area, arbitrary units
Enzymatic assay	147.9
Control assay without cytochrome P450 fatty acid decarboxylase	14.5
Control assay without flavodoxine reductase	16.8
Control assay without cytochrome P450 fatty acid decarboxylase and without flavodoxine reductase	12.5
Control assay without NADPH	8

Example 3: *In vivo* oxidative decarboxylation of isovaleric acid into isobutene catalyzed by cytochrome P450 fatty acid decarboxylase from *Jeotgalicoccus* sp.

BL21(DE3) competent cells were transformed with pET-25b(+) expression vector, coding for cytochrome P450 fatty acid decarboxylase from *Jeotgalicoccus* sp. ATCC 8456 and plated out onto LB agar plates supplemented with ampicillin (100 µg/ml).

BL21(DE3) strain transformed with empty pET-25b(+) vector was used as a negative control in the subsequent assays (control strain). Plates were incubated overnight at 30°C. Single transformants were used to inoculate LB medium, supplemented with ampicillin, followed by incubation at 30°C overnight. 1 ml of this overnight culture was used to inoculate 300 ml of ZYM-5052 auto-inducing media (Studier FW (2005), local citation), supplemented with 0.5 mM aminolevulinic acid. The cultures were grown for 20 hours at 30°C and 160 rpm shaking.

A volume of cultures corresponding to OD₆₀₀ of 30 was removed and centrifuged. The pellet was resuspended in 30 ml of MS medium (Richaud C., Mengin-Leucreulx D., Pochet S., Johnson E.J., Cohen G.N. and Marlière P, The Journal of Biological Chemistry, 268, (1993), 26827-26835), containing glucose (45 g/L) and MgSO₄ (1mM) and supplemented with 50 mM isovalerate. These cultures were then incubated in 160 ml bottles, sealed with a screw cap, at 30°C with shaking for 22 h. The pH value of the cultures was adjusted to 8.5 after 8 hours of incubation by using 30 % NH₄OH.

After an incubation period, the isobutene produced in the headspace was analysed by Gas Chromatography (GC) equipped Flame Ionization Detector (FID). One ml of the headspace gas phase was separated and analysed according to the method described in Example 2.

Figure 17 shows that a certain quantity of isobutene was produced with a control strain probably due to the spontaneous decomposition of isovalerate. The ratio of isobutene produced by *E.coli* strain, expressing P450 fatty acid decarboxylase, versus isobutene produced with a control strain was about 2.2 fold judging from isobutene peak areas (Figure 17). These results clearly indicate that a production of isobutene from isovalerate can be achieved *in vivo* by *E.coli* strain expressing cytochrome P450 fatty acid decarboxylase from *Jeotgalicoccus* sp. ATCC 8456.

Example 4: *In vitro* oxidative decarboxylation of isovaleric acid into isobutene catalyzed by cytochrome P450 fatty acid decarboxylase from *Macrococcus caseolyticus*

The pellet from 200 ml of cultured cells was resuspended in 50 ml of lysis buffer (100 mM potassium phosphate pH 7, 100 mM KCl) supplemented with 20 µl of lysonase (Merck-Novagen). Cell suspensions were then incubated for 10 minutes at room temperature followed by 20 minutes on ice. The amount of cytochrome P450 fatty acid decarboxylase in the total cell lysate was estimated on SDS-PAGE using gel densitometry.

0.5 M stock solution of 3-methylbutyrate (isovalerate) was prepared in water and adjusted to pH 7.0 with 10 M solution of NaOH.

Enzymatic assays were set up in 2 ml glass vials (Interchim) in the following conditions:

100 mM potassium phosphate buffer pH 7.0

100 mM NaCl

2 mM NADPH

50 mM isovalerate

0.2 mg/ml purified flavodoxin reductase from *E.coli*

Assays were started by adding 100µl of cell lysate containing the recombinant P450 fatty acid decarboxylase from *Macrococcus caseolyticus* (total volume 300 µl).

A series of control assays were performed in parallel (Table 2).

The vials were sealed and incubated for 60 minutes at 30°C. The assays were stopped by incubating for 1 minute at 80°C and the isobutene present in the reaction headspace was analysed by Gas Chromatography (GC) equipped with Flame Ionization Detector (FID).

For the GC headspace analysis, one ml of the headspace gas was separated in a Bruker GC-450 system equipped with a GS-alumina column (30 m x 0.53 mm) (Agilent) using isothermal mode at 130°C. Nitrogen was used as carrier gas with a flow rate of 6 ml/min.

The enzymatic reaction product was identified by comparison with an isobutene standard. Under these GC conditions, the retention time of isobutene was 2.45 min.

A significant production of isobutene from isovalerate was observed in the assay, contained cytochrome P450 fatty acid decarboxylase and redox partners (Table 2, Figure 18).

Table 2

Assay	Isobutene peak area, arbitrary units
Enzymatic assay	18
Control assay without cytochrome P450 fatty acid decarboxylase	2.5
Control assay without flavodoxine reductase	2.7
Control assay without cytochrome P450 fatty acid decarboxylase and without flavodoxine reductase	2.5
Control assay without NADPH	1.5

Example 5: Cloning and overexpression of recombinant phosphate butyryltransferases and butyrate kinases

Gene synthesis, cloning and expression of recombinant proteins

The sequences of phosphate butyryltransferase genes from *Bacillus subtilis* (strain 168) and *Enterococcus faecalis* MTUP9 (Uniprot Accession number: P54530 and A0A038BNC2, respectively) and butyrate kinase from *Lactobacillus casei* W56 and *Geobacillus* sp. GHH01 (Uniprot Accession number: K0N529 and L8A0E1, respectively) were generated by oligonucleotide concatenation to fit the codon usage of *E. coli* (genes were commercially synthesized by GeneArt®). The expression of corresponding proteins was conducted following the method described in Example 1. The cells were collected by centrifugation at 4°C, 10.000 rpm for 20 min and the pellets were stored at -80°C.

Protein purification and concentration

The pellets from 200 ml of cultured cells were thawed on ice and resuspended in 5 ml of 50 mM potassium phosphate buffer pH 7.5 containing 100 mM NaCl, 10 mM MgCl₂, 10 mM imidazole and 1 mM DTT. Twenty microliters of lysonase (Novagen)

were added. Cells were incubated 10 minutes at room temperature and then returned to ice for 20 minutes. Cell lysis was completed by sonication for 2 x 30 seconds. The bacterial extracts were then clarified by centrifugation at 4°C, 4000 rpm for 40 min. The clarified bacterial lysates were loaded onto a PROTINO-2000 Ni-TED column (Macherey-Nagel) allowing adsorption of 6-His tagged proteins. Columns were washed and the enzymes of interest were eluted with 6 ml of 50 mM potassium phosphate buffer pH 7.5 containing 100 mM NaCl and 250 mM imidazole. Eluates were then concentrated, desalted on an Amicon Ultra-4 10 kDa filter unit (Millipore) and enzymes were resuspended in 50 mM potassium phosphate buffer pH 7.5, containing 100 mM NaCl. The purity of proteins thus purified varied from 70% to 90% as estimated by SDS-PAGE analysis. Protein concentrations were determined by direct UV 280 nm measurement on the NanoDrop 1000 spectrophotometer (Thermo Scientific) or by Bradford assay (BioRad).

Example 6: Enzyme-catalyzed hydrolysis of 3-methylcrotonyl-CoA into 3-methylcrotonic acid and free coenzyme-A

The gene coding for acyl-CoA thioesterase II from *Pseudomonas putida* was synthesized according to the procedure as described in Example 1.

The vector pCA24N which contained the gene encoding acyl-CoA thioesterase 2 (TesB) from *Escherichia coli* was purchased from NAIST (Nara Institute of Science and Technology, Japan, ASKA collection). This vector provided contained a stretch of 6 histidine codons after the methionine initiation codon.

The corresponding enzymes were produced according to the procedure described in Example 1.

The enzymatic assays were conducted in a total reaction volume of 0.2 ml.

The standard reaction mixture contained:

50 mM HEPES pH 7.0

10 mM 3-methylcrotonyl-CoA (Sigma-Aldrich)

20 mM MgCl₂

20 mM NaCl

1 mg/ml of purified recombinant thioesterase.

Control assays were performed in which either no enzyme was added or no substrate was added.

The assays were incubated for 30 min with shaking at 30°C, the reactions were stopped by the addition of 0.1 ml of acetonitrile and the samples were then analyzed by HPLC-based procedure.

HPLC based analysis of the consumption of 3-methylcrotonyl-CoA and the formation of 3-methylcrotonic acid and free coenzyme A (CoA-SH)

HPLC analysis was performed using an 1260 Infinity LC System (Agilent), equipped with a column heating module and a UV detector (210 nm). 5 µl of samples were separated on Zorbax SB-Aq column (250 x 4.6 mm, 5 µm particle size, column temp. 30°C) with a mobile phase flow rate of 1.5 ml/min. The separation was performed using mixed A (H₂O containing 8.4 mM sulfuric acid) and B (acetonitrile) solutions in a linear gradient (0% B at initial time 0 min → 70% B at 8 min). Commercial 3-methylcrotonyl-CoA, 3-methylcrotonic acid (Sigma-Aldrich) and CoA-SH (Sigma-Aldrich) were used as references. In these conditions, the retention time of free coenzyme A (CoA-SH), 3-methylcrotonyl-CoA and 3-methylcrotonic acid were 4.05, 5.38 and 5.83 min, respectively.

No 3-methylcrotonic acid signal was observed in control assays.

Both studied thioesterases catalyzed the hydrolysis of 3-methylcrotonyl-CoA with the formation of 3-methylcrotonic acid. An example of a chromatogram obtained with acyl-CoA thioesterase II from *Pseudomonas putida* is shown in Figure 19.

The degree of production of 3-methylcrotonic acid as observed in the enzymatic assays is shown in Table 3.

Table 3

GENE NAMES	ORGANISM	UNIPROT ACCESSION NUMBER	3-METHYLCROTONIC ACID PRODUCED, MM
TESB	ESCHERICHIA COLI	P0AGG2	0.6
TESB	PSEUDOMONAS PUTIDA	Q88DR1	3.1

Example 7: Conversion of 3-methylcrotonyl-CoA and ADP into 3-methylcrotonic acid and ATP catalysed by the combined action of phosphate butyryltransferase from *Bacillus subtilis* and butyrate kinase from *Lactobacillus casei* or *Geobacillus* sp.

In the assays described in the following, the following enzymes were used:

Table 4

GENE NAMES	ORGANISM	UNIPROT ACCESSION NUMBER
PTB (YQIS)	BACILLUS SUBTILIS	P54530
BUK	LACTOBACILLUS CASEI	K0N529
BUK	GEOBACILLUS SP.	L8A0E1

The enzymatic assays were conducted in a total reaction volume of 0.2 ml.

The standard reaction mixture contained:

50 mM potassium phosphate buffer pH 7.5

4 mM 3-methylcrotonyl-CoA

4 mM ADP

10 mM MgCl₂

10 mM NaCl

0.2 mg/ml of purified phosphate butyryltransferase from *Bacillus subtilis*

0.2 mg/ml of purified butyrate kinase from *Lactobacillus casei* or *Geobacillus* sp.

A series of controls was performed in parallel (Assays C-H as shown in Table 5).

Table 5

	Assay composition							
	A	B	C	D	E	F	G	H
3-methylcrotonyl-CoA	+	+	+	+	+	+	+	+
ADP	+	+			+	+	+	+
phosphate butyryltransferase from <i>Bacillus subtilis</i>	+	+	+	+	+			
butyrate kinase from <i>Lactobacillus casei</i>	+		+			+		
butyrate kinase from <i>Geobacillus</i> sp		+		+			+	

Assays were then incubated for 20 min with shaking at 30° C.

After an incubation period, the reactions were stopped by heating the reaction medium 4 min at 90°C. The samples were centrifuged, filtered through a 0.22 µm filter and the clarified supernatants were transferred into a clean vial for further analysis. The consumption of ADP and 3-methylcrotonyl-CoA, and the formation of ATP, 3-methylcrotonic acid and free coenzyme A (CoA-SH) were followed by using HPLC-based methods.

HPLC-based analysis of ADP and ATP

HPLC analysis was performed using the 1260 Infinity LC System (Agilent) equipped with a column heating module and an RI detector. 2 µl of samples were separated on a Polaris C18-A column (150 x 4.6 mm, 5 µm particle size, column temp.30°C) with a mobile phase flow rate of 1.5 ml/min. The consumption of ADP and the formation of ATP were followed by HPLC.

HPLC-based analysis of ADP and ATP

HPLC analysis was performed using 1260 Infinity LC System (Agilent), equipped with column heating module and RI detector. 2 µl of samples were separated on Polaris C18-A column (150 x 4.6 mm, 5 µm particle size, column temp.30°C) with a mobile phase flow rate of 1.5 ml/min. The separation was performed using 8.4 mM sulfuric acid in H₂O/MeOH mixed solution (99/1) (V/V). In these conditions, the retention time of ADP and ATP were 2.13 min, 2.33 min, respectively (see Figure 20b).

HPLC based analysis of 3-methylcrotonyl-CoA, 3-methylcrotonic acid and free coenzyme A (CoA-SH)

HPLC analysis was performed using the 1260 Infinity LC System (Agilent) equipped with a column heating module and a UV detector (260 nm). 1 µl of samples were separated on a Zorbax SB-Aq column (250 x 4.6 mm, 5 µm particle size, column temp.30°C), with a mobile phase flow rate of 1.5 ml/min.

The consumption of 3-methylcrotonyl-CoA and the formation of 3-methylcrotonic acid and free coenzyme A (CoA-SH) were followed according to the procedure described in Example 6. Under these conditions, the retention time of 3-methylcrotonyl-CoA, 3-

methylcrotonic acid and free coenzyme A (CoA-SH) was 5.38 min, 5.73 min and 4.07 min, respectively.

Typical chromatograms obtained for the enzymatic assay A and enzyme-free assay H are shown in Figures 20a and 20b. The results of the HPLC analysis are summarized in Figure 21.

The obtained data indicate that 3-methylcrotonyl-CoA was converted into 3-methylcrotonic acid with the concomitant generation of ATP from ADP in a two-step reaction, catalyzed by two enzymes, respectively (assays A and B). Thus, the conversion occurred through the formation of the intermediate 3-methylcrotonyl phosphate followed by transfer of a phosphate group from this intermediate to ADP, thereby releasing ATP.

A significant production of 3-methylcrotonic acid without a simultaneous generation of ATP was observed when phosphate butyryltransferase was used alone (assay E). This production is due to a spontaneous hydrolysis of 3-methylcrotonyl phosphate generated by the action of phosphate butyryltransferase.

The production of 3-methylcrotonic acid was observed in the same manner for the control assays without ADP (assays C and D). This production was also due to a hydrolysis of 3-methylcrotonyl phosphate generated by the action of phosphate butyryltransferase.

Example 8: Conversion of 3-methylcrotonyl-CoA and ADP into 3-methylcrotonic acid and ATP catalysed by the combined action of the phosphate butyryltransferase from *Enterococcus faecalis* and butyrate kinase from *Lactobacillus casei* or *Geobacillus* sp.

In the assays described in the following, the following enzymes were used:

Table 6

GENE NAMES	ORGANISM	UNIPROT ACCESSION NUMBER
PTB	ENTEROCOCCUS FAECALIS	A0A038BNC2
BUK	LACTOBACILLUS CASEI	K0N529
BUK	GEOBACILLUS SP.	L8A0E1

The enzymatic assays were conducted in a total reaction volume of 0.2 ml.

The standard reaction mixture contained:

50 mM potassium phosphate buffer pH 7.5

4 mM 3-methylcrotonyl-CoA

4 mM ADP

10 mM MgCl₂

10 mM NaCl

0.2 mg/ml of purified phosphate butyryltransferase from *Enterococcus faecalis*

0.2 mg/ml of purified butyrate kinase from *Lactobacillus casei* or *Geobacillus sp.*

A series of controls was performed in parallel (Assays C-H Table 7).

Table 7

	Assay composition							
	A	B	C	D	E	F	G	H
3-methylcrotonyl-CoA	+	+	+	+	+	+	+	+
ADP	+	+			+	+	+	+
phosphate butyryltransferase from <i>Enterococcus faecalis</i>	+	+	+	+	+			
butyrate kinase from <i>Lactobacillus casei</i>	+		+			+		
butyrate kinase from <i>Geobacillus sp</i>		+		+			+	

Assays were then incubated for 20 min with shaking at 30° C.

After an incubation period, the reactions were stopped by heating the reaction medium 4 min at 90°C. The samples were centrifuged, filtered through a 0.22 µm filter and the clarified supernatants were transferred into a clean vial for further

analysis. The consumption of ADP and 3-methylcrotonyl-CoA and the formation of ATP and 3-methylcrotonic acid and free coenzyme A (CoA-SH) were followed by HPLC analysis according to the methods described in Example 7 and Example 6.

The results of the HPLC analysis are summarized in Figure 22.

The obtained data indicate that the 3-methylcrotonyl-CoA was converted into 3-methylcrotonic acid with the concomitant generation of ATP from ADP in a two-step reaction, catalyzed by two enzymes, respectively (assays A and B). Thus, the conversion occurred through the formation of the intermediate 3-methylcrotonyl phosphate followed by the transfer of a phosphate group from this intermediate on ADP, thereby releasing ATP.

A significant production of 3-methylcrotonic acid without simultaneous generation of ATP was observed when phosphate butyryltransferase was used alone (assay E). This production was due to a hydrolysis of 3-methylcrotonyl phosphate generated by the action of phosphate butyryltransferase.

The production of 3-methylcrotonic acid was observed in a similar manner for the control assays without ADP (assays C and D). This production was also due to a hydrolysis of 3-methylcrotonyl phosphate generated by the action of phosphate butyryltransferase.

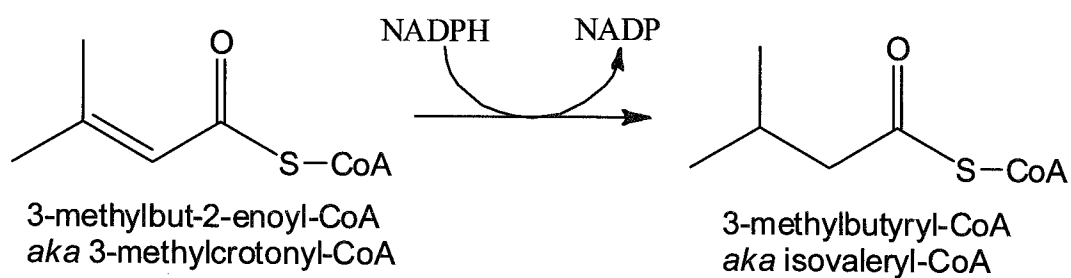
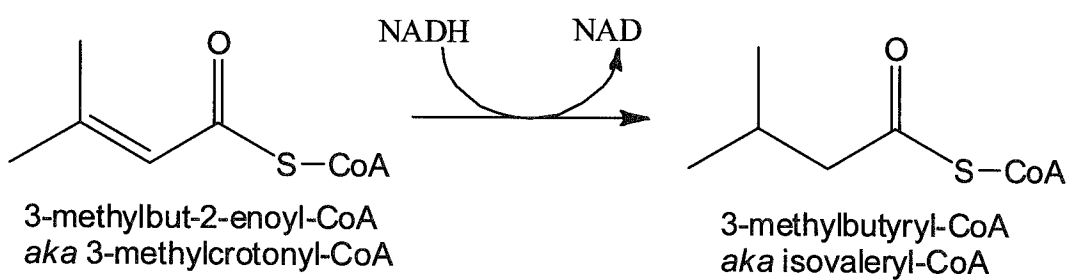
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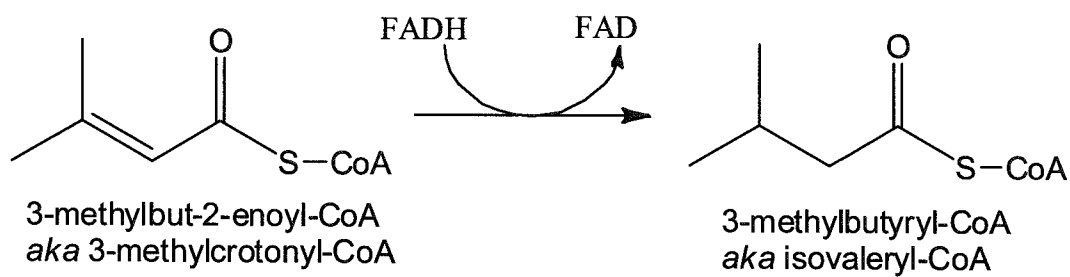
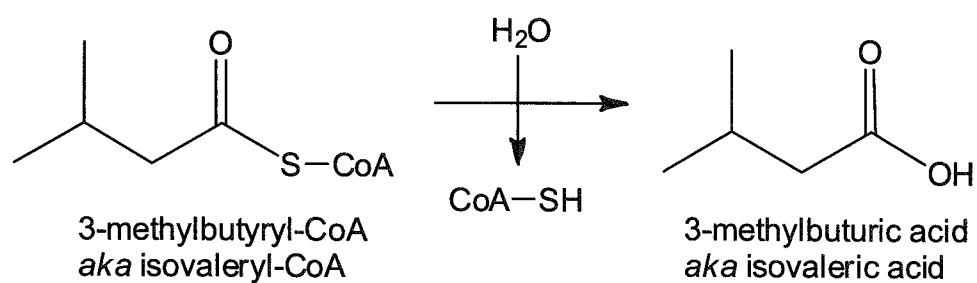
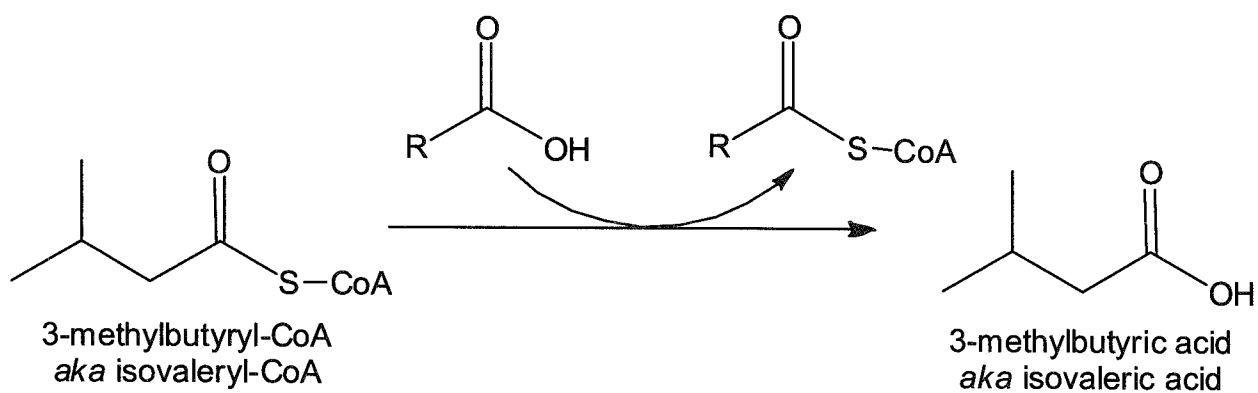
1. A method for the production of isobutene from 3-methylcrotonyl-CoA comprising the steps of:
 - (a) enzymatically converting 3-methylcrotonyl-CoA into 3-methylbutyric acid; and
 - (b) further enzymatically converting the thus produced 3-methylbutyric acid into isobutene.
2. The method of claim 1, wherein the enzymatic conversion of 3-methylcrotonyl-CoA into 3-methylbutyric acid according to step (a) is achieved by a method comprising the following steps:
 - (a1) enzymatically converting 3-methylcrotonyl-CoA into 3-methylbutyryl-CoA; and
 - (a2) further enzymatically converting the thus produced 3-methylbutyryl-CoA into 3-methylbutyric acid.
3. The method of claim 2, wherein the conversion of 3-methylcrotonyl-CoA into 3-methylbutyryl-CoA according to step (a1) is achieved by the use of an enzyme classified in EC 1.3.____.
4. The method of claim 3, wherein the enzyme is selected from the group consisting of
 - (i) an acyl-CoA dehydrogenase (NADP+) (EC 1.3.1.8);
 - (ii) an enoyl-[acyl-carrier-protein] reductase (NADPH, Si-specific) (EC 1.3.1.10);
 - (iii) a cis-2-enoyl-CoA reductase (NADPH) (EC 1.3.1.37);
 - (iv) a trans-2-enoyl-CoA reductase (NADPH) (EC 1.3.1.38);

- (v) an enoyl-[acyl-carrier-protein] reductase (NADPH, Re-specific) (EC 1.3.1.39);
 - (vi) crotonyl-CoA reductase (EC 1.3.1.86) ;
 - (vii) an enoyl-[acyl-carrier-protein] reductase (NADH) (EC 1.3.1.9) ;
 - (viii) a trans-2-enoyl-CoA reductase (NAD⁺) (EC 1.3.1.44) ; and
 - (ix) an isovaleryl-CoA dehydrogenase (EC 1.3.8.4).
5. The method of any one of claims 2 to 4, wherein the conversion of 3-methylbutyryl-CoA into 3-methylbutyric acid according to step (a2) is achieved by a method comprising the step of enzymatically converting 3-methylbutyryl-CoA into 3-methylbutyric acid in a hydrolysis reaction by making use of a thioester hydrolase (EC 3.1.2).
6. The method of any one of claims 2 to 4, wherein the conversion of 3-methylbutyryl-CoA into 3-methylbutyric acid according to step (a2) is achieved by a method comprising the step of enzymatically converting 3-methylbutyryl-CoA into 3-methylbutyric acid in a transferase reaction by making use of a CoA-transferase (EC 2.8.3).
7. The method of any one of claims 2 to 4, wherein the conversion of 3-methylbutyryl-CoA into 3-methylbutyric acid according to step (a2) is achieved by a method comprising the following steps:
- (i) enzymatically converting 3-methylbutyryl-CoA into 3-methylbutyryl phosphate; and
 - (ii) further enzymatically converting the thus produced 3-methylbutyryl phosphate into 3-methylbutyric acid.
8. The method of claim 7, wherein the conversion of 3-methylbutyryl-CoA into 3-methylbutyryl phosphate according to step (i) is achieved by making use of a phosphate butyryltransferase (EC 2.3.1.19) or a phosphate acetyltransferase (EC 2.3.1.8).

9. The method of claim 7 or 8, wherein the conversion of 3-methylbutyryl phosphate into 3-methylbutyric acid according to step (ii) is achieved by making use of a phosphotransferase (EC 2.7.2).
10. The method of claim 9, wherein the phosphotransferase (EC 2.7.2) is selected from the group consisting of a butyrate kinase (EC 2.7.2.7), a branched-chain-fatty-acid kinase (EC 2.7.2.14), a propionate kinase (EC 2.7.2.15) or an acetate kinase (EC 2.7.2.1).
11. The method of claim 1, wherein the enzymatic conversion of 3-methylcrotonyl-CoA into 3-methylbutyric acid according to step (a) is achieved by a method comprising the following steps:
 - (a1) enzymatically converting 3-methylcrotonyl-CoA into 3-methylcrotonic acid; and
 - (a11) further enzymatically converting the thus produced 3-methylcrotonic acid into 3-methylbutyric acid.
12. The method of claim 11, wherein the conversion of 3-methylcrotonyl-CoA into 3-methylcrotonic acid according to step (a1) is achieved by a method comprising the step of enzymatically converting 3-methylcrotonyl-CoA into 3-methylcrotonic acid in a hydrolysis reaction by making use of a thioester hydrolase (EC 3.1.2).
13. The method of claim 11, wherein the conversion of 3-methylcrotonyl-CoA into 3-methylcrotonic acid according to step (a1) is achieved by a method comprising the step of enzymatically converting 3-methylcrotonyl-CoA into 3-methylcrotonic acid in a transferase reaction by making use of a CoA-transferase (EC 2.8.3).
14. The method of claim 11, wherein the conversion of 3-methylcrotonyl-CoA into 3-methylcrotonic acid according to step (a1) is achieved by a method comprising the following steps:
 - (i) enzymatically converting 3-methylcrotonyl-CoA into 3-methylcrotonyl phosphate; and

- (ii) further enzymatically converting the thus produced 3-methylcrotonyl phosphate into 3-methylcrotonic acid.
15. The method of claim 14, wherein the conversion of 3-methylcrotonyl-CoA into 3-methylcrotonyl phosphate according to step (i) is achieved by making use of a phosphate butyryltransferase (EC 2.3.1.19) or a phosphate acetyltransferase (EC 2.3.1.8).
16. The method of claim 14 or 15, wherein the conversion of 3-methylcrotonyl phosphate into 3-methylcrotonic acid according to step (ii) is achieved by making use of a phosphotransferase (EC 2.7.2).
17. The method of claim 16, wherein the phosphotransferase (EC 2.7.2) is selected from the group consisting of a butyrate kinase (EC 2.7.2.7), a branched-chain-fatty-acid kinase (EC 2.7.2.14), a propionate kinase (EC 2.7.2.15) or an acetate kinase (EC 2.7.2.1).
18. The method of any one of claims 11 to 17, wherein the conversion of 3-methylcrotonic acid into 3-methylbutyric acid is achieved by making use of a 2-enoate reductase (EC 1.3.1.31).
19. The method of any one of claims 1 to 18, wherein the conversion of 3-methylbutyric acid into isobutene according to step (b) is achieved by an oxidative decarboxylation catalyzed by a cytochrome P450 or by a non-heme iron oxygenase.

**Figure 1****Figure 2**

**Figure 3****Figure 4****Figure 5**

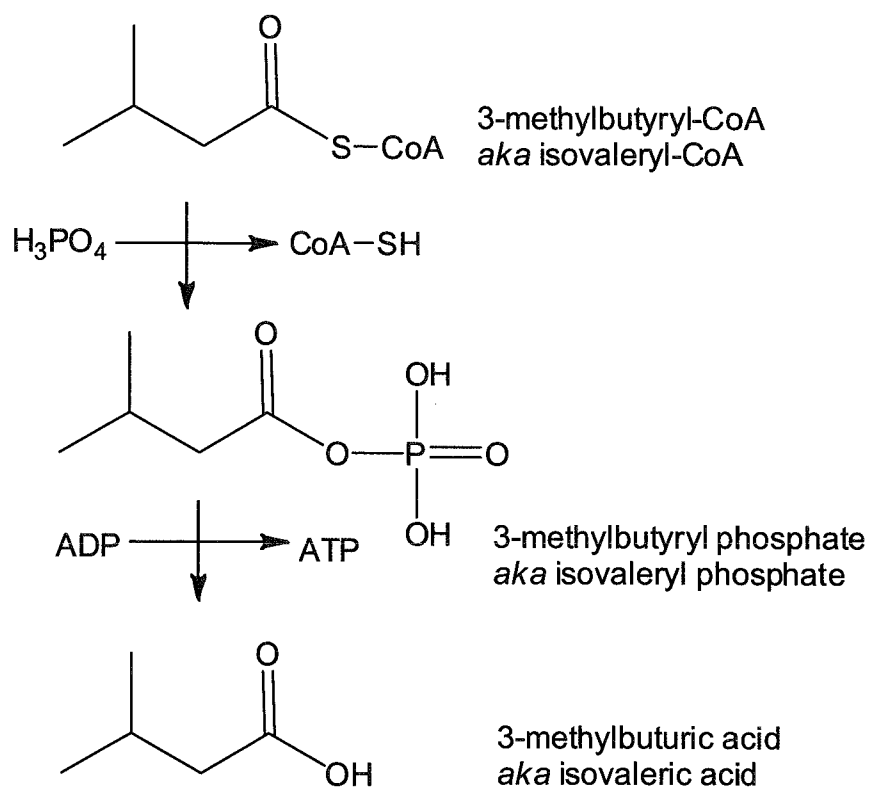


Figure 6

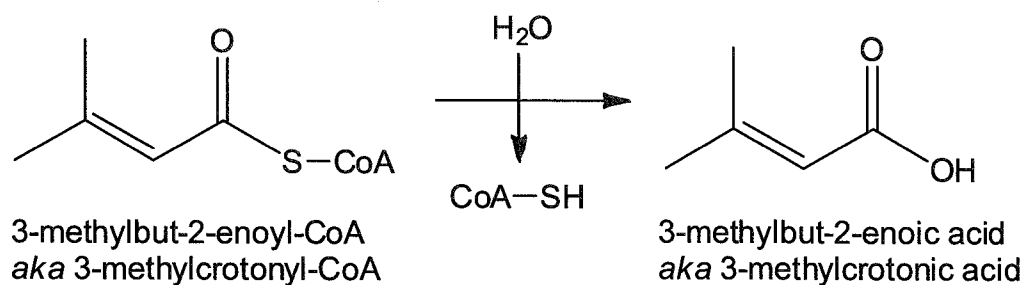
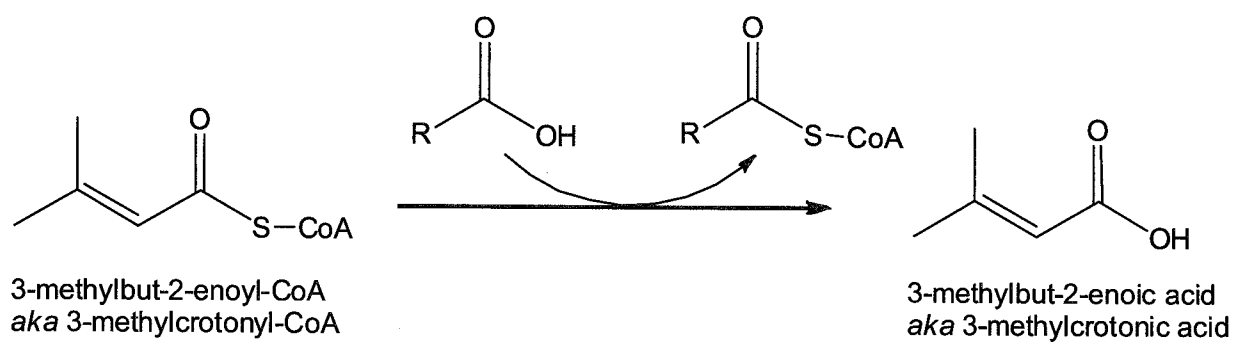
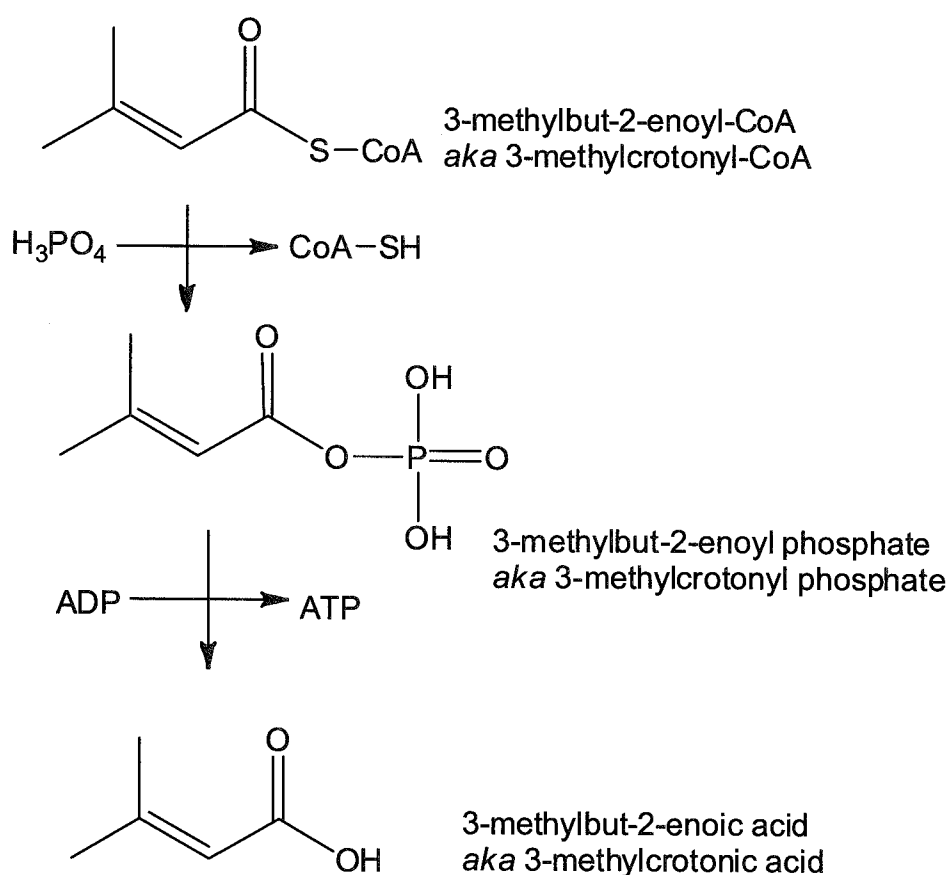
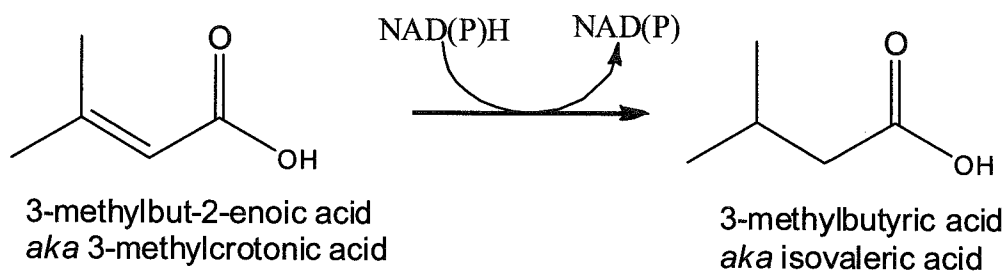
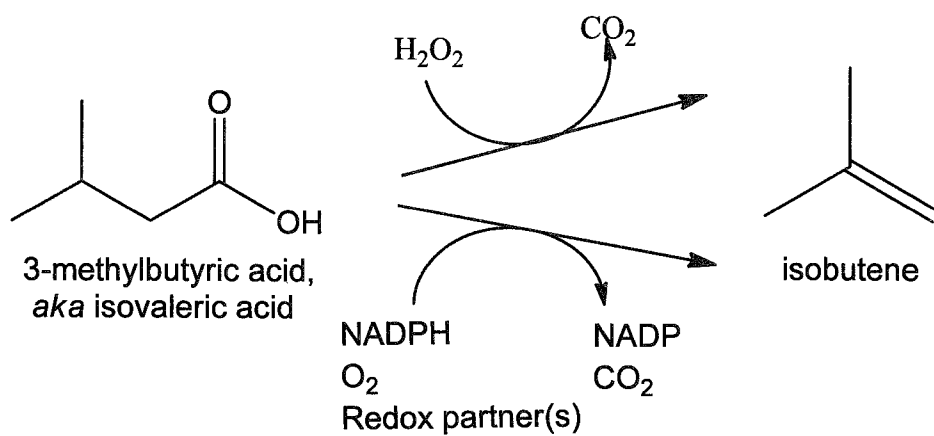
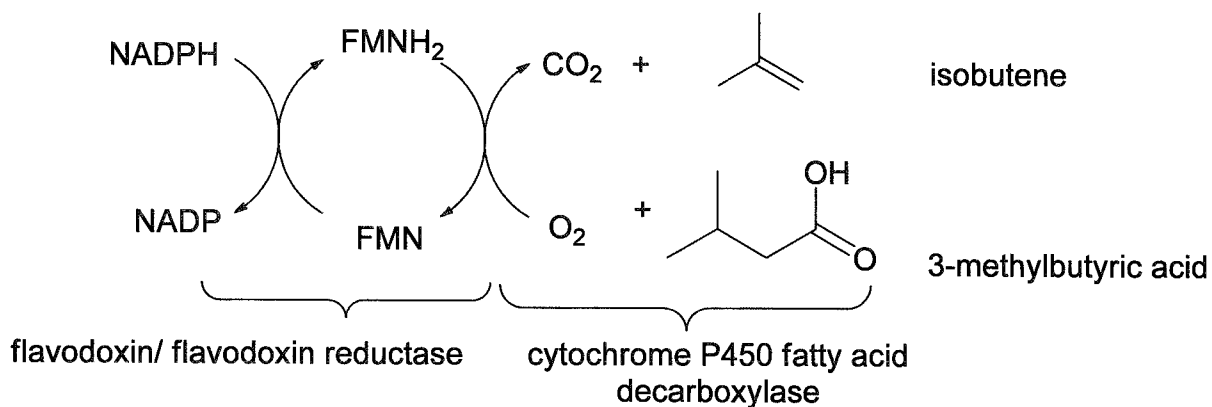


Figure 7

**Figure 8****Figure 9**

**Figure 10****Figure 11a****Abbreviations:**NADPH, H⁺ : reduced nicotinamide adenine dinucleotide phosphateNADP⁺ : oxidized nicotinamide adenine dinucleotide phosphateFMNH₂ : reduced riboflavin-5'-phosphate

FMN: oxidized riboflavin-5'-phosphate

Figure 11b

CC(=C)CC(=O)SCoA

 $\xrightarrow{\text{CO}_2}$
CC(=C)CC(=O)CC(=O)O

3-methylcrotonyl-CoA

 3-methylglutaconyl-CoA

Figure 12

CC(=C)CC(=O)OS(=O)(=O)C
 $\xrightarrow{\text{H}_2\text{O}}$
CC(O)CC(=O)OS(=O)(=O)C

3-methylglutaconyl-CoA

 3-hydroxy-3-methylglutaryl-CoA

Figure 13

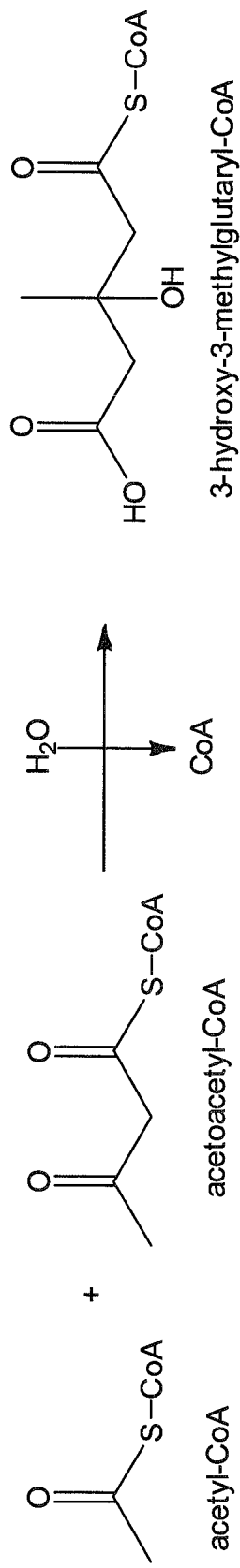


Figure 14

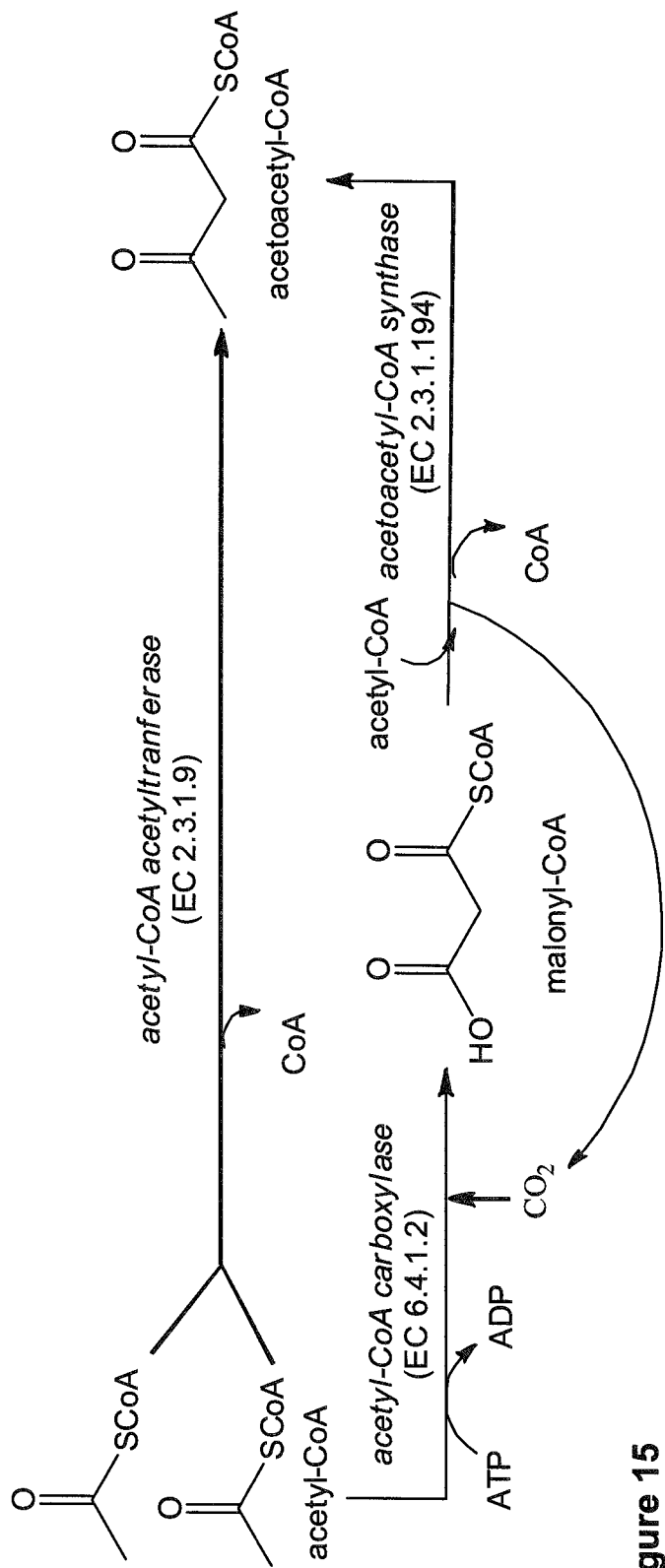


Figure 15

Gas
chromatography
signal, μV

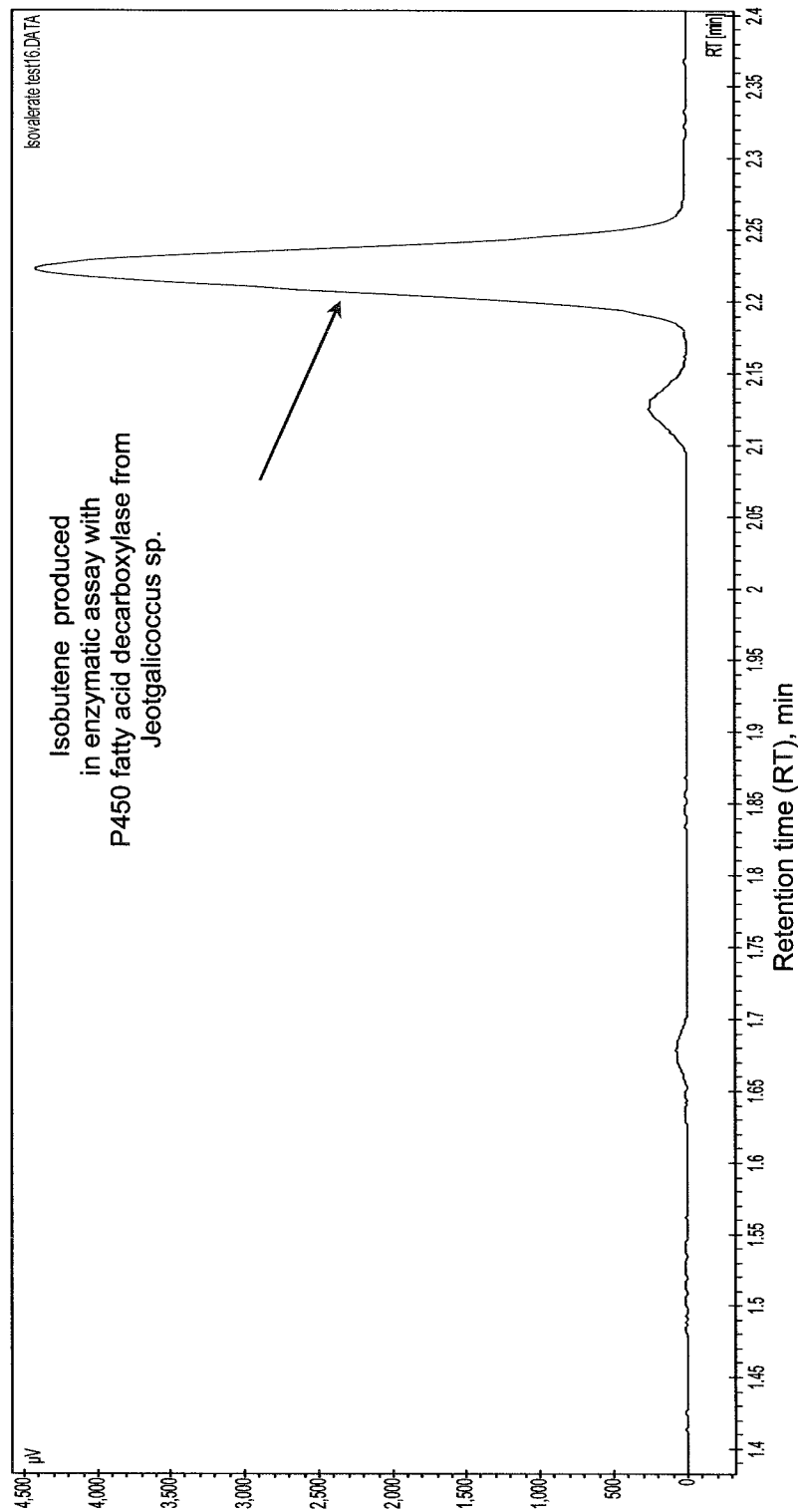


Figure 16

Gas chromatography
signal, μV

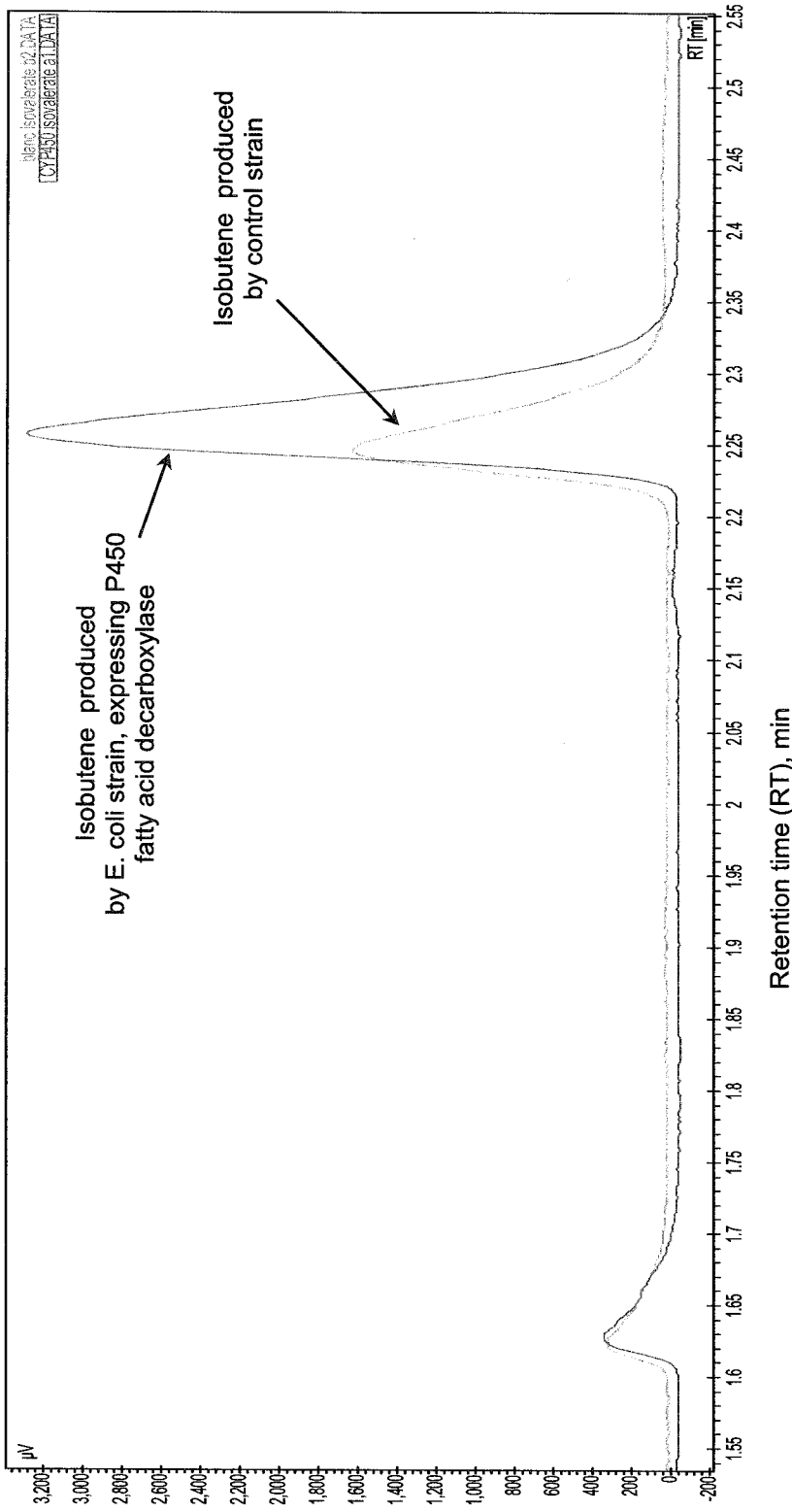
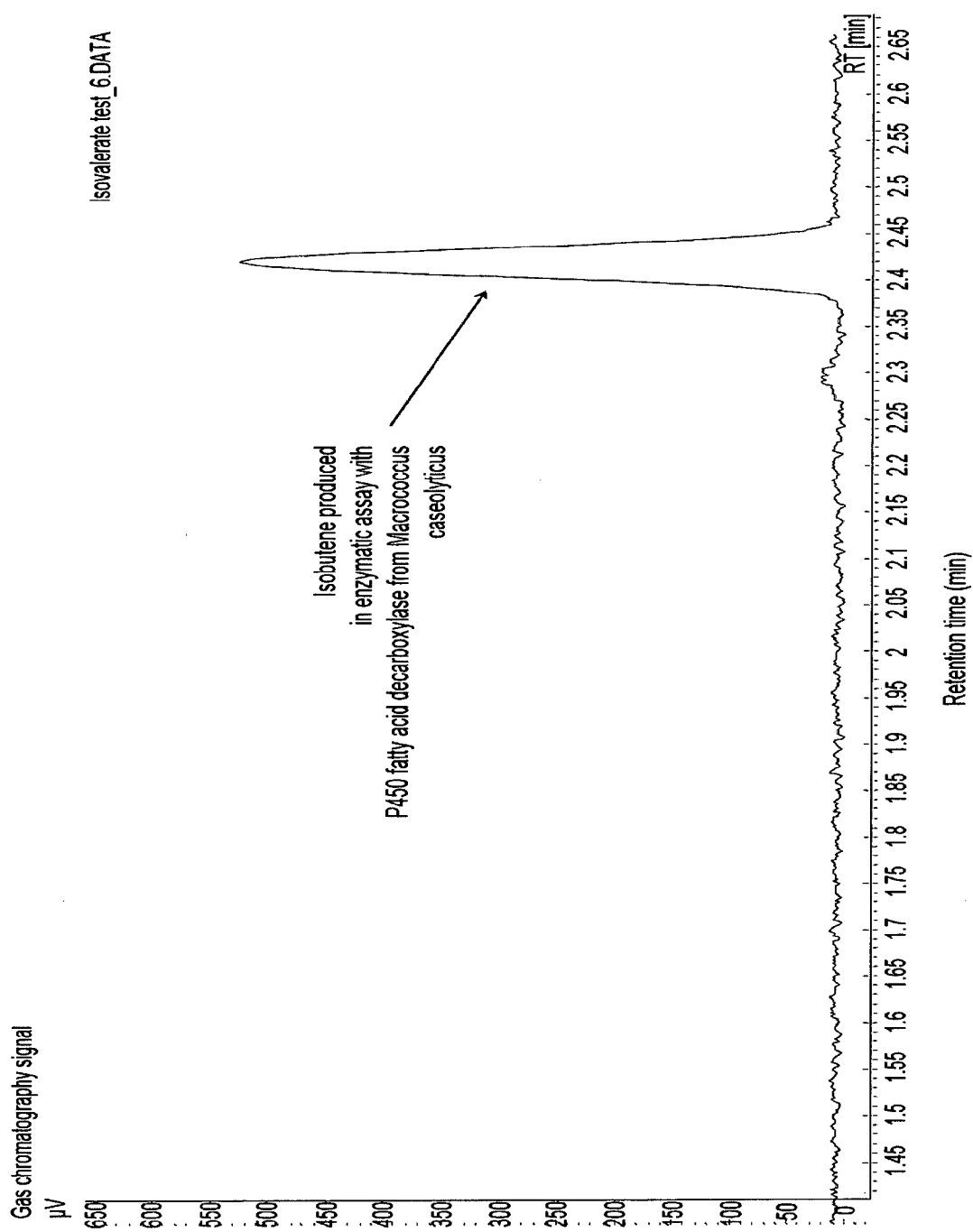


Figure 17

**Figure 18**

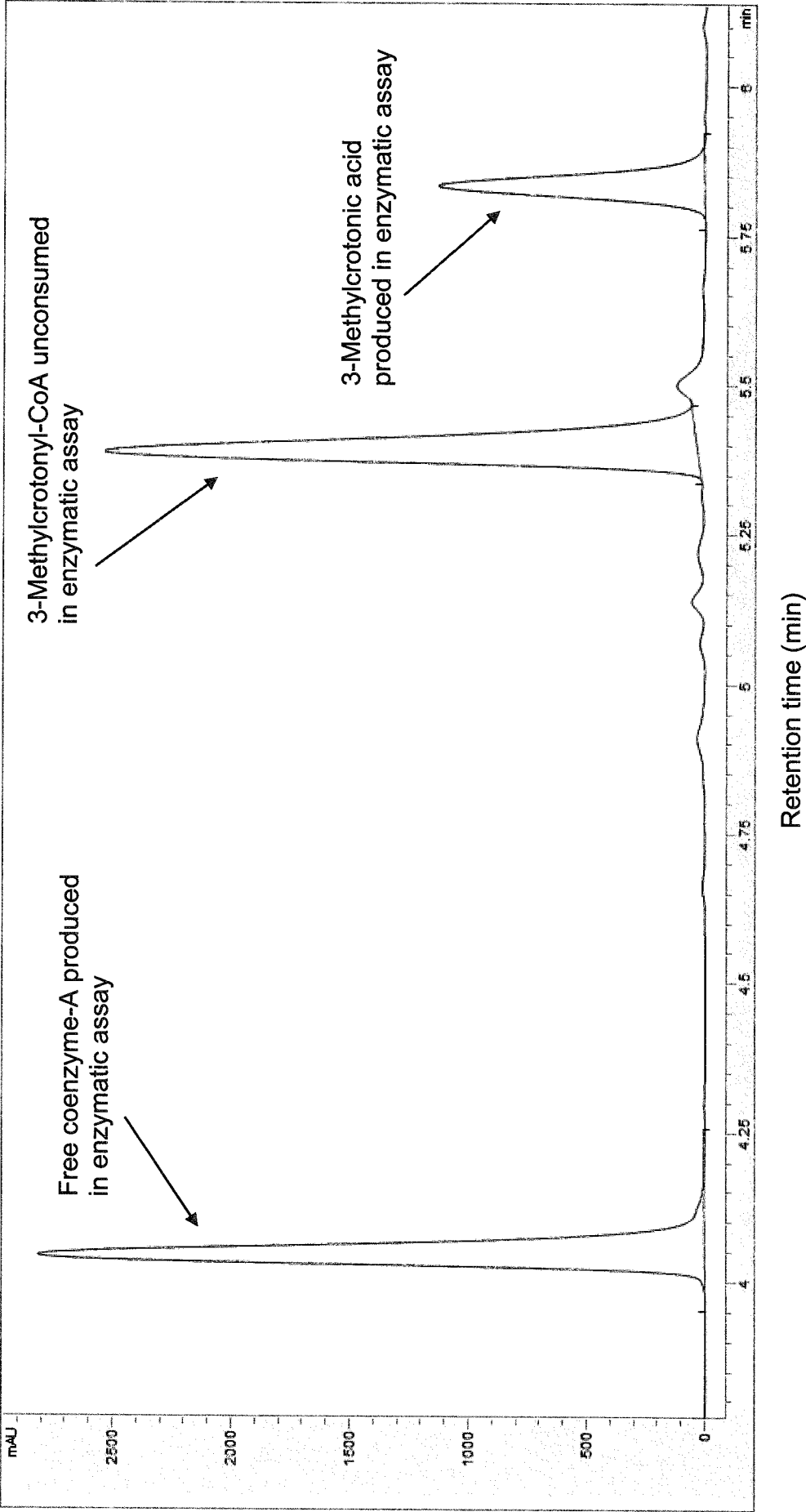


Figure 19

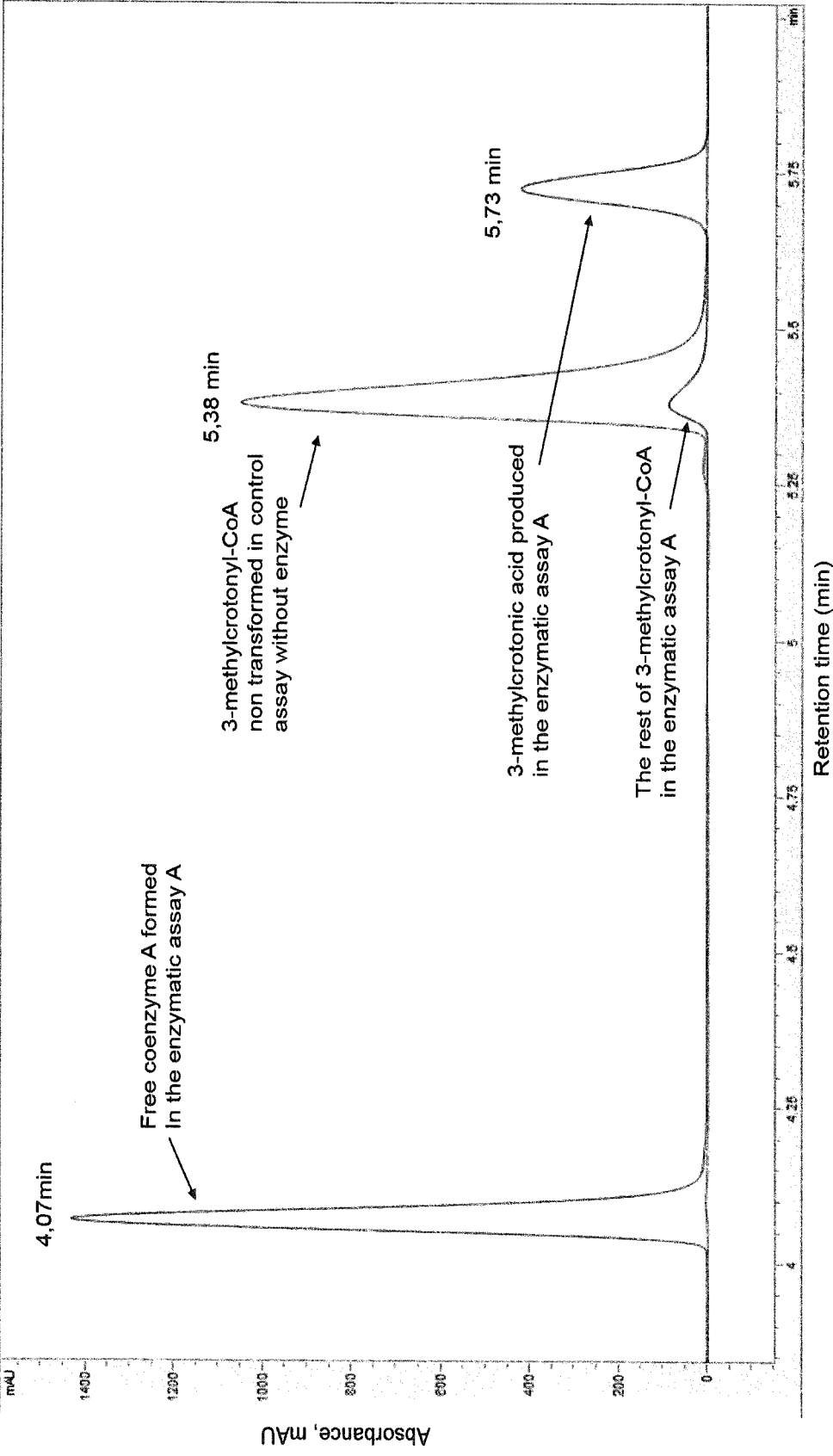


Figure 20a

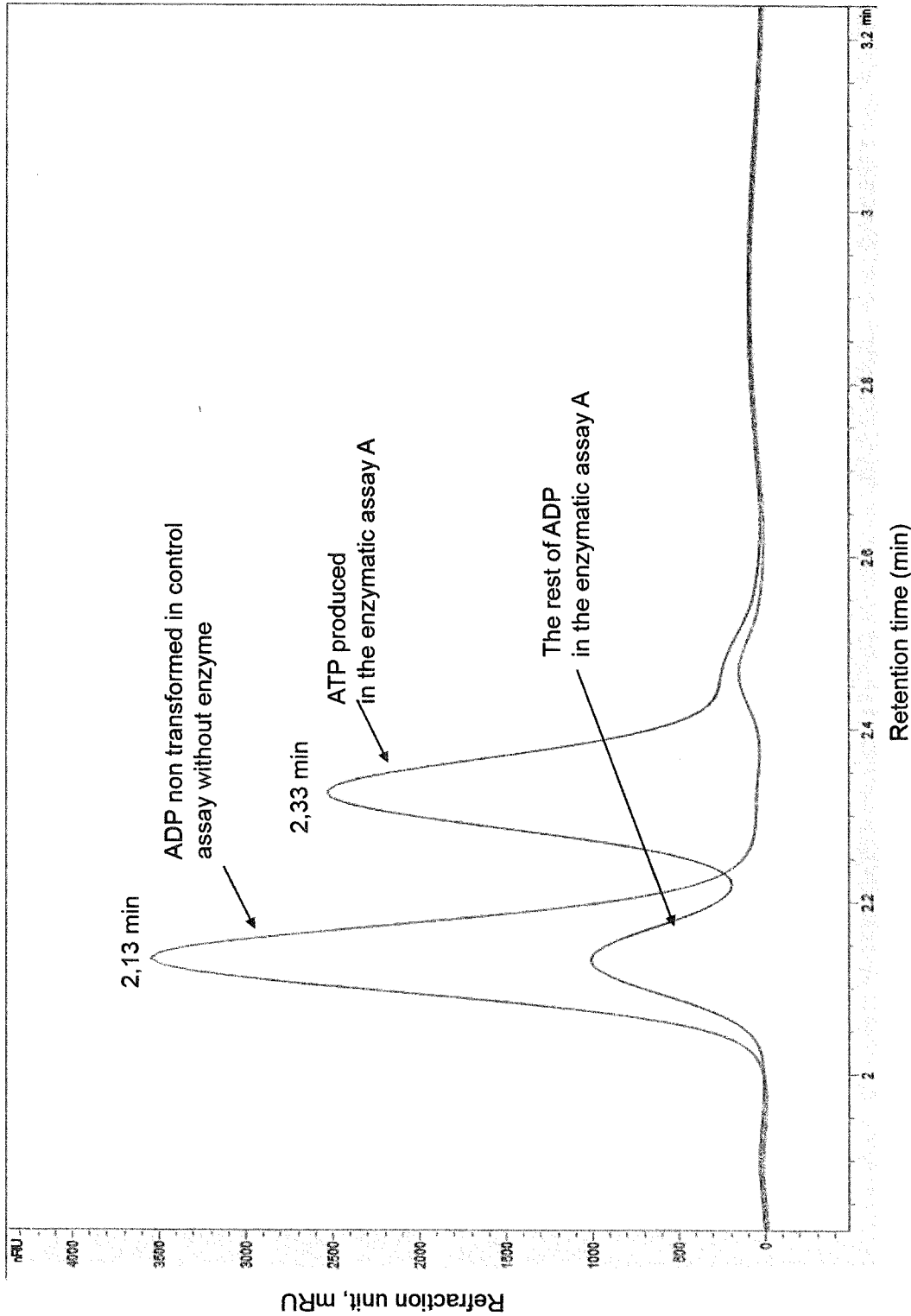


Figure 20b

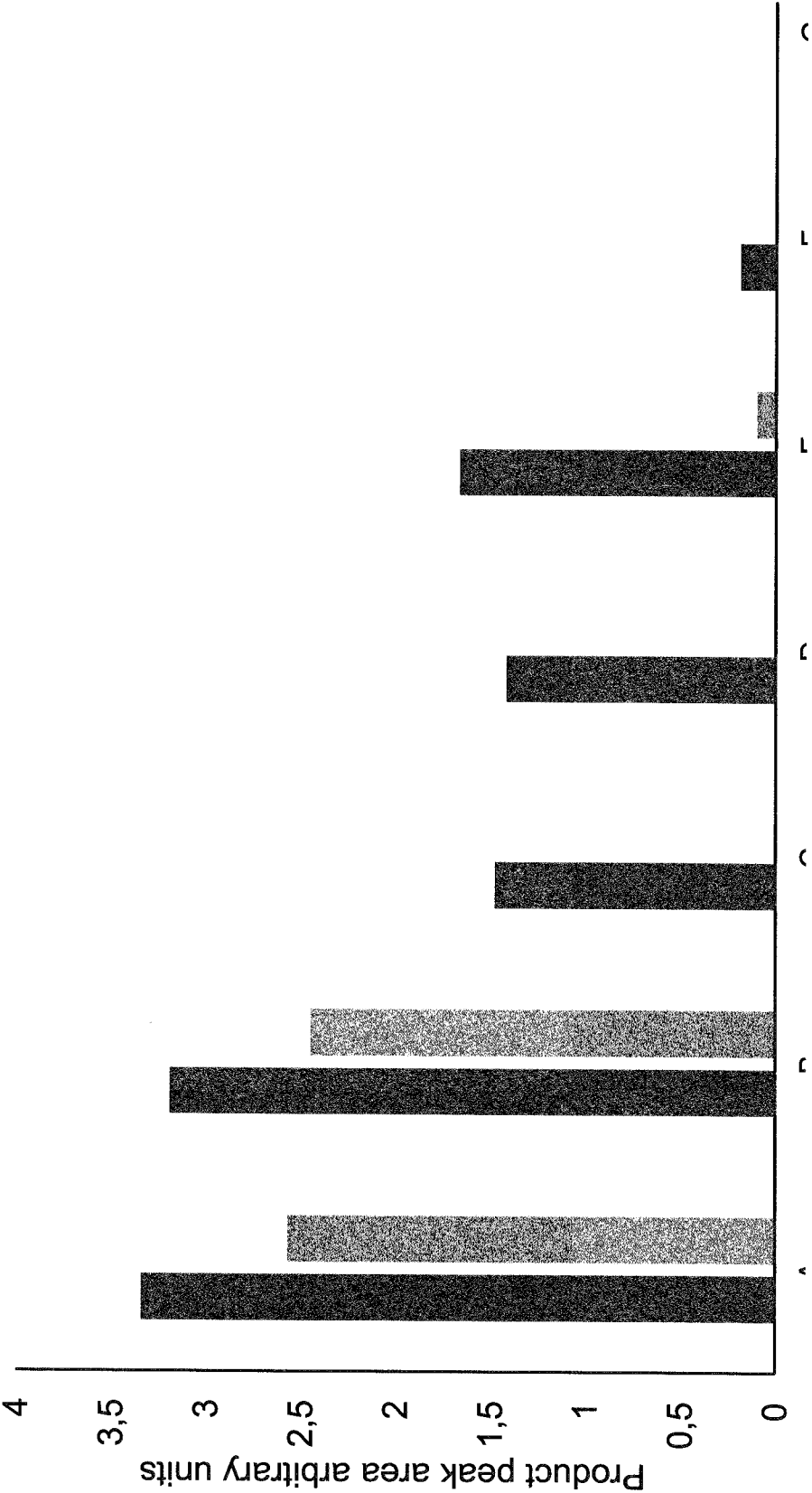


Figure 21

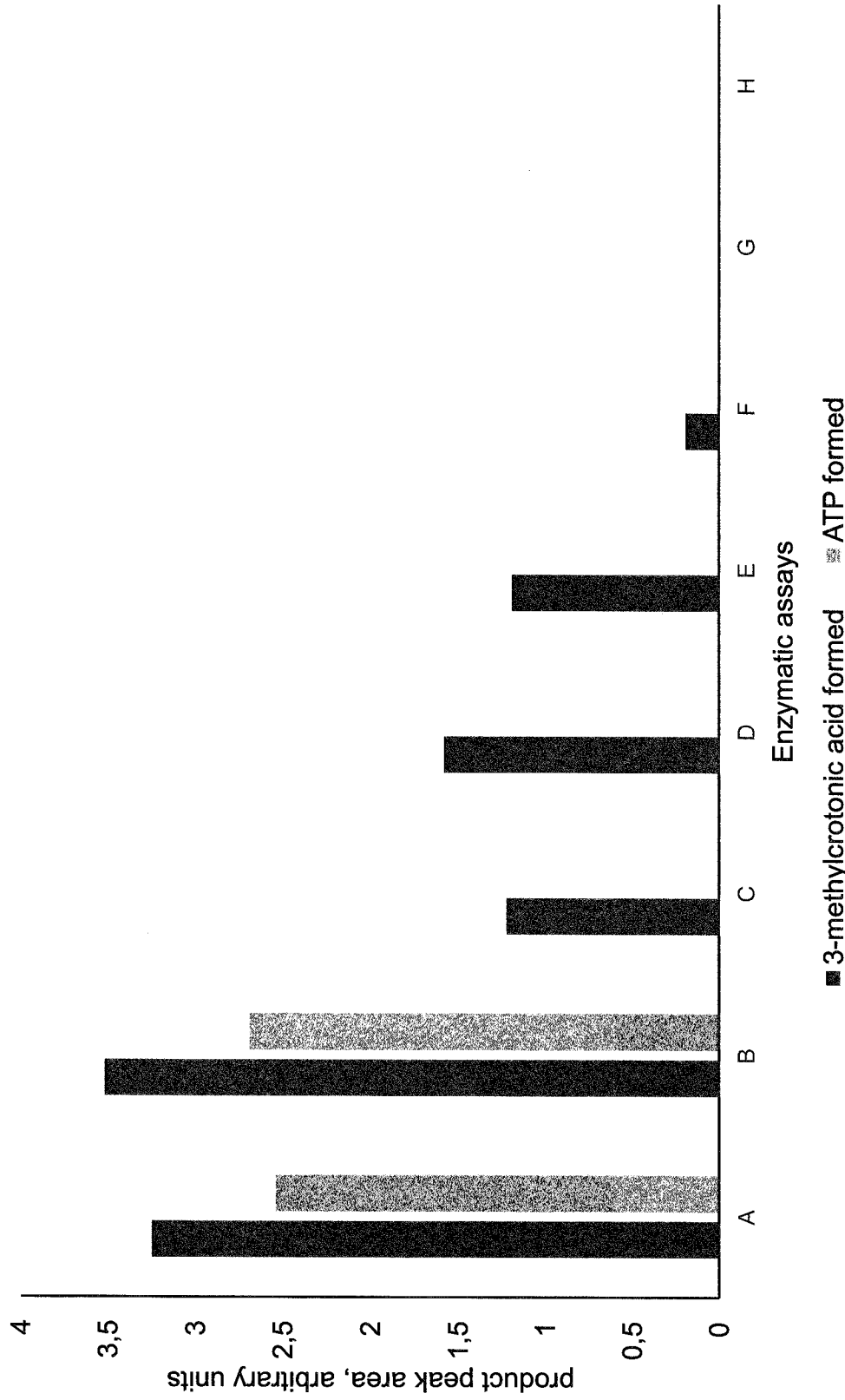


Figure 22

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2015/071182

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
- a. ☒ forming part of the international application as filed:
- ☒ in the form of an Annex C/ST.25 text file.
- ☐ on paper or in the form of an image file.
- b. ☐ furnished together with the international application under PCT Rule 13~~ter~~.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
- c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
- ☐ in the form of an Annex C/ST.25 text file (Rule 13~~ter~~.1(a)).
- ☐ on paper or in the form of an image file (Rule 13~~ter~~.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/071182

A. CLASSIFICATION OF SUBJECT MATTER INV. C12P5/02 C12N9/88 C12N9/10 C12N9/12 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) C12P C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2014/085612 A1 (INVISTA NORTH AMERICA S Á R L [US]) 5 June 2014 (2014-06-05) pages 1,15,16; figure 2 -----	1-19
A	FUJII T ET AL: "The relationship between isobutene-forming activity and a cytochrome P-450 in Rhodotorula minuta", JOURNAL OF FERMENTATION AND BIOENGINEERING, SOCIETY OF FERMENTATION TECHNOLOGY, JP, vol. 68, no. 3, 1 January 1989 (1989-01-01), pages 174-177, XP025778752, ISSN: 0922-338X, DOI: 10.1016/0922-338X(89)90132-3 [retrieved on 1989-01-01] the whole document ----- -/--	1-19
☒ 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 7 December 2015		Date of mailing of the international search report 16/12/2015
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Schneider, Patrick

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2015/071182

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Yi Liu ET AL: "R Hydrogen peroxide-independent production of [alpha]-alkenes by OleT JE P450 fatty acid decarboxylase", 24 February 2014 (2014-02-24), XP055107125, Retrieved from the Internet: URL:http://www.biotechnologyforbiofuels.com/content/pdf/1754-6834-7-28.pdf [retrieved on 2014-03-12] the whole document	1-19
A	DAVID S GOGERTY AND THOMAS A BOBIK: "Formation of Isobutene from 3-Hydroxy-3-Methylbutyrate by Diphosphomevalonate Decarboxylase", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 76, no. 24, 1 December 2010 (2010-12-01), pages 8004-8010, XP002680645, ISSN: 0099-2240, DOI: 10.1128/AEM.01917-10 [retrieved on 2010-10-22] the whole document	1-19
A	BIANCA N M VAN LEEUWEN ET AL: "Fermentative production of isobutene", APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, SPRINGER, BERLIN, DE, vol. 93, no. 4, 11 January 2012 (2012-01-11), pages 1377-1387, XP035013024, ISSN: 1432-0614, DOI: 10.1007/S00253-011-3853-7 the whole document	1-19

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2015/071182

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2014085612 A1	05-06-2014	CN 104903455 A	09-09-2015
		EP 2925871 A1	07-10-2015
		US 2014186913 A1	03-07-2014
		WO 2014085612 A1	05-06-2014
