

## **Method for the enzymatic production of isoprenol using mevalonate as a substrate**

The present invention relates to a method for the production of isoprenol using mevalonate as a substrate and enzymatically converting it into isoprenol. Moreover, the present invention relates to a method for the production of isoprene comprising the method for the production of isoprenol using mevalonate as a substrate and enzymatically converting it by a decarboxylation step into isoprenol and further comprising the step of converting the produced isoprenol into isoprene.

The present invention also relates to a method for the production of isoamyl alcohol comprising the method for the production of isoprenol using mevalonate as a substrate and enzymatically converting it by a decarboxylation step into isoprenol and further comprising the step of converting the produced isoprenol into isoamyl alcohol.

Isoprenol responds to the formula  $C_5H_{10}O$ . It can be used to produce prenol which is used in perfumes or as a building block in the pharmaceutical industry, e.g. in the production of citral, vitamin A and vitamin E. It is chemically produced by the condensation of isobutene and formaldehyde, leading to isoprenol further isomerised into prenol.

The biological route which is presently used to produce isoprenol involves the mevalonate pathway: mevalonate is produced, then diphosphorylated, then decarboxylated-dehydrated into isoprenyl-pyrophosphate, and finally dephosphorylated twice into isoprenol (US patent application 20080092829).

Isoprenol can be converted into isoprene which is a key compound for the tire industry, and also has many applications in the adhesives. It is produced chemically using several routes:

- Extractive distillation from oil (C5 stream)
- Dehydrogenation of iso-amylene
- Double dehydrogenation of isopentane

- Reaction of isobutene and formaldehyde
- Reaction of acetone and acetylene
- Propylene dimerization

WO 2009/076676 reports a metabolic pathway to isoprene. The pathway is based on the dephosphorylation-dehydration of downstream intermediates in the mevalonate pathway, i.e. isoprenyl-pyrophosphate or prenyl-pyrophosphate. This process has the drawback of requiring going through the whole mevalonate pathway: double phosphorylation of mevalonate, followed by a decarboxylation-dehydration into isoprenyl-pyrophosphate, further isomerised into prenyl-pyrophosphate, and finally double dephosphorylation/dehydration into isoprene.

Isoamyl alcohol is a very important chemical commonly used as solvents for fats, oils, resins and alkaloids. There is a demand for isoamyl alcohol in perfumery industry, for example in the manufacture of isoamyl salicylate used in soap and cosmetic fragrances. It is also used in the manufacture of phosphoric acid. Furthermore, it is used in the synthesis of pyrethroids. Commercial processes for the production of isoamyl alcohol include fractionation of fusel oils, chlorination of alkanes with subsequent hydrolysis to produce a mixture of isomers and a low pressure oxo-process or hydroformylation of n-butenes followed by hydrogenation of the resulting iso-valeraldehyde.

WO 2011/076261 describes a process for producing isoprenol by enzymatic conversion of mevalonate with an enzyme having the activity of a decarboxylase. Although the method described in WO 2011/076261 allows to produce isoprenol by enzymatically converting mevalonate, there is still a need for improvements, in particular as regards efficiency of the process so as to make it suitable for industrial purposes. The present application addresses this need.

Thus, in a first aspect, the present invention relates to a method for producing isoprenol from mevalonate. In particular, the present invention relates to a method for producing isoprenol from mevalonate through a biological process, in particular an enzymatic process, in which two types of enzymes are combined in order to increase the efficiency of the production rate. More specifically, the present invention relates to a method for producing isoprenol, characterized in that it comprises the conversion of mevalonate into isoprenol by

- (i) a first enzyme having an activity of converting mevalonate into mevalonate 3-

phosphate; and

- (ii) a second enzyme being different from the first enzyme and having an activity of converting said mevalonate 3-phosphate into isoprenol.

The present invention also relates to the use of at least two enzymes, wherein one enzyme is selected from (i) as specified above and the other enzyme is selected from (ii) as specified above or of a microorganism producing said combination of enzymes, for producing isoprenol from mevalonate.

The present invention also relates to organisms, preferably microorganisms, which produce at least two enzymes, wherein one enzyme is selected from (i) as specified above and the other enzyme is selected from (ii) as specified above.

The term "mevalonate" comprises mevalonic acid as well as the anion of mevalonic acid which is the predominant form in biological media. Mevalonic acid is a precursor in the biosynthetic pathway, known as the mevalonate pathway that produces terpenes and steroids. Mevalonate is the primary precursor of isoprenyl pyrophosphate that is in turn the basis for all terpenoids. The structural formula of mevalonic acid is shown in Figure 1. Mevalonic acid (3,5-dihydroxy-5-methylpentanoic acid) is chiral and exists in two enantiomers, R and S. In the present invention the term "mevalonic acid" encompasses both chiral forms, even if one of the two forms, for example the R form, is the one mainly produced naturally.

In the context of the present invention the term isoprenol comprises compounds which respond to the formula  $C_5H_{10}O$ . The IUPAC name of isoprenol is 3-methylbut-3-en-1-ol. Synonyms of isoprenol are, for example, 2-methyl-1-buten-4-ol, 3-buten-1-ol-3-methyl, 3-isopentenyl alcohol, 3-methyl-3-buten-1-ol, isobutenylcarbinol, isopropenylethyl alcohol and methallyl carbinol.

As mentioned above, WO 2011/076261 describes a process for producing isoprenol by enzymatic conversion of mevalonate with an enzyme having the activity of a decarboxylase. It has been described in WO 2011/076261 that generally the conversion of mevalonate into isoprenol by an enzyme having a decarboxylase activity, e.g. a mevalonate diphosphate (MDP) decarboxylase (E.C. 4.1.1.33), takes place by the conversion of mevalonate into mevalonate 3-phosphate which is then

dephosphorylated/decarboxylated to lead to isoprenol. The generic reaction carried out by MDP decarboxylase using mevalonate diphosphate is depicted in Figure 2. In the case of the natural reaction of mevalonate diphosphate decarboxylase with its natural substrate mevalonate 5-diphosphate, the first step of the reaction, i.e. the phosphorylation step, leads to an intermediate (3-phosphonoxy-3-methyl-5-diphosphopentanoate; see Figure 2A) which is unstable. The phosphorylation of mevalonate 5-diphosphate therefore facilitates the second step of the reaction, i.e. the concerted release of the phosphate (dephosphorylation) and of CO<sub>2</sub> (decarboxylation) to produce isopentenyl diphosphate (Byres et al., J. Mol. Biol. 371 (2007), 540-553). Since the two steps of the reaction are catalyzed by the same enzyme, it was assumed that when using mevalonate as a substrate the reaction would go through the same mechanism and would also involve an unstable intermediate which is immediately converted by the enzyme via the second reaction step. This is also supported by Dhe-Paganon et al. (Biochemistry 1994, 33, 13355-13362) which describes experiments carried out with the yeast mevalonate diphosphate decarboxylase as regards its mechanism of action and the mechanism of inhibition by fluorinated compounds. The authors concluded that the C<sub>3</sub>-hydroxyl of mevalonate diphosphate is phosphorylated by ATP, generating p-Mev-pp, which ionizes, leaving a carbocation on C<sub>3</sub> which is rapidly followed by decarboxylation. Thus, it was the understanding that the intermediate of the reaction catalyzed by mevalonate diphosphate decarboxylases is immediately converted by the enzyme into the final product.

However, it has surprisingly been found that when using mevalonate as a substrate the intermediate of the reaction, i.e. mevalonate 3-phosphate, is not unstable but transiently accumulates (see Example 3). In particular, it has been found that the intermediate is not immediately converted by the enzyme but is set free and is therefore available as a substrate for another enzyme converting it into the final product. Moreover, it has been found that different decarboxylases, in particular mevalonate diphosphate decarboxylases, catalyze the two above mentioned steps with different efficiencies, i.e. that some decarboxylases catalyze the first step with a higher efficiency than other decarboxylases and that some decarboxylases show a preference for the second step, i.e. the decarboxylation step. Due to this and the fact that the intermediate mevalonate 3-phosphate is not unstable and transiently accumulates it has now been found that it is possible to drastically increase the

efficiency of the conversion of mevalonate into isoprenol as described in WO 2011/076261 by combining enzymes which show high catalytic activities for the first and the second step of the reaction, respectively. Thus, the present invention in particular relates to a method for achieving a higher efficiency in the enzymatic production of isoprenol from mevalonate, i.e. a method for improving the efficiency of such an enzymatic production.

The term “an enzyme having an activity of converting mevalonate into mevalonate 3-phosphate” means an enzyme which can phosphorylate mevalonate into mevalonate 3-phosphate. The phosphate group comes preferably from an ATP molecule.

This activity can, e.g., be measured as described in the attached Examples, in particular Example 2. One possibility is thus to incubate the respective enzyme with mevalonate and ATP and to measure the production of ADP (which reflects the production of mevalonate 3-phosphate). Assays for measuring the production of ADP are known to the person skilled in the art. One of these methods is the pyruvate kinase/lactate dehydrogenase assay described in Example 2. In this case the assay measures the rate of NADH absorbance decrease at 340 nm which is proportional to the ADP quantity. Alternatively, the activity can be measured by directly measuring the produced mevalonate 3-phosphate, e.g. by mass spectrometry. Such an assay is described in Example 3. In a preferred embodiment the term “an enzyme having an activity of converting mevalonate into mevalonate 3-phosphate” means an enzyme which can convert mevalonate and ATP into mevalonate 3-phosphate and ADP. Even more preferably such an enzyme can catalyze the reaction of converting mevalonate into mevalonate 3-phosphate, preferably the reaction of converting mevalonate and ATP into mevalonate 3-phosphate and ADP, with a  $K_M$  of 10 mM or lower, e.g. with a  $K_M$  of 5 mM or lower, preferably of 1 mM or lower and even more preferably of 0.1 mM or lower. In a particularly preferred embodiment such an enzyme can catalyze the reaction of converting mevalonate into mevalonate 3-phosphate, preferably the reaction of converting mevalonate and ATP into mevalonate 3-phosphate and ADP, with a  $k_{cat}$  of at least  $0.2\text{ s}^{-1}$ , preferably with a  $k_{cat}$  of at least  $0.5\text{ s}^{-1}$ , particularly preferred with a  $k_{cat}$  of at least  $1.0\text{ s}^{-1}$ , more preferred of at least  $2.0\text{ s}^{-1}$  and even more preferred with a  $k_{cat}$  of at least  $5.0\text{ s}^{-1}$ .

In a particularly preferred embodiment the capacity to convert mevalonate and ATP into mevalonate 3-phosphate and ADP is measured in an assay as described in

Example 2 or as described in Example 3.

The term “an enzyme having an activity of converting said mevalonate 3-phosphate into isoprenol” means an enzyme which can catalyze a reaction by which there is a decarboxylation and dephosphorylation of the mevalonate 3-phosphate thereby leading to isoprenol.

This activity can, e.g., be measured as described in the appended Examples, in particular in Example 4 or 5. One possibility is thus to effect a combined enzyme assay in which the L200E mutant (SEQ ID NO: 16) of the *Th. acidophilum* mevalonate diphosphate decarboxylase is incubated with mevalonate and with the respective enzyme under conditions which allow the conversion of mevalonate into mevalonate 3-phosphate by the L200E mutant (SEQ ID NO: 16) of the *Th. acidophilum* mevalonate diphosphate decarboxylase and which in principle allow the decarboxylation and the dephosphorylation of mevalonate 3-phosphate into isoprenol and to detect the production of isoprenol, e.g. by gas chromatography. In a preferred embodiment such an assay is performed under the assay conditions described in Example 4 or 5. In a further preferred embodiment the term “an enzyme having an activity of converting mevalonate 3-phosphate into isoprenol” means an enzyme which is able to lead to an isoprenol production in such an assay which is at least half as high, more preferably at least as high as the isoprenol production which can be obtained by using in such an assay the L200E mutant (SEQ ID NO: 16) of the *Th. acidophilum* mevalonate diphosphate decarboxylase in combination with the mevalonate decarboxylase from *S. mitis* (SEQ ID NO: 10).

In a particularly preferred embodiment the capacity to produce isoprenol is measured in an assay as described in Example 4 or 5.

In one preferred embodiment an enzyme mentioned in (i) and (ii), above, is an enzyme which is considered by NCBI or an equivalent engine as having a COG3407 domain.

In a preferred embodiment of the method according to the invention the first enzyme (i) having an activity of converting mevalonate into mevalonate 3-phosphate is selected from the group consisting of

(A) a protein comprising the amino acid sequence as shown in SEQ ID NO: 1 or a

protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 1 and showing an activity of converting mevalonate into mevalonate 3-phosphate which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 1;

- (B) a protein comprising the amino acid sequence as shown in SEQ ID NO: 2 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 2 and showing an activity of converting mevalonate into mevalonate 3-phosphate which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 2;
- (C) a protein comprising the amino acid sequence as shown in SEQ ID NO: 3 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 3 and showing an activity of converting mevalonate into mevalonate 3-phosphate which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 3;
- (D) a protein comprising the amino acid sequence as shown in SEQ ID NO: 4 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 4 and showing an activity of converting mevalonate into mevalonate 3-phosphate which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 4; and
- (E) a protein comprising the amino acid sequence as shown in SEQ ID NO: 16 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 16 and showing an activity of converting mevalonate into mevalonate 3-phosphate which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 16.

SEQ ID NO: 1 shows the amino acid sequence of an enzyme from *Picrophilus torridus* DSM 9790 (GenBank accession number AAT43941; Swissprot/TrEMBL accession number Q6KZB1).

SEQ ID NO: 2 shows the amino acid sequence of an enzyme from *Thermoplasma*

acidophilum (GenBank accession number CAC12426; Swissprot/TrEMBL accession number Q9HIN1).

SEQ ID NO: 3 shows the amino acid sequence of an enzyme from *Thermoplasma volcanium* (GenBank accession number BAB59465; Swissprot/TrEMBL accession number Q97BY2).

SEQ ID NO: 4 shows the amino acid sequence of an enzyme from *Ferroplasma acidarmanus fer1* (GenBank accession number ZP\_05571615).

SEQ ID NO: 16 shows the amino acid sequence of the enzyme from *Thermoplasma acidophilum* (GenBank accession number CAC12426; Swissprot/TrEMBL accession number Q9HIN1) shown in SEQ ID NO: 2 in which the leucine (Leu) in position 200 is replaced by a glutamate (Glu).

In a further preferred embodiment of the method according to the invention the second enzyme (ii) having an activity of converting mevalonate 3-phosphate into isoprenol is selected from the group consisting of

- (a) a protein comprising the amino acid sequence as shown in SEQ ID NO: 5 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 5 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 5;
- (b) a protein comprising the amino acid sequence as shown in SEQ ID NO: 6 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 6 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 6;
- (c) a protein comprising the amino acid sequence as shown in SEQ ID NO: 7 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 7 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 7;
- (d) a protein comprising the amino acid sequence as shown in SEQ ID NO: 8 or a

protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 8 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 8;

- (e) a protein comprising the amino acid sequence as shown in SEQ ID NO: 9 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 9 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 9;
- (f) a protein comprising the amino acid sequence as shown in SEQ ID NO: 10 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 10 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 10;
- (g) a protein comprising the amino acid sequence as shown in SEQ ID NO: 11 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 11 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 11;
- (h) a protein comprising the amino acid sequence as shown in SEQ ID NO: 12 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 12 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 12;
- (i) a protein comprising the amino acid sequence as shown in SEQ ID NO: 13 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 13 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown

in SEQ ID NO: 13;

- (j) a protein comprising the amino acid sequence as shown in SEQ ID NO: 14 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 14 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 14;
- (k) a protein comprising the amino acid sequence as shown in SEQ ID NO: 15 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 15 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 15;
- (l) a protein comprising the amino acid sequence as shown in SEQ ID NO: 17 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 17 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 17;
- (m) a protein comprising the amino acid sequence as shown in SEQ ID NO: 18 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 18 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 18;
- (n) a protein comprising the amino acid sequence as shown in SEQ ID NO: 19 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 19 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 19;
- (o) a protein comprising the amino acid sequence as shown in SEQ ID NO: 20 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 20 and showing an activity of

converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 20;

- (p) a protein comprising the amino acid sequence as shown in SEQ ID NO: 21 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 21 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 21; and
- (q) a protein comprising the amino acid sequence as shown in SEQ ID NO: 22 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 22 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 22.

SEQ ID NO: 5 shows the amino acid sequence of an enzyme cloned from *Streptococcus gordonii*. SEQ ID NO: 6 shows the amino acid sequence of an enzyme from *Streptococcus gordonii* str. Challis substr. CH1 (GenBank accession number AAT43941; Swissprot/TrEMBL accession number A8UU9). SEQ ID NO: 7 shows the amino acid sequence of an enzyme from *Streptococcus infantarius* subsp. *infantarius* ATCC BAA-102 (GenBank accession number EDT48420.1; Swissprot/TrEMBL accession number B1SCG0). SEQ ID NO: 8 shows the amino acid sequence of *Dictyostelium discoideum* (GenBank accession number EAL68476.1; Swissprot/TrEMBL accession number Q54YQ9). SEQ ID NO: 9 shows the amino acid sequence of an enzyme from *Lactobacillus delbrueckii* (GenBank accession number CAI97800.1; Swissprot/TrEMBL accession number Q1GAB2). SEQ ID NO: 10 shows the amino acid sequence of an enzyme from *Streptococcus mitis* (strain B6) (GenBank accession number CBJ22986.1). SEQ ID NO: 11 shows the amino acid sequence of an enzyme from *Streptococcus gallolyticus* UCN34 (GenBank accession number CBI13757.1). SEQ ID NO: 12 shows the amino acid sequence of an enzyme from *Streptococcus sanguinis* SK36 (GenBank accession number ABN43791.1). SEQ ID NO: 13 shows the amino acid sequence of an enzyme from *Streptococcus* sp. M143 (GenBank accession number EFA24040.1).

SEQ ID NO: 14 shows the amino acid sequence of an enzyme from *Streptococcus suis* 89/1591 (GenBank accession number EEF63672.1). SEQ ID NO: 15 shows the amino acid sequence of an enzyme from *Streptococcus salivarius* SK126 (GenBank accession number EEK09252).

SEQ ID NO: 17 shows the amino acid sequence of *Methanosarcina mazei* (GenBank accession number AAM31457.1; Swissprot/TrEMBL accession number Q8PW40).

SEQ ID NO: 18 shows the amino acid sequence of *Sulfolobus tokodaii* (GenBank accession number BAK54434.1; Swissprot/TrEMBL accession number F9VNS6).

SEQ ID NO: 19 shows the amino acid sequence of *Streptococcus pneumonia* (GenBank accession number EDT95457.1; Swissprot/TrEMBL accession number B2DRT0).

SEQ ID NO: 20 shows the amino acid sequence of *Chloroflexus aggregans* (GenBank accession number ACL26234.1; Swissprot/TrEMBL accession number B8G8V9).

SEQ ID NO: 21 shows the amino acid sequence *Natromonas pharaonis* (GenBank accession number CAI48881.1; Swissprot/TrEMBL accession number Q3ISK5).

SEQ ID NO: 22 shows the amino acid sequence *Saccharomyces cerevisiae* (GenBank accession number CAA66158.1; Swissprot/TrEMBL accession number P32377).

In a preferred embodiment of the method according to the invention the first enzyme (i) is as defined in (E) above and the second enzyme (ii) is as defined in (a) or (b) mentioned above, even more preferably the second enzyme is as defined in (f), (n) or (q), mentioned above. As illustrated in the examples, the combination of these enzymes is particularly efficient at producing isoprenol compounds according to the present invention.

In another preferred embodiment of the method according to the invention the second enzyme (ii) having an activity of converting mevalonate 3-phosphate into isoprenol is selected from any one of the proteins listed in the following Table or from a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence of such a protein and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of said protein.

**Table 1**

| <b>Organism</b>               | <b>Ref sequence GenBank</b> |
|-------------------------------|-----------------------------|
| Methanosarcina mazei          | AAM31457.1                  |
| Methanocaldococcus jannaschii | AAB98390.1                  |
| Staphylococcus saprophyticus  | BAE19266.1                  |
| Streptococcus agalactiae      | EAO73731.1                  |
| Enterococcus faecalis         | AAO80711.1                  |
| Flavobacterium johnsoniae     | ABQ04421.1                  |
| Bdellovibrio bacteriovorus    | CAE79505.1                  |
| Chloroflexus aurantiacus      | A9WEU8.1                    |
| Legionella pneumophila        | CAH13175.1                  |
| Listeria monocytogenes        | EAL09343.1                  |
| Metallosphaera sedula         | ABP95731.1                  |
| Staphylococcus epidermidis    | AAO03959.1                  |
| Streptococcus thermophilus    | AAV60266.1                  |
| Bacillus coagulans            | EAY45229.1                  |
| Chloroflexus aggregans        | ACL26234.1                  |
| Lactobacillus brevis          | ABJ64001.1                  |
| Lactobacillus fermentum       | BAG27529.1                  |
| Lactobacillus plantarum       | CAD64155.1                  |
| Lactobacillus salivarius      | ABD99494.1                  |
| Lactococcus lactis sp. lactis | AAK04503.1                  |
| Dichelobacter nodosus         | ABQ14154.1                  |
| Flavobacterium psychrophilum  | CAL42423.1                  |
| Streptococcus pneumoniae      | EDT95457.1                  |
| Streptococcus pyogenes        | AAT86835.1                  |
| Streptococcus suis            | ABP91444.1                  |
| Staphylococcus haemolyticus   | BAE05710.1                  |
| Streptococcus equi            | ACG62435.1                  |
| Arabidopsis thaliana          | AAC67348.1                  |
| Borrelia afzelii              | ABH01961.1                  |
| Encephalitozoon cuniculi      | CAD25409.1                  |
| Streptomyces sp.              | BAB07791.1                  |
| Streptococcus agalactiae      | EAO73731.1                  |
| Streptococcus uberis          | CAR41735.1                  |
| Gallus gallus                 | XP_423130                   |
| Salmo salmar                  | ACI34234                    |

As mentioned above, not only the proteins having the specifically mentioned amino acid sequences listed in the respective SEQ ID NOs or in Table 1 can be used, but also proteins which are considered by NCBI or an equivalent engine as having a COG3407 domain and, more preferred, proteins the amino acid sequence of which shows a homology of at least 15% to the specifically mentioned amino acid sequence and which have a respective enzymatic activity at least as high as the activity of a protein having the specifically mentioned amino acid sequence. Preferred enzymes advantageously have at least x% homology, wherein x is selected from the group consisting of 20, 25, 30, 35, 40, 45, 50, 55 and 60. In a further preferred embodiment the enzyme has at least 65% sequence homology, preferably at least 70%, more preferably at least 75%, even more preferably, at least 80, 85, 90, 95, 96, 97, 98 or 99% homology to one of the sequences shown in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 18, 19, 20, 21 or 22 or to one of the sequences shown in Table 1. 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 percent of sequence homology can be determined by different methods and by means of software programs known to one of skill in the art, such as for example the CLUSTAL method or BLAST and derived software, or by using a sequence comparison algorithm such as that described by Needleman and Wunsch (J. Mol. Biol., 1970, 48:443) or Smith and Waterman (J. Mol. Biol., 1981, 147:195). 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.

Preferably, the degree of identity is calculated over the complete length of the sequence. Moreover, if the term "homology" is used in the context of the present

invention, this term preferably means "sequence identity".

Such proteins showing the indicated degree of homology can, e.g., be other enzymes which occur naturally or which have been prepared synthetically. They include in particular enzymes which can be selected for their ability to produce isoprenol according to the invention. Thus, a selection test comprises contacting the purified enzyme, or a microorganism producing the enzyme, with the substrate of the reaction and measuring the production of the respective compound, i.e. mevalonate 3-phosphate or isoprenol. Such tests are described in the experimental section. Such selection tests can also be used to screen for enzymes with an optimized enzymatic activity for the substrate, i.e. mevalonate, to be converted into mevalonate 3-phosphate or further into isoprenol, i.e. having an optimized activity with respect to mevalonate or mevalonate 3-phosphate.

Such methods are well-known in the art and include, e.g. protein engineering techniques such as random mutagenesis, massive mutagenesis, site-directed mutagenesis, DNA shuffling, synthetic shuffling, *in vivo* evolution, or complete synthesis of genes and subsequent screening for the desired enzymatic activity.

The enzyme used in the invention can thus be natural or synthetic, and produced by chemical, biological or genetic means. It can also be chemically modified, for example in order to improve its activity, resistance, specificity, purification, or to immobilize it on a support.

The combination of the enzymes as defined in (i) and (ii), hereinabove, is characterized in that it leads to a higher conversion rate of mevalonate into isoprenol, i.e. a higher production of isoprenol, than the conversion rate which would be achieved by either enzyme alone or the mere addition of the conversion rates which either enzyme achieves in isolation. Preferably, the conversion rate of the combination is at least 2 fold higher than the mere addition of the conversion rates achieved by the enzymes in isolation, more preferably at least 5-fold higher, even more preferably at least 8-fold higher, particularly preferred at least 10-fold higher and most preferred at least 15-fold higher.

It has been found that enzymes which are able to catalyze the above described reactions for converting mevalonate into isoprenol via mevalonate 3-phosphate are

often enzymes which can be classified in the phylogenetic superfamily of mevalonate diphosphate (MDP) decarboxylases (enzyme nomenclature EC 4.1.1.33). MDP decarboxylase is an enzyme involved in cholesterol biosynthesis. Said enzyme has been isolated from a variety of organisms including animals, fungi, yeasts and some bacteria. It can also be expressed by some plants (Lalitha et al., *Phytochemistry* 24 (11), (1985), 2569-2571). Many genes encoding this enzyme have been cloned and sequenced. These enzymes are generally composed of 300 to 400 amino acids and use ATP as co-substrate, which is converted during the reaction to ADP and inorganic phosphate. The phosphate group is transferred from the ATP molecule to the tertiary alcohol of mevalonate diphosphate, releasing ADP. The reaction intermediate phosphorylated on the 3-hydroxyl group then undergoes elimination of the phosphate group, in the physiological case releasing isopentenyl diphosphate Figure 2.

MDP decarboxylases belong to a large class of metabolite kinases, the GHMP kinase superfamily. Analysis of data on structural features of MDP decarboxylases accessible from Uniprot reveal that they share a common structural motif referenced in InterPRO database as IPR020568 (<http://www.ebi.ac.uk/interpro/entry/IPR020568>). Domain IPR020568 or ("domain 2 of the ribosomal protein S5") has a left-handed, 2-layer alpha/beta fold with a core structure consisting of beta(3)-alpha-beta-alpha. Domains with this fold are found in numerous kinases from the GHMP kinase family

Accordingly, in a preferred embodiment, the enzyme defined in (i) or (ii) above, is a MDP decarboxylase. In the context of the present invention a MDP decarboxylase is defined as an enzyme which can at least catalyze the conversion of 5-diphospho-3-phosphomevalonate into isopentenyl-5-diphosphate and CO<sub>2</sub> or which can at least catalyze the reaction of converting mevalonate diphosphate and ATP into 5-diphospho-3-phosphomevalonate and ADP. Preferably, such an enzyme can catalyze both reactions.

In another preferred embodiment the enzyme defined in (i) above, is an enzyme as defined in (i) (B) or (E). The sequence shown in SEQ ID NO: 2 represents an enzyme identified in *Thermoplasma acidophilum*. SEQ ID NO: 16 is a mutant of SEQ ID NO: 2 in which the leucine (Leu) residue at position 200 is replaced by a glutamate (Glu) residue. In Genbank this enzyme is classified as a mevalonate

diphosphate decarboxylase. However, it is known from Chen and Poulter (Biochemistry 49 (2010), 207-217) that in *Th. acidophilum* there exists an alternative mevalonate pathway which involves the action of a mevalonate-5-monophosphate decarboxylase. Thus, it is possible that the enzyme represented by SEQ ID NO: 2 actually represents a mevalonate-5-monophosphate decarboxylase.

The term “diphosphomevalonate decarboxylase” or “a protein/enzyme having the activity of a diphosphomevalonate decarboxylase” in the context of the present application also covers enzymes which are derived from a diphosphomevalonate decarboxylase, which are capable of catalyzing the decarboxylation of mevalonate but which only have a low affinity to their natural substrate, e.g. mevalonate diphosphate, or do no longer accept their natural substrate, e.g. mevalonate diphosphate. Such a modification of the preferred substrate allows to improve the conversion of mevalonate into isoprenol and to reduce the production of the possibly occurring by-product isoprenyl pyrophosphate. 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”, DNA shuffling or *in vivo* evolution.

For example, for genetic engineering in prokaryotic cells, a nucleic acid molecule encoding a diphosphomevalonate decarboxylase 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. The resulting diphosphomevalonate decarboxylase variants are then tested for their enzymatic activity and in particular for their capacity to prefer mevalonate as a substrate rather than mevalonate diphosphate.

Such methods for identifying variants with improved enzymatic properties as regards the production of isoprenol may also be carried out in the presence of a cofactor which allows for a steric and/or electronic complementation in the catalytic site of the enzyme due to the fact that the substrate mevalonate is shorter than the natural substrate mevalonate diphosphate. Examples for such a cofactor would be phosphono-phosphate or phosphonamido-phosphate (see Figure 3) or orthophosphate.

The modified version of the diphosphomevalonate decarboxylase accepting or preferring mevalonate or mevalonate 3-phosphate as a substrate but having a low affinity to its natural product mevalonate diphosphate as a substrate or no longer accepting its natural product mevalonate diphosphate as a substrate may be derived from a naturally occurring diphosphomevalonate decarboxylase or from an already modified, optimized or synthetically synthesized diphosphomevalonate decarboxylase.

The enzymes employed in the process according to the present invention can be natural versions of the proteins or synthetic proteins as well as proteins which have been chemically synthesized or produced in a biological system or by recombinant processes. The enzymes may also be chemically modified, for example in order to improve their stability, resistance, e.g. to temperature, for facilitating their purification or their immobilization on a support. The enzymes may be used in isolated form, purified form, in immobilized form, as a crude or partially purified extract obtained from cells synthesizing the enzyme(s), as chemically synthesized enzymes, as recombinantly produced enzymes, in the form of organism/microorganisms producing them etc.

The 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 one or more cofactors). In one embodiment, the enzymes employed in the method are used in purified form.

For carrying out the process *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 isoprenol. The production of isoprenol can be measured by methods known in the art, such as chromatography, e.g. thin layer chromatography or liquid or 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 one embodiment, the conversion occurs in the presence of a co-substrate, said co-substrate preferably being a compound containing a phosphoanhydride, and preferably being ATP, an rNTP, a dNTP or a mixture of several of these molecules, a polyphosphate, or pyrophosphate. The co-substrate can be added to the reaction and is preferably selected from the group consisting of ATP, an rNTP, a dNTP, a mixture of several rNTPs or dNTPs, a polyphosphate, and preferably pyrophosphate, or a compound containing a phosphoanhydride (represented by the general formula  $X-PO_3H_2$ ).

Although the decarboxylation step, i.e. the reaction defined as (ii) herein-above, does not require ATP consumption, it could be shown that the presence of ATP in the reaction could be beneficial. It is assumed that ATP might have an effect on the folding of the protein by the binding of ATP to the ATP-binding site of the diphosphomevalonate decarboxylase. In fact, this can be observed by eye: the purified enzyme has a tendency to precipitate, and the addition of ATP prevents this effect. It is considered that not only ATP but also other similar compounds like dATP, ADP, AMP or other NTPs or dNTPs have this effect. Thus, in a further embodiment, the method according to the present invention is carried with ATP, dATP, ADP, AMP or an NTP other than ATP or a dNTP as co-substrate.

In another embodiment, cofactors are added so as to best mimic the natural reaction or so as to provide steric or electronic complementation in the catalytic cleft. The structure of mevalonate leaves a space in the catalytic cleft empty during enzyme-substrate binding. Filling this space with a cofactor to replace the missing part of the substrate has the purpose of most closely mimicking the MDP molecule. As the

cofactor is not modified during the reaction, it will therefore be added only in catalytic amounts. Examples for such a cofactor would be phosphono-phosphate or phosphonamido-phosphate (see Figure 3) or orthophosphate.

Moreover, it is described for some diphosphomevalonate decarboxylase enzymes that they require monovalent and/or divalent cations. Thus, in a further embodiment, and if necessary, a suitable amount of a suitable monovalent (e.g.  $K^+$ ) and/or divalent cation is added to the reaction when carrying out the method according to the invention. The divalent cation is preferably  $Mg^{2+}$ ,  $Mn^{2+}$  or  $Co^{2+}$ , but it is possible to also use other divalent cations such as  $Ca^{2+}$ . Of course, the nature of the monovalent and/or divalent cation depends on the need of the diphosphomevalonate decarboxylase enzyme in question.

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. Thus, in such an embodiment of the invention, an organism, preferably a microorganism, that produces the enzymes specified in (i) and (ii) above is used. In a preferred embodiment, the (micro)organism is recombinant in that the enzymes specified in (i) and (ii) produced by the host are heterologous relative to the production host. The method can thus be carried out directly in the culture medium, without the need to separate or purify the enzymes. In an especially advantageous manner, a (micro)organism is used having the natural or artificial property of endogenously producing mevalonate, and also expressing or overexpressing the enzymes specified in (i) and (ii) above, natural or modified, so as to produce isoprenol directly from a carbon source present in solution.

In one embodiment the organism employed in the method according to the invention is an organism, preferably a microorganism, which has been genetically modified to contain a foreign nucleic acid molecule encoding an enzyme as defined above. The term "foreign" in this context means that the nucleic acid molecule does not naturally occur in said organism/microorganism. This means that it does not occur in the same structure or at the same location in the organism/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 organism/microorganism, i.e. a promoter which does naturally not occur in the respective organism/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 organism/microorganism in that the encoded enzyme is not endogenous to the organism/microorganism, i.e. are naturally not expressed by the organism/microorganism when it is not genetically modified. In other words, the encoded enzyme is heterologous with respect to the organism/microorganism. The foreign nucleic acid molecule may be present in the organism/microorganism in extrachromosomal form, e.g. as plasmid, or stably integrated in the chromosome. A stable integration is preferred.

For example, the method according to the invention can be carried out by using microorganisms which produce mevalonate, for example an *E. coli* strain which naturally produce mevalonate or which have been genetically modified so as to produce (or overproduce) mevalonate] and which have been genetically engineered such that they overexpress the enzymes as defined in (i) and (ii) above, said enzymes preferably originating from an organism different from the host microorganism. The genetic modification can consist, e.g. in integrating the corresponding genes encoding the enzymes into the chromosome, expressing the enzymes 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.

Thus, for carrying out the process in vivo use is made of a suitable organism/microorganism(s) which is/are capable of providing mevalonate. There are two alternate pathways that lead to isoprenyl-pyrophosphate. One is the mevalonate pathway, observed in eukaryotes and some prokaryotes, especially in the firmicute phylum. All these organisms thus produce mevalonate. Most of the bacteria, including *E. coli*, use the other pathway (DXP pathway) and are thus not producing

mevalonate. However, the latter can be genetically modified so as to produce mevalonate. For example, the implementation of the mevalonate pathway in *E. coli* has already been done successfully (Maury et al., FEBS Lett. 582 (2008), 4032). Overexpression of only the upstream part (thiolase, HMG-CoA synthase, HMG-CoA reductase) in organisms that have or that do not have the mevalonate pathway allows for the production of high levels of mevalonate.

The organisms used in the invention can be prokaryotes or eukaryotes, preferably, they are microorganisms such as bacteria, yeasts, fungi or molds, or plant cells or animal cells. In a particular embodiment, the microorganisms are bacteria, preferably of the genus *Escherichia* and even more preferably of the species *Escherichia coli*.

In another embodiment, the microorganisms are recombinant bacteria of the genus *Escherichia*, preferably of the species *Escherichia coli*, having been modified so as to endogenously produce mevalonate and to convert it into isoprenol.

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

In one embodiment the microorganism is a fungus, more preferably a fungus of the genus *Saccharomyces*, *Schizosaccharomyces*, *Aspergillus*, *Trichoderma*, *Pichia* or *Kluyveromyces* and even more preferably of the species *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Aspergillus niger*, *Trichoderma reesei*, *Pichia pastoris* or of the species *Kluyveromyces lactis*. In a particularly preferred embodiment the microorganism is a recombinant yeast producing produce mevalonate and to convert it into isoprenol due to the expression of the enzymes specified in (i) and (ii) above.

In another preferred embodiment, the method according to the invention makes use of a photosynthetic microorganism expressing the enzymes as specified in (i) and (ii) 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.

Even more preferably such a microorganism has the natural or artificial property of endogenously producing mevalonate. In this case the microorganism would be capable of producing isoprenol directly from CO<sub>2</sub> present in solution.

It is also conceivable to use in the method according to the invention one microorganism that produces an enzyme as defined in (i) above and another microorganism which produces an enzyme as defined in (ii) above. Moreover, in a further embodiment at least one of the microorganisms is capable of producing mevalonate or, in an alternative embodiment, a further microorganism is used in the method which is capable of producing mevalonate.

In another embodiment the method according to the invention makes use of a multicellular organism expressing the enzymes as defined in (i) and (ii) above. Examples for such organisms are plants or animals.

In a particular embodiment, the method involves culturing microorganisms in standard culture conditions (30-37°C at 1 atm, in a fermenter allowing aerobic growth of the bacteria) or non-standard conditions (higher temperature to correspond to the culture conditions of thermophilic organisms, for example).

When the process according to the invention is carried out in vivo by using an organism/microorganism providing the respective enzyme activities, the organism, preferably microorganism, is cultivated under suitable culture conditions allowing the occurrence of the enzymatic reaction. The specific culture conditions depend on the specific organism/microorganism employed but are well known to the person skilled in the art. The culture conditions are generally chosen in such a manner that they allow the expression of the genes encoding the enzymes for the respective reactions. Various methods are known to the person skilled in the art in order to improve and fine-tune the expression of certain genes at certain stages of the culture such as induction of gene expression by chemical inducers or by a temperature shift.

In another embodiment the organism employed in the method according to the invention is a plant. In principle any possible plant can be used, i.e. a monocotyledonous plant or a dicotyledonous plant. It is preferable to use a plant which can be cultivated on an agriculturally meaningful scale and which allows to produce large amounts of biomass. Examples are grasses like *Lolium*, cereals like rye, wheat, barley, oat, millet, maize, other starch storing plants like potato or sugar storing plants like sugar cane or sugar beet. Conceivable is also the use of tobacco

or of vegetable plants such as tomato, pepper, cucumber, egg plant etc. Another possibility is the use of oil storing plants such as rape seed, olives etc. Also conceivable is the use of trees, in particular fast growing trees such as eucalyptus, poplar or rubber tree (*Hevea brasiliensis*).

In another embodiment, the method according to the invention is characterized by the conversion of a carbon source such as glucose into mevalonate followed by the conversion of mevalonate into isoprenol.

In another embodiment, the method according to the invention comprises the production of isoprenol from atmospheric CO<sub>2</sub> or from CO<sub>2</sub> artificially added to the culture medium. In this case the method is implemented in an organism which is able to carry out photosynthesis, such as for example microalgae.

The present invention also relates to a method for producing isoprenol comprising the step of enzymatically converting mevalonate 3-phosphate into isoprenol by use of an enzyme which can catalyze the conversion via decarboxylation and dephosphorylation. As described above, the present application for the first time shows that the intermediate of the reaction leading from mevalonate to isoprenol is set free by the enzyme and can be used by another enzyme as a substrate. This opens up the possibility that the first and the second step of the reaction can be catalyzed by different enzymes thereby optimizing the overall reaction efficiency.

The method is preferably characterized in that the mevalonate 3-phosphate is provided as a substrate to the enzyme, i.e. is not produced by the enzyme itself from mevalonate but is taken up externally from the enzyme. Thus, the method is preferably characterized in that the mevalonate-3-phosphate is not produced in situ, wherein "in situ" means that the mevalonate-3-phosphate is not produced by the enzyme itself from mevalonate. Preferably, the mevalonate-3-phosphate is provided externally.

The present invention also relates to a composition comprising mevalonate-3-phosphate and an enzyme which can catalyze the conversion of mevalonate 3-phosphate into isoprenol via decarboxylation and dephosphorylation.

As regards the preferred enzyme to be used in such a method or composition, the same applies as has been set forth above in connection with (ii) of the method

according to the invention as described herein-above.

Moreover, also with respect to the other preferred embodiments described above for the method according to the invention, the same applies to the method for producing isoprenol from mevalonate 3-phosphate.

Moreover, the present invention also relates to a composition comprising (a) mevalonate; and (b) a (micro)organism as described herein above. Such a composition may further comprise a suitable culture medium or a carbon source that can be converted into mevalonate.

The present invention also relates to a composition comprising

- (a) mevalonate; and
- (b) (i) a first enzyme having an activity of converting mevalonate into mevalonate 3-phosphate; and
- (ii) a second enzyme being different from the first enzyme and having an activity of converting said mevalonate 3-phosphate into isoprenol.

For the preferred embodiments of the enzymes expressed by the (micro)organism and mentioned in (i) and (ii), above, the same applies as has already been set forth above in connection with the method according to the invention.

In a particularly preferred embodiment, the composition also comprises a co-substrate (such as ATP), a co-factor and/or monovalent and/or divalent cations (such as  $K^+$ ,  $Mn^{2+}$ ,  $Mg^{2+}$ ,  $Co^{2+}$  or  $Ca^{2+}$ ).

The present invention also relates to the use of a combination of at least two enzymes, wherein one enzyme is selected from the following (i) and the other enzyme is selected from the following (ii) or of an organism, preferably a microorganism, as described herein above or of a composition according to the invention, for producing isoprenol from mevalonate, wherein (i) and (ii) are as follows:

- (i) a first enzyme having an activity of converting mevalonate into mevalonate 3-phosphate; and
- (ii) a second enzyme being different from the first enzyme and having an activity of converting said mevalonate 3-phosphate into isoprenol.

As regards the preferred embodiments of the different components recited, the same

applies as has been set forth above in connection with the method according to the invention.

In addition the present invention also relates to a method for producing isoprene from mevalonate comprising the method for producing isoprenol according to the invention as described above and further comprising the step of converting the produced isoprenol into isoprene. The conversion of isoprenol into isoprene can be achieved by means and methods known to the person skilled in the art. In particular, the respective reaction is a dehydration reaction.

Moreover, the present invention also relates to a method for producing isoamyl alcohol from mevalonate comprising the method for producing isoprenol according to the invention as described above and further comprising the step of converting the produced isoprenol into isoamyl alcohol. The conversion of isoprenol into isoamyl alcohol can be achieved by means and methods known to the person skilled in the art. In particular, the respective reaction is a hydrogenation reaction.

- Figure 1      Chemical structure of mevalonic acid.
- Figure 2      Reaction of diphosphomevalonate decarboxylase on the physiological substrate 5-diphosphomevalonate and on the precursor mevalonate.
- Figure 3      Structure of phosphono-phosphate and phosphonoamido-phosphate
- Figure 4      Scheme of the ADP quantification assay, monitoring NADH consumption by the decrease of absorbance at 340 nm.
- Figure 5      Plot of the rate as a function of substrate concentration for the phosphotransferase reaction catalyzed by *Th. acidophilum* MDP decarboxylase (mutant L200E). Initial rates were computed from the kinetics over the 20 first minutes of the reaction.
- Figure 6      Electrospray MS spectrums of mevalonate phosphorylation reaction catalyzed by MDP decarboxylase from *Th. acidophilum* (a), control assay without enzyme (b).
- Figure 7      Screening of MDP decarboxylases in a complementation assay. Peak area ratios were obtained by dividing the isoprenol peak area of each enzymatic assay by the peak area of the sample without enzyme

(background noise).

- Figure 8 Combined effect of MDP decarboxylase enzymes from *Th. acidophilum* and *S. mitis* for converting mevalonate into isoprenol.
- Figure 9 Mass spectrums of commercial isoprenol (a) and isoprenol produced from mevalonate by combining action of two enzymes (b). The characteristic ions 68 and 56 representing, respectively, the loss of H<sub>2</sub>O and CH<sub>2</sub>O were observed in both spectrums.
- Figure 10 Plot of the rate of isoprenol production as a function of the *S. gordonii* MDP decarboxylase concentration.
- Figure 11 Combined effect of MDP decarboxylase enzymes from *Th. acidophilum* and *S. tokodaii* for converting mevalonate into isoprenol.
- Figure 12 Combined effect of MDP decarboxylase enzymes from *Th. acidophilum* and *D. discoideum* for converting mevalonate into isoprenol.

Other aspects and advantages of the invention will be described in the following examples, which are given for purposes of illustration and not by way of limitation

## Examples

### **Example 1: Cloning, expression and purification of enzymes**

A set of genes encoding representatives of the diphosphomevalonate decarboxylase (MDP decarboxylase) family across eukaryotic, prokaryotic and archaeal organisms was constructed and tested to identify the most active candidates for improving isoprenol production.

#### *Cloning, bacterial cultures and expression of proteins*

The genes encoding mevalonate diphosphate (MDP) decarboxylase EC 4.1.1.33 were cloned in the pET 25b vector (Novagen) in the case of eukaryotic genes and in pET 22b (Novagen) in the case of prokaryotic genes. A stretch of 6 histidine codons was inserted after the methionine initiation codon to provide an affinity tag for purification. Competent *E. coli* BL21(DE3) cells (Novagen) were transformed with these vectors according to the heat shock procedure. The transformed cells were grown with shaking (160 rpm) on ZYM-5052 auto-induction medium (Studier FW,

*Prot.Exp.Pur.* 41, (2005), 207-234) for 6h at 37°C and protein expression was continued at 28°C overnight (approximately 16 h). The cells were collected by centrifugation at 4°C, 10,000 rpm for 20 min and the pellets were frozen at -80°C.

#### *Protein purification and concentration*

The pellets from 200 ml of culture cells were thawed on ice and resuspended in 5 ml of Na<sub>2</sub>HPO<sub>4</sub> pH 8 containing 300 mM NaCl, 5 mM MgCl<sub>2</sub> 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 3 x 15 seconds. The bacterial extracts were then clarified by centrifugation at 4°C, 10,000 rpm for 20 min. The clarified bacterial lysates were loaded on PROTINO-1000 Ni-TED column (Macherey-Nagel) allowing adsorption of 6-His tagged proteins. Columns were washed and the enzymes of interest were eluted with 4 ml of 50 mM Na<sub>2</sub>HPO<sub>4</sub> pH 8 containing 300 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 250 mM imidazole. Eluates were then concentrated and desalted on Amicon Ultra-4 10 kDa filter unit (Millipore) and resuspended in 0.25 ml 50 mM Tris-HCl pH 7.5 containing 0.5 mM DTT and 5 mM MgCl<sub>2</sub>. Protein concentrations were quantified by direct UV 280 nm measurement on the NanoDrop 1000 spectrophotometer (Thermo Scientific). The purity of proteins thus purified varied from 50 % to 90 %.

#### **Example 2: Characterization of the phosphotransferase activity**

The release of ADP that is associated with isoprenol production from mevalonate was quantified using the pyruvate kinase/lactate dehydrogenase coupled assay (Figure 4). The MDP decarboxylases from *P. torridus* phylum and *S. mitis* enzyme were evaluated for their ability to phosphorylate mevalonate, releasing ADP.

The studied enzymatic reaction was carried out under the following conditions at 40°C:

50 mM Tris-HCl pH 7.5

10 mM MgCl<sub>2</sub>

100 mM KCl

5 mM ATP

0.4 mM NADH

1 mM Phosphoenolpyruvate

1.5 U/ml Lactate dehydrogenase

3 U/ml Pyruvate kinase

0 to 5 mM R,S- sodium Mevalonate

The pH was adjusted to 7.5.

Each assay was started by addition of particular enzyme (at a concentration from 0.025 to 1 mg/ml) and the disappearance of NADH was monitored by following the absorbance at 340 nm.

Figure 5 shows an example of a Michaelis-Menten plot corresponding to the data collected for the *Th. acidophilum* (L200E) enzyme. The kinetic parameters are shown in the following Table 1.

Table 1

| Enzyme                                                 | $K_M$ , mM | $k_{cat}$ , $s^{-1}$ | $k_{cat}/K_M$ , $mM^{-1} s^{-1}$ |
|--------------------------------------------------------|------------|----------------------|----------------------------------|
| <i>Ferroplasma acidarmanus</i>                         | 0.62       | 1.5                  | 2.5                              |
| <i>Picrophilus torridus</i>                            | 0.32       | 1.2                  | 3.8                              |
| <i>Thermoplasma volcanium</i>                          | 0.25       | 1.1                  | 4.4                              |
| <i>Thermoplasma acidophilum</i>                        | 0.32       | 1.5                  | 4.7                              |
| Mutant L200E of <i>Thermoplasma acidophilum</i> enzyme | 0.50       | 2.5                  | 5.0                              |
| <i>Streptococcus mitis</i>                             | 0.20       | $2 \times 10^{-3}$   | 0.01                             |

Assays with MDP decarboxylases from the *P. torridus* phylum as well as *Streptococcus mitis* enzyme gave rise to a reproducible increase in ADP production in the presence of mevalonate. The enzymes from the *P. torridus* phylum displayed higher phosphotransferase activities than the *Streptococcus mitis* enzyme.

### Example 3: Analysis of mevalonate phosphorylation by mass spectrometry

The mevalonate phosphorylation reactions were run under the following conditions:

50 mM Tris HCl pH 7.5

10 mM  $MgCl_2$

20 mM KCl

40 mM ATP

200 mM R,S-sodium Mevalonate

The assays were initiated by adding purified MDP decarboxylase (0.2 mg/ml) and incubated at 37°C. Control reactions were performed in which either no enzyme was added, or no substrate was added. Following incubation assays were processed by

mass spectrometry analysis in negative ion mode. Typically, an aliquot of 80  $\mu$ l reaction was removed, centrifuged and the supernatant was transferred to a clean vial. The product was then extracted with equal volume of ethyl acetate and diluted 1:5 (20%, vol/vol) with methanol. An aliquot of 10  $\mu$ l was directly injected into mass spectrometer. Detection was performed by a PE SCIEX API 2000 quadrupole spectrometer interfaced to an electrospray ionisation (ESI) source. MS analysis showed an  $[M-H]^-$  ion at  $m/z = 227.20$ , corresponding to mevalonate 3-phosphate (3-phosphonoxo-3-methyl-5-hydroxypentanoate), from the complete enzymatic assay (Figure 6a), but not from the control (Figure 6b).

#### **Example 4: Identification of enzyme combinations leading to an increased isoprenol production from mevalonate**

MDP decarboxylases were evaluated using a complementation assay. *Th. acidophilum* MDP decarboxylase (mutant L200E) was incubated together with each tested enzyme from the library.

The combined enzymatic assay was carried out under the following conditions:

50 mM Tris HCl pH 7.5

10 mM  $MgCl_2$

20 mM KCl

40 mM ATP

200 mM R,S-sodium Mevalonate

The pH was adjusted to 7.5

0.01 mg of the *Th. acidophilum* enzyme and 0.5 mg of the MDP decarboxylase to be tested were added to 0.1 ml of reaction mixture. Reaction mixture containing only 0.51 mg of the *Th. acidophilum* MDP decarboxylase (L200E) was used as reference. The assays were incubated without shaking at 37°C for 24 h in a sealed vial (Interchim). The isoprenol production was analyzed as follows. An aliquot of 50  $\mu$ l of liquid phase was removed and mixed with 100  $\mu$ l of ethyl acetate. 100  $\mu$ l of the upper ethyl acetate phase was transferred to a clean vial for analysis by gas chromatography. Commercial isoprenol was used as reference. The samples were analyzed on a Varian GC-430 gas chromatograph equipped with a flame ionization detector (FID). A 1  $\mu$ l sample was separated on the DB-WAX column (30 m, 0,32 x 0,50 $\mu$ m, Agilent) using the following gradient: 60°C for 2 minutes, increasing the temperature at 20°C/minute to a temperature of 220 °C and hold at final temperature

for 10 minutes. The retention time of isoprenol in these conditions was 7.38 min. This screening procedure led to the identification of several archaeal, prokaryotic and eukaryotic MDP decarboxylases increasing the isoprenol production yield in combined assay (Figure 7). The highest production of isoprenol was observed with enzymes from the *Streptococcus* genus, in particular with *S. mitis* MDP decarboxylase, and with the *S. cerevisiae* MDP decarboxylase.

**Example 5: Detailed study of isoprenol production from mevalonate by combining MDP decarboxylase from *Th. acidophilum* and MDP decarboxylase from *S. mitis***

The desired enzymatic reaction was carried out under the following conditions:

50 mM Tris HCl pH 7,5

10 mM MgCl<sub>2</sub>

20 mM KCl

40 mM ATP

200 mM R,S-sodium Mevalonate

0.01 mg of purified MDP decarboxylase from *Th. acidophilum* (mutant L200E) and 0.2 mg of purified MDP decarboxylase from *S. mitis* were added to 0.1 ml of reaction mixture. Control reactions were performed in which either no enzyme was added, or no ATP was added.

To validate the combined action of two enzymes, a series of additional controls were carried out. In one assay, MDP decarboxylase from *S. mitis* (0.21 mg) was the only enzyme using as the catalyst. In the other experiment, 0.21 mg of the *Th. acidophilum* (L200E) enzyme was added, lacking *S. mitis* decarboxylase. The assays were incubated in a sealed vial (Interchim) without shaking for 24 hours at 37°C. Isoprenol extraction and analysis were performed according to the procedure described in Example 4.

The highest production of isoprenol was observed in the reaction mixture contained decarboxylase from *S. mitis* and decarboxylase from *Th. acidophilum* (Figure 8). This indicated that the two enzymes present in the assay were performing complementarily the two steps of reaction producing isoprenol from mevalonate: transfer of the terminal phosphoryl group from ATP to the C3-oxygen of mevalonate followed by combined dephosphorylation-decarboxylation of the intermediate mevalonate 3-phosphate.

Gas chromatography-mass spectrometry (GC/MS) was then used to confirm the identity of the product detected by gas chromatography with flame ionization. The samples were analyzed on a Varian 3400Cx gas chromatograph equipped with Varian Saturn 3 mass selective detector. A mass spectrum of isoprenol obtained by enzymatic conversion of mevalonate was similar to this of commercial isoprenol (Figures 9a and 9b).

#### **Example 6: Effect of enzyme concentration on isoprenol production yield**

The effect of *S. mitis* MDP decarboxylase concentration was assessed under the following conditions:

50 mM Tris HCl pH 7,5

10 mM MgCl<sub>2</sub>

20 mM KCl

40 mM ATP

200 mM R,S-Mevalonate

0.01 mg of purified MDP decarboxylase from *Th. acidophilum* (mutant L200E) and a varying amount (from 0 to 0.4 mg) of purified MDP decarboxylase from *S. mitis* were added to 0.1 ml of reaction mixture. The mixtures were then incubated without shaking at 37°C for 24h in a sealed vial. Isoprenol extraction and analysis were performed according to the procedure described in Example 4. Increasing the *S. mitis* enzyme concentration resulted in an increase of the amount of produced isoprenol (Figure 10).

#### **Example 7: Detailed study of isoprenol production from mevalonate by combining MDP decarboxylase from *Th. acidophilum* and MDP decarboxylase from *S. tokodaii***

The studied reaction was carried out under the following conditions:

50 mM Tris-HCl pH 7.5

10 mM MgCl<sub>2</sub>

20 mM KCl

40 mM ATP

200 mM R,S -sodium mevalonate

0.01 mg of purified MDP decarboxylase from *Th. acidophilum* (L200E) and 0.4 mg

MDP of decarboxylase from *S. tokodaii* were added to 0.1 ml of reaction mixture. A series of controls were performed in parallel under the same conditions. In one assay with MDP decarboxylase from *S. tokodaii* (0.41 mg) alone, containing no enzyme from *Th. acidophilum* was performed. In the other experiment, 0.41 mg of the *Th. acidophilum* (L200E) enzyme was added to the reaction mixture, lacking *S. tokodaii* decarboxylase.

The assays were incubated in sealed vials (Interchim) for 24 hours at 37°C. Isoprenol extraction was performed according to the procedure described in Example 4. Commercial isoprenol was used as reference.

Isoprenol production was then analyzed by gas-chromatography using Bruker 430-GC gas chromatograph equipped with flame ionization detector (FID) according to the following procedure:

5 µl of sample was separated on DB-WAX column (30 m, 0.32 mm x 0.50 µm, Agilent Technologies) using the gradient described in Example 4.

The highest production of isoprenol was observed in the reaction mixture contained MDP decarboxylase *S.tokodaii* and MDP decarboxylase *Th.acidophilum* (Figure 11). This indicates that the combination of two enzymes significantly increases isoprenol yield.

**Example 8: Detailed study of isoprenol production from mevalonate by combining MDP decarboxylase from *Th. acidophilum* and MDP decarboxylase from *D. discoideum***

The studied assay was carried out according the protocol described in Example 7.

0.01 mg of purified MDP decarboxylase from *Th. acidophilum* (L200E) and 0.4 mg MDP of decarboxylase from *D. discoideum* were added to 0.1 ml of reaction mixture. A series of control were performed in parallel under the same conditions. Assay with MDP decarboxylase from *D. discoideum* (0.41 mg) alone, containing no enzyme from *Th. acidophilum* was performed. In the other experiment, 0.41 mg of the *Th. acidophilum* (L200E) enzyme was added to the reaction mixture, lacking *D. discoideum* decarboxylase.

Isoprenol production was analyzed as described in Example 7.

The highest production of isoprenol was observed in the reaction mixture contained

MDP decarboxylase *D. discoideum* and MDP decarboxylase *Th. acidophilum* (Figure 12). Thus, higher isoprenol yield can be achieved by combining action of two enzymes on mevalonate.

## Claims

1. A method for producing isoprenol, characterized in that it comprises the conversion of mevalonate into isoprenol by
  - (i) a first enzyme having an activity of converting mevalonate into mevalonate 3-phosphate; and
  - (ii) a second enzyme being different from the first enzyme and having an activity of converting said mevalonate 3-phosphate into isoprenol.
2. The method of claim 1 wherein
  - (i) the first enzyme having an activity of converting mevalonate into mevalonate 3-phosphate is selected from the group consisting of
    - (A) a protein comprising the amino acid sequence as shown in SEQ ID NO: 1 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 1 and showing an activity of converting mevalonate into mevalonate 3-phosphate which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 1;
    - (B) a protein comprising the amino acid sequence as shown in SEQ ID NO: 2 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 2 and showing an activity of converting mevalonate into mevalonate 3-phosphate which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 2;
    - (C) a protein comprising the amino acid sequence as shown in SEQ ID NO: 3 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ

ID NO: 3 and showing an activity of converting mevalonate into mevalonate 3-phosphate which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 3;

- (D) a protein comprising the amino acid sequence as shown in SEQ ID NO: 4 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 4 and showing an activity of converting mevalonate into mevalonate 3-phosphate which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 4; and
- (E) a protein comprising the amino acid sequence as shown in SEQ ID NO: 16 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 16 and showing an activity of converting mevalonate into mevalonate 3-phosphate which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 16.

3. The method of claim 1 or 2, wherein

- (ii) the second enzyme having an activity of converting mevalonate 3-phosphate into isoprenol selected from the group consisting of
  - (a) a protein comprising the amino acid sequence as shown in SEQ ID NO: 5 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 5 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 5;
  - (b) a protein comprising the amino acid sequence as shown in SEQ ID NO: 6 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 6 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the

- corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 6;
- (c) a protein comprising the amino acid sequence as shown in SEQ ID NO: 7 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 7 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 7;
  - (d) a protein comprising the amino acid sequence as shown in SEQ ID NO: 8 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 8 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 8;
  - (e) a protein comprising the amino acid sequence as shown in SEQ ID NO: 9 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 9 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 9
  - (f) a protein comprising the amino acid sequence as shown in SEQ ID NO: 10 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 10 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 10;
  - (g) a protein comprising the amino acid sequence as shown in SEQ ID NO: 11 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 11 and showing an activity of converting mevalonate

- 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 11;
- (h) a protein comprising the amino acid sequence as shown in SEQ ID NO: 12 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 12 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 12;
  - (i) a protein comprising the amino acid sequence as shown in SEQ ID NO: 13 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 13 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 13;
  - (j) a protein comprising the amino acid sequence as shown in SEQ ID NO: 14 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 14 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 14;
  - (k) a protein comprising the amino acid sequence as shown in SEQ ID NO: 15 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 15 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 15;
  - (l) a protein comprising the amino acid sequence as shown in SEQ ID NO: 17 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in

SEQ ID NO: 17 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 17;

- (m) a protein comprising the amino acid sequence as shown in SEQ ID NO: 18 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 18 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 18;
- (n) a protein comprising the amino acid sequence as shown in SEQ ID NO: 19 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 19 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 19;
- (o) a protein comprising the amino acid sequence as shown in SEQ ID NO: 20 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 20 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 20;
- (p) a protein comprising the amino acid sequence as shown in SEQ ID NO: 21 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 21 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 21; and
- (q) a protein comprising the amino acid sequence as shown in SEQ ID NO: 22 or a protein comprising an amino acid sequence which

is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 22 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 22.

4. The method of any one of claims 1 to 3 characterized in that the conversion step is carried out *in vitro*, in a cell-free system.
5. The method of any one of claims 1 to 3 characterized in that the method is carried out in the presence of a microorganism producing said enzymes as defined in (i) and (ii) of claim 1.
6. The method of claim 4 characterized by the use of a multicellular organism producing said enzymes as defined in (i) and (ii) of claim 1.
7. A composition comprising
  - (a) mevalonate; and
  - (b) at least two enzymes, wherein one enzyme is selected from (i) and the other enzyme is selected from (ii), wherein (i) and (ii) are as follows:
    - (i) a first enzyme having an activity of converting mevalonate into mevalonate 3-phosphate; and
    - (ii) a second enzyme being different from the first enzyme and having an activity of converting said mevalonate 3-phosphate into isoprenol.
8. A composition comprising
  - (a) mevalonate; and
  - (b) a multicellular organism or a microorganism which produces at least two enzymes, wherein one enzyme is selected from (i) and the other enzyme is selected from (ii), wherein (i) and (ii) are as follows:
    - (i) a first enzyme having an activity of converting mevalonate into mevalonate 3-phosphate; and
    - (ii) a second enzyme being different from the first enzyme and

having an activity of converting said mevalonate 3-phosphate into isoprenol.

9. Use of a combination of at least two enzymes, wherein one enzyme is selected from the following (i) and the other enzyme is selected from the following (ii) or of a composition of claim 7 or 8 or of a microorganism as defined in claim 8, for producing isoprenol from mevalonate, wherein (i) and (ii) are as follows:
- (i) a first enzyme having an activity of converting mevalonate into mevalonate 3-phosphate; and
  - (ii) a second enzyme being different from the first enzyme and having an activity of converting said mevalonate 3-phosphate into isoprenol.
10. The composition of claim 7 or 8 or the use of claim 9, wherein
- (i) the first enzyme having an activity of converting mevalonate into mevalonate 3-phosphate is selected from the group consisting of
    - (A) a protein comprising the amino acid sequence as shown in SEQ ID NO: 1 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 1 and showing an activity of converting mevalonate into mevalonate 3-phosphate which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 1;
    - (B) a protein comprising the amino acid sequence as shown in SEQ ID NO: 2 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 2 and showing an activity of converting mevalonate into mevalonate 3-phosphate which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 2;
    - (C) a protein comprising the amino acid sequence as shown in SEQ ID NO: 3 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 3 and showing an activity of converting mevalonate into

mevalonate 3-phosphate which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 3;

- (D) a protein comprising the amino acid sequence as shown in SEQ ID NO: 4 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 4 and showing an activity of converting mevalonate into mevalonate 3-phosphate which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 4; and
- (E) a protein comprising the amino acid sequence as shown in SEQ ID NO: 16 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 16 and showing an activity of converting mevalonate into mevalonate 3-phosphate which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 16.

- 11. The composition of claim 7, 8 or 10 or the use of claim 9 or 10, wherein
  - (ii) the second enzyme having an activity of converting mevalonate 3-phosphate into isoprenol selected from the group consisting of
    - (a) a protein comprising the amino acid sequence as shown in SEQ ID NO: 5 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 5 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 5;
    - (b) a protein comprising the amino acid sequence as shown in SEQ ID NO: 6 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 6 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the

- corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 6;
- (c) a protein comprising the amino acid sequence as shown in SEQ ID NO: 7 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 7 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 7;
  - (d) a protein comprising the amino acid sequence as shown in SEQ ID NO: 8 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 8 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 8;
  - (e) a protein comprising the amino acid sequence as shown in SEQ ID NO: 9 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 9 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 9
  - (f) a protein comprising the amino acid sequence as shown in SEQ ID NO: 10 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 10 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 10;
  - (g) a protein comprising the amino acid sequence as shown in SEQ ID NO: 11 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 11 and showing an activity of converting mevalonate

- 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 11;
- (h) a protein comprising the amino acid sequence as shown in SEQ ID NO: 12 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 12 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 12;
  - (i) a protein comprising the amino acid sequence as shown in SEQ ID NO: 13 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 13 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 13;
  - (j) a protein comprising the amino acid sequence as shown in SEQ ID NO: 14 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 14 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 14;
  - (k) a protein comprising the amino acid sequence as shown in SEQ ID NO: 15 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 15 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 15;
  - (l) a protein comprising the amino acid sequence as shown in SEQ ID NO: 17 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in

- SEQ ID NO: 17 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 17;
- (m) a protein comprising the amino acid sequence as shown in SEQ ID NO: 18 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 18 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 18;
  - (n) a protein comprising the amino acid sequence as shown in SEQ ID NO: 19 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 19 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 19;
  - (o) a protein comprising the amino acid sequence as shown in SEQ ID NO: 20 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 20 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 20;
  - (p) a protein comprising the amino acid sequence as shown in SEQ ID NO: 21 or a protein comprising an amino acid sequence which is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 21 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 21; and
  - (q) a protein comprising the amino acid sequence as shown in SEQ ID NO: 22 or a protein comprising an amino acid sequence which

is at least 15% identical to the amino acid sequence shown in SEQ ID NO: 22 and showing an activity of converting mevalonate 3-phosphate into isoprenol which is at least as high as the corresponding activity of the protein having the amino acid sequence shown in SEQ ID NO: 22.

12. A method for producing isprenol comprising the step of enzymatically converting mevalonate 3-phosphate into isoprenol by use of an enzyme which can catalyze the conversion via decarboxylation and dephosphorylation.
13. The method of any one of claims 1 to 6 or the method of claim 12, characterized in that the method is carried out with ATP, dATP, ADP, AMP, an NTP other than ATP or a dNTP as co-substrate.
14. A method for producing isoprene from mevalonate comprising the method of any one of claims 1 to 6, 12 or 13 and further comprising the step of converting the produced isoprenol into isoprene.
15. A method for producing isoamyl alcohol from mevalonate comprising the method of any one of claims 1 to 6, 12 or 13 and further comprising the step of converting the produced isoprenol into isoamyl alcohol.