3-buten-2-one can be further converted into 3-buten-2-ol and finally into 1,3-butadiene.

4-hydroxy-2-butanone can be dehydrated to make 3-buten-2-one, which can be converted into 3-buten-2-ol and finally into 1,3-butadiene.
Method for the enzymatic production of 3-buten-2-one

The present invention relates to a method for the production of 3-buten-2-one comprising the enzymatic conversion of 4-hydroxy-2-butanoate into 3-buten-2-one by making use of an enzyme catalyzing 4-hydroxy-2-butanoate dehydration, wherein said enzyme catalyzing 4-hydroxy-2-butanoate dehydration is (a) a 3-hydroxypropiony-CoA dehydratase (EC 4.2.1.116), (b) a 3-hydroxybutyryl-CoA dehydratase (EC 4.2.1.55), (c) an enoyl-CoA hydratase (EC 4.2.1.17), (d) a 3-hydroxyoctanoyl-[acyl-carrier-protein] dehydratase (EC 4.2.1.59), (e) a crotonyl-[acyl-carrier-protein] hydratase (EC 4.2.1.58), (f) a 3-hydroxydecanoyl-[acyl-carrier-protein] dehydratase (EC 4.2.1.60), (g) a 3-hydroxypalmitoyl-[acyl-carrier-protein] dehydratase (EC 4.2.1.74), or (i) a 3-methylglutaconyl-CoA hydratase (EC 4.2.1.18). The produced 3-buten-2-one can, e.g., be further converted in 3-buten-2-ol and finally into 1,3-butadiene.

Butadiene (1,3-butadiene) is a conjugated diene with the formula C₄H₆. It is an important industrial chemical used as a monomer in the production of synthetic rubber. There exist different possibilities to produce butadiene. Butadiene is, for example, produced as a by-product of the steam cracking process used to produce ethylene and other olefins. In this process butadiene occurs in the C₄ stream and is normally isolated from other by-products by extraction into a polar aprotic solvent, such as acetonitrile, from which it is then stripped. Butadiene can also be produced by the catalytic dehydrogenation of normal butane or it can be produced from ethanol. In the latter case, two different processes are in use. In a single-step process, ethanol is converted to butadiene, hydrogen and water at 400-450°C over a metal oxide catalyst (Kirshenbaum, I. (1978), Butadiene. In M. Grayson (Ed.), Encyclopedia of Chemical Technology, 3rd ed., Vol. 4, pp. 313-337. New York: John Wiley & Sons). In a two-step process, ethanol is oxidized to acetaldehyde which reacts with additional ethanol over a tantalum-promoted porous silica catalyst at 325-
350°C to yield butadiene (Kirshenbaum, I. (1978), loc cit). Butadiene can also be produced by catalytic dehydrogenation of normal butenes.

For the past two decades, genetic engineering technologies have made possible the modification of the metabolism of microorganisms, and hence their use to produce key substances which they would otherwise produce at a low yield. By enhancing naturally occurring metabolic pathways, these technologies open up new ways to bioproduce numerous compounds of industrial relevance. Several industrial compounds such as amino acids for animal feed, biodegradable plastics or textile fibres are now routinely produced using genetically modified organisms. There are presently attempts to provide also bio-processes using genetically modified microorganisms for the large-scale production of the major petrochemically derived molecules, in particular butadiene, since no microorganisms are known as natural producers of butadiene even in small quantities. Given the large amounts of rubber produced worldwide and the increasing environmental concerns and the limited resources for producing 1,3-butadiene using chemical processes, there is a need to provide alternative, environmentally-friendly and sustainable processes for the production of 1,3-butadiene and its precursor molecules acetoacetyl-CoA, 4-hydroxy-2-butanone, 3-buten-2-ol and, in particular, 3-buten-2-one. WO 2012/174439 describes methods for producing butadiene from butanols by enzymes which are able to catalyze the conversion of butanols to butadiene. Moreover, WO 2010/144746 describes non-naturally occurring microbial organism having an exogenous nucleic acid encoding a 3-oxobutanol dehydratase which is capable of dehydrating 1,3-oxobutanol to form butanone. However, given the importance of being able to produce compounds such as 1,3-butadiene by microorganisms, there is still a need to identify alternative routes or mechanisms for producing such compounds from renewable resources.

The present invention addresses this need and provides a process by which 3-buten-2-one (also referred to as methyl vinyl ketone) can be produced enzymatically starting from 4-hydroxy-2-butanone by employing certain enzymes. 3-buten-2-one can then further be converted enzymatically to 3-buten-2-ol which can then be further enzymatically converted to 1,3-butadiene. 4-hydroxy-2-butanone can be produced enzymatically from acetoacetyl-CoA while acetoacetyl-CoA can be provided starting from the metabolic intermediate acetyl-Coenzyme A (in the following also referred to
as acetyl-CoA) as described herein. The corresponding reactions are schematically shown in Figure 1.

Thus, the present invention relates to a method for the production of 3-buten-2-one comprising the enzymatic conversion of 4-hydroxy-2-butanone into 3-buten-2-one by making use of an enzyme catalyzing 4-hydroxy-2-butanone dehydration. Enzymes catalyzing 4-hydroxy-2-butanone dehydration which can be employed in this reaction are the following enzymes which are all classified as E.C. 4.2.1._ (i.e., hydro-lyases).

Enzymes catalyzing 4-hydroxy-2-butanone dehydration are enzymes which catalyze the following reaction:

$$\text{4-hydroxy-2-butanone} \leftrightarrow \text{3-buten-2-one (methyl vinyl ketone)} + \text{H}_2\text{O}$$

The term "dehydration" is generally referred to a reaction involving the removal of \text{H}_2\text{O}.

According to the present invention an enzyme catalyzing 4-hydroxy-2-butanone dehydration is selected from the group consisting of the following enzymes (a) to (i):

(a) a 3-hydroxypropionyl-CoA dehydratase (EC 4.2.1.16),
(b) a 3-hydroxybutyryl-CoA dehydratase (EC 4.2.1.55),
(c) an enoyl-CoA hydratase (EC 4.2.1.17),
(d) a 3-hydroxyoctanoyl-[acyl-carrier-protein] dehydratase (EC 4.2.1.59),
(e) a crotonyl-[acyl-carrier-protein] hydratase (EC 4.2.1.58),
(f) a 3-hydroxydecanoyl-[acyl-carrier-protein] dehydratase (EC 4.2.1.60),
(g) a 3-hydroxypalmitoyl-[acyl-carrier-protein] dehydratase (EC 4.2.1.61),
(h) a long-chain-enoyl-CoA hydratase (EC 4.2.1.74), and
(i) a 3-methylglutaconyl-CoA hydratase (EC 4.2.1.18).
All these enzymes which are capable of catalyzing 4-hydroxy-2-butanone dehydration have in common that they use a natural substrate having the following minimal structural motif:

\[
\begin{align*}
\text{OH} & \\
R^1 & \\
\text{O} & \\
R^2 & \\
\text{S} & \\
R^3 & 
\end{align*}
\]

wherein

- R\(^1\) is a hydrogen atom or an alkyl group or CH\(_2\)COO\(^-\);
- R\(^2\) is a hydrogen atom or a methyl group; and
- R\(^3\) is coenzyme A or acyl-carrier protein.

Thus, the above mentioned enzymes which can catalyze the dehydration of 4-hydroxy-2-butanone can be divided into two groups as follows:

I. R\(^3\) in the above shown formula is acyl-carrier protein
   
   This group includes EC 4.2.1.58, EC 4.2.1.59, EC 4.2.1.60 and EC 4.2.1.61. The enzymes of this group have in common that they catalyze a reaction of the following type:

   \[
   \text{3-hydroxyacyl-[acyl-carrier protein]} \rightleftharpoons \text{2-enoyl-[acyl-carrier protein]} + \text{H}_2\text{O}
   \]

   The enzymes of this group share a common structural motif which is referenced in the InterPro as InterPro IPR013114 (http://www.ebi.ac.uk/interpro/entry/IPR013114). The accession number for these enzymes in the Pfam database is PF 07977 (http://pfam.sanger.ac.uk/family/PF079771).

II. R\(^3\) in the above shown formula is coenzyme A

   This group includes EC 4.2.1.116, EC 4.2.1.55, EC 4.2.1.17, EC 4.2.1.74 and EC 4.2.1.18

   The enzymes of this group share a common structural motif which is referenced in the InterPRO database as InterPro IPR001753 (http://www.ebi.ac.uk/interpro/entry/IPR001753) and IPR0018376.
In one embodiment of the method according to the invention the conversion of 4-hydroxy-2-butanone into 3-buten-2-one is achieved by the use of a 3-hydroxypropionyl-CoA dehydratase (EC 4.2.1.16). 3-hydroxypropionyl-CoA dehydratases (EC 4.2.1.16) catalyze the following reaction:

\[
3\text{-hydroxypropionyl-CoA} \rightleftharpoons \text{acryloyl-CoA} + \text{H}_2\text{O}
\]

The enzyme is known from various bacteria and archae. Thus, in a preferred embodiment of the invention a bacterial 3-hydroxypropionyl-CoA dehydratase (EC 4.2.1.16) is used, preferably a 3-hydroxypropionyl-CoA dehydratase from a bacterium or an archaeabacterium of a genus selected from the group consisting of Metallosphaera, Sulfolobus and Brevibacillus and most preferably from a species selected from the group consisting of Metallosphaera cuprina, Metallosphaera sedula, Sulfolobus tokodaii and Brevibacillus laterosporus. Examples for such bacterial 3-hydroxypropionyl-CoA dehydratases are the enzymes from Metallosphaera cuprina (Uniprot F4FZ85), Metallosphaera sedula (Uniprot A4YI89, Teufel et al., J. Bacteriol. 191 (2009), 4572-4581), Sulfolobus tokodaii (Uniprot F9VNG3) and Brevibacillus laterosporus (Uniprot F7TTZ1). Amino acid and nucleotide sequences for these enzymes are available. Examples for corresponding amino acid sequences are provided in SEQ ID NOs: 1 to 4 wherein SEQ ID NO:1 is the amino acid sequence of 3-hydroxypropionyl-CoA dehydratase of M. cuprina, SEQ ID NO:2 is the amino acid sequence of 3-hydroxypropionyl-CoA dehydratase of M. sedula, SEQ ID NO:3 is the amino acid sequence of a 3-hydroxypropionyl-CoA dehydratase of S. tokodaii and SEQ ID NO:4 is the amino acid sequence of a 3-hydroxypropionyl-CoA dehydratase of Brevibacillus laterosporus.
In a preferred embodiment, the 3-hydroxypropionyl-CoA dehydratase employed in the method of the invention has an amino acid sequence as shown in any one of SEQ ID NOs: 1 to 4 or shows an amino acid sequence which is at least \( x\% \) homologous to any of SEQ ID NOs: 1 to 4 and has the activity of catalyzing the conversion of 4-hydroxy-2-butanone into 3-buten-2-one, with \( x \) being an integer between 30 and 100, preferably 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99.

As shown in the appended Examples, it was found that different tested 3-hydroxypropionyl-CoA dehydratases from different organisms are capable of using 4-hydroxy-2-butanone as a substrate and converting it into 3-buten-2-one. Thus, in principle any 3-hydroxypropionyl-CoA dehydratase can be employed in the method according to the invention. However, it is not only possible to employ in the method of the invention a 3-hydroxypropionyl-CoA dehydratase for converting 4-hydroxy-2-butanone into 3-buten-2-one but also enzymes which show the structural and functional similarities as described above, i.e. enzymes as listed in items (b) to (f), above.

Thus, in another embodiment of the method according to the invention the conversion of 4-hydroxy-2-butanone into 3-buten-2-one is achieved by the use of a 3-hydroxybutyryl-CoA dehydratase (EC 4.2.1.55). 3-hydroxybutyryl-CoA dehydratases (EC 4.2.1.55) catalyze the following reaction:

\[
\text{3-hydroxybutyryl-CoA} \rightleftharpoons \text{crotonyl-CoA} + \text{H}_2\text{O}
\]

\[
\text{OH} \quad \text{O} \quad \text{S-CoA} \rightleftharpoons \text{O} \quad \text{S-CoA}
\]

This reaction corresponds to a Michael elimination. 3-hydroxybutyryl-CoA dehydratase belongs to the family of lyases, specifically the hydro-lyases, which cleave carbon-oxygen bonds. The systematic name of this enzyme class is (3R)-3-hydroxybutanoyl-CoA hydro-lyase (crotonoyl-CoA-forming). Other names in common use include D-3-hydroxybutyryl coenzyme A dehydratase, D-3-hydroxybutyryl-CoA dehydratase, enoyl coenzyme A hydratase, and (3R)-3-hydroxybutanoyl-CoA hydro-
lyase. This enzyme participates in the butanoate metabolism. Enzymes belonging to
this class and catalyzing the above shown conversion of 3-hydroxybutyryl-Coenzyme
A into crotonyl-Coenzyme A have been described to occur, e.g. in rat (Rattus
norvegicus), in Rhodospirillum rubrum, in Sulfolobus acidocaldarius and in Acidianus
hospitalis. Nucleotide and/or amino acid sequences for such enzymes have been
determined, e.g. for Aeropyrum pernix. In principle, any 3-hydroxybutyryl-CoA
dehydratase (EC 4.2.1 .55) which can catalyze the conversion of 4-hydroxy-2-
butanone into 3-buten-2-one can be used in the context of the present invention. In a
preferred embodiment of the invention a 3-hydroxybutyryl-CoA dehydratase from an
archaebacterium is used, preferably a 3-hydroxybutyryl-CoA dehydratase from an
archaebacterium of a genus selected from the group consisting of Sulfolobus and
Acidianus and most preferably from a species selected from the group consisting of
S. acidocaldarius and Acidianus hospitalis. Examples for such bacterial 3-
hydroxybutyryl-CoA dehydratases are the enzymes from Sulfolobus acidocaldarius
(Uniprot Q4J8D5) and from Acidianus hospitalis ((Uniprot F4B9R3). Examples for
corresponding amino acid sequences are provided in SEQ ID NOs: 5 and 6 wherein
SEQ ID NO:5 is the amino acid sequence of 3-hydroxybutyryl-CoA dehydratase of
Sulfolobus acidocaldarius and SEQ ID NO:6 is the amino acid sequence of 3-
hydroxybutyryl-CoA dehydratase of Acidianus hospitalis.
In a preferred embodiment, the 3-hydroxybutyryl-CoA dehydratase employed in
the method of the invention has an amino acid sequence as shown in SEQ ID NO: 5 or 6
or shows an amino acid sequence which is at least x% homologous to SEQ ID NO: 5
or 6 and has the activity of catalyzing the conversion of 4-hydroxy-2-butanone into 3-
buten-2-one, with x being an integer between 30 and 100, preferably 35, 40, 45, 50,
55, 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99.

In another embodiment of the method according to the invention the conversion of 4-
hydroxy-2-butanone into 3-buten-2-one is achieved by the use of an enoyl-CoA
hydratase (EC 4.2.1 .17). Enoyl-CoA hydratases (EC 4.2.1 .17) catalyze the following
reaction:

\[(3S)-3\text{-hydroxyacyl-CoA} \rightleftharpoons \text{trans-2(or 3)-enoyl-CoA} + \text{H}_2\text{O}\]
Enoyl-CoA hydratase is an enzyme that normally hydrates the double bond between the second and third carbons on acyl-CoA. However, it can also be employed to catalyze the reaction in the reverse direction. This enzyme, also known as crotonase, is naturally involved in metabolizing fatty acids to produce both acetyl-CoA and energy. Enzymes belonging to this class have been described to occur, e.g. in rat (Rattus norvegicus), humans (Homo sapiens), mouse (Mus musculus), wild boar (Sus scrofa), Bos taurus, E.coli, Clostridium acetobutylicum and Clostridium aminobutyricum. Nucleotide and/or amino acid sequences for such enzymes have been determined, e.g. for rat, humans and Bacillus subtilis. In principle, any enoyl-CoA hydratase (EC 4.2.1.17) which can catalyze the conversion of 4-hydroxy-2-butanone into 3-buten-2-one can be used in the context of the present invention.

In another embodiment of the method according to the invention the conversion of 4-hydroxy-2-butanone into 3-buten-2-one is achieved by the use of a 3-hydroxyoctanoyl-[acyl-carrier-protein] dehydratase (EC 4.2.1.59). 3-hydroxyoctanoyl-[acyl-carrier-protein] dehydratases (EC 4.2.1.59) catalyze the following reaction:

\[(3R)-3\text{-hydroxyoctanoyl-[acyl-carrier protein]} \rightleftharpoons \text{oct-2-enoyl-[acyl-carrier protein]} + \text{H}_2\text{O}\]

This enzyme belongs to the family of lyases, specifically the hydro-lyases, which cleave carbon-oxygen bonds. The systematic name of this enzyme class is (3R)-3-hydroxyoctanoyl-[acyl-carrier-protein] hydro-lyase (oct-2-enoyl-[acyl-carrier protein]-forming). Other names in common use include D-3-hydroxyoctanoyl-[acyl carrier protein] dehydratase, D-3-hydroxyoctanoyl-acyl carrier protein dehydratase, beta-hydroxyoctanoyl-acyl carrier protein dehydrase, beta-hydroxyoctanoyl thioester dehydratase, beta-hydroxyoctanoyl-ACP-dehydrase, and (3R)-3-hydroxyoctanoyl-[acyl-carrier-protein] hydro-lyase. 3-hydroxyoctanoyl-[acyl-carrier-protein] dehydratases has been described to exist, e.g., in E. coli (Mizugaki et al., Biochem. Biophys. Res. Commun. 33 (1968), 520-527). In principle, any 3-hydroxyoctanoyl-[acyl-carrier-protein] dehydratase which can catalyze the conversion of 4-hydroxy-2-butanone into 3-buten-2-one can be used in the context of the present invention. In a preferred embodiment the enzyme from E. coli is used in a method according to the present invention.
In another embodiment of the method according to the invention the conversion of 4-hydroxy-2-butanone into 3-buten-2-one is achieved by the use of a crotonoyl-[acyl-carrier-protein] hydratase (EC 4.2.1.58). Crotonoyl-[acyl-carrier-protein] hydratases (EC 4.2.1.58) catalyze the following reaction:

\[
(3R)-3\text{-hydroxybutanoyl-[acyl-carrier-protein]} \rightleftharpoons \text{but-2-enoyl-[acyl-carrier-protein]} + \text{H}_2\text{O}
\]

This enzyme belongs to the family of lyases, specifically the hydro-lyases, which cleave carbon-oxygen bonds. Other names in common use include (3R)-3-hydroxybutanoyl-[acyl-carrier-protein] hydro-lyase, beta-hydroxybutyryl acyl carrier protein dehydratase, beta-hydroxybutyryl acyl carrier protein (ACP) dehydratase, beta-hydroxybutyryl acyl carrier protein dehydratase, enoyl acyl carrier protein hydratase, crotonyl acyl carrier protein hydratase, 3-hydroxybutyryl acyl carrier protein dehydratase, beta-hydroxybutyryl acyl carrier, and protein dehydratase. This enzyme participates in fatty acid biosynthesis. Crotonoyl-[acyl-carrier-protein] hydratase has been described to exist, e.g., in E. coli and Arabidopsis thaliana. In principle, any crotonoyl-[acyl-carrier-protein] hydratase which can catalyze the conversion of 4-hydroxy-2-butanone into 3-buten-2-one can be used in the context of the present invention. In a preferred embodiment the enzyme from E. coli is used in a method according to the present invention.

In another embodiment of the method according to the invention the conversion of 4-hydroxy-2-butanone into 3-buten-2-one is achieved by the use of a 3-hydroxydecanoyl-[acyl-carrier-protein] dehydratase (EC 4.2.1.60). 3-hydroxydecanoyl-[acyl-carrier-protein] dehydratases (EC 4.2.1.60) catalyze the following reactions:

1. \[
(3R)-3\text{-hydroxydecanoyl-[acyl-carrier protein]} \rightleftharpoons \text{a trans-dec-2-enoyl-[acyl-carrier protein]} + \text{H}_2\text{O}
\]
The enzyme has been described to exist, e.g., in Pseudomonas aeruginosa, Pseudomonas fluorescens, Toxoplasma gondii, Plasmodium falciparum, Helicobacter pylori, Corynebacterium ammoniagenes, Enterobacter aerogenes, E. coli, Proteus vulgaris and Salmonella enterica. In principle, any 3-hydroxydecanoyl-[acyl-carrier-protein] dehydratase which can catalyze the conversion of 4-hydroxy-2-butanone into 3-buten-2-one can be used in the context of the present invention. In a preferred embodiment the enzyme from E. coli is used in a method according to the present invention.

In another embodiment of the method according to the invention the conversion of 4-hydroxy-2-butanone into 3-buten-2-one is achieved by the use of a 3-hydroxypalmitoyl-[acyl-carrier-protein] dehydratase (EC 4.2.1.61). 3-hydroxypalmitoyl-[acyl-carrier-protein] dehydratases (EC 4.2.1.61) catalyze the following reaction:

\[
(3R)-3\text{-hydroxypalmitoyl-[acyl-carrier-protein]} \overset{\text{EC 4.2.1.61}}{\longrightarrow} \text{hexadec-2-enoyl-[acyl-carrier-protein]} + \text{H}_2\text{O}
\]

This enzyme belongs to the family of lyases, specifically the hydro-lyases, which cleave carbon-oxygen bonds. Other names in common use include D-3-hydroxypalmitoyl-[acyl-carrier-protein] dehydratase, beta-hydroxypalmitoyl-acyl carrier protein dehydratase, beta-hydroxypalmitoyl thioester dehydratase, beta-hydroxypalmitoyl-ACP dehydratase, and (3R)-3-hydroxypalmitoyl-[acyl-carrier-protein] hydro-lyase. 3-hydroxypalmitoyl-[acyl-carrier-protein] dehydratase has been described to exist, e.g., in Candida albicans, Yarrowia lipolytica, S. cerevisiae, s. pombe, Cochliobolus carbonum, Mus musculus, Rattus norvegicus, Bos taurus, Gallus gallus and Homo sapiens. In principle, any 3-hydroxypalmitoyl-[acyl-carrier-protein] dehydratase which can catalyze the conversion of 4-hydroxy-2-butanone into 3-buten-2-one can be used in the context of the present invention.
In another embodiment of the method according to the invention the conversion of 4-hydroxy-2-butanone into 3-buten-2-one is achieved by the use of a long-chain-enoyl-CoA hydratase (EC 4.2.1.74). Long-chain-enoyl-CoA hydratases (EC 4.2.1.74) catalyze the following reaction:

\[(3S)-3\text{-hydroxyacyl-CoA} \rightleftharpoons \text{trans-2-enoyl-CoA} + \text{H}_2\text{O}\]

This enzyme belongs to the family of lyases, specifically the hydro-lyases, which cleave carbon-oxygen bonds. The systematic name of this enzyme class is long-chain-(3S)-3-hydroxyacyl-CoA hydro-lyase. This enzyme is also called long-chain enoyl coenzyme A hydratase and it participates in fatty acid elongation in mitochondria and fatty acid metabolism. This enzyme occurs in a number of organisms, e.g., in Rattus norvegicus (Wu et al., Org. Lett. 10 (2008), 2235-2238), Sus scrofa and Cavia porcellus (Fong and Schulz, J. Biol. Chem. 252 (1977), 542-547; Schulz, Biol. Chem. 249 (1974), 2704-2709) and in principle any long-chain-enoyl-CoA hydratase which can catalyze the conversion of 4-hydroxy-2-butanone into 3-buten-2-one can be employed in the method of the invention.

In another embodiment of the method according to the invention the conversion of 4-hydroxy-2-butanone into 3-buten-2-one is achieved by the use of a 3-methylglutaconyl-CoA hydratase (EC 4.2.1.18). 3-methylglutaconyl-CoA hydratases (EC 4.2.1.18) catalyze the following reaction:

\[(S)-3\text{-hydroxymethyl glutaryl-CoA} \rightleftharpoons \text{trans-3-methylglutaconyl-CoA} + \text{H}_2\text{O}\]

This enzyme occurs in a number of organisms in particular in bacteria, plants and animals. The enzyme has been described, e.g., for Pseudomonas putida, Acinetobacter sp. (SwissProt accession number Q3HW12), Catharanthus roseus, Homo sapiens (SwissProt accession number Q13825), Bos taurus and Ovis aries and in principle any 3-methylglutaconyl-CoA hydratase which can catalyze the
conversion of 4-hydroxy-2-butanone into 3-buten-2-one can be employed in the method of the invention. The term "3-methylglutaconyl-CoA hydratase" also covers the enzyme encoded by the gene LiuC (Li et al., Angew. Chem. Int. Ed. 52 (2013), p. 1304-1308; Uniprot number Q1D5Y4) from Myxococcus xanthus, preferably from strain DK 1622. The amino acid sequence of this enzyme is shown in SEQ ID NO:25. Although this gene was annotated as a 3-hydroxybutyryl-CoA dehydratase, Li et al. (loc. cit.) showed that its natural substrate is 3-hydroxymethylglutaryl-CoA. In a particularly preferred embodiment any protein can be employed in a method according to the present invention which comprises an amino acid as shown in SEQ ID NO:25 or an amino acid sequence which is at least x% homologous SEQ ID NO: 25 and which has the activity of a 3-methylglutaconyl-CoA hydratase/3-hydromethylglutaryl-CoA dehydratase and which shows the activity of converting 4-hydroxy-2-butanone into 3-buten-2-one, with x being an integer between 30 and 100, preferably 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99.

As described above, the 4-hydroxy-2-butanone to be converted into 3-buten-2-one can be provided by the enzymatic conversion of acetoacetyl-CoA into 4-hydroxy-2-butanone. Thus, the present invention also relates to a method for the production of 3-buten-2-one comprising the enzymatic conversion of 4-hydroxy-2-butanone into 3-buten-2-one by making use of an enzyme catalyzing 4-hydroxy-2-butanone dehydration as described above wherein said method further comprises the enzymatic conversion of acetoacetyl-CoA into said 4-hydroxy-2-butanone by making use of an acetoacetyl-CoA reductase.

The enzymatic conversion of acetoacetyl-CoA into 4-hydroxy-2-butanone by making use of an acetoacetyl-CoA reductase may be accomplished by a two step conversion which comprises (1) the enzymatic conversion of acetoacetyl-CoA into acetoacetalddehyde and (2) the subsequent enzymatic conversion of acetoacetalddehyde into 4-hydroxy-2-butanone.

The first step may occur according to the following scheme:

\[ \text{Acetoacetyl-CoA} + \text{NADH} + \text{H}^+ \rightarrow \text{Acetoacetalddehyde} + \text{NAD}^+ \]

or
Acetoacetyl-CoA + NADPH + $H^+$ $\rightarrow$ Acetoacetaldehyde + NADP$^+$

The second step may occur according to the following scheme:

Acetoacetaldehyde + NADH + $H^+$ $\rightarrow$ 4-Hydroxy-2-butanone + NAD$^+$

or

Acetoacetaldehyde + NADPH + $H^+$ $\rightarrow$ 4-Hydroxy-2-butanone + NADP$^+$

In principle any enzyme which can catalyze such a conversion can be employed. Examples for corresponding enzymes are hydroxymethylglutaryl-CoA reductase (EC 1.1.1.34) or short-chain dehydrogenases/fatty acyl-CoA reductase.

Thus, in one embodiment of the method according to the invention the conversion of acetoacetyl-CoA into 4-hydroxy-2-butanone is achieved by employing a hydroxymethylglutaryl-CoA reductase (EC 1.1.1.34). This enzyme normally catalyzes the following reaction:

$$(S)$-3-hydroxy-methylglutaryl-CoA+2NADPH+H^+ $\rightarrow$ (R)-mevalonate + CoA + 2 NADP^+

Enzymes belonging to this class and catalyzing the above shown conversion occur in organisms of all kingdoms, i.e. plants, animals, fungi, bacteria etc. and have extensively been described in the literature. Nucleotide and/or amino acid sequences for such enzymes have been determined for numerous organisms, in particular bacterial organisms. In principle, any hydroxymethylglutaryl-CoA reductase (EC 1.1.1.34) which can catalyze the conversion of acetoacetyl-CoA into 4-hydroxy-2-butanone can be used in the context of the present invention.

Alternatively or in addition, the above described conversion of acetoacetyl-CoA into 4-hydroxy-2-butanone can also be achieved by using an enzyme referred to as a short-chain dehydrogenases/fatty acyl-CoA reductase. The term "short-chain dehydrogenase/fatty acyl-CoA reductase" or "short-chain dehydrogenases/reductase (SDR)" in the context of the present invention refers to enzymes which are characterized by the following features:
1. They catalyze a two-step reaction in which fatty acyl-CoA is reduced to fatty alcohol.

2. They show a substrate specificity for acyl-CoA containing an aliphatic chain from 8 to 20 carbon atoms.

Preferably such enzymes are furthermore characterized by the feature that they show a specific motif in their primary structure, i.e. amino acid sequence, namely they show two specific glycine motifs for NADP(H) binding.

The short-chain dehydrogenase/fatty acyl-CoA reductase or short-chain dehydrogenases/reductases (SDR) enzymes constitute a family of enzymes, most of which are known to be NAD- or NADP-dependent oxidoreductase (Jornvall H. et al., Biochemistry 34 (1995), 6003-6013). Recently, a novel bacterial NADP-dependent reductase from Marinobacter aquaeolei VT8 was characterized (Willis et al., Biochemistry 50 (2011), 10550-10558). This enzyme catalyzes the four-electron reduction of fatty acyl-CoA substrates to the corresponding fatty alcohols.

The enzymatic conversion of fatty acyl-CoA into fatty alcohol occurs through an aldehyde intermediate according to the following scheme:

```
R-CO-S-CoA  \( \rightarrow \)  R-CO-H  \( \rightarrow \)  R-CH\textsubscript{2}OH
```

The enzyme displays activity on fatty acyl-CoA substrates ranging from 8 to 20 carbons in length (both saturated and unsaturated) as well as on fatty aldehyde substrates. Characteristically, proteins of this family possess two NAD(P)(H)-binding motifs, which have the conserved sequence GXGX(1-2X)G (Willis et al., Biochemistry 50 (2011), 10550-10558; Jornvall H. et al., Biochemistry 34 (1995), 6003-6013). The first pattern, GTGFIG, is identified near the N-terminus and the second signature sequence, GXXXGXG, is located between residues 384-390.

In principle any "short-chain dehydrogenase/fatty acyl-CoA reductase" or "short-chain dehydrogenases/reductases (SDR)" can be applied in the method according to the invention.

Preferably, the short-chain dehydrogenase/fatty acyl-CoA reductase is a short-chain dehydrogenase/fatty acyl-CoA reductase from a marine bacterium, preferably from
the genus Marinobacter or Hahella, even more preferably from the species Marinobacter aquaeolei, more preferably Marinobacter aquaeolei VT8, Marinobacter manganoxydans, Marinobacter algicola, Marinobacter sp. ELB17 or Hahella chejuensis. Examples of such enzymes are the short-chain dehydrogenase/fatty acyl-CoA reductase from Marinobacter aquaeolei VT8 (Uniprot accession number A1U3L3; Willis et al., Biochemistry 50 (2011), 10550-10558), the short-chain dehydrogenase from Marinobacter manganoxydans (Uniprot accession number G6YQS9), the short-chain dehydrogenase from Marinobacter algicola (Uniprot accession number A6EUH6), the short-chain dehydrogenase from Marinobacter sp. ELB17 (Uniprot accession number A3JCC5) and the short-chain dehydrogenase from Hahella chejuensis (Uniprot accession number Q2SCE0).

The sequence of the short-chain dehydrogenase/fatty acyl-CoA reductase from Marinobacter aquaeolei VT8 is shown in SEQ ID NO: 7. The sequence of the short-chain dehydrogenase/fatty acyl-CoA reductase from Marinobacter manganoxydans is shown in SEQ ID NO: 8. The sequence of the short-chain dehydrogenase/fatty acyl-CoA reductase from Marinobacter sp. ELB17 is shown in SEQ ID NO: 9. The sequence of the short-chain dehydrogenase/fatty acyl-CoA reductase from Marinobacter algicola is shown in SEQ ID NO: 10. The sequence of the short-chain dehydrogenase/fatty acyl-CoA reductase from Hahella chejuensis is shown in SEQ ID NO: 11. The sequence of the short-chain dehydrogenase/fatty acyl-CoA reductase from Marinobacter santoriniensis NKSG1 is shown in SEQ ID NO: 12. The sequence of the short-chain dehydrogenase/fatty acyl-CoA reductase from Marinobacter lipolyticus SM19 is shown in SEQ ID NO: 13. The sequence of the short-chain dehydrogenase/fatty acyl-CoA reductase from Marinobacter hydrocarbonoclasticus ATCC 49840 is shown in SEQ ID NO: 14. In a particularly preferred embodiment any protein can be employed in a method according to the present invention which shows an amino acid sequence as shown in any one of SEQ ID NOs: 7 to 11 and SEQ ID NOs: 20 to 24 or an amino acid sequence which is at least x% homologous to any of SEQ ID NOs: 7 to 11 and SEQ NOs: 20 to 24 and which has the activity of a short-chain dehydrogenase/fatty acyl-CoA reductase, i.e., an activity of converting acetoacetyl-CoA into 4-hydroxy-2-
butanone, with \( x \) being an integer between 30 and 100, preferably 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99.

As is evident from the appended Examples, it was found that different tested short-chain dehydrogenase/reductase proteins from different organisms are capable of using acetoacetyl-CoA as a substrate and converting it into 4-hydroxy-2-butanone.

The present invention also relates to a method as described herein above, in which acetoacetyl-CoA is converted into 4-hydroxy-2-butanone which is then further converted into 3-buten-2-one and which further comprises the step of enzymatically providing the acetoacetyl-CoA. This can be achieved by the enzymatic conversion of two molecules acetyl-CoA into one molecule of acetoacetyl-CoA. Such methods have already been described, e.g., in WO 2013/057194. Thus, according to the present invention, acetyl-CoA can, for example, be converted into acetoacetyl-CoA by the following reaction:

\[
2 \text{acetyl-CoA} \rightarrow \text{acetoacetyl-CoA} + \text{CoA}
\]

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

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

\[
\text{acetyl-CoA} + \text{malonyl-CoA} \rightarrow \text{acetoacetyl-CoA} + \text{CoA} + \text{CO}_2
\]
This reaction is catalyzed by an enzyme called acetoacetyl-CoA synthase (EC 2.3.1.194). The gene encoding this enzyme was identified in the mevalonate pathway gene cluster for terpenoid production in a soil-isolated Gram-positive Streptomyces sp. Strain CL190 (Okamura et al., PNAS USA 107 (2010), 11265-11270, 2010). Moreover a biosynthetic pathway using this enzyme for acetoacetyl-CoA production was recently developed in E. coli (Matsumoto K et al., Biosci. Biotechnol. Biochem, 75 (2011), 364-366).

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

In another preferred embodiment, in the methods of the invention further comprising the enzymatic conversion of acetyl-CoA into acetoacetyl-CoA, the enzymatic conversion of acetyl-CoA into acetoacetyl-CoA comprises two enzymatic steps of

(i) enzymatically converting acetyl-CoA into malonyl-CoA; and
(ii) enzymatically converting malonyl-CoA and acetyl-CoA into acetoacetyl-CoA.

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

\[
\text{Acetyl-CoA + ATP + CO}_2 \rightarrow \text{Malonyl-CoA + ADP}
\]

Preferably, the enzymatic conversion of malonyl-CoA and acetyl-CoA into acetoacetyl-CoA is achieved by the use of an acetoacetyl-CoA synthase (EC 2.3.1.194).

In principle, any acetyl-CoA acetyltransferase (EC 2.3.1.9), acetyl-CoA carboxylase (EC 6.4.1.2) and/or acetoacetyl-CoA synthase (EC 2.3.1.194) can be applied in the method according to the invention.
The 3-buten-2-one produced according to any method as described herein can be further converted into 3-buten-2-ol, which in itself may serve as a substrate for the production of 1,3-butadiene as described further below. Thus, a method according to the present invention as described above may further include the step of the enzymatic conversion of the produced 3-buten-2-one into 3-buten-2-ol by making use of a 3-buten-2-one reductase. Enzymes which can act as a 3-buten-2-one reductase, are, e.g., enzymes which are classified as EC 1.1.1 or enzymes which can be classified into E.C. 1.1.1 due to their characteristics, such as short-chain dehydrogenases/fatty acyl-CoA reductases, in particular those from marine bacteria.

In principle any possible enzyme classified in EC 1.1.1 which can catalyze the conversion of 3-buten-2-one into 3-buten-2-ol can be employed in a method according to the invention. Examples of preferred enzymes are alcohol dehydrogenases, such as alcohol dehydrogenases classified as EC 1.1.1.1, alcohol dehydrogenase (NADP+) classified as EC 1.1.1.2 and allyl alcohol dehydrogenases classified as EC 1.1.1.54, as well as carbonyl reductases (EC 1.1.1.184).

Alcohol dehydrogenases are enzymes which are a group of dehydrogenase enzymes that occur in many organisms and facilitate the interconversion between alcohols and aldehydes or ketones. In one preferred embodiment, the 3-buten-2-one reductase catalyzing the enzymatic conversion of 3-buten-2-one into 3-buten-2-ol is an alcohol dehydrogenase (EC 1.1.1.1). In principle, any alcohol dehydrogenase classified as EC 1.1.1.1 which can catalyze the conversion of 3-buten-2-one into 3-buten-2-ol can be employed in a method according to the present invention. Alcohol dehydrogenases (EC 1.1.1.1) are a group of dehydrogenase enzymes that occur in many organisms and facilitate the interconversion between alcohols and aldehydes or ketones with the reduction of nicotinamide adenine dinucleotide (NAD+ to NADH).

In humans and many other animals, they serve to break down alcohols that otherwise are toxic, and they also participate in generation of useful aldehyde, ketone, or alcohol groups during biosynthesis of various metabolites. In yeast, plants, and many bacteria, some alcohol dehydrogenases catalyze the opposite reaction as part of fermentation to ensure a constant supply of NAD+. Enzymes belonging to this class and catalyzing the above shown conversion occur in organisms of all kingdoms, i.e. plants, animals, fungi, bacteria etc. and have extensively been
described in the literature. For example, enzymes from Homo sapiens, Mus musculus, Drosophila melanogaster, Sacharomyces cerevisiae, Schizosaccharomyces pombe, Rhodobacter ruber, Pseudomonas aeruginosa, and Brassica napus have been identified and characterized. Nucleotide and/or amino acid sequences for such enzymes have been determined for numerous organisms, in particular bacterial organisms. In principle, any alcohol dehydrogenase (EC 1.1.1.1) which can catalyze the conversion of 3-buten-2-one into 3-buten-2-ol can be used in the context of the present invention. Indeed, as exemplified in the examples, it was found that different tested alcohol dehydrogenases from different organisms are capable of using 3-buten-2-one as a substrate and converting it into 3-buten-2-ol.

In another embodiment, the 3-buten-2-one reductase catalyzing the enzymatic conversion of 3-buten-2-one into 3-buten-2-ol is an alcohol dehydrogenase (NADP+) (EC 1.1.1.2). In principle, any alcohol dehydrogenase (NADP+) classified as EC 1.1.1.2 which can catalyze the conversion of 3-buten-2-one into 3-buten-2-ol can be employed in a method according to the present invention. Alcohol dehydrogenases (NADP+) (EC 1.1.1.2) are a group of dehydrogenase enzymes that occur in many organisms and facilitate the interconversion between alcohols and aldehydes or ketones with the reduction of nicotinamide adenine dinucleotide (NADP+ to NADPH). Enzymes belonging to this class and catalyzing the above shown conversion occur in organisms of all kingdoms, i.e. plants, animals, fungi, bacteria etc. and have extensively been described in the literature. For example, enzymes from Bos taurus, Mus musculus, Homo sapiens, Lactobacillus brevis, Escherichia coli, Sacharomyces cerevisiae, and Drosophila melanogaster have been identified and characterized. Nucleotide and/or amino acid sequences for such enzymes have been determined for numerous organisms. In principle, any alcohol dehydrogenase (NADP+) (EC 1.1.1.2) which can catalyze the conversion of 3-buten-2-one into 3-buten-2-ol can be used in the context of the present invention. As shown in the Examples, it was found that an alcohol dehydrogenase (NADP+) is capable of using 3-buten-2-one as a substrate and converting it into 3-buten-2-ol.

In a preferred embodiment, an alcohol dehydrogenase employed in a method according to the invention is of bacterial origin. More preferably it is an alcohol dehydrogenase from a bacterium of the genus Rhodococcus, Thermoanaerobium or
Lactobacillus, even more preferably from the species Rhodococcus ruber, Thermoanaerobium brockii, Lactobacillus brevis or Lactobacillus kefiri.

In particularly preferred embodiments the alcohol dehydrogenase is a secondary alcohol dehydrogenase derived from Rhodococcus ruber (Uniprot Q8KLT9; Karabec et al., Chem. Commun. 46 (2010), 6314-6316), an alcohol dehydrogenase derived from Thermoanaerobacter brockii (Thermoanaerobium brockii) (Uniprot P14941; Keinan et al., J. Am. Chem. Soc. 108 (1986), 162-169), an R-specific alcohol dehydrogenase derived from Lactobacillus brevis (Uniprot Q84EX5; Schlieben et al., J. Mol. Biol. 349 (2005), 801-813), an NADH-dependent (S)-specific alcohol dehydrogenase derived from Lactobacillus kefiri (Uniprot B5AXN9; Chen et al., Appl. Biochem. Biotechnol. 160 (2010), 19-29) or an NADH-dependent (S)-specific alcohol dehydrogenase derived from Lactobacillus brevis (Uniprot C2D0H5).

Also preferred is an NADPH dependent R-specific alcohol dehydrogenase derived from Lactobacillus kefiri (Uniprot Q6WVP7; Bradshaw et al., J. Org. Chem. 57 (1992), 1532-1536).

Amino acid and nucleotide sequences for the above-mentioned enzymes are available. Examples for corresponding amino acid sequences are provided in SEQ ID NOs: 12 to 17 wherein SEQ ID NO:12 is the amino acid sequence of a secondary alcohol dehydrogenase derived from Rhodococcus ruber, SEQ ID NO:13 is the amino acid sequence of an alcohol dehydrogenase derived from Thermoanaerobacter brockii (Thermoanaerobium brockii) (Uniprot P14941), SEQ ID NO:14 is the amino acid sequence of an R-specific alcohol dehydrogenase derived from Lactobacillus brevis (Uniprot Q84EX5), SEQ ID NO:15 is the amino acid sequence of an NADH-dependent (S)-specific alcohol dehydrogenase derived from Lactobacillus kefiri (Uniprot B5AXN9) and SEQ ID NO:16 is the amino acid sequence of an NADH-dependent (S)-specific alcohol dehydrogenase derived from Lactobacillus brevis (Uniprot C2D0H5).

SEQ ID NO:17 is the amino acid sequence of an NADPH dependent R-specific alcohol dehydrogenase derived from Lactobacillus kefiri (Uniprot Q6WVP7).

In a particularly preferred embodiment any protein can be employed in a method according to the present invention which shows an amino acid sequence as shown in any one of SEQ ID NOs: 12 to 17 or an amino acid sequence which is at least x% homologous to any of SEQ ID NOs: 12 to 17 and which has the activity of converting
3-buten-2-one into 3-buten-2-ol, with \( x \) being an integer between 30 and 100, preferably 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99.

In another preferred embodiment, the conversion of 3-buten-2-one into 3-buten-2-ol is achieved by employing an allyl alcohol dehydrogenase (EC 1.1.1.54). Allyl alcohol dehydrogenases (EC 1.1.1.54) catalyze the following reaction:

\[
\text{Acrolein} + \text{NADPH} + \text{H}^+ \rightleftharpoons \text{allyl alcohol} + \text{NADP}^+
\]

\[
\begin{array}{c}
\text{\cong} \\
\text{CH} \quad \text{CH} \\
\text{O} \quad \text{O} \\
& + \text{NADPH} + \text{H}^+ \rightleftharpoons \\
\text{\cong} \\
\text{CH} \quad \text{CH} \\
\text{OH} \\
& + \text{NADP}^+
\end{array}
\]

In principle, any allyl alcohol dehydrogenase classified as EC 1.1.1.54 which can catalyze the conversion of 3-buten-2-one into 3-buten-2-ol can be employed in a method according to the present invention. This enzyme belongs to the family of oxidoreductases, specifically those acting on the CH-OH group of donor with NAD\(^+\) or NADP\(^+\) as acceptor. The systematic name of this enzyme class is often referred to as allyl-alcohol:NADP\(^+\) oxidoreductase. Enzymes belonging to this class and catalyzing the above shown conversion have been described to occur in various organisms and have been described in the literature. For example, enzymes from Pseudomonas putida and Escherichia coli have been identified and characterized. In principle, any allyl alcohol dehydrogenase (EC 1.1.1.54) which can catalyze the conversion of 3-buten-2-one into 3-buten-2-ol can be used in the context of the present invention.

In another preferred embodiment, the 3-buten-2-one reductase catalyzing the enzymatic conversion of 3-buten-2-one into 3-buten-2-ol is a carbonyl reductase (EC 1.1.1.184).

A carbonyl reductase (EC 1.1.1.184) catalyzes the following reaction:

\[
\text{Ketone} + \text{NADPH} + \text{H}^+ \rightleftharpoons \text{secondary alcohol} + \text{NADP}^+
\]

This enzyme belongs to the family of oxidoreductases, specifically those acting on the CH-OH group of donor with NAD\(^+\) or NADP\(^+\) as acceptor. The systematic name
of this enzyme class is secondary-alcohol:NADP+ oxidoreductase. Other names in common use include aldehyde reductase 1, prostaglandin 9-ketoreductase, xenobiotic ketone reductase, NADPH-dependent carbonyl reductase, ALR3, carbonyl reductase, nonspecific NADPH-dependent carbonyl reductase, aldehyde reductase 1, and carbonyl reductase (NADPH). This enzyme participates in arachidonic acid metabolism, and has been shown to catabolize S-Nitrosoglutathione, as a means to degrade NO in an NADPH dependent manner. Enzymes belonging to this class and catalyzing the above shown conversion have been described to occur in many organisms and have extensively been described in the literature. For example, enzymes from Rattus norvegicus, Homo sapiens, Drosophila melanogaster, Saccharomyces cerevisiae and Candida magnoliae have been identified and characterized. In principle, any carbonyl reductase which can catalyze the enzymatic conversion of 3-buten-2-one into 3-buten-2-ol can be used in the context of the present invention. As is evident from the appended examples, it was found that a carbonyl reductase showed the reduction activity towards 3-buten-2-one in the presence of NADPH as co-factor.

In a preferred embodiment the carbonyl reductase employed in a method according to the invention is derived from a mammal, preferably from a rodent, even more preferably from a rodent of the genus Rattus and most preferably from Rattus norvegicus. An example for such an enzyme is the carbonyl reductase from Rattus norvegicus (Uniprot B2GV72; Okamoto et al., IUBMB Life 48 (1999), 543-547). Amino acid and nucleotide sequences for carbonyl reductases are available. SEQ ID NO:18 is the amino acid sequence of a carbonyl reductase derived from Rattus norvegicus (Uniprot B2GV72).

In a particularly preferred embodiment any protein can be employed in a method according to the present invention which shows an amino acid sequence as shown in SEQ ID NO: 18 or an amino acid sequence which is at least x% homologous to any of SEQ ID NOs: 18 and which has the activity of catalyzing the conversion of 3-buten-2-one into 3-buten-2-ol, with x being an integer between 30 and 100, preferably 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99.

Alternatively, in another aspect, the 3-buten-2-one reductase catalyzing the enzymatic conversion of 3-buten-2-one into 3-buten-2-ol, may be a short chain
dehydrogenase/fatty acyl-CoA reductase. These enzymes, i.e., the short-chain
dehydrogenases/fatty acyl-CoA reductase, in particular fatty acyl-CoA reductases
derived from marine bacteria, have already been described above and the same as
has been set forth above also applies here, in particular as regards the preferred
embodiments.
In the Examples it is shown that short-chain dehydrogenases/fatty acyl-CoA
reductases can be employed for converting 3-buten-2-one into 3-buten-2-ol. In
preferred embodiments of the method according to the invention an enzyme
originating from a bacterium is employed, preferably from a marine bacterium,
preferably from the genus Marinobacter or Hahella, most preferably from the species
Hahella chejuensis. In a particularly preferred embodiment, the enzyme employed is
the one showing the amino acid sequence as reflected in Uniprot Q2SCEO.

The 3-buten-2-ol produced by a method as described herein-above may be further
converted into 1,3-butadiene, in particular via an enzymatic conversion, i.e. by
making use of an alkenol dehydratase. Thus, the present invention also relates to a
method for the production of 1,3-butadiene comprising:
(a) producing 3-buten-2-ol according to a method of the invention as described
herein-above, and
(b) the step of enzymatically converting the thus produced 3-buten-2-ol into 1,3-
butadiene.

An enzyme which is capable of converting 3-buten-2-ol into 1,3-butadiene is referred
to in this context as an alkenol dehydratase.
An example of an alkenol dehydratase to be employed in a method according to the
present invention is the enzyme which has been designated "linalool dehydratase-
isomerase" and which has been identified in Castellaniella defragrans (formerly
Alcaligenes defragrans) strain 65Phen (Brodkorb et al., J. Biol. Chem. 285 (2010),
30436-30442). Linalool dehydratase-isomerase is a bifunctional enzyme which is
involved in the anaerobic degradation of monoterpenes. The native enzyme has
been found to have a molecular mass of 160 kDa and is assumed to be a
homotetramer of 40 kDa subunits. The enzyme catalyzes \textit{in vitro} two reactions in
both directions depending on the thermodynamic driving forces. On the one hand,
the enzyme catalyzes the isomerisation of the primary allyl alcohol geraniol into its
stereoisomer linalool which bears a tertiary allyl alcohol motif. On the other hand, the
enzyme catalyzes the water secession (dehydration) from the tertiary alcohol linaiool to the corresponding acyclic monoterpenic beta-myrcene, a molecule bearing a conjugated diene motif. In Castellaniella defragrans the protein is expressed as a precursor protein with a signal peptide for a periplasmatic location which is cleaved after transport through the membrane. The enzyme is classified as EC 4.2.1.127. A linaiool dehydratase-isomerase has the capacity to catalyze the following reaction under anaerobic conditions:

\[ \text{Linaiool} \rightarrow \beta-\text{myrcene} + \text{H}_2\text{O} \]

This activity can, e.g., be measured with an assay as described in Brodkorb et al. (loc. cit.). In such an assay, vials are prewarmed at 35 °C, anoxic protein solution is transferred into the vials and DTT is added to 2 mM. The reaction mixtures are sealed with a butyl septum and the headspace is flushed with CO\(_2\)/N\(_2\) (10/90 (v/v)). The reaction is started by adding a distinct concentration of linaiool and incubated at 35 °C. The conversion of linaiool into myrcene is assessed by investigating the production of myrcene, e.g. by gas chromatography.

In a preferred embodiment, a linaiool dehydratase-isomerase also has the capacity to catalyze the isomerisation of geraniol into linaiool under anaerobic conditions:

\[ \beta-\text{myrcene} \rightarrow \text{geraniol} \]

This activity can, e.g., be measured with an assay as described in Brodkorb et al. (loc. cit.). In such an assay, vials are prewarmed at 35° C, anoxic protein solution is transferred into the vials and DTT is added to 2 mM. The reaction mixtures are sealed with a butyl septum and the headspace is flushed with CO\(_2\)/N\(_2\) (10/90 (v/v)). The reaction is started by adding a distinct concentration of geraniol and incubated at 35° C. The conversion of geraniol into linaiool is assessed by investigating the production of myrcene, i.e. the product of the second reaction catalyzed by the enzyme, e.g. by gas chromatography.

Geraniol, linaiool and myrcene are acyclic C\(_{10}\)-terpenoids produced by plants, belonging to the class of allyl alcohols and hydrocarbons, respectively. Luddecke and Harder (Z. Naturforsch. 66c (2011), 409-412) reported on a high substrate
specificity of linalool dehydratase-isomerase. It has been shown that linalool dehydratase-isomerase can act on but-3-en-2-ol and convert it into butadiene.

An example of a sequence of an alkenol dehydratase which can be employed in the method according to the present invention is given in SEQ ID NO: 19. A sequence for an alkenol dehydratase is also accessible in the UniProtKB/TrEMBL database under accession number E1XUJ2. These sequences represent an alkenol dehydratase which is classified as a linalool dehydratase-isomerase. In a preferred embodiment the method according to the present invention makes use of an alkenol dehydratase comprising the amino acid sequence shown in SEQ ID NO: 19 or a sequence which is at least x % identical to SEQ ID NO: 19 and which is able to catalyze the conversion of 3-buten-2-ol into 1,3-butadiene, with x being an integer between 30 and 100, preferably 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99.

The term "an alkenol dehydratase" as used in the present invention therefore in particular refers to an enzyme which shows the above indicated degree of sequence identity with SEQ ID NO: 19 and which can catalyze the conversion of 3-buten-2-ol into 1,3-butadiene. By using the sequence of SEQ ID NO: 19 or corresponding encoding nucleotide sequences, it is possible for the skilled person to identify further alkenol dehydratases which can catalyze the above indicated conversion.

Finally, the present invention also relates to a method for the production of 3-buten-2-ol comprising the enzymatic conversion of 4-hydroxy-2-butanone into 3-buten-2-one by making use of an enzyme catalyzing 4-hydroxy-2-butanone dehydration and the enzymatic conversion of 3-buten-2-one into 3-buten-2-ol by making use of a 3-buten-2-one reductase. The enzyme catalyzing 4-hydroxy-2-butanone dehydration is not particularly limited to a specific enzyme as described herein above but can be any possible enzyme which can catalyze this conversion. In a preferred embodiment, the enzyme catalyzing 4-hydroxy-2-butanone dehydration is an enzyme as described above. Moreover, the enzyme converting 3-buten-2-one into 3-buten-2-ol by making use of a 3-buten-2-one reductase is not particularly limited to a specific enzyme but may, in a preferred embodiment, be an enzyme as described above in the context of the fourth aspect of the present invention.
When referring to "homology" in connection with amino acid or nucleotide sequences, reference is preferably made to sequence identity. The degree of sequence identity can be determined according to methods well known in the art using preferably suitable computer algorithms such as CLUSTAL.

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

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

Preferably, the degree of identity is calculated over the complete length of the sequence. When the sequences which are compared do not have the same length, the degree of identity either refers to the percentage of residues in the shorter sequence which are identical to residues in the longer sequence or to the percentage of residues in the longer sequence which are identical to residues in the shorter sequence. Preferably, it refers to the percentage of residues in the shorter sequence which are identical to residues in the longer sequence.

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

For carrying out the method in vitro the substrates for the reaction and the enzymes are incubated under conditions (buffer, temperature, cosubstrates, cofactors etc.) allowing the enzymes to be active and the enzymatic conversion to occur. The reaction is allowed to proceed for a time sufficient to produce the respective product.
The production of the respective products can be measured by methods known in the art, such as gas chromatography possibly linked to mass spectrometry detection. The enzymes may be in any suitable form allowing the enzymatic reaction to take place. They may be purified or partially purified or in the form of crude cellular extracts or partially purified extracts. It is also possible that the enzymes are immobilized on a suitable carrier. In a preferred embodiment, at least one of the enzymes employed in such an in vitro reaction according to the present invention is a non-naturally occurring enzyme, e.g., a variant of an enzyme which does not as such occur in nature. Such variants include, for example, mutants, in particular prepared by molecular biological methods, which show improved properties, such as a higher enzyme activity, higher substrate specificity, higher temperature resistance and the like.

In one embodiment of the method according to the invention the substrate which is used in such an in vitro method is 4-hydroxy-2-butanone which is converted by the use of the above-mentioned enzymes to 3-buten-2-one. In another embodiment, the substrate used in such an in vitro method is acetoacetyl-CoA which is first converted into 4-hydroxy-2-butanone as described above which is then in turn converted into 3-buten-2-one as described above. In another embodiment, the substrate used in such an in vitro method is acetyl-CoA which is first converted into acetoacetyl-CoA as described above which is then converted into 4-hydroxy-2-butanone as described above which is then in turn converted into 3-buten-2-one as described above.

The in vitro method according to the invention may be carried out in a one-pot-reaction, i.e. the substrate is combined in one reaction mixture with the above described enzymes necessary for the conversion into 3-buten-2-one, 3-buten-2-ol or 1,3-butadiene, respectively, and the reaction is allowed to proceed for a time sufficient to produce the respective product. Alternatively, the method may also be carried out by effecting one or more enzymatic steps in a consecutive manner, i.e. by first mixing the substrate with one or more enzymes and allowing the reaction to proceed to an intermediate and then adding one or more further enzymes to convert the intermediate further either into an intermediate or into butadiene.

The in vitro method according to the invention furthermore may comprise the step of collecting the product(s), preferably the gaseous products, in particular 1,3-butadiene, degassing out of the reaction, i.e. recovering the products from the culture or, in the case of gaseous products the products which degas, e.g., out of the culture.
Thus, in one embodiment, the method is carried out in the presence of a system for collecting a gaseous product, such as butadiene, under gaseous form during the reaction.

As a matter of fact, 1,3-butadiene, adopts the gaseous state at room temperature and atmospheric pressure. The method according to the invention therefore does not necessarily require extraction of the product from the reaction mixture, a step which is always very costly when performed at industrial scale. The evacuation and storage of butadiene and its possible subsequent physical separation from other gaseous substances as well as its chemical conversion can be performed according to any method known to one of skill in the art. For example, butadiene can be separated from \( \text{CO}_2 \) by the condensation of \( \text{CO}_2 \) at low temperatures. \( \text{CO}_2 \) can also be removed by polar solvents, e.g. ethanolamine. Moreover, it can be isolated by adsorption on a hydrophobic membrane.

In another embodiment the method according to the invention is carried out in culture, in the presence of an organism, preferably a microorganism, producing at least one of the enzymes described above which are necessary to produce 3-buten-2-one according to one of the methods of the invention. Moreover, in another embodiment the method according to the invention is carried out in culture, in the presence of an organism, preferably a microorganism, producing at least one of the enzymes described above which are necessary to produce 3-buten-2-ol according to one of the methods of the invention. Moreover, in another embodiment the method according to the invention is carried out in culture, in the presence of an organism, preferably a microorganism, producing at least one of the enzymes described above which are necessary to produce 1,3-butadiene according to one of the method of the invention.

Thus, in such embodiments of the invention, an organism, preferably a microorganism, that produces at least one of the enzymes specified in the description, above, is used. It is possible to use a (micro)organism which naturally produces one or more of the required enzymes and to genetically modify such a (micro)organism so that it expresses also those enzymes which it does not naturally express.

If a (micro)organism is used which naturally expresses one of the required enzyme activities, it is possible to modify such a (micro)organism so that this activity is
overexpressed in the (micro)organism. This can, e.g., be achieved by effecting mutations in the promoter region of the corresponding gene so as to lead to a promoter which ensures a higher expression of the gene. Alternatively, it is also possible to mutate the gene as such so as to lead to an enzyme showing a higher activity.

By using (micro)organisms which express the enzymes which are necessary for achieving the enzymatic conversions as described above, it is possible to carry out the method according to the invention directly in the culture medium, without the need to separate or purify the enzymes.

In one embodiment the organism employed in the method according to the invention is an organism, preferably a microorganism, which has been genetically modified to contain one or more foreign nucleic acid molecules encoding one or more of the enzymes as described 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. is 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 a plasmid, or stably integrated in the chromosome. A stable integration is preferred. Thus, the genetic modification can consist, e.g. in integrating the corresponding gene(s) encoding the enzyme(s) into the chromosome, or in expressing the enzyme(s) from a plasmid containing a promoter upstream of the enzyme-coding sequence, the promoter and coding sequence preferably originating from different organisms, or any other method known to one of skill in the art.

Genetically modified organisms are organisms which do not naturally occur, i.e., which cannot be found in nature, and which differ substantially from naturally occurring organisms due to the introduction of a foreign nucleic acid molecule. Such non-naturally occurring organisms are preferably employed in a method according to the present invention carried out in vivo.

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 or Bacillus and even more preferably of the species Escherichia coli or Bacillus subtilis.

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

In one embodiment the microorganism is a fungus, more preferably a fungus of the genus Saccharomyces, Schizosaccharomyces, Aspergillus, Trichoderma, Pichia or Kluveromyces and even more preferably of the species Saccharomyces cerevisiae, Schizosaccharomyces pombe, Aspergillus niger, Trichoderma reesei, Pichia pastoris or of the species Kluyveromyces lactis.

In another embodiment, the method according to the invention makes use of a photosynthetic microorganism expressing at least the enzymes which are necessary for achieving the enzymatic conversions as described above. Preferably, the microorganism is a photosynthetic bacterium, or a microalgae. In a further embodiment the microorganism is an algae, more preferably an algae belonging to the diatomeae.

In another embodiment, it is possible to use a microorganism which belongs to the group of acetogenic bacteria which are capable of converting CO (or CO₂+H₂) to produce acetyl-CoA via the so-called Wood-Ljungdahl pathway (Kopke et al.; PNAS
A fermentation process using such microorganisms is known as syngas fermentation. Strictly mesophilic anaerobes such as C. ljungdahlii, C. aceticum, Acetobacterium woodii, C. autoethanogenum, and C. carboxydevorin, are frequently being used in syngas fermentation (Munasingheet et al.; Bioresource Technology 101 (2010), 5013-5022).

It is also conceivable to use in the method according to the invention a combination of (micro)organisms wherein different (micro)organisms express different enzymes as described above.

In another embodiment the method according to the invention makes use of a multicellular organism expressing at least one of the enzymes which are necessary for achieving the enzymatic conversions as described above. Examples for such organisms are plants or animals.

In a particular embodiment, the method according to the invention 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).

In a further embodiment the method of the invention is carried out under microaerophilic conditions. This means that the quantity of injected air is limiting so as to minimize residual oxygen concentrations in the gaseous effluents containing butadiene.

In another embodiment the method according to the invention furthermore comprises the step of collecting the product either from the culture or, in the case of a gaseous product, like, 1,3-butadiene degassing out of the reaction, from the culture off-gas. Thus in a preferred embodiment, the method is carried out in the presence of a system for collecting a product, for example, a gaseous product, such as 1,3-butadiene, under gaseous form during the reaction.

As a matter of fact, 1,3-butadiene adopts the gaseous state at room temperature and atmospheric pressure. The method according to the invention therefore does not necessarily require extraction of butadiene from the liquid culture medium, a step which is always very costly when performed at industrial scale. The evacuation and storage of butadiene and its possible subsequent physical separation and chemical
conversion can be performed according to any method known to one of skill in the
art and as described above.

In a particular embodiment, the method also comprises detecting a gaseous product,
such as butadiene, which is present in the gaseous phase. The presence of, e.g.,
butadiene in an environment of air or another gas, even in small amounts, can be
detected by using various techniques and in particular by using gas chromatography
systems with infrared or flame ionization detection, or by coupling with mass
spectrometry.

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

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).

As described above, it is possible to use in the method according to the invention a (micro)organism which is genetically modified so as to contain a nucleic acid molecule encoding at least one of the which are necessary for achieving the enzymatic conversions as described above. Such a nucleic acid molecule encoding an enzyme as described above can be used alone or as part of a vector. The nucleic acid molecules can further comprise expression control sequences operably linked to the polynucleotide comprised in the nucleic acid molecule. The term "operatively linked" or "operably linked", as used throughout the present description, refers to a linkage between one or more expression control sequences and the coding region in
the polynucleotide to be expressed in such a way that expression is achieved under conditions compatible with the expression control sequence.

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

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

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

In addition, it is possible to insert different mutations into the polynucleotides by methods usual in molecular biology (see for instance Sambrook and Russell (2001), Molecular Cloning: A Laboratory Manual, CSH Press, Cold Spring Harbor, NY, USA), leading to the synthesis of polypeptides possibly having modified biological properties. The introduction of point mutations is conceivable at positions at which a modification of the amino acid sequence for instance influences the biological activity or the regulation of the polypeptide.

Moreover, mutants possessing a modified substrate or product specificity can be prepared. Preferably, such mutants show an increased activity. Furthermore, the introduction of mutations into the polynucleotides encoding an enzyme as defined above allows the gene expression rate and/or the activity of the enzymes encoded by said polynucleotides to be optimized.

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


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

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

The present invention also relates to an organism, preferably a microorganism, which is able to express the above described enzymes required for the conversion of 4-hydroxy-2-butanone into 3-buten-2-one and for the conversion of 3-buten-2-one into 3-buten-2-ol. Thus, the present invention relates to an organism or microorganism which expresses

(i) an enzyme catalyzing 4-hydroxy-2-butanone dehydration as defined above, and

(ii) a 3-buten-2-one reductase as defined above.
In a preferred embodiment, the above organism is an organism wherein the 3-buten-2-one reductase is
(a) a carbonyl reductase (EC 1.1.1.184),
(b) an alcohol dehydrogenase, or
(c) a short chain dehydrogenase/fatty acyl-CoA reductase as defined above.

In a further aspect, the organisms is an organism which further expresses an alkenol dehydratase enzyme as defined above.

In yet a further aspect, the organism is an organism which further expresses
(i) an acetoacetyl-CoA reductase; or
(ii) an acetoacetyl-CoA reductase and enzymes capable of converting acetyl-CoA into acetoacetyl-CoA as defined above.

As regards the preferred embodiments of the enzymes which the organism expresses, the same applies as has been set forth above in connection with the method according to the invention.

In one embodiment an organism according to the present invention is a recombinant organism in the sense that it is genetically modified due to the introduction of at least one nucleic acid molecule encoding at least one of the above mentioned enzymes. Preferably such a nucleic acid molecule is heterologous with regard to the organism which means that it does not naturally occur in said organism. Accordingly, the organism according to the present invention is preferably a non-naturally occurring organism which differs from a naturally occurring organism due to the introduction of a foreign nucleic acid molecule.

The microorganism is preferably a bacterium, a yeast or a fungus. In another preferred embodiment the organism is a plant or non-human animal. As regards other preferred embodiments, the same applies as has been set forth above in connection with the method according to the invention.

The present invention also relates to a composition comprising an organism according to the invention and, optionally, acetoacetyl-CoA, 4-hydroxy-2-butanone, 3-buten-2-one and/or 3-buten-2-ol.
Thus, the present invention relates in a preferred embodiment to a composition comprising an organism, preferably a microorganism, producing at least one of the enzymes described above which are necessary to produce 3-buten-2-one according to a method of the invention. Optionally, such a composition further comprises acetoacetyl-CoA and/or 4-hydroxy-2-butanoate. Moreover, in another embodiment, the present invention relates to a composition comprising an organism, preferably a microorganism, producing at least one of the enzymes described above which are necessary to produce 3-buten-2-ol according to a method of the invention. Optionally, such a composition further comprises acetoacetyl-CoA, 4-hydroxy-2-butanoate and/or 3-buten-2-one. Moreover, in another embodiment, the present invention relates to a composition comprising an organism, preferably a microorganism, producing at least one of the enzymes described above which are necessary to produce 1,3-butadiene according to a method of the invention. Optionally, such a composition further comprises acetoacetyl-CoA, 4-hydroxy-2-butanoate, 3-buten-2-one and/or 3-buten-2-ol.

As regards preferred embodiments of the above compositions, the same applies as has been set forth above in connection with the method according to the invention.

The present invention also relates to the use of an enzyme catalyzing 4-hydroxy-2-butanoate dehydration as described herein above for the production of 3-buten-2-one from 4-hydroxy-2-butanoate.

The present invention also relates to the use of a combination of at least one enzyme catalyzing 4-hydroxy-2-butanoate dehydration as described above and of at least one enzyme catalyzing the enzymatic conversion of acetoacetyl-CoA into 4-hydroxy-2-butanoate as described above for the production of 3-buten-2-one from acetoacyl-CoA.

The present invention also relates to the use of a combination of at least one enzyme catalyzing 4-hydroxy-2-butanoate dehydration as described above and of at least one enzyme catalyzing the enzymatic conversion of acetoacetyl-CoA into 4-hydroxy-2-butanoate as described above and of at least one enzyme catalyzing the enzymatic conversion of acetyl-CoA into acetoacetyl-CoA as described above for the production of 3-buten-2-one from acetyl-CoA.
As regards the preferred embodiments of the enzymes applied in the uses for the production of 3-buten-2-one, the same applies as has been set forth above in connection with the method according to the invention.

The present invention also relates to the use of a combination of at least one enzyme catalyzing the enzymatic conversion of 3-buten-2-one into 3-buten-2-ol as described above in combination with at least one enzyme catalyzing 4-hydroxy-2-butanone dehydration as described above for the production of 3-buten-2-ol from 4-hydroxy-2-butanone.

The present invention also relates to the use of a combination of at least one enzyme catalyzing the enzymatic conversion of acetoacetyl-CoA into 4-hydroxy-2-butanone as described above for the production of 3-buten-2-ol from acetoacetyl-CoA.

The present invention also relates to the use of a combination of at least one enzyme catalyzing the enzymatic conversion of acetyl-CoA into acetoacetyl-CoA as described above for the production of 3-buten-2-ol from acetyl-CoA.

As regards the preferred embodiments of the enzymes applied in the uses for the production of 3-buten-2-one, the same applies as has been set forth above in connection with the method according to the invention.

The present invention also relates to the use of a combination of at least one enzyme catalyzing the enzymatic conversion of 3-buten-2-ol into 1,3-butadiene as described and at least one enzyme catalyzing the enzymatic conversion of 3-buten-2-one into 3-buten-2-ol as described above for the production of 1,3-butadiene from 3-butene-2-one.
The present invention also relates to the use of a combination of at least one enzyme catalyzing the enzymatic conversion of 3-buten-2-ol into 1,3-butadiene as described above and at least one enzyme catalyzing the enzymatic conversion of 3-buten-2-one into 3-buten-2-ol as described above in combination with at least one enzyme catalyzing 4-hydroxy-2-butane dehydration into 3-buten-2-one as described above for the production of 1,3-butadiene from 4-hydroxy-2-butane.

The present invention also relates to the use of a combination of at least one enzyme catalyzing the enzymatic conversion of 3-buten-2-ol into 1,3-butadiene as described above and at least one enzyme catalyzing the enzymatic conversion of 3-buten-2-one into 3-buten-2-ol as described above in combination with at least one enzyme catalyzing 4-hydroxy-2-butane dehydration into 3-buten-2-one as described above and at least one enzyme catalyzing the enzymatic conversion of acetoacetyl-CoA into 4-hydroxy-2-butanol as described above for the production of 1,3-butadiene from acetoacetyl-CoA.

The present invention also relates to the use of a combination of at least one enzyme catalyzing the enzymatic conversion of 3-buten-2-ol into 1,3-butadiene as described above and at least one enzyme catalyzing the enzymatic conversion of 3-buten-2-one into 3-buten-2-ol as described above in combination with at least one enzyme catalyzing 4-hydroxy-2-butanol dehydration into 3-buten-2-one as described above and at least one enzyme catalyzing the enzymatic conversion of acetoacetyl-CoA into 4-hydroxy-2-butanol as described above and at least one enzyme catalyzing the enzymatic conversion of acetyl-CoA into acetoacetyl-CoA as described above for the production of 1,3-butadiene from acetyl-CoA.

As regards the preferred embodiments of the enzymes applied in the uses for the production of 1,3-butadiene, the same applies as has been set forth above in connection with the method according to the invention.

The present invention also relates to the use of at least one enzyme catalyzing the enzymatic conversion of acetoacetyl-CoA into 4-hydroxy-2-butanol as described above for the production of 4-hydroxy-2-butanol from acetoacetyl-CoA.

The present invention also relates to the use of a combination of at least one enzyme catalyzing the enzymatic conversion of acetoacetyl-CoA into 4-hydroxy-2-butanol as described above and at least one enzyme for the enzymatic conversion...
of acetyl-CoA into acetoacetyl-CoA as described above for the production of 4-hydroxy-2-butanoate from acetyl-CoA.

As regards the preferred embodiments of the enzymes applied in the uses for the production of 4-hydroxy-2-butanoate, the same applies as has been set forth above in connection with the method according to the invention.

**Figure 1:** shows an artificial metabolic pathway for 1,3-butadiene production from acetyl-CoA via 3-buten-2-ol.

**Figure 2:** shows a chromatogram obtained for the enzyme-catalyzed reduction of acetoacetyl-CoA with the short chain alcohol dehydrogenase-like protein from H. chejuensis as outlined in Example 3. The assay was performed with 32 mM acetoacetyl-CoA and incubated for 2 hours.

**Figure 3:** shows a chromatogram obtained for the enzymatic dehydration of 4-hydroxy-2-butanoate with 3-hydroxypropionyl-CoA dehydratase from S. tokodaii as outlined in Example 5. The assay was performed with 100 mM 4-hydroxy-2-butanoate and incubated for 24 hours.

**Figure 4:** shows 3-buten-2-one production by enzymatic dehydration of 4-hydroxy-2-butanoate. A significant production of 3-buten-2-one was observed for several 3-hydroxyacyl-CoA dehydratases.

**Figure 5:** shows a chromatogram obtained for the enzymatic reduction 3-buten-2-one to 3-buten-2-ol catalyzed by alcohol dehydrogenase from Thermoanaerobium brockii as outlined in Example 8. The assay was performed with 20 mM 3-buten-2-one and incubated for 4 hours.

**Figure 6:** shows GC/FID chromatograms obtained for enzymatic and enzyme-free assays with 80 mM 3-buten-2-ol and linalool dehydratase-isomerase after 22 hours incubation.

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**

*Gene synthesis, cloning and expression of recombinant proteins*

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

Competent *E. coli* BL21(DE3) cells (Novagen) were transformed with these vectors according to standard heat shock procedure. The transformed cells were grown with shaking (160 rpm) using 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 or 20°C overnight (approximately 16 h). The cells were collected by centrifugation at 4°C, 10,000 rpm for 20 min and the pellets were stored at -80°C.

*Protein purification and concentration*

The pellets from 200 ml of culture cells were thawed on ice and resuspended in 5 ml of Na$_2$HPO$_4$ pH 8 containing 300 mM NaCl, 5 mM MgCl$_2$ 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$_2$HPO$_4$ pH 8 containing 300 mM NaCl, 5 mM MgCl$_2$, 1 mM DTT, 250 mM imidazole. Eluates were then concentrated, desalted on Amicon Ultra-4 10 kDa filter unit (Millipore) and enzymes were resuspended in buffers compatible with downstream enzyme activity assay. 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 60 % to 90 %.

Example 2: Continuous spectrophotometric assay of acetoacetyl-CoA reductase activity

The genes of short-chain dehydrogenase/fatty acyl-CoA reductases were synthesized and corresponding enzymes were further produced according to the procedure described in Example 1. Purified enzymes were resuspended in 50 mM potassium phosphate buffer pH 7.5. Standard reaction mixture for assays of acetoacetyl-CoA reductase activity contained 5 mM acetoacetyl-CoA, 100 mM NaCl and 0.1-0.4 mM NADPH in 50 mM potassium phosphate buffer pH 7.5. Each assay was started by adding 1 mg/ml of enzyme. Control assays were performed in which either no enzyme was added, or no substrate was added. Reactions were conducted at 37°C. Each sample was continuously monitored for the decrease of NADPH at 340 nm on a SpectraMax Plus384 UVA/is Microplate Reader (Molecular Device). Several enzymes demonstrated acetoacetyl-CoA reductase activity with NADPH as cofactor (Table 1).

Table 1

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity, nmol/min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short-chain dehydrogenase/reductase (Fatty alcohol forming acyl-CoA reductase) from Marinobacter aquaeolei VT8</td>
<td>2.3</td>
</tr>
<tr>
<td>Short chain alcohol dehydrogenase-like protein from Hahella chejuensis</td>
<td>0.9</td>
</tr>
<tr>
<td>Short chain alcohol dehydrogenase-like protein from Marinobacter manganoxydans</td>
<td>1.7</td>
</tr>
</tbody>
</table>

The products of the enzymatic reduction of acetoacetyl-CoA were next analyzed by high-performance liquid chromatography (HPLC).
Example 3: HPLC-based analysis of products of the enzymatic reduction of acetoacetyl-CoA

The enzymatic assays were carried out under the following conditions:
50 mM potassium phosphate  pH 7.5
100 mM NaCl
60 mM NADPH
0-32 mM acetoacetyl-CoA

The assays were initiated with the addition of 150 µg of purified dehydrogenase/reductase to 150 µL of reaction mixture. Incubations were run for 0, 0.5, 1, 2 and 4 hours with shaking at 37° C. The reactions were stopped by heating at 65°C for 5 minutes, centrifuged and 120 µL of the clarified supernatants were transferred into a clean vial. Commercial 4-hydroxy-2-butanone (Sigma) was used as reference. HPLC analyses were performed using a 1260 Infinity LC System (Agilent), equipped with refractometer detector and column heating module. 10 µL of samples were separated on Zorbax SB-Aq column (250 x 4.6 mm, 5 µm particle size, column temp. 75°C) with a mobile phase flow rate of 1.5 ml/min. The mobile phase consisted of aqueous sulfuric acid (8.4 mM). Retention time of 4-hydroxy-2-butanone under these conditions was 3.2 min.

The HPLC analysis showed that 4-hydroxy-2-butanone was formed by enzyme-catalyzed reduction of acetoacetyl-CoA. A typical chromatogram obtained with short chain alcohol dehydrogenase-like protein from H. chejuensis is shown on Figure 2.

These data indicate that the short-chain dehydrogenase/reductase catalyzes the four-electron reduction of acetoacetyl-CoA to 4-hydroxy-2-butanone.

Example 4: Kinetic parameters of acetoacetyl-CoA reduction

Enzyme assays were carried out in a total volume of 150 µL potassium phosphate (50 mM, pH 7.5), containing 100 mM NaCl and pure enzyme (150 µg). Kinetic parameters were determined with acetoacetyl-CoA as variable substrate (0-32 mM), and NADPH concentration was kept constant (60 mM). The amount of 4-hydroxy-2-
butanone that forms for each assay was measured using HPLC-based procedure as described in Example 3.
Kinetic parameters obtained for purified short-chain dehydrogenase/fatty acyl-CoA reductases are presented in Table 2.

### Table 2

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$, mM</th>
<th>$k_{cat}$, s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short-chain dehydrogenase/reductase (Fatty alcohol forming acyl-CoA reductase) from Marinobacter aquaeolei VT8</td>
<td>8</td>
<td>0.10</td>
</tr>
<tr>
<td>Short chain alcohol dehydrogenase-like protein from Hahella chejuensis</td>
<td>5</td>
<td>0.07</td>
</tr>
</tbody>
</table>

### Example 5: Enzyme-catalyzed dehydration of 4-hydroxy-2-butanone to 3-buten-2-one

Sequences of 3-hydroxypropionyl-CoA and 3-hydroxybutyryl-CoA dehydratases inferred from the genome of archaea and bacteria were generated according to the procedure described in Example 1. The genes thus synthesized were cloned in a pET25b(+) expression vector (vectors were constructed by GeneArt AG) and the proteins were produced according to the procedure described in Example 1.

The purified dehydratases were evaluated for their ability to dehydrate 4-hydroxy-2-butanone.

The studied enzymatic reaction was carried out under the following conditions at 37°C:

- 50 mM Tris-HCl pH 7.5
- 20 mM MgCl$_2$
- 100 mM 4-hydroxy-2-butanone

Reaction volume was 0.4 ml
Each assay was started with the addition of a specific enzyme at a final concentration of 4 mg/ml. Control assays were performed in which either no enzyme was added, or no substrate was added. Test tubes were incubated for 23 hours with shaking at 37°C.
C. Reaction products were extracted with an equal volume of ethyl acetate and then analyzed by HPLC. Commercial 3-buten-2-one (Sigma) was used as reference. HPLC analysis were performed using a 1260 Infinity LC System (Agilent), equipped with column heating module and refractometer and UV detector. 10 µl of samples were separated on Zorbax SB-Aq column (250 x 4.6 mm, 5 µm particle size, column temp. 30°C) with a mobile phase flow rate of 1.5 ml/min. The mobile phase consisted of 98:2 (v/v) H_{2}O/Acetonitrile containing 8.4 mM sulfuric acid. Retention time of 3-buten-2-one under these conditions was 5.4 min.

Example of typical chromatogram obtained for 3-hydroxypropionyl-CoA dehydratase from S. tokodaii is presented in Figure 3. Significant production of 3-buten-2-one was observed in enzymatic assay with several 3-hydroxyacyl-CoA dehydratases (Figure 4).

**Example 6: Kinetic parameters of the dehydration of 4-hydroxy-2-butanone to 3-buten-2-one**

Kinetic parameters of the reaction were determined for purified recombinant 3-hydroxypropionyl-CoA dehydratase from Sulfolobus tokodaii under the following conditions:

100 mM Tris-HCl pH 7.5
20 mM MgCl_{2}
0-300 mM 4-Hydroxy-2-butanone
4 mg/ml 3-Hydroxypropionyl-CoA dehydratase S. tokodaii

Test tubes were incubated for 0, 20, 60, 120 and 180 min with shaking at 37°C. The formation of 3-buten-2-one was quantified using commercial 3-buten-2-one according to the procedure described in Example 4. Hydroxypropionyl-CoA dehydratase from S. tokodaii was found to have a $K_{M}$ higher than 0.4 M and a $k_{cat}$ of $7.5 \times 10^{-3}$ s^{-1}.

**Example 7: Screening of a collection of reductases using 3-buten-2-one as substrate and NADPH as cofactor**
A set of 10 genes encoding representatives of the reductase/alcohol dehydrogenase across eukaryotic and prokaryotic organisms was constructed and tested to identify the potential candidates for 3-buten-2-ol production from 3-buten-2-one. The genes were synthesized and corresponding enzymes were then produced according to the procedure described in Example 1.

For the reductase assay, a reaction mixture containing 50 mM Tris-HCl pH 7.5, 0.4 mM NADPH, 50 mM NaCl, 20 mM 3-buten-2-one and 0.001 - 1 mg/ml enzyme in a total volume of 120 µl was used and the reaction was carried out at 37°C for 20 min. Assays with alcohol dehydrogenase from Thermoanaerobium brockii and short chain alcohol dehydrogenase-like protein from Hahella chejuensis were conducted in 50 mM potassium phosphate buffer pH 7.5 instead of Tris-HCl buffer. Enzyme, substrate and cofactor negative control reactions were performed in parallel. Each sample was continuously monitored for the decrease of NADPH at 340 nm on a SpectraMax Plus384 UV/Vis Microplate Reader (Molecular Device). Several enzymes demonstrated reductase activity with 3-buten-2-one as substrate and NADPH as cofactor (Table 3).

### Table 3

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity, µmol/min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secondary alcohol dehydrogenase Rhodococcus ruber (Uniprot Q8KLT9)</td>
<td>0.069</td>
</tr>
<tr>
<td>Alcohol dehydrogenase Thermoanaerobacter brockii (Thermoanaerobium brockii) (Uniprot P14941)</td>
<td>0.098</td>
</tr>
<tr>
<td>R-specific alcohol dehydrogenase Lactobacillus kefiri (Uniprot Q6WVP7)</td>
<td>26.0</td>
</tr>
<tr>
<td>R-specific alcohol dehydrogenase Lactobacillus brevis (Uniprot Q84EX5)</td>
<td>29.0</td>
</tr>
<tr>
<td>S-specific alcohol dehydrogenase Lactobacillus kefiri (Uniprot B5AXN9)</td>
<td>0.03</td>
</tr>
<tr>
<td>S-specific alcohol dehydrogenase Lactobacillus</td>
<td>0.03</td>
</tr>
</tbody>
</table>
Example 8: HPLC-based analysis of the enzyme-catalyzed reduction of 3-buten-2-one by alcohol dehydrogenase from Thermoanaerobium brockii

The studied enzymatic reaction was carried out under the following conditions at 37°C:
- 50 mM Tris-HCl pH 7.5
- 50 mM NaCl
- 1 mM DTT
- 40 mM NADPH
- 0.1 mM ZnCl₂
- 0-40 mM 3-buten-2-one
- 1 mg/ml alcohol dehydrogenase from Th. brockii

Reaction volume was 200 µl.

The assays were incubated for various time periods (0, 20, 40, 60 120 and 240 min) with shaking at 37°C. The reactions were stopped by heating at 65°C for 5 minutes, reaction mixtures were centrifuged and 150 µl of the clarified supernatant were transferred into a clean vial. The reaction products were then extracted with an equal volume of ethyl acetate. 150 µl of the upper ethyl acetate phase was transferred into a clean vial for HPLC analysis. Commercial 3-buten-2-ol (Sigma) was used as reference. HPLC analysis was performed using a 1260 Infinity LC System (Agilent), equipped with column heating module and refractometer and UV detector. 10 µl of samples were separated on Zorbax SB-Aq column (250 x 4.6 mm, 5 µm particle size, column temp. 30°C) with a mobile phase flow rate of 1.5 ml/min. The mobile phase consisted of 98:2 (v/v) H₂O/acetonitrile containing 8.4 mM sulfuric acid. Retention time of 3-buten-2-ol and 3-buten-2-one under these conditions was 3.9 and 5.4 min, respectively.
Significant and reproducible production of 3-buten-2-ol was observed in enzymatic assay. An example of a typical chromatogram is presented in Figure 5. These data indicate that the reduction of 3-buten-2-one can be achieved using alcohol dehydrogenase in the presence of NADPH as cofactor, leading to the production of 3-buten-2-ol.

Secondary alcohol dehydrogenase from Th. brockii was found to have a $K_M$ of 100 mM and a $k_{cat}$ of at least 0.5 s$^{-1}$.

**Example 9: Kinetic parameters of the reduction of 3-buten-2-one to 3-buten-2-ol by R-specific alcohol dehydrogenase from Lactobacillus kefiri**

The studied enzymatic reaction was carried out under the following conditions at 37°C:

- 50 mM Tris-HCl pH 7.5
- 50 mM NaCl
- 1 mM DTT
- 40 mM NADPH
- 0-40 mM 3-buten-2-one
- 1 mg/ml alcohol dehydrogenase from L. kefiri.

Reaction volume was 200 µl.

The assays were incubated for 0, 10, 20, 40, 60 and 120 min with shaking at 37°C. The formation of 3-buten-2-ol was quantified according to the protocol described in Example 8. R-specific alcohol dehydrogenase from Lactobacillus kefiri was found to have a $K_M$ of 19 mM and a $k_{cat}$ of 4 s$^{-1}$.

**Example 10: Kinetic parameters of the reduction of 3-buten-2-one to 3-buten-2-ol by secondary alcohol dehydrogenase from Rhodococcus ruber**

The studied enzymatic reaction was carried out under the following conditions at 37°C:

- 50 mM Tris-HCl pH 7.5
- 50 mM NaCl
1 mM DTT
40 mM NADPH
0-40 mM 3-buten-2-one
1 mg/ml alcohol dehydrogenase from R. ruber.
Reaction volume was 200 µl.
The assays were incubated for 0, 10, 20, 40, 60 and 120 min with shaking at 37°C.
The formation of 3-buten-2-ol was quantified according to the protocol described in Example 8. Secondary alcohol dehydrogenase from Rhodococcus ruber was found to have a $K_m$ of 180 mM and a $k_{cat}$ of at least 0.03 s⁻¹.

Example 11: Cloning and expression in E.coli of the gene for linalool dehydratase-isomerase

Cloning and bacterial culture
The sequence of linalool dehydratase-isomerase inferred from the genome of Castellaniella defragrans (formerly Alcaligenes defragrans) was generated by oligonucleotide concatenation to fit the codon usage of E. coli. A stretch of 6 histidine codons was inserted after the methionine initiation codon to provide an affinity tag for purification. The gene thus synthesized was cloned in a pET25b(+) expression vector (the vector was constructed by GeneArt AG). Competent E. coli BL21(DE3) cells (Novagen) were transformed with this vector according to the heat shock procedure. As negative control, E. coli BL21(DE3) strain was transformed with empty vector. 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 6 hours at 37°C and protein expression was continued at 18°C overnight (approximately 12 hours). The cells were collected by centrifugation at 4°C, 10,000 rpm for 20 min and the pellets were frozen at -80°C.

Preparation of cell lysate
The pellets from 100 ml of culture cells were thawed on ice and resuspended in 4 ml of 50 mM Tris-HCl pH 7.5. 10 µl of lysonase (Novagen) were then added. Cells were incubated for 10 minutes at room temperature and then returned to ice for 20 minutes. Protein concentration was determined using the Bradford method (Biorad).
Example 12: 1,3-butadiene production from 3-buten-2-ol

The enzymatic assays were carried out under the following conditions:

- 50 mM Tris HCl pH 7.5
- 2 mM D,L-Dithiothreitol
- 0-80 mM 3-buten-2-ol

The pH was adjusted to 7.5.

0.25 ml of cell lysate containing recombinant linalool dehydratase-isomerase was added to 0.5 ml of reaction mixture. An enzyme-free control reaction containing lysate of E. coli cells transformed with empty vector was carried out in parallel. Assays were incubated at 37°C for 1-22 hours in a 2 ml sealed glass vial (Interchim) with shaking. 1,3-butadiene production was analyzed by GC/FID procedure. For this purpose one ml of the headspace phase was then collected and injected into a gas chromatograph Varian 450-GC equipped with a flame ionization detector (FID). Nitrogen was used as carrier gas with a flow rate of 1.5 ml/min. Volatile compounds were chromatographically separated on Rt-Alumina Bond/Na₂SO₄ column (Restek) using an isothermal mode at 130°C. The enzymatic reaction product was identified by comparison with 1,3-butadiene standard (Sigma). Under these GC conditions, the retention time for butadiene was 7.6 min. A significant production of 1,3-butadiene was observed in enzymatic assay with linalool dehydratase-isomerase. No butadiene signal was observed in enzyme-free control assay (Figure 6). The turnover number for this conversion amounted to about 10⁻⁴ s⁻¹ substrate molecule per enzyme active site.
CLAIMS

1. A method for the production of 3-buten-2-one comprising the enzymatic conversion of 4-hydroxy-2-butanone into 3-buten-2-one by making use of an enzyme catalyzing 4-hydroxy-2-butanone dehydration, wherein said enzyme catalyzing 4-hydroxy-2-butanone dehydration is
   (a) a 3-hydroxypropionyl-CoA dehydratase (EC 4.2.1.16),
   (b) a 3-hydroxybutyryl-CoA dehydratase (EC 4.2.1.55),
   (c) an enoyl-CoA hydratase (EC 4.2.1.17),
   (d) a 3-hydroxyoctanoyl-[acyl-carrier-protein] dehydratase (EC 4.2.1.59),
   (e) a crotonyl-[acyl-carrier-protein] hydratase (EC 4.2.1.58),
   (f) a 3-hydroxydecanoyl-[acyl-carrier-protein] dehydratase (EC 4.2.1.60),
   (g) a 3-hydroxypalmitoyl-[acyl-carrier-protein] dehydratase (EC 4.2.1.61),
   (h) a long-chain-enoyl-CoA hydratase (EC 4.2.1.74), or
   (i) a 3-methylglutaconyl-CoA hydratase (EC 4.2.1.18).

2. The method of claim 1, wherein the 4-hydroxy-2-butanone dehydratase is
   (a) a 3-hydroxypropionyl-CoA dehydratase of M. cuprina (SEQ ID NO:1),
   (b) a 3-hydroxypropionyl-CoA dehydratase of S. tokodaii (SEQ ID NO:3),
   (c) a 3-hydroxypropionyl-CoA dehydratase of M. sedula (SEQ ID NO:2),
   (d) a 3-hydroxybutyryl-CoA dehydratase of S. acidocaldarius (SEQ ID NO:5),
   (e) a 3-hydroxybutyryl-CoA dehydratase of A. hospitalis (SEQ ID NO:6), or
   (f) a 3-hydroxypropionyl-CoA dehydratase of B. laterosporus (SEQ ID NO:4).

3. The method of claim 1 or 2, further comprising the enzymatic conversion of acetoacetyl-CoA into said 4-hydroxy-2-butanone by making use of an acetoacetyl-CoA reductase.
4. The method of claim 3 wherein said acetoacetyl-CoA reductase is
   (i) a hydroxymethylglutaryl-CoA reductase (EC 1.1.1.34); or
   (ii) a short chain dehydrogenase/fatty acyl-CoA reductase.

5. The method of any one of claims 1 to 4, further comprising the enzymatic
   conversion of acetyl-CoA into acetoacetyl-CoA.

6. A method for the production of 3-buten-2-ol comprising
   (a) the production of 3-buten-2-one according to a method of any one of
       claims 1 to 5 and further comprising
   (b) the enzymatic conversion of the thus produced 3-buten-2-one into 3-
       buten-2-ol by making use of a 3-buten-2-one reductase.

7. The method of claim 6, wherein said 3-buten-2-one reductase is
   (i) a carbonyl reductase (EC 1.1.1.184),
   (ii) an alcohol dehydrogenase, or
   (iii) a short chain dehydrogenase/fatty acyl-CoA reductase.

8. A method for the production of 1,3-butadiene comprising
   (a) the production of 3-buten-2-ol according to a method of claim 6 or 7;
       and
   (b) enzymatically converting the thus produced 3-buten-2-ol into 1,3-
       butadiene by the use of an alkenol dehydratase.

9. An organism or microorganism which expresses
   (i) an enzyme as defined in claim 1 or 2 catalyzing 4-hydroxy-2-butanoone
       dehydration, and
   (ii) a 3-buten-2-one reductase.

10. The organism or microorganism of claim 9, wherein the 3-buten-2-one
    reductase is
    (a) a carbonyl reductase (EC 1.1.1.184),
    (b) an alcohol dehydrogenase, or
    (c) short chain dehydrogenase/fatty acyl-CoA reductase.
11. The organism or microorganism of claim 9 or 10 further expressing:
   (i) an alkenol dehydratase.

12. The organism or microorganism of any one of claims 9 to 11, further expressing
   (i) an acetoacetyl-CoA reductase; or
   (ii) an acetoacetyl-CoA reductase and an enzyme capable of converting
        acetyl-CoA into acetoacetyl-CoA.

13. A composition comprising the organism or microorganism of any one of claims
    9 to 12 and, optionally, acetoacetyl-CoA, 4-hydroxy-2-butanone, 3-buten-2-one
    and/or 3-buten-2-ol.

14. Use of an enzyme as defined in claim 1 or 2 catalyzing 4-hydroxy-2-butanone
    dehydration for the production of 3-butene-2-one from 4-hydroxy-2-butanone.

15. A method for the production of 3-buten-2-ol comprising the enzymatic
    conversion of 4-hydroxy-2-butanone into 3-buten-2-one by making use of an
    enzyme catalyzing 4-hydroxy-2-butanone dehydration and the enzymatic
    conversion of the thus produced 3-buten-2-one into 3-buten-2-ol by making
    use of a 3-buten-2-one reductase.
4-hydroxy-2-butanone

Figure 2
3-buten-2-one peak area, arbitrary units

Control assay without enzyme
3-hydroxypropionyl-CoA dehydratase M. cuprina
3-hydroxypropionyl-CoA dehydratase S. tokodaii
3-hydroxypropionyl-CoA dehydratase M. sedula
3-hydroxybutyryl-CoA dehydratase S. acidocaldarius
3-hydroxybutyryl-CoA dehydratase A. hospitalis
3-hydroxypropionyl-CoA dehydratase B. laterosporus

Figure 4
Gas chromatography signal μV

1,3-butadiene produced in enzymatic assay with linalool dehydratase-isomerase

No butadiene in enzyme-free control assay

Figure 6