

- 1 -

Method for the enzymatic production of 2-hydroxy-2-methyl carboxylic acids

The present invention relates to a method for the enzymatic production of 2-hydroxy-2-methyl carboxylic acids from 3-hydroxy carboxylic acids, where a 3-hydroxy carboxylic acid is produced in an aqueous reaction solution and/or is added to this reaction solution and is incubated. The aqueous reaction solution comprises a unit having 3-hydroxy-carboxylate-CoA mutase activity which has both 3-hydroxy-carbonyl-CoA ester-producing and 3-hydroxy-carbonyl-CoA ester-isomerizing activity and converts the 3-hydroxy carboxylic acid into the corresponding 2-hydroxy-2-methyl carboxylic acid which is isolated as acid or in the form of its salts. In a preferred embodiment, the unit having 3-hydroxy-carboxylate-CoA mutase activity comprises an isolated cobalamin-dependent mutase and where appropriate a 3-hydroxy-carbonyl-CoA ester-producing enzyme or enzyme system or is a microorganism including them. The invention preferably relates to a biotechnological process for producing 2-hydroxy-2-methyl carboxylic acids, where microorganisms which have the desired mutase activity are cultured in an aqueous system with the aid of simple natural products and intracellularly formed 3-hydroxy-carbonyl-CoA esters are converted into the corresponding 2-hydroxy-2-methyl carboxylic acids. The invention likewise encompasses the production of unsaturated 2-methyl carboxylic acids, where the 2-hydroxy-2-methyl carboxylic acids obtained are converted by dehydration into the corresponding unsaturated 2-methyl carboxylic acids (methacrylic acid and higher homologs).

In a preferred embodiment of the invention, the 3-hydroxy-carbonyl-CoA thioester-producing and 3-hydroxy-carbonyl-CoA thioester-isomerizing microorganism used

is the strain HCM-10 (DSM 18028).

Methacrylic acid and homologous unsaturated 2-methyl
carboxylic acids are widely used in the production of
5 acrylic glass sheets, injection-molded products,
coatings and many other products.

A plurality of processes for the production of
methacrylic acid and its homologs have been disclosed.
10 However, the vast majority of the commercial production
worldwide is based on a method of hydrolyzing the amide
sulfates of methacrylic acid and its homologs, which
are produced from the corresponding 2-hydroxy nitriles
(W. Bauer, "Metharylic acid and derivatives", in:
15 Ullmann's Encyclopedia of Industrial Chemistry, 5th
edition, editors: B. Elvers, S. Hawkins, G. Schulz,
VCH, New York, 1990, Vol. A16, pp. 441-452; A. W.
Gross, J. C. Dobson, "Methacrylic acid and
derivatives", in Kirk-Othmer Encyclopedia of Chemical
20 Technology, 4th edition, editors: J. I. Kroschwitz, M.
Howe-Grant, John Wiley & Sons, New York, 1995, Vol. 16,
pp. 474-506). This method requires, for example, about
1.6 kg of sulfuric acid for the production of 1 kg of
methacrylic acid. For this reason, alternative methods
25 for the commercial production of methacrylic acid
without the requirement of recovering the sulfuric acid
(and the high energy costs associated therewith) would
be advantageous.

30 US 3,666,805 and US 5,225,594 have disclosed the
chemical conversion of 2-hydroxy isobutyric acid to
methacrylic acid. This comprises dehydrating 2-hydroxy
isobutyric acid by using metal oxides, metal
hydroxides, ion exchange resins, alumina, silicon
35 dioxide, amines, phosphines, alkali metal alkoxides and
alkali metal carboxylates. Usual reaction temperatures
are between 160°C and 250°C. This method made possible
methacrylic acid yields of up to 96%.

An alternative method for the production of methacrylic acid and its homologs is based on the hydrolysis of 2-hydroxy nitriles to the corresponding 2-hydroxy-2-methyl carboxylic acids, utilizing nitrile-hydrolyzing enzymes. The latter are nitrilase or a combination of nitrile hydratase and amidase (A. Banerjee, R. Sharma, U. C. Banerjee, 2002, "The nitrile-degrading enzymes: current status and future prospects", Appl. Microbiol. Biotechnol., 60:33-44). This method is protected by a plurality of patents (US 6,582,943 B1). A severe disadvantage of this method is the instability of the nitriles in the neutral pH range required for an efficient nitrile-hydrolyzing enzyme activity. The decomposition of the nitriles in the reaction mixture results in accumulation of ketones and cyanides, both of which inhibit the nitrile-hydrolyzing enzyme activities.

A general disadvantage of both methods, i.e. of the currently dominating method based on amide sulfates and of the enzymatic nitrile-hydrolyzing method, is the need for 2-hydroxy nitriles. The latter must first be prepared from environmentally harmful reactants, namely ketones and cyanide.

For this reason, methods for the production of methacrylic acid and its homologs, which are based on simple environmentally benign reactants, would be advantageous.

It was therefore the object of the invention to search for alternative possibilities of producing 2-hydroxy-2-methyl carboxylic acids and to provide methods which are based, where possible, on the application of simple, environmentally benign reactants, consume little energy and produce few waste products.

The object is achieved by an enzymatic method for the production of 2-hydroxy-2-methyl carboxylic acids from

3-hydroxy carboxylic acids. According to the invention, said 3-hydroxy carboxylic acid is produced in and/or added to an aqueous reaction solution which has a unit having 3-hydroxy-carboxylate-CoA mutase activity. A
5 unit having 3-hydroxy-carboxylate-CoA mutase activity means for the purpose of the invention a unit comprising a cobalamin-dependent mutase and, where appropriate, a 3-hydroxy-carbonyl-CoA ester-producing enzyme or enzyme system or a biological system
10 comprising or producing them, which have 3-hydroxy-carboxylate-CoA mutase activity and exhibit both 3-hydroxy-carbonyl-CoA ester-producing and 3-hydroxy-carbonyl-CoA ester-isomerizing activity. After incubation the correspondingly converted 2-hydroxy-2-
15 methyl carboxylic acid is then isolated as acid or in the form of its salts.

The invention preferably relates to a biotechnological process for the production of 2-hydroxy-2-methyl
20 carboxylic acids with the use of microorganisms. Said microorganisms usually have 3-hydroxy-carbonyl-CoA ester-synthesizing activity and are capable of producing or comprise such a cobalamin-dependent mutase and, due to the 3-hydroxy-carboxylate-CoA mutase
25 activity, are capable of converting intracellularly 3-hydroxy-carbonyl-CoA esters formed from simple natural products (from reactants such as, for example, sugars and/or alcohols and/or organic acids and their derivatives) to the corresponding 2-hydroxy-2-methyl-
30 carbonyl CoA esters.

The method of the invention is characterized in particular in that microorganisms which produce or comprise the cobalamin-dependent mutase and have 3-
35 hydroxy-carboxylate-CoA mutase activity are used in aqueous systems for converting 3-hydroxy carboxylic acids to the corresponding 2-hydroxy-2-methyl carboxylic acid.

In a preferred variant method, microorganisms which comprise 3-hydroxy-carboxylate-CoA mutase activity and have both 3-hydroxy-carbonyl-CoA thioester-producing and 3-hydroxy-carbonyl-CoA thioester-isomerizing activity are cultured in an aqueous system with renewable raw materials or waste products deriving from the consumption of renewable raw materials as carbon and energy sources. In the process, the intracellularly formed 3-hydroxy-carboxylate-CoA thioesters are converted to the corresponding 2-hydroxy-2-methyl carboxylic acids. The reaction is preferably carried out with the addition of external 3-hydroxy carboxylic acid. The corresponding 2-hydroxy-2-methyl carboxylic acid is then isolated as acid or in the form of its salts.

This novel biotechnology method which utilizes the production of 3-hydroxy carboxylic acids from simple natural products and their isomerization to 2-hydroxy-2-methyl carboxylic acids is capable of solving the problem specified above.

In a preferred embodiment of the invention, the method comprises the following steps

- (a) 3-hydroxy carboxylic acids are produced from simple natural products and then converted to 2-hydroxy-2-methyl carboxylic acids in a suitable biological system which has 3-hydroxy-carbonyl-CoA ester-synthesizing activity and mutase activity, and
- (b) the 2-hydroxy-2-methyl carboxylic acids are isolated as free acids or as their corresponding salts.

The 2-hydroxy-2-methyl carboxylic acids obtained in this way may be used advantageously for producing C2-C3-unsaturated iso-alkenoic acids (methacrylic acid and its homologs), possibly by dehydration of the acids produced in (a) and (b) or their corresponding salts. These reactions are depicted below:

- simple natural products (e.g. renewable raw materials or waste products deriving from the consumption of renewable raw materials, such as, for example, sugars, organic acids or alcohols) → 3-hydroxy carboxylic acids → 2-hydroxy-2-methyl carboxylic acids (e.g. by strain HCM-10)
- 2-hydroxy-2-methyl carboxylic acids → methacrylic acid and homologs (e.g. in the presence of NaOH and a temperature of 185°C).

10

The reaction conditions (pH, ion concentration, oxygen/carbon dioxide requirements, trace elements, temperatures and the like) are of course chosen here in such a way that the microorganisms are enabled to optimally convert 3-hydroxy carboxylic acids to 2-hydroxy-2-methyl carboxylic acids. Under these process conditions, the cobalamin-dependent mutase may have higher stability and efficacy in the natural micro environment, i.e. inside the cell, than the isolated enzyme. In addition, cell propagation and thus an increase in mutase concentration may be possible under suitable conditions. The enzymatic conversion by means of microorganisms thus constitutes, where appropriate, an important advantage regarding reliability, automation and simplicity as well as quality and yield of the final product of the method..

For the enzymatic conversion according to the invention of 3-hydroxy carboxylic acids to 2-hydroxy-2-methyl carboxylic acids, it is also possible to introduce into the reaction solution the unit having 3-hydroxy-carboxylate-CoA mutase activity, i.e. a cobalamin-dependent mutase, preferably in combination with a CoA ester-synthesizing activity, in a purified, concentrated and/or isolated form, it being possible for the enzymes to be of natural origin, for example. The enzymes may of course be recombinantly produced enzymes from a genetically modified organism.

For the purpose of the invention, the enzymes are used in the method of the invention as catalysts both in the form of intact microbial cells and in the form of permeabilized microbial cells. Further possible uses
5 are those in the form of components (one or more) from microbial cell extracts, but also in a partially purified or purified form. Where appropriate, other CoA ester-synthesizing enzymes, for example CoA transferase or CoA sythetases, are used according to the invention.
10 The enzymatic catalysts may be immobilized or attached to a dissolved or undissolved support material.

In a preferred variant embodiment, particular cell compartments or parts thereof separated from one
15 another or combined, i.e. carbohydrate structures, lipids or proteins and/or peptides and also nucleic acids, which are capable of influencing the unit having mutase activity in a positive or negative way may be combined or separated. In order to utilize such an
20 influence consciously, for example, crude extracts are prepared from the microorganisms in an expert manner for example, which extracts are centrifuged, where appropriate, to be able to carry out a reaction of the invention with the sediment or the supernatant.

25 3-hydroxy carboxylic acids (for example 3-hydroxy butyric acid) or more specifically their intracellular CoA thioester 3-hydroxy-carbonyl-CoA, may readily be produced from simple natural products by a large number
30 of bacteria strains. These acids are the basic building blocks/monomers for the common bacterial carbon and energy storage substance, poly-3-hydroxyalkanoate. Rearrangements of the carbon within the skeleton of carboxylic acids are likewise common in bacterial as
35 well as in other biological systems. However, no biological system for converting 3-hydroxy-carbonyl-CoA esters to the corresponding 2-hydroxy-2-methyl-carbonyl-CoA esters has previously been identified. The invention is based on the surprising finding that

systems with cobalamin-dependent mutase activity have both properties.

Microorganisms comprising cobalamin-dependent mutases are, for example, *Methylibium petroleiphilum* PM1, *Methylibium* sp. R8 (strain collection UFZ, Leipzig, Germany), the β -proteobacterial strain HCM-10, *Xanthobacter autotrophicus* Py2, *Rhodobacter sphaeroides* (ATCC17029) or *Nocardioides* sp. JS614.

10

A preferably suitable biological system has been found in the strain HCM-10. Said strain has been deposited in accordance with the Budapest Treaty on the deposit of microorganisms for the purposes of patent procedure at the Deutschen Sammlung von Microorganismen und Zellkulturen GmbH [German collection of microorganisms and cell cultures], Brunswick, Germany, under No. DSM 18028 on 13.03.2006.

20 Using this preferred biological system, it has been possible to achieve a particularly good yield of 2-hydroxy-2-methyl carboxylic acids, in particular 2-hydroxy isobutyric acid. However, the enzymatic conversion by microorganisms is not at all limited to this strain. Any organisms capable of converting 3-hydroxy carboxylic acids to 2-hydroxy-2-methyl carboxylic acids may be used according to the invention.

30 They may be microorganisms which firstly possess the same gene or gene product or secondly have an analogous gene resulting in gene products having a similar or analogous activity. I.e., 3-hydroxy-carbonyl-CoA mutase activities of other origin are likewise covered by the invention. The invention also includes transformed systems which have a or a similar 3-hydroxy-carbonyl-CoA mutase activity as strain HCM-10 or that of other origin.

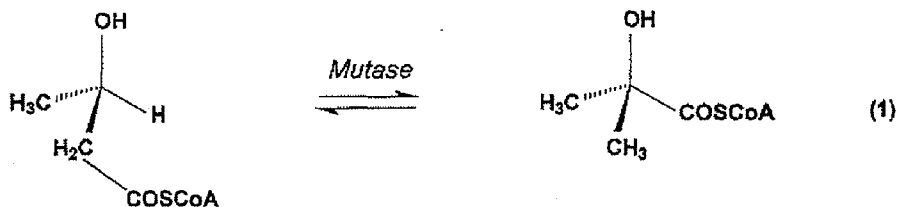
35 This may include mutants, genetically modified and

isolated modifications of the microorganisms, for example organisms which have the desired cobalamin-dependent mutase activity owing to the introduction of a mutase-encoding nucleotide sequence.

5

The preferably used biological system (strain HCM-10 - DSM 18028) produces 3-hydroxy-carbonyl-CoA esters as thioesters from simple natural products such as sugars and/or organic acids and/or alcohols and their derivatives. In the preferred system used herein, the 3-hydroxy-carbonyl-CoA esters are converted by the cobalamin-dependent carbon skeleton-rearranging mutase to 2-hydroxy-2-methyl-carbonyl-CoA esters, as depicted by way of example for the case of (R)-3-hydroxy-butyryl CoA in equation 1. The CoA thioester is hydrolyzed in the system and the acid is secreted into the culture medium.

10
15



20

(R)-3-hydroxybutyryl CoA 2-hydroxyisobutyryl CoA

Preference is given to using as enzyme catalysts in the method of the invention the microorganism strains comprising cobalamin-dependent mutases, HCM-10 (DSM 18028), *Xanthobacter autotrophicus* Py2, *Rhodobacter sphaeroides* (ATCC17029) or *Nocardioides* sp. JS614, their crude extracts or parts. The strains used according to the invention preferably produce the proteins with the sequences SEQ ID NO: 2 and/or SEQ ID NO: 4 or comprise the nucleic acid sequences SEQ ID NO: 1 and/or SEQ ID NO: 3 (HCM-10), the proteins with the sequences SEQ ID NO: 5 and/or SEQ ID NO: 6, or comprise the nucleic acid sequences SEQ ID NO: 7 and/or SEQ ID

25
30

NO: 8 (*Xanthobacter autotrophicus* Py2), the proteins with the sequences SEQ ID NO: 9 and/or SEQ ID NO: 10, or comprise the nucleic acid sequences SEQ ID NO: 11 and/or SEQ ID NO: 12 (*Rhodobacter sphaeroides* ATCC 17029) or the proteins with the sequences SEQ ID NO: 13 and/or SEQ ID NO: 14, or comprise the nucleic acid sequences SEQ ID NO: 15 and/or SEQ ID NO: 16 (*Nocardioides* sp. JS614). For the purposes of the invention, said proteins may also be used in a concentrated, isolated or synthetically produced form.

In a further preferred variant embodiment of the invention, the enzyme catalysts, in particular microorganisms, crude extracts, parts thereof and/or the concentrated or isolated enzymes are used in an immobilized form. Immobilization renders enzymes, cell organelles and cells insoluble and limited in reaction space. For example, they may be immobilized in a polymer matrix (e.g. alginate, polyvinyl alcohol or polyacrylamide gels). They may also be immobilized on dissolved or undissolved support materials (e.g. celite) to facilitate catalyst recovery and reuse. Methods of cell immobilization in a polymer matrix or on a dissolved or undissolved support are known to the skilled worker and have been described in detail previously. The enzyme activities may likewise be isolated from the microbial cells. They may then be used directly as catalyst or in an immobilized form in a polymer matrix or on a dissolved or undissolved support. The methods required for this are known to the skilled worker and, for example, described in *Methods in Biotechnology, Vol. 1: Immobilization of enzymes and cells*, editor: G. F. Bickerstaff, Humana Press, Totowa, New Jersey, 1997.

35

3-hydroxy carboxylic acids are converted to 2-hydroxy-2-methyl carboxylic acids preferably within the framework of a continuous process which may be carried out in a flow reactor in which microbial growth and

thus product formation takes place. However, a continuous process may also mean any system of growing cells and catalyzing enzymes, which is supplied with nutrient solution and from which culture solution, including enzymatically formed 2-hydroxy-2-methyl carboxylic acid is removed. According to the invention, the process may also be carried out as semicontinuous or batch process.

As explained above, 3-hydroxy carboxylic acid which is the starting material for 2-hydroxy-2-methyl carboxylic acid is produced preferably by enzymatic conversion of carbohydrates and/or organic acids and/or alcohols or their derivatives. In the context of the invention, use is made, aside from the cobalamin-dependent mutase, where appropriate furthermore of CoA ester-synthesizing enzymes which are present in or added to the microorganism. This involves the conversion of hydrocarbons and/or carbohydrates and/or organic acids and/or alcohols or derivatives thereof to the 3-hydroxy carboxylic acid and of the 3-hydroxy carboxylic acid to the 2-hydroxy-2-methyl carboxylic acid in a single process step, i.e. conversion of the starting substrates up to 3-hydroxy carboxylic acid and the enzymatic conversion reactions of 3-hydroxy carboxylic acid to the corresponding 2-hydroxy-2-methyl carboxylic acid are carried out at the same time or with a slight time delay in one and the same reaction solution.

In a very particular embodiment of the invention, a substrate with a tert-butyl radical as carbon source and energy source is used for culturing, with preference being given to tert-butyl alcohol being the sole carbon and energy source in a basal medium.

35

The method of the invention is preferably useful for the production of 2-hydroxy-2-methyl propanoic acid (2-hydroxy isobutyric acid). The preferred production of 2-hydroxy isobutyric acid is furthermore characterized

in that 3-hydroxy butyric acid is added externally.

The method may be carried out aerobically, preferably with the use of intact cells, or else unaerobically, for example with gassing with nitrogen, preferably when
5 extracts or purified enzymes are used.

The invention also relates to nucleic acid molecules coding for an enzyme having the activity of a
10 cobalamin-dependent mutase, selected from the group consisting of

- a) nucleic acid molecules coding for a protein comprising the amino acid sequences indicated under Seq. No. 2 and/or Seq. No. 4;
- 15 b) nucleic acid molecules comprising the nucleotide sequence depicted under Seq. No. 1 and/or Seq. No. 3.

An enzyme of the invention has been shown to be
20 preferably a heterodimeric protein which comprises the sub units described under Seq. No. 2 and Seq. No. 4 and thus has excellent enzyme activity.

A nucleic acid molecule may be a DNA molecule, preferably cDNA or genomic DNA and/or an RNA molecule.
25 Both nucleic acids and proteins may be isolated from natural sources, preferably from DSM 18028, but also, for example, from *Methylibium petroleiphilum* PM1, *Methylibium* sp. R8 (strain collection UFZ Leipzig, Germany), *Xanthobacter autotrophicus* Py2, *Rhodobacter*
30 *sphaeroides* (ATCC17029) or *Nocardioides* sp. JS614 or they may be synthesized by known methods.

Mutations may be generated in the nucleic acid
35 molecules used according to the invention by means of molecular biology techniques known per se, thereby enabling further enzymes with analogous or similar properties to be synthesized, which are likewise used in the method of the invention. Mutations may be

deletion mutations which result in truncated enzymes. Modified enzymes with similar or analogous properties may likewise be generated by other molecular mechanisms such as, for example, insertions, duplications, 5 transpositions, gene fusion, nucleotide exchange or else gene transfer between different microorganism strains.

Such nucleic acid molecules may be identified and 10 isolated using the nucleic acid molecules or parts thereof. The molecules hybridizing with the nucleic acid molecules also comprise fragments, derivatives and allelic variants of the above-described nucleic acid molecules, which code for an enzyme usable according to 15 the invention. Fragments here mean parts of nucleic acid molecules, which are long enough to encode the enzyme described. Derivative means sequences of these molecules, which differ from the sequences of the above-described nucleic acid molecules in one or more 20 positions but which have a high degree of homology to these sequences. Homology here means a sequence identity of at least 40%, in particular an identity of at least 60%, preferably over 80% and particularly preferably over 90%, 95%, 97% or 99%, at the nucleic 25 acid level. The encoded enzymes here have a sequence identity to the amino acid sequences specified of at least 60%, preferably of at least 80%, particularly preferably of at least 95%, very particularly preferably at least 99%, at the amino acid level. The 30 deviations here may be the result of deletion, substitution, insertion or recombination. They may be naturally occurring variations, for example sequences from other organisms, or else mutations which may occur naturally or by specific mutagenesis (UV rays, X rays, 35 chemical agents or others). The variants may also be synthetically produced sequences. These variants have particular common characteristics such as, for example, enzyme activity, active enzyme concentration, subunits, functional groups, immunological reactivity,

conformation and/or physical properties such as the migration behavior in gel electrophoresis, chromatographic behavior, solubility, sedimentation coefficients, pH optimum, temperature optimum, spectroscopic properties, stability and/or others.

The invention furthermore also relates to the novel proteins with the sequence No. 2 and 4 and to a heterodimeric protein comprising the sequence No. 2 and sequence No. 4 and their at least 99% homologs.

SEQ ID NO: 1 depicts the 1644 bp nucleotide sequence for the large subunit of the cobalamin-dependent mutase from DSM 18028.

SEQ ID NO: 2 depicts the 548 aa amino acid sequence of the large subunit of the cobalamin-dependent mutase from DSM 18028.

SEQ ID NO: 3 depicts 369 bp of the partial nucleotide sequence for the small subunit of the cobalamin-dependent mutase from DSM 18028.

SEQ ID NO: 4 depicts the 123 aa partial sequence of the subunit of the cobalamin-dependent mutase from DSM 18028.

SEQ ID NO: 5 and 6 depict the 562 and 135 aa, respectively, amino acid sequences of a cobalamin-dependent mutase from *Xanthobacter autotrophicus* Py2.

SEQ ID NO: 7 and 8 depict the 1689 and 408 bp, respectively, of the nucleotide sequence for the cobalamin-dependent mutases from *Xanthobacter autotrophicus* Py2.

SEQ ID NO: 9 and 10 depict the 563 and 135 aa, respectively, amino acid sequences of a cobalamin-dependent mutase from *Rhodobacter sphaeroides* ATCC 17029.

SEQ ID NO: 11 and 12 depict the 1692 and 408 bp, respectively, of the nucleotide sequence for the cobalamin-dependent mutases from *Rhodobacter sphaeroides* ATCC 17029.

SEQ ID NO: 13 and 14 depict the 569 and 164 aa, respectively, amino acid sequences of a cobalamin-dependent mutase from *Nocardoides* sp. JS614.

- 5 SEQ ID NO: 15 and 16 depict the 1710 and 495 bp, respectively, of the nucleotide sequence for the cobalamin-dependent mutases from *Nocardoides* sp. JS614.

10 The 2-hydroxy-2-methyl carboxylic acids produced according to the invention may be isolated by treating the culture medium (after removing undissolved components such as microbial cells) by previously disclosed methods. Examples of such methods are, among others, concentration, ion exchange, distillation, 15 electrodialysis, extraction and crystallization. The product may be isolated as salt or (after acidification) as protonated 2-hydroxy-2-methyl carboxylic acid.

20 2-hydroxy-2-methyl carboxylic acids (or their corresponding salts) may be dehydrated by a multiplicity of methods to give the corresponding unsaturated 2-methyl carboxylic acids. C2-C3-unsaturated isoalkenoic acids are produced by 25 dehydrating the 2-hydroxy-2-methyl carboxylic acid produced, using the known methods of the prior art. The 2-hydroxy-2-methyl carboxylic acids may be dehydrated using metal oxides, metal hydroxides, ion exchange resins, alumina, silicon dioxide, amines, phosphines, 30 alkali metal alkoxides and alkali metal carboxylates. Reaction temperatures are usually between 160°C and 250°C. Thus, for example, methacrylic acid is produced by dehydrating 2-hydroxy isobutyric acid in the presence of NaOH at temperatures of approx. 185°C.

35

The methacrylic acid produced by this process and its homologs are appropriately applied in a whole number of industrial sectors, for example as additives and in coatings. In contrast to the previously known methods,

the method combines the desired advantages of a low temperature process, the use of environmentally benign reactants and lower waste production.

- 5 The invention will be described in more detail below on the basis of exemplary embodiments but is not intended to be limited thereto.

Material and methods

10

Microbial enzyme catalyst

- Microbial cells of strain HCM-10 (DSM 18028), characterized by a 3-hydroxy-carbonyl-CoA ester-producing and 3-hydroxy-carbonyl-CoA ester-isomerizing
15 activity, or the protein subunits with sequence No. 2 and No. 4 isolated therefrom.

Growth of the microbial enzyme catalysts

- The microbial strain used for the production of 2-
20 hydroxy-2-methyl carboxylic acids was isolated as described hereinbelow. Stock cultures are stored in 20% strength glycerol solution in liquid nitrogen.

- Strain HCM-10 was concentrated from ground water on a
25 basal medium (table 1) containing tert-butyl alcohol as sole carbon and energy source.

The strain belongs phylogenetically to the Rubrivivax-Leptothrix group.

Table 1

Basal medium (mg/L)			
NH ₄ Cl	761.4	Biotin	0.02
KH ₂ PO ₄	340.25	Folic acid	0.02
K ₂ HPO ₄	435.45	Pyridoxine-HCl	0.1
CaCl ₂ × 6 H ₂ O	5.47	Thiamin-HCl	0.05
MgSO ₄ × 7 H ₂ O	71.2	Riboflavin	0.05
ZnSO ₄ × 7 H ₂ O	0.44	Nicotinic acid	0.05
MnSO ₄ × H ₂ O	0.615	DL-Ca-pantothenate	0.05
CuSO ₄ × 5 H ₂ O	0.785	p-aminobenzoic acid	0.05
CoCl ₂ × 6 H ₂ O	0.2	Liponic acid	0.05
Na ₂ MoO ₄ × 2 H ₂ O	0.252		
FeSO ₄ × 7 H ₂ O	4.98	pH	7.0

Strain HCM-10 was grown aerobically under the following
 5 conditions (table 2) for assaying 3-hydroxy-carbonyl-CoA mutase activity.

Table 2

Strain	Substrate	Medium	Temperature (°C)	Time (d)
HCM-10	tert-butyl alcohol (0.5 g/L)	Basal medium	25	7

10

The cells were used immediately after harvesting. Intact cells may be used without further pretreatment such as, for example, permeabilization. Moreover, the cells may be used in a permeabilized form (for example
 15 by treatment with toluene, detergents or by freeze-thaw cycles) in order to improve the rates of diffusion of substances into the cells and out of the cells.

The concentration of 2-hydroxy isobutyric acid and 3-
 20 hydroxy butyric acid in the culture liquid or in the reaction mixture were determined by gas chromatography after acidic methanolysis, utilizing an FFAP and an FID

detector.

Example 1:

Conversion of 3-hydroxy butyric acid to 2-hydroxy
5 isobutyric acid by strain HCM-10

A suspension of 1 g (dry mass) of cells of strain HCM-
10 in 100 ml of basal medium was introduced into 120 ml
serum bottles. This suspension was admixed with 50 mg
10 of 3-hydroxy butyric acid, and the suspension was
incubated on a rotary shaker at 30°C. After 0.3 h of
aerobic incubation, the suspension was gassed with
nitrogen and incubated with shaking at 30°C for another
4.4 h. At various times, samples were taken and the 2-
15 hydroxy isobutyric acid content and 3-hydroxy butyric
acid content in the cell-free supernatant were
determined after centrifugation of the suspension. 2-
hydroxy isobutyric acid was found to be the sole
product released in the anaerobic phase. In contrast,
20 3-hydroxy butyric acid was evidently completely
degraded in the aerobic initial phase (fig. 1). The
yield of 2-hydroxy isobutyric acid was in this case
5.1%, with approx. 80% of 3-hydroxy butyric acid
remaining in the reaction liquid.

25

Example 2:

Conversion of 3-hydroxy butyric acid to 2-hydroxy
isobutyric acid by a crude extract of strain HCM-10

30 Cell-free crude extract of strain HCM-10 was prepared
by disintegrating the cells in a ball mill, and cell
debris was subsequently removed by centrifugation.
Cell-free crude extract at a concentration of 10 mg of
protein in 5 ml of 50 mM potassium phosphate buffer
35 (contains 1 mM $MgCl_2$ at pH 7.2) was introduced into
sealable 10 ml glass vessels. To this extract were then
added 0.01 mM coenzyme B12, 1 mM coenzymeA, 1 mM ATP
and 4.25 mg of 3-hydroxy butyric acid. The reaction
liquid was gassed with nitrogen, the reaction vessel

was sealed tightly and incubated with shaking at 30°C for 2 h. The reaction products were analyzed as illustrated above. The yield of 2-hydroxy isobutyric acid was in this case 9%, with approx. 88% of 3-hydroxy butyric acid remaining in the reaction liquid (fig. 2).

Example 3:

Dehydration of 2-hydroxy isobutyric acid to methacrylate

10

A solution of 2-hydroxy isobutyric acid (1 mg/5 ml) produced according to the procedure carried out in example 2 was admixed with NaOH (0.06 mg) with stirring. The solution was incubated with stirring and cooling at reflux under reduced pressure (300 torr) at 185-195°C. Further aliquots of 0.5 mg of 2-hydroxy isobutyric acid per 5 ml were added every hour over a period of 5 h, said aliquots additionally containing 0.4 percent by weight of p-methoxyphenol in order to prevent polymerization of methacrylate. The reaction was stopped after 24 h of incubation. The conversion of 2-hydroxy isobutyric acid to methacrylate was 97%. Methacrylic acid was removed from the reaction mixture by distillation.

Claims

1. A method for the enzymatic production of 2-hydroxy-2-methyl carboxylic acids from 3-hydroxy carboxylic acids, characterized in that a 3-hydroxy carboxylic acid is produced in and/or added to an aqueous reaction solution comprising a unit having 3-hydroxy-carboxylate-CoA mutase activity, which has both 3-hydroxy-carbonyl-CoA ester-producing and 3-hydroxy-carbonyl-CoA ester-isomerizing activity, and, after incubation, the correspondingly converted 2-hydroxy-2-methyl carboxylic acid is isolated as acid or in the form of its salts.
2. A method as claimed in claim 1, characterized in that the unit having 3-hydroxy-carboxylate-CoA mutase activity comprises an isolated cobalamin-dependent mutase.
3. A method as claimed in claim 1 or 2, characterized in that the unit having 3-hydroxy-carboxylate-CoA mutase activity comprises a 3-hydroxy-carbonyl-CoA ester-producing enzyme or enzyme system.
4. A method as claimed in any of claims 1 to 3, characterized in that the unit having 3-hydroxy-carboxylate-CoA mutase activity is a microorganism or a crude extract thereof.
5. A method as claimed in claim 1 or 4, characterized in that microorganisms producing or comprising a cobalamin-dependent mutase, having 3-hydroxy-carboxylate-CoA mutase activity and having both 3-hydroxy-carbonyl-CoA ester-producing and 3-hydroxy-carbonyl-CoA ester-isomerizing activity are used in aqueous systems for converting 3-

hydroxy carboxylic acids to the corresponding 2-hydroxy-2-methyl carboxylic acid.

- 5 6. A method as claimed in any of claims 1 to 5, characterized in that
 - 10 a) microorganisms with 3-hydroxy-carboxylate-CoA mutase activity, which have both 3-hydroxy-carbonyl-CoA thioester-producing activity and 3-hydroxy-carbonyl-CoA thioester-isomerizing activity, are cultured in an aqueous system with renewable raw materials or waste products deriving from the consumption of renewable raw materials as carbon and energy sources, whereby intracellular 3-hydroxy-carboxylate-CoA
 - 15 thioesters are synthesized and converted to the corresponding 2-hydroxy-2-methyl carboxylic acids, and
 - 20 b) the corresponding 2-hydroxy-2-methyl carboxylic acid is isolated as acid or in the form of its salts.
- 25 7. A method as claimed in any of claims 1 to 6, characterized in that the reaction is carried out with the addition of external 3-hydroxy carboxylic acid.
- 30 8. A method as claimed in any of claims 1 to 7, characterized in that the microorganism is selected from the bacteria strain HCM-10 DSM 18028, *Xanthobacter autotrophicus* Py2, *Rhodobacter sphaeroides* (ATCC17029) or *Nocardioides* sp. JS614.
- 35 9. A method as claimed in any of claims 1 to 8, characterized in that intact cells of the microorganisms are used unchanged, permeabilized or fixed to a support.
10. A method as claimed in any of claims 1 to 8, characterized in that cell extracts and/or the

5 cobalamin-dependent mutase and, where appropriate, the other enzymes such as, for example, CoA ester-synthesizing enzymes, after partial or complete isolation from the microorganisms are used, where appropriate in a purified form.

- 10 11. A method as claimed in claim 10, characterized in that cell-free crude extracts of the microorganisms are used.
- 15 12. A method as claimed in any of claims 1 to 11, characterized in that the proteins with the sequences SEQ ID NO: 2 and/or SEQ ID NO: 4, with the sequences SEQ ID NO: 5 and/or SEQ ID NO: 6, the proteins with the sequences SEQ ID NO: 9 and/or SEQ ID NO: 10, or the proteins with the sequences SEQ ID NO: 13 and/or SEQ ID NO: 14 are used and their at least 60% homologs.
- 20 13. A method as claimed in any of claims 1 to 12, characterized in that a sugar and/or an alcohol and/or an organic acid and/or a hydrocarbon or their derivatives are used as carbon and energy sources for enzymatic conversion during culturing.
- 25 14. A method as claimed in any of claims 1 to 13, characterized in that a substrate with a tert-butyl radical is used as carbon source and energy source for culturing.
- 30 15. A method as claimed in any of claims 1 to 14, characterized in that tert-butyl alcohol is used as sole carbon source and energy source in a basal medium for culturing.
- 35 16. A method as claimed in any of claims 1 to 15, characterized in that the enzymatic conversion of a sugar and/or an alcohol and/or an organic acid and/or a hydrocarbon or their derivatives to the

3-hydroxy carboxylic acid and of the 3-hydroxy carboxylic acid to the 2-hydroxy-2-methyl carboxylic acid is carried out in a single method step.

5

17. A method as claimed in any of claims 1 to 16, characterized in that 2-hydroxy isobutyric acid is produced with the addition of external 3-hydroxy butyric acid.

10

18. A method for the production of C2-C3-unsaturated isoalkenoic acids, characterized in that a 2-hydroxy-2-methyl carboxylic acid produced as claimed in any of claims 1 to 17 is dehydrated.

15

19. A method for the production of methacrylic acid, characterized in that a 2-hydroxy isobutyric acid produced as claimed in any of claims 1 to 17 is dehydrated.

20

20. A microorganism strain HCM-10 - DSM 18028.

21. A nucleic acid molecule coding for an enzyme having the activity of a cobalamin-dependent mutase, selected from the group consisting of

25

a) nucleic acid molecules coding for a protein comprising the amino acid sequences indicated under Seq. No. 2 and/or Seq. No. 4;

30

b) nucleic acid molecules comprising the nucleotide sequence depicted under Seq. No. 1 and/or Seq. No. 3.

35

22. A nucleic acid molecule as claimed in claim 21, characterized in that it codes for a protein which, as an oligomeric enzyme composed of two different subunits, comprises the amino acid sequences indicated under Seq. No. 2 and Seq. No. 4.

23. A nucleic acid molecule as claimed in claim 21 or 22, which is a DNA molecule.
- 5 24. A nucleic acid molecule as claimed in claim 23, which is a cDNA or genomic DNA.
25. A nucleic acid molecule as claimed in claim 21 or 22, which is an RNA molecule.
- 10 26. A protein having the activity of a cobalamin-dependent mutase encoded by a nucleic acid molecule as claimed in claims 21 and 22.
- 15 27. A protein with the sequence No. 2 and its at least 99% homologs.
28. A protein with the sequence No. 4 and its at least 99% homologs.
- 20 29. A protein as heterodimeric enzyme comprising sequence No. 2 and sequence No. 4