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(71) Applicant: **METABOLIC EXPLORER** [FR/FR]; Biopole Clermont-limagne, 63360 Saint Beauzire (FR).

(72) Inventors: **SOUCAILLE, Philippe**; Chant du Coucou, 31450 Deyme (FR). **DISCHERT, Wanda**; 67 rue de Coulogne, 63270 Vic-le-Comte (FR). **DUMON-SEIGNOVERT, Laurence**; 12 rue Marguerite de Valois, 63430 Pont du Château (FR).

(74) Agent: **REGIMBEAU**; 139 rue Vendôme, 69477 Lyon Cedex 06 (FR).

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(54) **Title**: METHIONINE HYDROXY ANALOG (MHA) PATHWAYS FOR PRODUCTION BY FERMENTATION

(57) **Abstract**: The present invention relates to a method for the production of 2-Hydroxy-4-(Methylthio) Butyric Acid (HMBA), an analog of the essential amino acid methionine and to a recombinant microorganism useful for the production by fermentation of 2-Hydroxy-4-(Methylthio) Butyric Acid. The microorganism of the invention is modified in a way that the HMBA biosynthesis is improved by enhancing production of enzymes catalyzing the conversion of methionine into HMBA.

METHIONINE HYDROXY ANALOG (MHA) PATHWAYS FOR PRODUCTION BY FERMENTATION

FIELD OF THE INVENTION

The present invention relates to a method for the production of 2-Hydroxy-4-(Methylthio) Butyric Acid (HMBA), an analog of the essential amino acid methionine. It is also an object of the invention to provide a genetically modified microorganism for the production of HMBA. The microorganism of the invention is modified in a way that the HMBA biosynthesis is improved by enhancing production of enzymes catalyzing the conversion of methionine into HMBA.

BACKGROUND OF THE INVENTION

Nutritivity-improving feedstuffs additives are nowadays an indispensable constituent of animal nutrition. One of the most important of these additives is the essential amino acid methionine, which occupies a prominent position as a feedstuffs additive above all in poultry rearing. In fact, methionine is the first limiting amino acid in corn and soybean meal based diets of poultry, and probably also in high-yielding cows and second or third amino acid in diets of pigs (Dilger and Baker, 2007). The racemic form of 2-Hydroxy-4-(Methylthio) Butyric Acid (HMBA), commonly referred to as "hydroxymethionine" or "methionine hydroxy analogue" (MHA) is a methionine substitute which has been known for a long time and is chiefly used as a feedstuffs additive in animal nutrition, in particular in the rearing of poultry. This MHA can be used instead of methionine and, like this, improves the yield of breast meat on poultry. It is furthermore also used pharmaceutically in the form of its calcium salt in treatment of renal insufficiency.

The methionine hydroxy analogue contains a hydroxyl radical on the α-carbon of the methionine molecule rather than an amino group. It has the formula (1):

$$CH_3SCH_2CH_2CH\ (\mathbf{OH})\ COOH \qquad (1)$$

In contrast with the amino acid, it is not used directly by the organism in protein synthesis, because it must be anabolically converted into the amino acid to be used as such. HMBA is not used in the pure form, but in various forms according to its application, namely:

- a mixture of calcium and ammonium salts of HMBA (US 2,745,745 and US 2,938,053),
- acidic aqueous solutions (US 4,353,924),
- calcium salts of HMBA, obtained by the process described in US 3,175,000.

It is known for a long time that HMBA can be prepared by a chemical process. It is synthesized by hydratation and successive hydrolysis of 2-hydroxy-4-methylthiobutyronitrile (HMBN) in a sulfuric acid medium. This process is described in EP0874811, but not exclusively. Several patents applications from NOVUS International (WO1998/032872), MONSANTO Company (EP0142488), or Rhone Poulenc Animal Nutrition S.A. (US 6,180,359) describe hydrolysis of HMBN into HBMA by a two-stage process. All these technologies rely approximately on the same raw material (3-methylthiopropionaldehyde or MMP) and key intermediates (2-hydroxy-4-methylthio-hydroxybutyronitrile or HMBN and 2-hydroxy-4-(methylthio)butyronitrile or MMP-CN). The general process for the preparation starts from 3-methylthiopropionaldehyde (MMP) which is reacted with hydrogen cyanide (HCN) or sodium cyanide (NaCN) to give the 2-hydroxy-4-methylthiobutyronitrile (HMBN). The MMP-CN formed is then conventionally hydrolysed with strong inorganic acid such as hydrochloric or sulphuric acid. In a subsequent stage, after dilution with water, the hydrolysis is completed at a higher temperature. The HMBA is then extracted with organic solvent which is not very miscible with water, such as ketone, and then the solvent is removed by electroporation.

During the past few years, new methods have emerged involving enzymes or biological material. Aventis Animal Nutrition S. A. has for instance described and patented a method for the preparation of HMBA by enzymatic hydrolysis of the 2-hydroxy-4-methylthiobutyronitrile intermediate. The invention is based on bioconversion of HMBN after contacting the molecule with immobilized biological material having nitrilase activity (US 6,180,359). A similar process was described by Novus with the enzymatic conversion of 2-hydroxy-4-(methylthio)-butanenitrile to 2-hydroxy-4-(methylthio)-butaneamide or 2-hydroxy-4-(methylthio)-butanoic acid or salts (WO1998/032872).

The Japanese company Sumitomo has recently protected another technology to produce the methionine hydroxy analogue in three steps; the first two are chemical whereas the third one is a bioconversion. The invention is described in the patent application WO2006/041209 and comprises in step (A) a reaction between 1,2-epoxy-3-butene and water to obtain 3-butene-1,2-diol, which reacts in step (B) with methanethiol to obtain 4-(methylthio)butane-1,2-diol, which is finally oxidized in step (C) by interacting with microbial cells having the required activity to convert the 4-(methylthio)butane-1,2-diol into 2-hydroxy-4-(methylthio)butyric acid.

Another invention from ADISSEO combines production of HMBA by fermentation and chemistry, the former process to obtain the intermediate 2,4-dihydroxybutyrate (2,4DHB) which is converted chemically into HMBA (WO2014/009435).

A completely new invention was disclosed in the patent application WO2012/090022 which describes the bio-production of the hydroxymethionine by fermentation of a

recombinant microorganism modified to produce L-methionine under conditions of nitrogen limitation. This is the only example of one-step production of L-2-hydroxy-4-(methylthio) butyric acid by fermentation from a renewable raw material.

From growing oil prices the need for the HMBA production from renewable resources arises. Optimization of production microorganisms often requires rational engineering of metabolic network. Another possibility is the implementation of novel enzymatic systems that catalyze the production of HMBA. Using both approaches, the inventors were able to identify pathways for the production of the methionine hydroxyl analog (MHA) by fermentation from a renewable raw material.

BRIEF DESCRIPTION OF THE INVENTION

The present invention is related to a method for the production of 2-hydroxy-4-(methylthio) butyric acid wherein in a first enzymatic step methionine is converted into 4-methylthio-2-oxobutanoic acid and in a second enzymatic step 4-methylthio-2-oxobutanoic acid is converted into 2-hydroxy-4-(methylthio) butyric acid.

The invention relates more particularly to a genetically modified microorganism for the production of 2-hydroxy-4-(methylthio) butyric acid expressing a transaminase or oxidoreductase for the conversion of methionine into 4-methylthio-2-oxobutanoic acid and an oxidoreductase and/or transferase and/or hydrolase and/or lyase and/or ligase for the conversion of 4-methylthio-2-oxobutanoic acid into 2-hydroxy-4-(methylthio) butyric acid. Method for the production of 2-hydroxy-4-(methylthio) butyric acid comprising culturing the genetically modified microorganism in an appropriate culture medium comprising a source of carbon, a source of sulfur and a source of nitrogen and recovering 2-hydroxy-4-(methylthio) butyric acid from the culture medium is also an object of the invention.

DESCRIPTION OF THE DRAWINGS

Figure 1. 2-hydroxy-4-(methylthio) butyric acid production pathway N°1

A can either be a transaminase enzyme or an oxidoreductase enzyme acting on CH-NH2 groups.

B is an oxidoreductase and/or a transferase, and/or an hydrolase, and/or a ligase and/or a lyase.

Figure 2. 2-hydroxy-4-(methylthio) butyric acid production pathway N°2.

C is a lyase enzyme acting on carbon-nitrogen bond.

D can either be a carbon-oxygen lyase or a carbon-oxygen dehydratase.

DETAILED DESCRIPTION OF THE INVENTION

Before describing the present invention in detail, it is to be understood that this invention is not limited to particularly exemplified methods and may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments of the invention only, and is not intended to be limiting, which will be limited only by the appended claims.

All publications, patents and patent applications mentioned herein are cited for the purpose of describing and disclosing the protocols, reagents and vectors that are reported in the publications and that might be used in connection with the invention.

Furthermore, the practice of the present invention employs, unless otherwise indicated, conventional microbiological and molecular biological techniques within the skill of the art. Such techniques are well known to the skilled worker, and are explained fully in the literature.

It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to "a microorganism" includes a plurality of such microorganisms, and a reference to "an endogenous gene" is a reference to one or more endogenous genes, and so forth. Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any materials and methods similar or equivalent to those described herein can be used to practice or test the present invention, the preferred materials and methods are now described.

In the claims that follow and in the description of the invention, except where the context requires otherwise due to express language or necessary implication, the word "comprise" or variations such as "comprises" or "comprising" is used in an inclusive sense, i.e. to specify the presence of the stated features but not to preclude the presence or addition of further features in various embodiments of the invention.

The term “methionine” means “L-methionine” or “D-methionine”. Similarly, the following terms “hydroxymethionine” meaning “L-hydroxymethionine” or “D-hydroxymethionine”, “methionine hydroxy analogue” meaning “L-methionine hydroxy analogue” or “D-methionine hydroxy analogue” , “MHA” meaning “L-MHA” or “D-MHA”, “2-Hydroxy-4-(methylthio) butyric acid” meaning “L-2-Hydroxy-4-(methylthio) butyric acid” or “D-2-Hydroxy-4-(methylthio) butyric acid”, “2-Hydroxy-4-(methylthio)

butanoic acid" meaning "L-2-Hydroxy-4-(methylthio) butanoic acid" or "D-2-Hydroxy-4-(methylthio) butanoic acid", "HMTBA" or "HMBA" or "DL-2-Hydroxy-4-(methylmercapto) butanoic acid" are used interchangeably to designate the product.

The term "microorganism", as used herein, refers to a living microscopic organism, which may be a single cell, or a multicellular organism and which can generally be found in nature. In the context of the present invention, the microorganism is preferably a bacterium, yeast or fungus. More preferably, the microorganism of the invention is selected among *Enterobacteriaceae*, *Bacillaceae*, *Streptomycetaceae, Corynebacteriaceae* and yeast. Even more preferably, the microorganism of the invention is a species of *Escherichia, Klebsiella, Thermoanaerobacterium, Corynebacterium or Saccharomyces*. Yet, even more preferably, the microorganism of the invention is selected from *Escherichia coli, Klebsiella pneumoniae, Thermoanaerobacterium thermosaccharolyticum, Corynebacterium glutamicum* and *Saccharomyces cerevisiae.* Most preferably, the microorganism of the invention is either the species *Escherichia coli* or *Corynebacterium glutamicum.*

The term "recombinant microorganism", "genetically modified microorganism", or "genetically engineered microorganism", as used herein, refers to a microorganism as defined above that is not found in nature and therefore genetically differs from its natural counterpart. In other words, it refers to a microorganism that is modified by introduction and/or by deletion and/or by modification of its genetic elements. Such modification can be performed by genetic engineering, by forcing the development and evolution of new metabolic pathways by culturing the microorganism under specific selection pressure, or by combining both methods (see, e.g. WO2005/073364 or WO2008/116852).

A microorganism genetically modified for the production of 2-Hydroxy-4-(methylthio) butanoic acid according to the invention therefore means that said microorganism is a recombinant microorganism as defined above that is capable of producing 2-Hydroxy-4-(methylthio) butanoic acid. In other words, said microorganism has been genetically modified to allow higher productions of 2-Hydroxy-4-(methylthio) butanoic acid than the non-modified microorganism.

According to the invention, the amount of 2-Hydroxy-4-(methylthio) butanoic acid produced by the recombinant microorganism, and particularly the 2-Hydroxy-4-(methylthio) butanoic acid yield (ratio of 2-Hydroxy-4-(methylthio) butanoic acid

produced per carbon source, in gram/gram or mol/mol), is higher in the modified microorganism compared to the corresponding unmodified microorganism.

The terms "microorganism for the production of methionine" or "methionine-producing microorganism" or "microorganism modified to produce methionine" or "microorganism optimized for the production of methionine" and expression derived thereof designate a microorganism as defined above producing higher levels of methionine than non-modified microorganism. Microorganisms optimized for methionine production are well known in the art, and have been disclosed in particular in patent applications WO2005/111202, WO2007/077041, WO2009/043803, WO2010/020681, WO2011/073738, WO2011/080542, WO2011/080301, WO2012/055798, WO2013/001055, WO2013/190343, WO2015/028675 and WO2015/028674. Preferably, the modified microorganisms of the invention for the production of 2-Hydroxy-4-(methylthio) butanoic acid are further optimized for methionine production. The inventors have observed that if more methionine is produced by the microorganisms, also more 2-Hydroxy-4-(methylthio) butanoic acid is produced.

As further explained below, the microorganism of the invention can be genetically modified by modulating the expression level of one or more endogenous genes, and/or by expressing one or more heterologous genes in said microorganism.

By "modulating", it is meant herein that the expression level of said gene is up-regulated, downregulated, or even completely abolished by comparison to its natural expression level. Such modulation can therefore result in an enhancement of the activity of the gene product, or alternatively, in a lower or null activity of the endogenous gene product.

By "gene", it is meant herein a nucleic acid molecule or polynucleotide that codes for a particular protein (i.e. polypeptide), or in certain cases, for a functional or structural RNA molecule. In the context of the present invention, the genes referred herein encode proteins, such as enzymes. Genes according to the invention are either endogenous genes or exogenous.

The term "endogenous gene" refers herein to a gene that is naturally present in the microorganism.

An endogenous gene can be overexpressed by introducing heterologous sequences which favour upregulation in addition to endogenous regulatory elements or by substituting those endogenous regulatory elements with such heterologous sequences, or by introducing one or more supplementary copies of the endogenous gene into the chromosome or a plasmid within the microorganism. Endogenous gene activity and/or

expression level can also be modified by introducing mutations into their coding sequence to modify the gene product. A deletion of an endogenous gene can also be performed to inhibit totally its expression within the microorganism. Another way to modulate the expression of an endogenous gene is to exchange its promoter (i.e. wild type promoter) with a stronger or weaker promoter to up or down regulate the expression level of this gene. Promoters suitable for such purpose can be homologous or heterologous and are well-known in the art. It is within the skill of the person in the art to select appropriate promoters for modulating the expression of an endogenous gene.

In addition, or alternatively, a microorganism can be genetically modified to express one or more exogenous genes, provided that said genes are introduced into the microorganism with all the regulatory elements necessary for their expression in the host microorganism. The modification or "transformation" of microorganisms with exogenous DNA is a routine task for those skilled in the art. In the context of the present invention, the term "overexpression" or "overexpressing" is also used herein in relation to the expression of exogenous genes in the microorganism.

By "exogenous gene" or "heterologous gene", it is meant herein that said gene is not naturally occurring in the microorganism. In order to express an exogenous gene in a microorganism, such gene can be directly integrated into the microorganism chromosome, or be expressed extra-chromosomally by plasmids or vectors within the microorganism. A variety of plasmids, which differ in respect of their origin of replication and of their copy number in a cell, are well known in the art and can be easily selected by the skilled practitioner for such purpose. Exogenous genes according to the invention are advantageously homologous genes.

In the context of the invention, the term "homologous gene" or "homolog" not only refers to a gene inherited by two species (i.e. microorganism species) by a theoretical common genetic ancestor, but also includes genes which may be genetically unrelated that have, nonetheless, evolved to encode proteins which perform similar functions and/or have similar structure (i.e. functional homolog). Therefore the term "functional homolog" refers herein to a gene that encodes a functionally homologous protein.

Using the information available in databases such as Uniprot (for proteins), Genbank (for genes), or NCBI (for proteins or genes), those skilled in the art can easily determine the sequence of a specific protein and/or gene of a microorganism, and identify based on this sequence the one of equivalent genes, or homologs, in another microorganism. This routine work can be performed by a sequence alignment of a specific gene sequence of a microorganism with gene sequences or the genome of other microorganisms, which can be found in the above mentioned databases. Such sequence alignment can advantageously be performed using the BLAST algorithm developed by Altschul *et al.* (1990). Once a sequence homology has been established between those sequences, a consensus sequence

can be derived and used to design degenerate probes in order to clone the corresponding homolog gene of the related microorganism. These routine methods of molecular biology are well known to those skilled in the art.

It shall be further understood that, in the context of the present invention, should an exogenous gene encoding a protein of interest be expressed in a specific microorganism, a synthetic version of this gene is preferably constructed by replacing non-preferred codons or less preferred codons with preferred codons of said microorganism which encode the same amino acid. It is indeed well-known in the art that codon usage varies between microorganism species, which may impact the expression level of the protein of interest. To overcome this issue, codon optimization methods have been developed, and are extensively described by Graf *et al.* (2000), Deml *et al.* (2001) and Davis & Olsen (2011). Several software have notably been developed for codon optimization determination such as the GeneOptimizer® software (Lifetechnologies) or the OptimumGene™ software of (GenScript). In other words, the exogenous gene encoding a protein of interest is preferably codon-optimized for expression in a specific microorganism.

The microorganism according to the invention can also be genetically modified to increase or decrease the activity of one or more proteins.

The term “decrease the activity” or “attenuation of activity” according to the invention could be employed for an enzyme or a gene and denotes, in each case, the partial or complete suppression of the expression of the corresponding gene, which is then said to be ‘decreased’ or ‘attenuated’. This suppression of expression can be either an inhibition of the expression of the gene, a deletion of all or part of the promoter region necessary for the gene expression, a deletion in the coding region of the gene, or the exchange of the wildtype promoter by a weaker natural or synthetic promoter. Preferentially, the attenuation of a gene is essentially the complete deletion of that gene, which can be replaced by a selection marker gene that facilitates the identification, isolation and purification of the strains according to the invention. A gene is inactivated preferentially by the technique of homologous recombination (Datsenko & Wanner, 2000). Decreasing the activity of a protein can mean either decreasing its specific catalytic activity and/or decreasing expression of the protein in the cell by way of mutation, suppression, insertion or modification of single or multiple residues in a polynucleotide leading to alterations arising within a protein-encoding region of a gene as well as alterations in regions outside of a protein-encoding sequence such as, but not limited to, regulatory or promoter sequences. The alteration may be a mutation of any type and for instance: a point mutation, a frame-shift mutation, a nonsense mutation, an insertion or a deletion of part or all of a gene as described above.

The term "increased activity" or "enhanced activity" designates an enzymatic activity that is superior to the enzymatic activity of the non modified microorganism. The man skilled in the art knows how to measure the enzymatic activity of said enzyme.

Increasing such activity can be obtained by improving the protein catalytic efficiency, by decreasing protein turnover, by decreasing messenger RNA (mRNA) turnover, by increasing transcription of the gene, or by increasing translation of the mRNA.

Improving the protein catalytic efficiency means increasing the kcat and/or decreasing the Km for a given substrate and/or a given cofactor, and/or increasing the Ki for a given inhibitor. kcat, Km and Ki are Michaelis-Menten constants that the man skilled in the art is able to determine (Segel, 1993). Decreasing protein turnover means stabilizing the protein. Methods to improve protein catalytic efficiency and/or decrease protein turnover are well known from the man skilled in the art. Those include rational engineering with sequence and/or structural analysis and directed mutagenesis, as well as random mutagenesis and screening. Mutations can be introduced by site-directed mutagenesis by conventional methods such as Polymerase Chain Reaction (PCR), by random mutagenesis techniques, for example via mutagenic agents (Ultra-Violet rays or chemical agents like nitrosoguanidine (NTG) or ethylmethanesulfonate (EMS)) or DNA shuffling or error-prone PCR. Stabilizing the protein can also be achieved by adding a "tag" peptide sequence either at the N-terminus or the C-terminus of the protein. Such tags are well known in the art, and include, among others, the Glutathione-S-Transferase (GST).

Decreasing mRNA turnover can be achieved by modifying the gene sequence of the 5'-untranslated region (5'-UTR) and/or the coding region, and/or the 3'-UTR (Carrier and Keasling, 1999).

Increasing the transcription of a gene, whether endogenous or exogenous, can be achieved by increasing the number of its copies within the microorganism and/or by using a promoter leading to a higher level of expression of the gene compared to the wild type promoter. In the context of the present invention, the terms "overexpress", "overexpression" or "overexpressing" are also used to designate an increase in transcription of a gene in a microorganism.

As indicated above, to increase the number of copies of a gene in the microorganism, said gene can be encoded chromosomally or extra-chromosomally. When the gene of interest is to be encoded on the chromosome, several copies of the gene can be introduced on the chromosome by methods of genetic recombination, which are well-known to in the art (e.g. gene replacement). When the gene is to be encoded extra-chromosomally in the microorganism, it can be carried by different types of plasmid that differ in respect to their origin of replication depending on the microorganism in which they can replicate, and by their copy number in the cell. The microorganism transformed by said plasmid can contain 1 to 5 copies of the plasmid, or about 20 copies of it, or even up to 500 copies of it,

depending on the nature of the plasmid. Examples of low copy number plasmids which can replicate in *E.coli* include, without limitation, the pSC101 plasmid (tight replication), the RK2 plasmid (tight replication), as well as the pACYC and pRSF1010 plasmids, while an example of high copy number plasmid which can replicate in *E.coli* is pSK bluescript II.

Promoters which can increase the expression level of a gene are also well-known to the skilled person in the art, and can be homologous (originating from same species) or heterologous (originating from a different species or artificial promoter). Examples of such promoters include, without limitation, the promoters P*trc*, P*tac*, P*lac*, and P_R and P_L of the lambda phage. These promoters can also be induced ("inducible promoters") by a particular compound or by specific external condition like temperature or light.

Increasing translation of the mRNA can be achieved by modifying the Ribosome Binding Site (RBS). A RBS is a sequence on mRNA that is bound by the ribosome when initiating protein translation. It can be either the 5' cap of a mRNA in eukaryotes, a region 6-7 nucleotides upstream of the start codon AUG in prokaryotes (called the Shine-Dalgarno sequence), or an internal ribosome entry site (IRES) in viruses. By modifying this sequence, it is possible to change the protein translation initiation rate, to proportionally alter its production rate, and control its level activity inside the cell. It is also possible to optimize the strength of a RBS sequence to achieve a targeted translation initiation rate by using the software RBS CALCULATOR (Salis, 2011). It is within the skill of the person in the art to select the RBS sequence based on the nature of the mRNA.

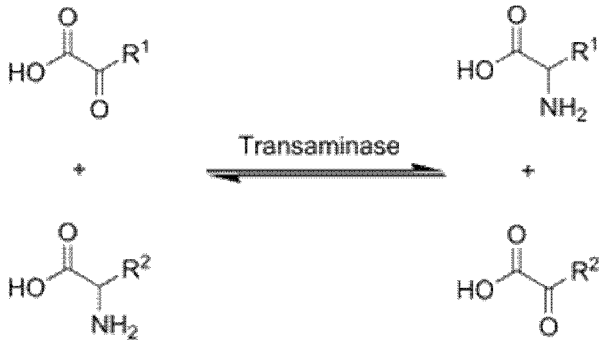
The present invention is directed to a method for the production of 2-hydroxy-4-(methylthio) butyric acid wherein in a first enzymatic step methionine is converted into 4-methylthio-2-oxobutanoic acid (KMTB) and in a second enzymatic step 4-methylthio-2-oxobutanoic acid is directly or indirectly converted into 2-hydroxy-4-(methylthio) butyric acid (Cf. Fig 1).

The term "convert" or "conversion" designates the enzymatic step that transforms a product A into the product B and catalysed by enzyme(s). This conversion can be direct or indirect. If the conversion of product A into product B is catalysed by a single enzyme, the conversion is direct. By contrast if the conversion of product A into product B is catalysed by several successive enzymes the conversion is indirect. In the case of the invention, the conversion of methionine into 4-methylthio-2-oxobutanoic acid is direct and the conversion of 4-methylthio-2-oxobutanoic acid into 2-hydroxy-4-(methylthio) butyric acid is direct or indirect.

In a first aspect of the invention, methionine is converted into 4-methylthio-2-oxobutanoic acid by one enzyme having transaminase or oxidoreductase activity.

In one preferred embodiment, methionine is converted into 4-methylthio-2-oxobutanoic acid by one enzyme having transaminase activity.

The reaction between methionine and 4-methylthio-2-oxobutanoic acid is a transamination performed by a transaminase (EC 2.6.1), also named amino-transferase or nitrogenous groups-transferring enzyme, catalysing the exchange of the amino (NH_2) group and the keto (=O) group between an amino acid and an α-keto acid also named 2-oxo acid, in one way or in the other as schematized below:



Most of these transaminases require the coenzyme pyridoxal-phosphate converted into pyridoxamine in the first phase of the reaction when an amino acid is converted into a keto acid. Enzyme-bound pyridoxamine in turn reacts with pyruvate, oxaloacetate, or alpha-ketoglutarate, giving alanine, aspartic acid, or glutamic acid, respectively.

In this pathway, the 4-methylthio-2-oxobutanoic acid compound (KMTB) is formed from methionine amino acid by one transaminase having the following enzymatic activity:

L-Methionine + 2-oxoglutarate ⇔ 4-Methylthio-2-oxobutanoate + L-glutamate

2-Oxoglutaramate + L-Methionine ⇔ 4-Methylthio-2-oxobutanoate + L-Glutamine

L-Methionine + Phenylpyruvate ⇔ L-Phenylalanine + 4-Methylthio-2-oxobutanoate

L-methionine + 2-oxo-3-phenylpropanoate ⇔ 2-oxo-4-methylthiobutanoate + L-phenylalanine

D/L-methionine + pyruvate ⇔ 4-methylthio-2-oxobutanoate + L-alanine

4-Methyl-2-oxopentanoate + L-Methionine ⇔ L-Leucine + 4-Methylthio-2-oxobutanoate

alpha-Ketovaline + L-Methionine ⇔ L-Valine + 4-Methylthio-2-oxobutanoate

Methionine + (S)-3-Methyl-2-oxopentanoate ⇔ L-Isoleucine + 4-Methylthio-2-oxobutanoate

L-methionine + glyoxylate ⇔ 4-methylthio-2-oxobutanoate + glycine

L-methionine + a 2-oxo acid ⇔ 2-oxo-4-methylthiobutanoate + an L-amino acid

L-methionine + indole-3-pyruvate ⇔ L-tryptophan + 2-oxo-4-methylthiobutanoate

Several transaminases can catalyze such reactions even if it is not known in the literature. The transaminases candidates with the EC number 2.6.1 able to produce KMTB from L-methionine and their characteristics are summarized into the table 1 below.

Table 1: Transaminase enzymes EC 2.6.1 able to produce KMTB from methionine

EC-number	*Name*	*Amines Acceptors*	*Amines Donors*	*Reactions described for these enzymes*
2.6.1.1	aspartate transaminase	Oxaloacetate	L-Aspartate	L-aspartate + 2-oxoglutarate ⇔ oxaloacetate + L-glutamate
2.6.1.15	glutamine—pyruvate transaminase	2-oxoglutaramate Phenylpyruvate	Glutamine Phenylalanine	L-glutamine + pyruvate ⇔ 2-oxoglutaramate + L-alanine L-Methionine can act as donor; glyoxylate can act as acceptor.
2.6.1.28	tryptophan—phenylpyruvate transaminase	2-oxo-3-phenylpropanoate	L-phenylalanine	L-tryptophan + phenylpyruvate ⇔ (indol-3-yl)pyruvate + L-phenylalanine Valine, leucine and isoleucine can replace tryptophan as amino donor.
2.6.1.41	D-methionine—pyruvate transaminase	Pyruvate	L-alanine	D-methionine + pyruvate ⇔ 4-methylthio-2-oxobutanoate + L-alanine Oxaloacetate can replace pyruvate
2.6.1.42	branched-chain-amino-acid transaminase	4-methyl-2-oxopentanoate a-ketovaline 3-methyl-2-oxopentanoate	L-leucine L-valine L-isoleucine	L-leucine + 2-oxoglutarate ⇔ 4-methyl-2-oxopentanoate + L-glutamate Also acts on L-isoleucine and L-valine.
2.6.1.57	aromatic-amino-acid transaminase	2-oxoglutarate	L-glutamate	An aromatic amino acid + 2-oxoglutarate ⇔ an aromatic oxo acid + L-glutamate L-Methionine can also act as donor, oxaloacetate can act as acceptor.
2.6.1.64	glutamine—phenylpyruvate transaminase	Phenylpyruvate	Phenylalanine	L-glutamine + phenylpyruvate ⇔ 2-oxoglutaramate + L-phenylalanine L-Methionine, L-histidine and L-tyrosine can act as donors. The enzyme has little activity on pyruvate and glyoxylate
2.6.1.73	methionine—glyoxylate transaminase	Glyoxylate	Glycine	L-methionine + glyoxylate ⇔ 4-methylthio-2-oxobutanoate + glycine L-Glutamate can also act as donor.
2.6.1.99	L-tryptophan—pyruvate aminotransferase	Indole-3-pyruvate	L-tryptophan	L-tryptophan + pyruvate ⇔ indole-3-pyruvate + L-alanine

More preferably, the transaminase acting on methionine is an aspartate aminotransferase.

In another preferred embodiment, methionine is converted into 4-methylthio-2-oxobutanoic acid by one enzyme having oxidoreductase activity which acts on the CH-NH_2 groups with NAD^+ or $NADP^+$ as acceptor (enzyme EC 1.4.1) or with oxygen as acceptor (enzyme EC 1.4.3).

An oxidoreductase is an enzyme that catalyzes the transfer of electrons from one molecule, the reductant, also called the electron donor, to another the oxidant, and also called the electron acceptor. This group of enzymes usually utilizes $NADP^+$ or NAD^+ as cofactor, but the oxygen is also electrons acceptors for such enzymes. Proper names of oxidoreductases are formed as "donor:acceptor oxidoreductase"; however, other names are much more common. The common name is "donor dehydrogenase" when possible. Common names are also sometimes formed as "acceptor reductase", such as NAD^+ reductase. "Donor oxidase" is a special case where O_2 is the acceptor.

In the present invention, the 4-methylthio-2-oxobutanoic acid compound (also named as KMTB) is formed from methionine amino acid by one oxidoreductase acting on CH-NH_2 group of donors having the following enzymatic activity:

$NAD^+ + H_2O$ + Methionine ⇔ 4-Methylthio-2-oxobutanoate + NH_3 + NADH + H^+

Méthionine + H_2O + $NADP^+$ ⇔ 4-Methylthio-2-oxobutanoate + NH_3 + NADPH + H^+

In such reactions the cofactor of the enzyme is NAD^+ or $NADP^+$.

Methionine + H_2O + O_2 ⇔ NH_3 + H_2O_2 + 4-Methylthio-2-oxobutanoate

In such reactions the cofactor of the enzyme is the oxygen.

Several oxidoreductases can catalyze such reactions even if it is not known in the literature. The oxidoreductases candidates with EC number EC 1.4.1 and 1.4.3 able to convert the methionine into 4-Methylthio-2-oxobutanoate (KMTB) and their characteristics are summarized below in table 2 below.

Table 2: Oxidoreductase enzymes EC 1.4.1 or 1.4.3 acting CH-NH_2 group of donors able to produce KMTB from methionine

EC Number	*Name*	*Reactions described for these enzymes*
1.4.1.20	Phenylalanine Dehydrogenase	L-phenylalanine + H2O + NAD+ ⇔ phenylpyruvate + NH3 + NADH.
1.4.1.2	Glutamate Dehydrogenase	L-glutamate + H2O + NAD+ ⇔ 2-oxoglutarate + NH3 + NADH
1.4.3.2	L-amino-acid oxidase	2-hydroxy acid + O2 ⇔ 2-oxo acid + H2O2 L-amino acid + H2O + O2 ⇔ a 2-oxo acid + NH3 + H2O2.

In a second step of the invention, 4-methylthio-2-oxobutanoic acid is converted directly or indirectly into 2-hydroxy-4-(methylthio) butyric acid by at least one enzyme having activity chosen among oxidoreductase, transferase, hydrolase, lyase and ligase activities.

In one embodiment 4-methylthio-2-oxobutanoic acid is converted into 2-hydroxy-4-(methylthio) butyric acid by at least one enzyme having oxidoreductase activity.

As described above, an oxidoreductase is an enzyme that catalyses the transfer of electrons from one molecule, the reductant, also called the electron donor, to another the oxidant, and also called the electron acceptor. In this aspect of the invention the oxidoreductase is an oxidoreductase acting on the CH-OH group of donors (EC 1.1 enzyme), an oxidoreductase acting on the aldehyde or oxo group of donors (EC 1.2 enzyme), an oxidoreductase acting on the CH-CH group of donors (EC 1.3 enzyme) or an oxidoreductase acting on the CN-NH_2 group of donors (EC 1.4 enzyme). Examples of oxidoreductases acting on the CH-OH group of donors include, without limitation, EC 1.1.1 enzymes (oxidoreductases with NAD^+ or $NADP^+$ as acceptor, also known as $NAD^+/NADP^+$ oxidoreductase), EC 1.1.2 enzymes (oxidoreducatses with a cytochrome as acceptor), EC 1.1.3 enzymes (oxidoreductase with oxygen as acceptor) and EC 1.1.99 enzymes (oxidoreductases with unknown physiological acceptor). Examples of oxidoreductases acting on the aldehyde group or oxo group of donors comprise, without limitation, EC 1.2.1 enzymes (oxidoreductases with NAD^+ or $NADP^+$ as acceptor).

In this embodiment, the oxidoreductase is preferably an aspartate-semialdehyde dehydrogenase and/or an inosine-5'-monophosphate (IMP) dehydrogenase.

In another embodiment 4-methylthio-2-oxobutanoic acid is converted into 2-hydroxy-4-(methylthio) butyric acid by at least one enzyme having transferase activity.

A transferase is an enzyme (EC 2 enzyme) that transfer specific functional group (e.g. a methyl or glycosyl group) from one molecule (called the donor) to another (called the acceptor).

In this embodiment of the invention, the transferase is preferably transferring phosphorus containing group (EC 2.7 enzyme) or a sulphur containing group (EC 2.8 enzyme). Most preferably the enzyme is a cysteine desulfurase.

In another embodiment 4-methylthio-2-oxobutanoic acid is converted into 2-hydroxy-4-(methylthio) butyric acid by at least one enzyme having hydrolase activity.

An hydrolase is an enzyme (EC 3 enzyme) that catalyses the hydrolysis of a chemical bond.

In this embodiment of the invention, the hydrolase is preferably acting on a carbon-nitrogen bond other than peptide bonds (EC 3.5 enzyme).

In a further embodiment, 4-methylthio-2-oxobutanoic acid is converted into 2-hydroxy-4-(methylthio) butyric acid by at least one enzyme having lyase activity.

A lyase is an enzyme that catalyzes the breaking (an "elimination" reaction) of various chemical bonds by means other than hydrolysis (a "substitution" reaction) and oxidation, often forming a new double bond or a new ring structure. The reverse reaction is also possible. Lyases differ from other enzymes in that they require only one substrate for the reaction in one direction, but two substrates for the reverse reaction. Lyases are classified as EC 4 in the EC number classification of enzymes.

In this further embodiment, the lyase is preferably a carbon-oxygen lyase also named dehydratase (EC 4.2 enzyme) that cleaves carbon-oxygen bonds or a carbon-nitrogen lyase (EC 4.3 enzyme) that cleaves carbon-nitrogen bonds. Most preferably the enzyme is an aconitase hydratase B.

In a last embodiment, 4-methylthio-2-oxobutanoic acid is converted into 2-hydroxy-4-(methylthio) butyric acid by at least one enzyme having ligase activity.

A ligase is an enzyme that can catalyze the joining of two large molecules by forming a new chemical bond, usually with accompanying hydrolysis of a small pendant chemical group on one of the larger molecules or an enzyme catalyzing the linking together of two compounds, e.g., enzymes that catalyze joining of C-O, C-S, C-N, etc.

In this embodiment of the invention, the ligase is preferably forming carbon-nitrogen bonds (EC 3.5 enzyme).

Several enzymes can catalyze the conversion of 4-methylthio-2-oxobutanoic acid into 2-hydroxy-4-(methylthio) butyric acid even if it is not known in the literature. The candidates able to make the conversion and their characteristics are summarized below in table 3.

Table 3: Enzymes able to produce 2-hydroxy-4-(methylthio) butyric acid from KMTB.

EC-number	***Name***	***Reactions described for these enzymes***
1.2.1.11	Aspartate-semialdehyde dehydrogenase	L-aspartate 4-semialdehyde + phosphate + NADP+ ⇔ L-4-aspartyl phosphate + NADPH + H+
1.1.1.205	IMP dehydrogenase	IMP + NAD+ + H2O ⇔ XMP + NADH + H+
1.3.5.1	succinate dehydrogenase	succinate + a quinone = fumarate + a quinol
1.4.1.13	glutamate synthase (NADPH)	2 L-glutamate + NADP+ ⇔ L-glutamine + 2-oxoglutarate + NADPH + H+
1.4.1.4	glutamate dehydrogenase (NADP+)	L-glutamate + H2O + NADP+ ⇔ 2-oxoglutarate + NH3 + NADPH + H+
1.1.1.3 *2.7.2.4*	Homoserine dehydrogenase Aspartokinase	L-homoserine + NAD(P)+ ⇔ L-aspartate 4-semialdehyde + NAD(P)H. ATP + L-aspartate ⇔ ADP + 4-phospho-L-aspartate.
2.7.1.167 *2.7.7.70*	D-glycero-β-D-manno-heptose-7-phosphate kinase D-glycero-β-D-manno-heptose 1-phosphate adenylyltransferase	D-glycero-beta-D-manno-heptose 7-phosphate + ATP ⇔ D-glycero-beta-D-manno-heptose 1,7-bisphosphate + ADP D-glycero-β-D-manno-heptose 1-phosphate + ATP ⇔ ADP-D-glycero-β-D-manno-heptose + diphosphate
2.8.1.7	cysteine desulfurase	L-cysteine + acceptor ⇔ L-alanine + S-sulfanyl-acceptor
3.5.99.10	2-iminobutanoate/2-iminopropanoate deaminase	2-iminobutanoate + H2O ⇔ 2-oxobutanoate + NH3. 2-iminopropanoate + H2O ⇔ pyruvate + NH3
4.2.1.3	aconitate hydratase	citrate ⇔ isocitrate
4.3.1.1	aspartate ammonia-lyase	L-aspartate ⇔ fumarate + NH3
6.3.1.2	Glutamine synthetase	ATP + L-glutamate + NH3 ⇔ ADP + phosphate + L-glutamine.

In a most preferred embodiment, 4-methylthio-2-oxobutanoic acid is converted into 2-hydroxy-4-(methylthio) butyric acid by at least one enzyme selected among the group consisting of aspartate-semialdehyde dehydrogenase, inosine-5'-monophosphate dehydrogenase, cysteine desulfurase, aconitase hydratase B and any combination thereof.

The present invention is also directed to a genetically modified microorganism for the production of 2-hydroxy-4-(methylthio) butyric acid wherein the genetically modified microorganism overexpresses (i) at least one gene encoding enzyme having transaminase or oxidoreductase activity, and (ii) at least one gene encoding enzyme having activity chosen among the group consisting of oxidoreductase, transferase, hydrolase, lyase and ligase.

The gene encoding enzyme having transaminase or oxidoreductase activity which is overexpressed in the recombinant microorganism of the invention is as detailed above (Table 1 or 2). These genes are listed in table 4 for the conversion of methionine into 4-methylthio-2-oxobutanoic acid.

Table 4: Candidates genes encoding enzymes for the reaction of conversion of methionine into 4-methylthio-2-oxobutanoic acid.

EC number	***Gene name***	***Enzyme name***	***Origin (Genus Species)***	***Database (gene and/or protein)***	***Accession number in the database (gene and/or protein***	***SEQ ID N° (gene and/or protein***
EC 2	***Transferases***					
EC 2.6	***Transferring nitrogenous groups***					
EC 2.6.1	***Transaminases***					
2.6.1.1	***aspC***	Aspartate aminotransferase	*Escherichia coli*	*Genbank Uniprot*	945553 P00509	9 21
2.6.1.57	***tyrB***	Aromatic-amino-acid aminotransferase	*Escherichia coli*	*Genbank Uniprot*	948563 P04693	22
2.6.1.2	***alaC***	Glutamate-pyruvate aminotransferase	*Escherichia coli*	*Genbank Uniprot*	946850 P77434	23
2.6.1.2	***alaA***	Glutamate-pyruvate aminotransferase	*Escherichia coli*	*Genbank Uniprot*	946772 P0A959	24
2.6.1.66	***avtA***	Valine--pyruvate aminotransferase	*Escherichia coli*	*Genbank Uniprot*	948087 P09053	25
2.6.1.87	***arnB***	UDP-4-amino-4-deoxy-L-arabinose_oxoglutarate aminotransferase	*Escherichia coli*	*Genbank Uniprot*	947375 P77690	26
2.6.1.57	***DDB_G0272014***	Aromatic amino acid aminotransferase	*Dictyostelium discoideum*	*Genbank Uniprot*	8618270 Q86AG8	27
2.6.1.57	***SPAC56E4.03***	Aromatic amino acid aminotransferase	*Schizosaccharomyces pombe*	*Genbank Uniprot*	2543350 O14192	28
2.6.1.57	***ARO8***	Aromatic amino acid aminotransferase 1	*Saccharomyces cerevisiae*	*Genbank Uniprot*	852672 P53090	29

2.6.1.57	***ARO9***	Aromatic amino acid aminotransferase 2	*Saccharomyces cerevisiae*	*Genbank Uniprot*	856539 P38840	30
2.6.1.88	***BCAT4***	Methionine aminotransferase BCAT4	*Arabidopsis thaliana*	*Genbank Uniprot*	821508 Q9LE06	31
2.6.1.57	***phhC***	Aromatic-amino-acid aminotransferase	*Pseudomonas aeruginosa*	*Genbank Uniprot*	880839 P43336	32
2.6.1.57	***tyrB***	Tyrosine aminotransferase	*Klebsiella pneumoniae*	*Genbank Uniprot*	NA O85746	33
2.6.1.57	***tyrB***	Aromatic-amino-acid aminotransferase	*Paracoccus denitrificans*	*Genbank Uniprot*	NA P95468	34
2.6.1.57	***tyrB***	Aromatic-amino-acid aminotransferase	*Salmonella typhimurium*	*Genbank Uniprot*	1255774 P74861	35
2.6.1	***mtnE***	Transaminase MtnE	*Bacillus subtilis*	*Genbank Uniprot*	939330 O31665	36
2.6.1	***F5I6.11***	Methionine-specific aminotransferase	*Arabidopsis thaliana*	*Genbank Uniprot*	844376 Q9C969	37
EC 1	**Oxidoreductase**					
EC 1.4	**Acting on the CH-NH2 group of donors**					
EC 1.4.1	**With NAD+ or NADP+ as acceptor**					
1.4.1.20	***pdh***	Phenylalanine dehydrogenase	*Thermoactinomyces intermedius*	*Genbank Uniprot*	NA P22823	38
1.4.1.2	***gdh***	NAD-specific glutamate dehydrogenase	*Clostridium symbiosum (Bacteroides symbiosus)*	*Genbank Uniprot*	NA P24295	39
1.4.1.20	***pdh***	Phenylalanine dehydrogenase	*Lysinibacillus sphaericus (Bacillus sphaericus)*	*Genbank Uniprot*	NA P23307	40
EC 1.4.3	**With oxygen as acceptor**					
1.4.3.16	***nadB***	L-aspartate oxidase	*Escherichia coli*	*Genbank Uniprot*	947049 P10902	41
1.4.3.2	***NA***	L-amino acid oxidase	*Rhodococcus opacus*	*Genbank Uniprot*	NA Q8VPD4	42
1.4.3.2	***lao1***	L-amino acid oxidase	*Cupriavidus necator*	*Genbank Uniprot*	NA Q0KDC9	43
1.4.3.2	***NA***	L-amino-acid oxidase	*Calloselasma rhodostoma (Malayan pit viper) (Agkistrodon rhodostoma)*	*Genbank Uniprot*	NA P81382	44
1.4.3.2	***NA***	L-amino-acid oxidase	*Ophiophagus hannah (King cobra)*	*Genbank Uniprot*	NA P81383	45

In a preferred embodiment the conversion of methionine into 4-methylthio-2-oxobutanoic acid is catalysed by an enzyme having transaminase activity encoded by at least one endogenous or exogenous gene disclosed in table 4 and overexpressed in said recombinant microorganism.

Preferentially the enzyme having transaminase activity is an aspartate aminotransferase.

More preferably *aspC* gene encoding enzyme having aspartate aminotransferase activity is overexpressed in said recombinant microorganism.

Preferentially the microorganism of the invention overexpresses at least one gene encoding for a transaminase having at least 60%, preferably at least 70%, more preferably at least 85% and even more preferably 90% amino acids identity with complete protein encoded by gene *aspC* from *Escherichia coli*. More preferentially, the microorganism of the invention overexpresses *aspC* gene from *Escherichia coli* (i.e. 100% amino acids sequence identity with the protein sequence encoded by said gene).

The gene encoding enzyme having oxidoreductase and/or transferase and/or hydrolase and/or lyase and/or ligase activities which is overexpressed in the recombinant microorganism of the invention is as detailed above and in table 3. These genes are listed in table 5 for the conversion of 4-methylthio-2-oxobutanoic acid into 2-hydroxy-4-(methylthio) butyric acid.

Table 5: Candidates genes encoding enzymes for the reaction of conversion of 4-methylthio-2-oxobutanoic acid into 2-hydroxy-4-(methylthio) butyric acid

EC number	***Gene name***	***Enzyme name***	***Origin (Genus Species)***	***Database (gene and/or protein)***	***Accession number in the database (gene and/or protein)***	***SEQ ID N°***
EC 1	***Oxidoreductases***					
EC 1.1	***Acting on the CH-OH group of donors***					
1.1	***eutG***	Ethanolamine utilization protein EutG	*Escherichia coli*	*Genbank Uniprot*	946233 P76553	46
1.1	***YihU***	Uncharacterized oxidoreductase YihU	*Escherichia coli*	*Genbank Uniprot*	948372 P0A9V8	47
EC 1.1.1	***With NAD+ or NADP+ as acceptor***					
1.1.1	***ydiJ***	Uncharacterized protein YdiJ	*Escherichia coli*	*Genbank Uniprot*	946189 P77748	48
1.1.1.290	***pdxB***	Erythronate-4-phosphate dehydrogenase	*Escherichia coli*	*Genbank Uniprot*	946785 P05459	49
1.1.1.	***CTBP1***	C-terminal-binding protein 1	*Human*	*Genbank Uniprot*	1487 Q13363	50
1.1.1	***YdfG***	NADP-dependent 3-hydroxy acid dehydrogenase YdfG	*Escherichia coli*	*Genbank Uniprot*	946085 P39831	51
1.1.1	***ybiC***	Uncharacterized oxidoreductase YbiC	*Escherichia coli*	*Genbank Uniprot*	945412 P30178	52
1.1.1.1	***yiaY***	Probable alcohol dehydrogenase	*Escherichia coli*	*Genbank Uniprot*	948102 P37686	53
1.1.1.1	***adhE***	Aldehyde-alcohol dehydrogenase	*Escherichia coli*	*Genbank Uniprot*	945837 P0A9Q7	54

1.1.1.3 *2.7.2.4*	***metL***	Bifunctional aspartokinase/homoserine dehydrogenase 2	*Escherichia coli*	*Genbank* *Uniprot*	948433 P00562	55
1.1.1.23	***hisD***	Histidinol dehydrogenase	*Escherichia coli*	*Genbank* *Uniprot*	P06988 946531	56
1.1.1.28	***ldhA***	D-lactate dehydrogenase	*Escherichia coli*	*Genbank* *Uniprot*	946315 P52643	57
1.1.1.44	***gnd***	6-phosphogluconate dehydrogenase, decarboxylating	*Escherichia coli*	*Genbank* *Uniprot*	946554 P00350	58
1.1.1.60	***garR***	2-hydroxy-3-oxopropionate reductase	*Escherichia coli*	*Genbank* *Uniprot*	947631 P0ABQ2	59
	ygbJ	Uncharacterized oxidoreductase YgbJ	*Escherichia coli*	*Genbank* *Uniprot*	947200 Q46888	60
1.1.1.60	***glxR***	2-hydroxy-3-oxopropionate reductase	*Escherichia coli*	*Genbank* *Uniprot*	945146 P77161	61
1.1.1.69	***idnO***	Gluconate 5-dehydrogenase	*Escherichia coli*	*Genbank* *Uniprot*	947109 P0A9P9	62
1.1.1.77	***fucO***	Lactaldehyde reductase	*Escherichia coli*	*Genbank* *Uniprot*	947273 P0A9S1	63
1.1.1.79 *1.1.1.81*	***ghrB***	Glyoxylate/hydroxypyruvate reductase B	*Escherichia coli*	*Genbank* *Uniprot*	948074 P37666	64
1.1.1.79 *1.1.1.81*	***ghrA***	Glyoxylate/hydroxypyruvate reductase A	*Escherichia coli*	*Genbank* *Uniprot*	946431 P75913	65
1.1.1.103	***tdh***	L-threonine 3-dehydrogenase	*Escherichia coli*	*Genbank* *Uniprot*	948139 P07913	66
1.1.1.127	***kduD***	2-dehydro-3-deoxy-D-gluconate 5-dehydrogenase	*Escherichia coli*	*Genbank* *Uniprot*	947323 P37769	67
1.1.1.130	***dlgD***	2,3-diketo-L-gulonate reductase	*Escherichia coli*	*Genbank* *Uniprot*	948096 P37672	68
1.1.1.169	***panE***	2-dehydropantoate 2-reductase	*Escherichia coli*	*Genbank* *Uniprot*	945065 P0A9J4	69
1.1.1.205	***guaB***	Inosine-5'-monophosphate dehydrogenase	*Escherichia coli*	*Genbank* *Uniprot*	946985 P0ADG7	13 70
1.1.1.262	***pdxA***	4-hydroxythreonine-4-phosphate dehydrogenase	*Escherichia coli*	*Genbank* *Uniprot*	944919 P19624	71
1.1.1.274	***dkgA***	2,5-diketo-D-gluconic acid reductase A	*Escherichia coli*	*Genbank* *Uniprot*	947495 Q46857	72
1.1.1.346	***dkgB***	2,5-diketo-D-gluconic acid reductase B	*Escherichia coli*	*Genbank* *Uniprot*	944901 P30863	73
EC 1.1.2	***With a cytochrome as acceptor***					
1.1.2	***dld***	D-lactate dehydrogenase, cytochrome-type (Dld)	*Archaeoglobus fulgidus*	*Genbank* *Uniprot*	1483609 O29853	74
1.1.2.3	***lldD***	L-lactate dehydrogenase [cytochrome]	*Escherichia coli*	*Genbank* *Uniprot*	948121 P33232	75
EC 1.1.3	***With oxygen as acceptor***					
1.1.3.15	***lhgO***	L-2-hydroxyglutarate oxidase LhgO	*Escherichia coli*	*Genbank* *Uniprot*	948069 P37339	76

1.1.3.15	***glcD*** ***glcE*** ***glcF***	Glycolate oxidase	*Escherichia coli*	*Genbank* *Uniprot* *Genbank* *Uniprot* *Genbank* *Uniprot*	947353 P0AEP9 2847718 P52073 2847717 P52074	77 78 79
1.1.3.15	***Hao2***	Hydroxyacid oxidase 2	*Rattus norvegicus*	*Genbank* *Uniprot*	84029 Q07523	80
1.1.3.15	***NA***	Peroxisomal (S)-2-hydroxy-acid oxidase	*Spinacia oleracea (Spinach)*	*Genbank* *Uniprot*	NA P05414	81
EC 1.1.99	***With unknown physiological acceptors***					
1.1.99.6	***dld***	D-lactate dehydrogenase	*Sulfolobus tokodaii*	*Genbank* *Uniprot*	1458598 Q974L0	82
EC 1	***Oxidoreductases***					
EC 1.2	***Acting on the aldehyde or oxo group of donors***					
EC 1.2.1	***With NAD+ or NADP+ as acceptor***					
1.2.1.11	***asd***	Aspartate-semialdehyde dehydrogenase	*Escherichia coli*	*Genbank* *Uniprot*	947939 P0A9Q9	12 83
1.2.1.88	***putA***	Bifunctional protein PutA	*Escherichia coli*	*Genbank* *Uniprot*	945600 P09546	84
EC 1	***Oxidoreductases***					
EC 1.3	***Acting on the CH-CH group of donors***					
1.3.5.1	***sdhA***	Succinate dehydrogenase flavoprotein subunit	*Escherichia coli*	*Genbank* *Uniprot*	945402 P0AC41	85
EC 1	***Oxidoreductases***					
EC 1.4	***Acting on the CH-NH2 group of donors***					
1.4.1.4	***gdhA***	NADP-specific glutamate dehydrogenase	*Escherichia coli*	*Genbank* *Uniprot*	946802 P00370	86
EC 1	***Oxidoreductases***					
EC 1.17	***Acting on CH or CH2 groups***					
1.17.1.8	***dapB***	4-hydroxy-tetrahydrodipicolinate reductase	*Escherichia coli*	*Genbank* *Uniprot*	944762 P04036	87
EC 2	***Transferases***					
EC 2.7	***Transferring phosphorus-containing groups***					
2.7.1.167 *2.7.7.70*	***hldE***	Bifunctional protein HldE	*Escherichia coli*	*Genbank* *Uniprot*	947548 P76658	88
EC 2.8	***Transferring sulfur-containing groups***					
2.8.1.7	***iscS***	Cysteine desulfurase IscS	*Escherichia coli*	*Genbank* *Uniprot*	947004 P0A6B7	14 89
EC 3	***Hydrolases***					
EC 3.5	***Acting on carbon-nitrogen bonds, other than peptide bonds***					
EC 3.5.99	***on other compounds***					
3.5.99.10	***ridA***	2-iminobutanoate/2-iminopropanoate deaminase	*Escherichia coli*	*Genbank* *Uniprot*	948771 P0AF93	90
EC 4	***Lyases***					
EC 4.2	***Carbon-oxygen lyase***					
4.2.1.3	***acnB***	Aconitate hydratase B	*Escherichia coli*	*Genbank* *Uniprot*	944864 P36683	15 91
EC 4.3	***Carbon-nitrogen lyases***					
4.3.1.1	***aspA***	Aspartate ammonia-lyase	*Escherichia coli*	*Genbank* *Uniprot*	948658 P0AC38	92
EC 6	***Ligases***					

EC 6.3	***Forming carbon-nitrogen bonds***					
EC 6.3.1	***Acid—ammonia (or amine) ligases (amide synthases)***					
6.3.1.2	***glnA***	Glutamine synthetase	*Escherichia coli*	*Genbank Uniprot*	948370 P0A9C5	93

In a preferred embodiment the conversion of 4-methylthio-2-oxobutanoic acid into 2-hydroxy-4-(methylthio) butyric acid is catalysed by at least one enzyme having aspartate-semialdehyde dehydrogenase, inosine-5'-monophosphate dehydrogenase, cysteine desulfurase and aconitase hydratase B activity encoded by at least one endogenous or exogenous gene disclosed in table 5 and overexpressed in said recombinant microorganism.

More preferably at least one gene chosen among *asd, guaB, iscS* and *acnB* and catalyzing the conversion of 4-methylthio-2-oxobutanoic acid into 2-hydroxy-4-(methylthio) butyric acid is overexpressed in said recombinant microorganism.

Preferentially the microorganism of the invention overexpresses at least one gene chosen among *asd, guaB, iscS and acnB* having at least 60%, preferably at least 70%, more preferably at least 85% and even more preferably 90% amino acids identity with complete protein encoded by gene *asd* from *Escherichia coli,* gene *guaB* from *Escherichia coli,* gene *iscS* from *Escherichia coli* and gene *acnB* from *Escherichia coli*. More preferentially, the microorganism of the invention overexpresses at least one gene chose among *asd, guaB, gltB, iscS and acnB* genes from *Escherichia coli* (i.e. 100% amino acids sequence identity with the protein sequence encoded by said gene).

Sequence identity between amino acid sequences can be determined by comparing a position in each of the sequences which may be aligned for the purposes of comparison. When a position in the compared sequences is occupied by the same amino acid, then the sequences are identical at that position. A degree of sequence identity between proteins is a function of the number of identical amino acid residues at positions shared by the sequences of said proteins.

To determine the percentage of identity between two amino acid sequences, the sequences are aligned for optimal comparison. For example, gaps can be introduced in the sequence of a first amino acid sequence for optimal alignment with the second amino acid sequence. The amino acid residues at corresponding amino acid positions are then compared. When a position in the first sequence is occupied by the same amino acid residue as the corresponding position in the second sequence, the molecules are identical at that position.

The percentage of identity between the two sequences is a function of the number of identical positions shared by the sequences. Hence % identity = number of identical positions / total number of overlapping positions X 100.

Optimal alignment of sequences may be conducted by the global homology alignment algorithm of Needleman and Wunsch (1970), by computerized implementations of this algorithm or by visual inspection. The best alignment (i.e., resulting in the highest percentage of identity between the compared sequences) generated by the various methods is selected.

In other words, the percentage of sequence identity is calculated by comparing two optimally aligned sequences, determining the number of positions at which the identical amino acid occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions and multiplying the result by 100 to yield the percentage of sequence identity.

In a most preferred embodiment of the invention, the genetically modified microorganism overexpresses *aspC* gene and *asd* gene.

The method for the production of 2-hydroxy-4-(methylthio) butyric acid according to the present invention is related to the production of L-2-hydroxy-4-(methylthio) butyric acid or D-2-hydroxy-4-(methylthio) butyric acid from respectively L-methionine or D-methionine.

Similarly, the genetically modified microorganism of the invention is for the production of L-2-hydroxy-4-(methylthio) butyric acid or D-2-hydroxy-4-(methylthio) butyric acid from respectively L-methionine or D-methionine.

More preferentially the invention is directed to a method for the production of L-2-hydroxy-4-(methylthio) butyric acid from L-methionine and to a genetically modified microorganism for the production of L-2-hydroxy-4-(methylthio) butyric acid from L-methionine.

Preferably the microorganism of the invention is further genetically modified to improve methionine production, in particular L-methionine production, one of the precursors of the 2-hydroxy-4-(methylthio) butyric acid, in particular L-2-hydroxy-4-(methylthio) butyric acid.

The recombinant microorganism of the invention or used according to the invention is able to produce the methionine amino acid. More preferably the recombinant microorganism of the invention or used according to the invention is optimized for the production of L-methionine.

Genes involved in methionine production in a microorganism are well known in the art, and comprise genes involved in the methionine specific biosynthesis pathway as well as genes involved in precursor-providing pathways and genes involved in methionine consuming pathways.

Efficient production of methionine requires the optimisation of the methionine specific pathway and several precursor–providing pathways. L-Methionine producing strains have been described in patent applications WO2005/111202, WO2007/077041 and

WO2009/043803, WO2010/020681, WO2011/073738, WO2011/080542, WO2011/080301, WO2012/055798, WO2013/001055, WO2013/190343, WO2015/028675 and WO2015/028674 which are incorporated as reference into this application.

The patent application WO2005/111202 describes a L-methionine producing strain that overexpresses homoserine succinyltransferase alleles with reduced feed-back sensitivity to its inhibitors SAM and methionine (called *metA**). This application describes also the combination of theses alleles with a deletion of the methionine repressor MetJ responsible for the down-regulation of the methionine regulon. In addition, the application describes combinations of the two modifications with the overexpression of aspartokinase/homoserine dehydrogenase (coded by the *thrA* gene) encoding for an enzyme with reduced feed-back inhibition to threonine (*thrA**).

For improving the production of L-methionine, the microorganism of the invention may exhibit:

- an increased expression of at least one gene selected in the group consisting of:
  - *cysP* which encodes a periplasmic sulphate binding protein, as described in WO2007/077041 and in WO2009/043803,
  - *cysU* which encodes a component of sulphate ABC transporter, as described in WO2007/077041 and in WO2009/043803,
  - *cysW* which encodes a membrane bound sulphate transport protein, as described in WO2007/077041 and in WO2009/043803,
  - *cysA* which encodes a sulphate permease, as described in WO2007/077041 and in WO2009/043803,
  - *cysM* which encodes an O-acetyl serine sulfhydralase, as described in WO2007/077041 and in WO2009/043803,
  - *cysI* and *cysJ* encoded respectively the alpha and beta subunits of a sulfite reductase as described in WO2007/077041 and in WO2009/043803. Preferably *cysI* and *cysJ* are overexpressed together,
  - *cysH* which encodes an adenylylsulfate reductase, as described in WO2007/077041 and in WO2009/043803,
  - *cysE* which encodes a serine acyltransferase, as described in WO2007/077041,
  - *serA* which encodes a phosphoglycerate dehydrogenase, as described in WO2007/077041 and in WO2009/043803,
  - *serB* which encodes a phosphoserine phosphatase, as described in WO2007/077041 and in WO2009/043803,
  - *serC* which encodes a phosphoserine aminotransferase, as described in WO2007/077041 and in WO2009/043803,

- *metA* or *metA* alleles which encode an homoserine succinyltransferases with reduced feed-back sensitivity to S-adenosylmethionine and/or methionine (*metA**) as described in WO2005/111202,
- *thrA* or *thrA* alleles which encode aspartokinases/homoserine dehydrogenase with reduced feed-back inhibition to threonine (*thrA**), as described in WO2009/043803 and WO2005/111202,
- *ptsG* which encodes the glucose-specific phosphoenolpyruvate (PEP) phosphotransferase system (PTS) permease, as described in WO 2013/001055,
- *metH* which encodes a cobalamin-dependent methionine synthase, as described in WO2015/028674,
- *fldA* which encodes an essential flavodoxin containing FMN as a prosthetic group which interacts with Fpr and MetH proteins, as described in WO2015/028674,
- *fpr* which encodes a flavodoxin NADP+ reductase required for the activation of the methionine synthase MetH as described in WO2015/028674. The reductase uses non covalently bound FAD as a cofactor,
- *pntAB* operon which encodes respectively the α-subunit and the an inner membrane protein with nine predicted transmembrane domains of the membrane bound proton translocating pyridine nucleotide transhydrogenase, as described in WO 2012/055798,
- *metNIQ* operon which encodes for the subunits of the ABC transporter involved in the uptake of methionine,
- *pyc* which encodes pyruvate carboxylase as described in patent application WO 2012/055798.

- and/or an inhibition of the expression of at least one of the following genes:
  - *pykA* which encodes a pyruvate kinase, as described in WO2007/077041 and in WO2009/043803,
  - *pykF* which encodes a pyruvate kinase, as described in WO2007/077041 and in WO2009/043803,
  - *purU* which encodes a formyltetrahydrofolate deformylase, as described in WO2007/077041 and in WO2009/043803,
  - *yncA* which encodes a N-acetyltransferase, as described in WO 2010/020681,
  - *metJ* which encodes a repressor of the methionine biosynthesis pathway, as described in WO2005/111202,
  - *udhA* which encodes a soluble pyridine nucleotide transhydrogenase which catalyses essentially the oxidation of NADPH into $NADP^+$ via the reduction of NAD^+ into NADH as described in patent application WO 2012/055798.

- *dgsA* which encodes a transcriptional dual regulator that controls the expression of a number of genes encoding enzymes of the phosphotransferase (PTS) and phosphoenolpyruvate (PEP) systems, as described in WO 2013/001055,
- *sgrS* which encodes a small RNA which regulates post-transcriptionally the abundance of PtsG, as described in WO 2013/001055
- *sgrT* which encodes a regulator which plays a role in the glucose-phosphate stress response, regulating the activity of PtsG, as described in WO 2013/001055
- *metE* which encodes an independent cobalamin methionine synthase, as described in WO 2013/190343,
- *ygaZH* operon which encodes a member of the branched chain amino acid exporter (LIV-E) family responsible for export of L-valine and L-methionine,

- and/or an increasing of the C1 metabolism that leads to an improved methionine production.

According to the invention, "increasing C1 metabolism" relates to the increase of the activity of at least one enzyme involved in the C1 metabolism chosen among MetF, GcvTHP, Lpd, GlyA, MetE or MetH. For increasing enzyme activity, the corresponding genes of these different enzymes may be overexpressed or modified in their nucleic sequence to expressed enzyme with improved activity or their sensitivity to feed-back regulation may be decreased.

In a preferred embodiment of the invention, the one carbon metabolism is increased by enhancing the activity of methylenetetrahydrofolate reductase MetF and/or the activity of glycine cleavage complex GcvTHP and/or the activity of serine hydroxymethyltransferase GlyA.

In a specific embodiment of the invention, the activity of MetF is enhanced by overexpressing the gene *metF* and/or by optimizing the translation.

In a specific embodiment of the invention, overexpression of *metF* gene is achieved by expressing the gene under the control of a strong promoter belonging to the *Ptrc* family promoters, or under the control of an inducible promoter, like a temperature inducible promoter P_R as described in application WO2011/073738.

According to another embodiment of the invention, optimisation of the translation of the protein MetF is achieved by using a RNA stabiliser. Other means for the overexpression of a gene are known to the expert in the field and may be used for the overexpression of the *metF* gene.

In another aspect of the invention, the activity of the pyruvate carboxylase is enhanced. Increasing activity of pyruvate carboxylase is obtained by overexpressing the corresponding gene or modifying the nucleic sequence of this gene to express an enzyme with improved activity. In another embodiment of the invention, the *pyc* gene is introduced

on the chromosome in one or several copies by recombination or carried by a plasmid present at least at one copy in the modified microorganism. The *pyc* gene originates from *Rhizobium etli*, *Bacillus subtilis, Pseudomonas fluorescens, Lactococcus lactis* or *Corynebacterium* species. In a preferred embodiment, the microorganism of the invention overexpresses *pyc* gene from *Rhizobium etli*.

Genes may be expressed under control of an inducible promoter. Patent application WO2011/073738 describes a L-methionine producing strain that expresses a *thrA* allele with reduced feed-back inhibition to threonine and *cysE* gene under the control of an inducible promoter. This application is incorporated as reference into this application. In a specific embodiment of the invention, the *thrA* or allele, *pyc*, *pntAB* or *ptsG* genes are under control of a temperature inducible promoter. In a most preferred embodiment, the temperature inducible promoter used belongs to the family of P_R or P_L promoters.

In a particular embodiment of the invention, the overexpressed genes are at their native position on the chromosome or are integrated at a non-native position. For an optimal L-methionine production, several copies of the gene may be required, and these multiple copies are integrated into specific loci, whose modification does not have a negative impact on methionine production.

Examples for locus into which a gene may be integrated, without disturbing the metabolism of the cell, are disclosed in patent applications WO2011/073122, WO2011/073738 and WO2012/055798.

The present invention is also related to a method for the fermentative production of the methionine hydroxy analogue, comprising culturing a recombinant microorganism modified to produce methionine in a culture medium comprising a source of carbon; wherein in said microorganism methionine is converted into the 4-(methylsulfanyl)but-2-enoic acid by the action of an ammonia lyase, and the intermediate product 4-(methylsulfanyl)but-2-enoic acid is converted into 2-Hydroxy-4-(methylthio) butanoic acid (hydroxymethionine) by the action of a carbon-oxygen lyase also called hydro-lyase or dehydratase (Fig. 2).

A lyase is an enzyme that catalyzes the breaking (an "elimination" reaction) of various chemical bonds by means other than hydrolysis (a "substitution" reaction) and oxidation, often forming a new double bond or a new ring structure. The reverse reaction is also possible. Lyases differ from other enzymes in that they require only one substrate for the reaction in one direction, but two substrates for the reverse reaction. Lyases are classified as EC 4 in the EC number classification of enzymes.

The reaction which converts the L-methionine into 4-(methylsulfanyl)but-2-enoic acid can be performed by an ammonia lyase from the class EC 4.3. Enzymes responsible of this reaction are able to cleave carbon-nitrogen bonds, as for instance enzymes listed in table 6 below.

Table 6: Lyase enzymes acting carbon-nitrogen bonds able to produce 4-(methylsulfanyl)but-2-enoic acid from L-methionine

Accesion Number in Uniprot database	***Gene Name***	***Enzyme Name / Function***	***EC Number***	***Origin (Genus species)***
P0AC38	***aspA***	Aspartate ammonia-lyase	4.3.1.1	*Escherichia Coli*
P0AEJ6 P19636	***eutB*** ***eutC***	Ethanolamine ammonia-lyase heavy chain Ethanolamine ammonia-lyase light chain	4.3.1.7	*Escherichia Coli*

The reaction which converts the 4-(methylsulfanyl)but-2-enoic acid into hydroxymethionine can be performed by a carbon-oxygen lyase or dehydratase from the class EC 4.2. Enzymes responsible of this reaction are able to cleave carbon-oxygen bonds, as for instance enzymes listed in table 7 below.

Table 7: Lyase enzymes acting carbon-oxygen bonds able to produce L-hydroxymethionine from 4-(methylsulfanyl)but-2-enoic acid.

Accesion Number in Uniprot database	***Gene Name***	***Enzyme Name / Function***	***EC Number***	***Origin (Genus species)***
P77608	***mhpD***	2-keto-4-pentenoate hydratase	4.2.1.80	*Escherichia Coli*
P25516	***acnA***	Aconitate hydratase 1	4.2.1.3	*Escherichia Coli*
P36683	***acnB***	2-methylisocitrate dehydratase	4.2.1.99	*Escherichia Coli*
P05042	***fumC***	Fumarate hydratase class II	4.2.1.2	*Escherichia Coli*
P0AC33	***fumA***	Fumarate hydratase class I, aerobic	4.2.1.2	*Escherichia Coli*
P14407	***fumB***	Fumarate hydratase class I, anaerobic	4.2.1.2	*Escherichia Coli*
P75764	***YbhJ***	Uncharacterized protein YbhJ		*Escherichia Coli*

The invention relates also to a method for the production of 2-hydroxy-4-(methylthio) butyric acid by bioconversion comprising:

- mixing in an appropriate reaction mixture known by the man skilled in the art (NAD(P)H, buffer and salts) and purified enzyme having activity chosen among the group consisting of: transaminase, oxidoreductase, transferase, hydrolase, lyase and ligase with a methionine solution,
- incubating the mixture at 37°C for a sufficient time until the end of the reaction, and
- recovering 2-hydroxy-4-(methylthio) butyric acid from the mixture.

Finally, the present invention relates to a method for the production of 2-hydroxy-4-(methylthio) butyric acid comprising:

- culturing the genetically modified microorganism described above in an appropriate culture medium comprising a source of carbon, a source of sulphur and a source of nitrogen,
- recovering 2-hydroxy-4-(methylthio) butyric acid from the culture medium.

More preferentially the invention is directed to a method for the production of L-2-hydroxy-4-(methylthio) butyric acid from L-methionine comprising culturing a genetically modified microorganism for the production of L-2-hydroxy-4-(methylthio) butyric acid from L-methionine.

According to the invention, the terms "fermentative process", "culture" or "fermentation" are used interchangeably to denote the growth of a given microorganism on an appropriate culture medium containing a carbon source, a source of sulfur and a source of nitrogen. The growth is generally performed in fermenters with an appropriate growth medium adapted to the microorganism being used.

In the fermentative process of the invention, the source of carbon is used simultaneously for:

- biomass production: growth of the microorganism by converting inter alia the carbon source of the medium, and,
- hydroxymethionine and/or methionine production: transformation of the same carbon source into hydroxymethionine and/or methionine by the biomass.

The two steps are concomitant, and the transformation of the source of carbon by the microorganism to grow results in the L-hydroxymethionine and/or L-methionine production in the medium, since the microorganism comprises a metabolic pathway allowing such conversion.

An "appropriate culture medium" means herein a medium (e.g., a sterile, liquid media) comprising nutrients essential or beneficial to the maintenance and/or growth of the microorganism such as carbon sources or carbon substrates; nitrogen sources, for example peptone, yeast extracts, meat extracts, malt extracts, urea, ammonium sulfate, ammonium chloride, ammonium nitrate and ammonium phosphate; phosphorus sources, for example monopotassium phosphate or dipotassium phosphate; trace elements (e.g., metal salts) for example magnesium salts, cobalt salts and/or manganese salts; as well as growth factors such as amino acids and vitamins.

The term "source of carbon" according to the invention denotes any source of carbon that can be used by those skilled in the art to support the normal growth of a microorganism, which can be hexoses such as glucose, galactose or lactose; pentoses; monosaccharides; disaccharides such as sucrose (molasses), cellobiose or maltose;

oligosaccharides such as starch or its derivatives; hemicelluloses; glycerol and combinations thereof. An especially preferred carbon source is glucose. Another preferred carbon source is sucrose.

In a particular embodiment of the invention, the carbon source is derived from renewable feed-stock. Renewable feed-stock is defined as raw material required for certain industrial processes that can be regenerated within a brief delay and in sufficient amount to permit its transformation into the desired product. Vegetal biomass treated or not, is an interesting renewable carbon source.

The source of carbon is fermentable, i.e. it can be used for growth by microorganisms.

The term "source of sulfur" according to the invention refers to sulfate, thiosulfate, hydrogen sulfide, dithionate, dithionite, sulfite, methylmercaptan, dimethylsulfide, dimethyl disulfide and other methyl capped sulphides or a combination of the different sources. Preferred sulfur source in the culture medium is sulfate or thiosulfate or a mixture thereof. Another preferred sulfur source is dimethyl disulfide.

Those skilled in the art are able to define the culture conditions and the composition of culture medium for the microorganisms according to the invention. In particular the bacteria are fermented at a temperature between 20°C and 55°C, preferentially between 25°C and 40°C, and more specifically about 30°C for *C. glutamicum* and about 37°C for *E. coli*.

As an example of known culture medium for *E. coli*, the culture medium can be of identical or similar composition to an M9 medium (Anderson, 1946, *Proc. Natl. Acad. Sci. USA* **32**:120-128), an M63 medium (Miller, 1992; A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York) or a medium such as defined by Schaefer *et al.* (1999, *Anal. Biochem.* **270**: 88-96).

As an example of known culture medium for *C. glutamicum,* the culture medium can be of identical or similar composition to BMCG medium (Liebl *et al.*, 1989, *Appl. Microbiol. Biotechnol.* **32**: 205-210) or to a medium such as described by Riedel *et al.* (2001, *J. Mol. Microbiol. Biotechnol.* **3**: 573-583).

The method of the invention can be performed either in a batch process, in a fed-batch process or in a continuous process, and under aerobic, micro-aerobic or anaerobic conditions.

A fermentation "under aerobic conditions" means that oxygen is provided to the culture by dissolving gas into the liquid phase of the culture. This can be achieved by (1) sparging oxygen containing gas (e.g. air) into the liquid phase, or (2) shaking the vessel containing the culture medium in order to transfer the oxygen contained in the head space

into the liquid phase. The main advantage of the fermentation under aerobic conditions is that the presence of oxygen as an electron acceptor improves the capacity of the strain to produce more energy under the form of ATP for cellular processes, thereby improving the general metabolism of the strain.

Micro-aerobic conditions can be used herein and are defined as culture conditions wherein low percentages of oxygen (e.g. using a mixture of gas containing between 0.1 and 10% of oxygen, completed to 100% with nitrogen) are dissolved into the liquid phase.

By contrast, "anaerobic conditions" are defined as culture conditions wherein no oxygen is provided to the culture medium. Strictly anaerobic conditions can be obtained by sparging an inert gas like nitrogen into the culture medium to remove traces of other gas. Nitrate can be used as an electron acceptor to improve ATP production by the strain and improve its metabolism.

In the invention, the fermentation is done in fed-batch mode. This refers to a type of fermentation in which supplementary growth medium is added during the fermentation, but no culture is removed until the end of the batch (except small volumes for samplings and HPLC/GCMS analysis). The process comprises two main steps; the first one which is a series of pre cultures in appropriate batch mineral medium and fed-batch mineral medium. Subsequently, a fermentor filled with appropriate minimal batch medium is used to run the culture with different fed-batch medium according to the desire production.

The culture may be performed in such conditions that the microorganism is limited or starved for an inorganic substrate, in particular phosphate and/or potassium. Subjecting an organism to a limitation of an inorganic substrate defines a condition under which growth of the microorganisms is governed by the quantity of an inorganic chemical supplied that still permits weak growth. Starving a microorganism for an inorganic substrate defines the condition under which growth of the microorganism stops completely due, to the absence of the inorganic substrate.

The term "source of nitrogen" corresponds to either an ammonium salt or ammoniac gas. Nitrogen comes from an inorganic (e.g., $(NH_4)_2SO_4$) or organic (e.g., urea or glutamate) source. In the invention sources of nitrogen in culture are $(NH_4)_2HPO_4$, $(NH_4)_2S_2O_3$ and NH_4OH.

In a specific aspect of the invention, the recombinant microorganism is cultivated under conditions of nitrogen limitation. The term "conditions of nitrogen limitation" refers to a culture medium having a limited concentration of nitrogen compared to the needs of the microorganism of production, wherein the nitrogen may be supplied from an inorganic (e.g., $(NH_4)_2SO_4$) or organic (e.g., urea or glutamate) source, and the term "conditions of nitrogen starvation" refers to a medium having no nitrogen source at all. Conditions of

nitrogen limitation are well described in patent application WO 2012/090022 which is incorporated as reference into this application.

In a preferred embodiment of the invention the fermentation process is done in two successive steps in a bio-reactor system: a first step of growth of the microorganisms for about 10h to 20h in an appropriate culture medium comprising a source of carbon, a source of sulphur and nitrogen, preferably for about 15h to 20h and a second step of culture of the microorganisms for about 10h to 20h in nitrogen limitation conditions in an appropriate culture medium, preferably for about 10h to 15h.

According to a preferred embodiment, the method of the invention further comprises a step of recovering the 2-hydroxy-4-(methylthio) butyric acid from the culture medium.

The action of "recovering 2-hydroxy-4-(methylthio) butyric acid from the culture medium" designates the action of recovering and purifying 2-hydroxy-4-(methylthio) butyric acid. "Recovering" means recovering the first product directly obtained from the fermentative process (fermentation must) which contains the product of interest (in this case hydroxymethionine) and other co-products of the fermentation.

The "purifying" step consists of specifically purify the product of interest (in this case hydroxymethionine) in order to obtain pure hydroxymethionine.

2-hydroxy-4-(methylthio) butyric acid might be recovered by techniques and means well known by the man skilled in the art like distillation, ion-exchange chromatographic methods, precipitation, crystallisation or complexation with salts and particularly with calcium salts or ammonium salts.

In a specific embodiment of the invention, the 2-hydroxy-4-(methylthio) butyric acid is recovered from the fermentation broth (culture medium) by solvent extraction. Preferably the solvent used in this extraction is substantially water-immiscible. Suitable solvents are chosen among ketones such as acetone, methyl ethyl ketone, methyl amyl ketone, methyl isoamyl ketone, methyl isopropyl ketone, methyl isobutyl ketone, ethyl butyl ketone, diisobutyl ketone; ethers such as isopropyl ether, tetrahydrofurane and dimethoxyethane, secondary alcohols such as 2-propanol, aldehydes such as n-butyraldehyde and esters such as ethyl acetate, n-butyl acetate, n-proyl acetate and isopropyl acetate. Preferred solvents are chosen among ketone, ethers and secondary alcohols.

In another embodiment of the invention the purification may be a combination of solvent extraction and other techniques like distillation or crystallisation.

Preferably, step of polishing with active charcoal or ion exchange resin may be used after the steps of 2-hydroxy-4-(methylthio) butyric acid purification to removed organic and/or mineral impurities.

Optionally, biomass and other insoluble parts are removed, before or during the recovering step, by means well known by the man skilled in the art as flocculation, decanting, centrifugation or membrane technique like microfiltration, ultrafiltration, nanofiltration or reverse osmosis.

Each steps for the recovering of 2-hydroxy-4-(methylthio) butyric acid may be advantageously achieved in different conditions of temperature and pH.

EXAMPLES

PROTOCOLS

Several protocols have been used to construct methionine producing strains described in the following examples.

Protocol 1 (Chromosomal modifications by homologous recombination, selection of recombinants and antibiotic cassette excision) and protocol 2 (Transduction of phage P1) used in this invention have been fully described in patent application WO2013/001055.

Protocol 3: Construction of recombinant plasmids

Recombinant DNA technology is well known by the man skilled in the art. Briefly, the DNA fragments of interest were PCR amplified using oligonucleotides and appropriate genomic DNA as matrix (that the person skilled in the art is able to define). The resulting DNA fragments and the chosen plasmid were digested with compatible restriction enzymes (that the person skilled in the art is able to define), then ligated and transformed into competent cells. Transformants were analysed and recombinant plasmids of interest were verified by DNA sequencing.

EXAMPLE 1: MEASUREMENT OF ENZYMATIC ACTIVITIES INVOLVED IN THE PRODUCTION OF METHIONINE HYDROXY ANALOG

Crude extract preparation:

About 25 mg of *E. coli* biomass collected by centrifugation were resuspended in 1 ml of 100 mM Potassium phosphate pH 7.6, 0.1 mM DTT, 0.1mM pyridoxal-5′-phosphate (PLP) and a protease inhibitor cocktail. The cells suspension was applied to a Precellys® glass beads kit (0.1 mm) and cells were lysed using a Precellys 24 Bertin Technologies during two cycles of 10 sec at 5000 rpm. After lysis, cellular debris were removed by centrifugation at 12 000g for 30 min at 4°C. The crude extracts were desalted using a Zeba desalt spin column 5 ml (Thermo).

Metabolic pathway n°1 for MHA production,

REACTION A

The first step of the MHA pathway converts the L-methionine into 4-Methylthio-2-oxobutanoic acid (KMTB). This can be done by two different kinds of reactions catalysed by two different types of enzymes; i) a methionine amino transferase (MetAT) or ii) a methionine oxydoreductase.

Reaction A: Methionine aminotransferase assay (MetAT)

Several couples of molecules listed in table 8 were tested as amino acceptor or amino donors for the reaction MetAT according to the protocol presented below for α-ketoglutarate (amino acceptor) and glutamate (amino donor).

Table 8: Couples of amino acceptor and donor for the methionine aminotransferase reaction

Amino Acceptor	Amino Donor
Phenylpyruvate	Phenylalanine
2-oxoglutaramate	Glutamine
2-oxo-3-phenylpropanoate	L-phenylalanine
Pyruvate	L-alanine
4-methyl-2-oxopentanoate	L-leucine
α-ketovaline	L-valine
3-methyl-2-oxopentanoate	L-isoleucine
2-oxoglutarate	L-glutamate
Phenylpyruvate	Phenylalanine
Glyoxylate	Glycine
Indole-3-pyruvate	L-tryptophan

Methionine aminotransferase activity assay with the amino acceptor α-ketoglutarate and the amino donor glutamate

The methionine aminotransferase activity was measured at 37°C in the reverse direction using a coupled enzymatic assay. The assay was carried out with a solution of 20 mM potassium phosphate buffer pH 7.5, 2 mM NAD^+, 2 mM Thiamine pyrophosphate, 1mM $MgCl_2$, 1 mM DTT, 10 mM 4-Methylthio-2-oxobutanoic acid, 0.5 mM sodium CoA, 0.3 units α-ketoglutarate dehydrogenase from porcine heart, 10 mM glutamate acid neutralized in a final volume of 1 ml. The mixture was incubated for 5 min at 37°C and then the reaction was initiated by the addition of 50µg of crude extract of the strain of interest. The reduction of NAD^+ was monitored at 340 nm on a spectrophotometer. (Epsilon 340 nm =

6290 M^{-1} cm^{-1}). The activity of the blank solution without any crude extract was subtracted from the activity detected in the assay.

One unit of enzyme activity was defined as the amount of enzyme that consumed 1 µmol substrate per minute under the conditions of the assay. Specific enzyme activity was expressed as units per mg of protein.

In parallel the reaction mix used in the assays was analysed by GC-MS after derivatization in two stages (oximation and silylation) to confirm and quantify the products of the reaction; α-ketoglutarate and L-methionine. A standard of methionine prepared in the matrix was used for the quantification.

Table 9: Methionine aminotransferase activity measured in crude extracts of strain 5 corresponding to strain 1 described in patent application WO2012/090022 and renamed strain 5 in this application.

	Specific activity (mUI/mg)	**L-Methionine detection by GC-MS analysis**
Methionine aminotransferase (MetAT) activity	**260**	**+**

(+ Product quantity measured superior or equal to 150 µM)

These results show that the enzyme catalysing reaction A of pathway 1, which produces KMTB from L-methionine, is present and well active in strain 1 described in patent application WO2012/090022 and renamed strain 5 in this application. This reaction is the first step of the conversion of L-methionine in L-hydroxymethionine.

Reaction A: Methionine oxydoreductase

The reaction A of the pathway N°1 can also be performed by an oxydoreductase. The activity assay used for this reaction is described below.

Methionine oxydoreductase activity assay

The standard reaction mixture for oxidative deamination was carried out with 20 mM potassium phosphate buffer pH 7.5, 10 mM of L-methionine, 2 mM of NAD^+ in a final volume of 1.0 ml. The reaction mixture was incubated for 5 min at 37°C and then the reaction was initiated by the addition of 50 µg of crude extract.

The assay reaction mixture for reductive amination was carried out with 20 mM potassium phosphate buffer pH 9, 10 mM 4-Methylthio-2-oxobutanoic acid, 0.2 mM of NADH in a final volume of 1.0 ml. The reaction mixture was incubated for 5 min at 37°C and then the reaction was initiated by the addition of 50µg of crude extract.

The reduction or oxidation of NAD was monitored at 340 nm on a spectrophotometer. (Epsilon 340 nm = 6290 M^{-1} cm^{-1}). The activity detected in control assay, lacking the substrate, was subtracted from the activity detected in the assay.

One unit of enzyme activity was defined as the amount of enzyme that consumed 1 µmol substrate per minute under the conditions of the assay. Specific enzyme activity was expressed as units per mg of protein.

REACTION B

The second step of the MHA pathway converts the 4-Methylthio-2-oxobutanoic acid (KMTB) into L-hydroxymethionine (L-methionine hydroxy analogue MHA). This can be done by two different kinds of enzymatic reaction assays, i) a 4-Methylthio-2-oxobutanoic acid/hydroxymethionine transhydrogenase (KMTB-HOT) assay or ii) a 4-Methylthio-2-oxobutanoic acid reductase (KMTBr) assay.

Reaction B: 4-Methylthio-2-oxobutanoic acid/hydroxymethionine transhydrogenase assay (KMTB-HOT)

The KMTB-HOT activity was tested with different molecules of hydroxyacid in order to find out the best one for the reaction.

The 4-Methylthio-2-oxobutanoic acid/hydroxymethionine transhydrogenase activity assay was carried out in a solution of 20 mM potassium phosphate buffer pH 7.5, 0.1mM Pyridoxal 5-phosphate (PLP), 10 mM 4-Methylthio-2-oxobutanoic acid (KMTB), 6mM NAD(P)H, 10 mM hydroxyacid neutralized in a final volume of 100µl. The solution mixture was incubated for 10 min at 37°C and then the reaction was initiated by the addition of 100 µg of crude extract.

The reaction products (hydroxymethionine and ketoacid) of the 4-Methylthio-2-oxobutanoic acid/hydroxymethionine transhydrogenase assays were analysed by GC-MS after derivatization in two stages (oximation and silylation). Standard of hydroxymethionine and ketoacid prepared in the matrix were used for the quantification.

Table 10: 4-Methylthio-2-oxobutanoic acid/hydroxymethionine transhydrogenase (KMTB-HOT) activity measured in crude extract of strain 1 described in patent application WO2012/090022 and renamed strain 5 in this application.

	Hydoxyacid of the enzymatic assay	Hydroxymethionine detection by GC-MS analysis	
4-Methylthio-2-oxobutanoic acid/hydroxymethionine transhydrogenase (KMTB-HOT) activity	**NAD(P)H**	**0mM**	**6mM**
	2-Hydroxybutyrate	++	+++
	2-hydroxyisovalerate	+	++
	2-Hydroxyglutarate	-	-
	3-hydroxybutyrate	-	-

(+++: Product quantity measured superior to 150 µM, ++: Product quantity measured superior to 50 µM, - : Product not detected)

These results show that the enzyme 4-Methylthio-2-oxobutanoic acid/hydroxymethionine transhydrogenase catalysing reaction B of pathway 1, which produces hydroxymethionine (HMTBA) from 4-Methylthio-2-oxobutanoic acid (KMTB) is present and well active in strain 1 described in patent application WO2012/090022 and renamed strain 5 in this application. The preferential hydroxyacid for this reaction is the 2-hydroxybutyrate. This reaction can be performed with or without NAD(P)H cofactor but the presence of NAD(P)H enhances the production of hydroxymethionine.

This reaction is one of the two possible reactions for this conversion; it corresponds to the second step of the conversion of L-methionine in L-hydroxymethionine.

Reaction B: 4-Methylthio-2-oxobutanoic acid reductase assay (KMTBr)

The 4-Methylthio-2-oxobutanoic acid activity was assayed by measuring the initial rate of NAD(P)H oxidation with a spectrophotometer at a wavelength of 340 nm (Epsilon 340 nm = 6290 M-1 cm-1) and at a constant temperature of 37°C. The reaction mixture using 4-Methylthio-2-oxobutanoic acid (KMTB) as substrate was carried out in a solution of 20 mM potassium phosphate buffer pH 7.5, 0.1mM Pyridoxal 5-phosphate (PLP), 0.25 mM NAD(P)H, about 150 µg of crude extract in a final volume of 1ml. The reaction mixture was incubated for 5 min at 37°C and then the reaction was initiated by the addition of the substrate 4-Methylthio-2-oxobutanoic acid (KMTB) at a final concentration of 10 mM. In order to take into account non-specific oxidations of NAD(P)H, the blank assay, lacking the substrate, was run in parallel and the value measured for it was subtracted to the value measured for the assay containing the crude extract of the strain of interest..

One unit of enzyme activity was defined as the amount of enzyme that consumed 1 µmol substrate per minute under the conditions of the assay. Specific enzyme activity was expressed as units per mg of protein.

In parallel the reaction mix of the assays was analysed by GC-MS after derivatization in two stages (oxymation and silylation) to confirm and quantify the product of the reaction: hydroxymethionine. A standard of hydroxymethionine prepared in the matrix was used for the quantification.

Table 11: 4-Methylthio-2-oxobutanoic acid reductase activity (KMTBr) measured in crude extract of strain 1 described in patent application WO2012/090022 and renamed strain 5 in this application.

	specific activity (mUI/mg)	**Hydroxymethionine detection by GC-MS analysis**
4-Methylthio-2-oxobutanoic acid reductase activity	**30**	+

(+: Product quantity measured superior to 5 µM)

These results show that the enzyme catalyzing the 4-Methylthio-2-oxobutanoic acid reductase activity, reaction B of pathway 1, which produces L-hydroxymethionine (HMTBA) from 4-Methylthio-2-oxobutanoic acid (KMTB) is present in strain 1 described in patent application WO2012/090022 and renamed strain 5 in this application. This reaction is one of the two possible reactions for this conversion; it corresponds to the second step of the conversion of L-methionine in hydroxymethionine.

EXAMPLE 2: IDENTIFICATION OF ENZYMES INVOLVED IN CONVERSION OF L-METHIONINE INTO 4-METHYLTHIO-2-OXOBUTANOIC ACID

The *Escherichia coli* BW25113 mutant strains listed in table 12 below come from the Keio mutant collection (Baba *et al.*, 2006). The *Escherichia coli* MG1655 mutant strains were obtained by transferring the deletion by phage transduction (according to Protocol 2) from each BW25113 mutant strain to MG1655 *metA**11 strain. In case of the double mutant strain MG1655 *metA**11 *DybdL*::Cm *DaspC*::Km, to delete *ybdL* (SEQ ID NO: 18) into MG1655 *metA**11 *DaspC*::Km (pKD46) strain, Protocol 1 has been used with primers Ome 0589-DybdLF and Ome 0590-DybdLF (SEQ ID NO: 1 and SEQ ID NO: 2) to amplify the chloramphenicol resistance cassette from plasmid pKD3.

Chloramphenicol recombinants were selected and verified by PCR with appropriate primers. To construct the strain MG1655 *metA**11 P*trc*01/RBS01-*aspC*::Cm, an artificial promoter and ribosome binding site (SEQ ID NO: 3) were inserted in front of *aspC* gene (SEQ ID NO: 9) by using homologous recombination strategy described by Datsenko & Wanner, 2000 (according to Protocol 1). More precisely, a PCR product carrying the artificial promoter and ribosome binding site and the chloramphenicol resistance gene together with FRT sites, surrounded by sequences homologous to *aspC* gene and to the up-stream region of *aspC* on the chromosome (SEQ ID NO: 4 and SEQ ID NO: 5), was generated and introduced into MG1655 *metA**11 (pKD46) strain. The chloramphenicol resistant transformants were verified by PCR with the appropriate primers.

The methionine aminotransferase activity was measured in crude extracts of strains described in table 12 below.

Table 12: Methionine aminotransferase (MetAT) activity measured in crude extracts of strains with deleted targeted genes or overexpressed targeted gene.

Strains	Specific Activity MetAT mUI/mg
Deletions	
Reference strain BW25113	87
BW25113 D*hisC::Km*	87
BW25113 D*gabT::Km*	79
BW25113 D*argD*::*Km*	82
BW25113 *DaspC::Km*	**27**
BW25113 *DgdhA::Km*	83
BW25113 *DilvE::Km*	85
Reference strain MG1655	72
MG1655 *metA*11 DybdL::Km*	88
MG1655 *metA*11 DaspC::Km*	**24**
MG1655 *metA*11 DybdL::Cm DaspC::Km*	**23**
Overexpression	
Reference strain MG1655	72
MG1655 *metA*11 Ptrc01/RBS01-aspC::Cm*	**231**

The amounts of L-methionine detected by GC-MS analysis were consistent with the measured MetAT activities for each strain. For instance we detected only few amount of L-methionine for the strain BW25113 D*aspC*::Km.

The results show in table 12 above show that the enzyme catalysing the methionine aminotransferase activity, which produces KMTB from L-methionine, is not encoded by genes *hisC, gabT, argD, gdhA, ilvE* or *ybdl*. The gene encoding MetAT activity is *aspC*, since its deletion leads to a strong reduction (more than a factor of 3) of the MetAT activity measured in strains BW25113 D*aspC*::Km and MG1655 *metA*11 DaspC*::Km compared to the respective reference strains. Moreover, the overexpression of *aspC* gene increases the MetAT activity (more than a factor 3) measured for the strain MG1655 *metA*11 Ptrc01/RBS01-aspC*::Cm.

EXAMPLE 3: IDENTIFICATION OF ENZYMES INVOLVED IN CONVERSION OF 4-METHYLTHIO-2-OXOBUTANOIC ACID INTO L-HYDROXYMETHIONINE

The *Escherichia coli* BW25113 mutant strains listed in table 13 below comes from the Keio mutant collection (Baba *et al.*, 2006). The *Escherichia coli* MG1655 mutant strains were obtained by transferring the deletion by phage transduction from each BW25113 mutant strain to MG1655, MG1655 *metA**11 or MG1655 *metA**11 D*metJ* strains. To construct the MG1655 *metA**1 P*trc*01/ARN01/RBS01-*asd*::Km strain, artificial promoter, mRNA stabilizing sequence and ribosome binding site (sequence contained in the oligonucleotide SEQ ID N°35 in patent application WO2009/043803) were inserted in front of *asd* gene by using homologous recombination strategy described by Datsenko & Wanner, 2000 (according to Protocol 1). More precisely, a PCR product carrying the artificial promoter and ribosome binding site and the kanamycin resistance gene together with FRT sites, surrounded by sequences homologous to *asd* gene (SEQ ID NO: 12) and to the up-stream region of *asd* on the chromosome (SEQ ID NO: 7 and SEQ ID NO: 8), was generated and introduced into MG1655 *metA**11 (pKD46) strain. The kanamycin resistant transformants were verified by PCR with the appropriate primers.

Table 13: 4-Methylthio-2-oxobutanoic acid/hydroxymethionine transhydrogenase (KMTB-HOT) activity measured in crude extracts of strains with deleted targeted gene or overexpressed targeted gene.

Strains	Specific activity KMTB-HOT (mUI/mg)
Deletions	
Reference strain BW25113	81
BW25113 *DleuB::Km*	≈
BW25113 *DygdH::Km*	≈
BW25113 *DmetB::Km*	≈
BW25113 *DhdhA::Km*	≈
BW25113 *DpurH::Km*	≈
BW25113 *DprpD::Km*	≈
BW25113 *DgltD::Km*	≈
BW25113 *DguaB::Km*	**- - (35)**
BW25113 *DiscS::Km*	**- - (36)**
BW25113 *DacnB::Km*	**- - (45)**
Reference strain MG1655	99
MG1655 *DgltA::Km*	≈
MG1655 *DaldA::Cm*	≈
MG1655 *metA*11 DmetJ DmetF::Km*	≈
MG1655 *Dasd::Cm*	**- - (37)**
MG1655 *metA*11 DgatZ::Km*	≈
Overexpression	
Reference strain MG1655	99
MG1655 metA*11 Ptrc01/ARN01/RBS01-*asd*::Km	**191**

(≈ indicates that the activity measured is similar to the reference strain and -.- indicates that the activity measured is lower than the reference strain)

The results presented in table 13 above demonstrate that the enzyme catalysing the 4-Methylthio-2-oxobutanoic acid/hydroxymethionine transhydrogenase activity (KMTB-HOT), which produces HMTB from KMTB is not encoded by genes *leuB, ygdH, metB, hdhA, purH, prpD, gltD, gltA, aldA, metJ* or *gatZ*. In fact, the deletion of these genes either did not change the KMTB-HOT activity or even increased it. The genes encoding enzymes involved in the KMTB-HOT activity are *guaB*, *iscS, acnB* and *asd,* since their deletion in backgrounds BW25113 or MG1655 (for *asd*) drastically reduced the measured activity (more than a factor 2). Moreover, the overexpression of *asd* confirmed that Asd enzyme is able to convert KMTB into L-methionine since the activity was double in strain MG1655 *metA*11* Ptrc01/ARN01/RBS01-*asd*::Km compared to the reference strain MG1655.

EXAMPLE 4: CONSTRUCTION OF STRAINS FOR PRODUCTION OF L-METHIONINE HYDROXY ANALOG BY FERMENTATION

To produce the L-methionine hydroxy analogue (MHA), a couple of enzymes involved in pathway n°1 and described above were overproduced in different *E. coli* recombinant L-methionine producing strains. The first enzyme is involved in reaction A to allow conversion of methionine into 4-methylthio-2-oxobutanoic acid, and the second enzyme is involved in reaction B to allow conversion of 4-methylthio-2-oxobutanoic acid into L-hydroxymethionine (see Fig. 1 and Tables 4 and 5).

The L-methionine producing strains used as recipient for the overproduction enzymes A and B from the MHA biosynthesis pathway n°1 of the invention were described in previous patent applications. They are the following:

- Genetic background A: Strain MG1655 *metA*11 DmetJ Ptrc-metF Ptrc-metH (pME101-thrA*1-cysE)*, described in patent application WO2007/077041, and named strain 1 in this application.
- Genetic background B: Strain MG1655 *met*A*11 *ΔmetJ Ptrc-metH Ptrc36-ARNmst17-metF PtrcF-cysPUWAM PtrcF-cysJIH Ptrc09-gcvTHP ΔpykA ΔpykF ΔpurU (pME101-thrA*1-cysE-PgapA-metA*11) (pCC1BAC-serB-serA-serC)* and renamed strain 3 in this application.
  Its mother strain, *MG1655 metA*11 ΔmetJ Ptrc-metH Ptrc36-ARNmst17-metF PtrcF-cysPUWAM PtrcF-cysJIH Ptrc09-gcvTHP ΔpykA ΔpykF ΔpurU (pCC1BAC-serB-serA-serC)*, called strain 2, is described in patent application WO2009/043803. To obtain the strain 3 and more precisely the plasmid pME101-*thrA**1-*cysE*-P*gapA-metA**11, *metA**11 gene together with the promoter of *gapA* gene (Thouvenot, *et al.* 2004) were cloned downstream of *cysE* gene into the pME101-*thrA**1-*cysE* plasmid, with a methodology similar to the one described in the patent application WO2011/073122 for the construction of the plasmid *pCL1920-TTadc-CI857-PlambdaR*(-35)-thrA*1-cysE-PgapA-metA*11*. Then to obtain strain 3, the plasmid pME101-*thrA**1-*cysE*-P*gapA-metA**11 was introduced into strain 2.
- Genetic background C: Strain 10 described in patent application WO2012/055798 and renamed strain 4 in this application.
- Genetic background D: Strain 1 described in patent application WO2012/090022, and renamed strain 5 in this application.
- Genetic background E: Strain 17 described in patent application WO2013/001055, and renamed strain 6 in this application.

- Genetic background F: Strain 2 described in patent application EP13306189.5, and renamed strain 7 in this application.

To increase the expression of a gene, the man skilled in the art knows different techniques:
- Increasing the number of copies of the gene of interest in the microorganism. The gene is encoded chromosomally or extra-chromosomally. When the gene is located on the chromosome, several copies of the gene can be introduced on the chromosome by methods of recombination, known by the expert in the field (including gene replacement). When the gene is located extra-chromosomally, it may be carried by different types of plasmids that differ with respect to their origin of replication and thus their copy number in the cell. These plasmids are present in the microorganism either up to 1 to 5 copies, or about 20 copies, or up to 500 copies, depending on the nature of the origin of replication: low copy number plasmids with tight regulated replication (e.g for *E. coli* pSC101, RK2, F), replication origin giving low copy number of plasmids (e.g for *E. coli* p15A, pMB1, pRSF1010) or replication origin giving high copy number of plasmids (e.g for *E. coli* ColE1, pM1*, pSK bluescript II). Examples of suitable *E. coli* expression vectors include pTrc99A, pACYC184, pBR322, pBBRs, pUCs, pKC30, pRep4, pHS1, pHS2, pPLc236, pCC1BAC, pCL1920 etc… (Studier *et al.*, 1990).
- Using a promoter leading to a high overexpression of the gene. The man skilled in the art knows which promoters are the most convenient, for example promoters P*trc* (Brosius *et al.*, 1985), P*tac* (de Boer *et al.*, 1983), P*lac* (Dickson *et al.*, 1975) are widely used in such cases.

Overexpression for reaction A

More precisely for reaction A, *aspC* gene of *E. coli* (SEQ ID N°9) was overexpressed. To overexpress it, a copy of this gene has been integrated into the chromosome at *ybeH* locus (SEQ ID NO: 19) and an artificial promoter, mRNA stabilizing sequence and ribosome binding site (SEQ ID NO: 6) were added upstream of the translational start of the *aspC* gene, by using homologous recombination strategy described by Datsenko & Wanner, 2000 (according to Protocol 1). Briefly, a PCR product carrying the artificial promoter, mRNA stabilizing sequence and ribosome binding site, the *aspC* gene and the kanamycin resistance gene together with FRT sites, surrounded by sequences homologous to *ybeH* locus (SEQ ID NO: 10 and SEQ ID NO: 11), was generated and introduced into the MG1655 *metA*11* (pKD46) strain. The kanamycin resistant transformants MG1655 *metA*11 DybeH::aspC::Km* were verified by PCR with the appropriate primers.

Overexpressions for reaction B

More precisely for reaction B, *asd* or *guaB* or *iscS* or *acnB* gene of *E. coli* (SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14 and SEQ ID NO: 15 respectively) was overexpressed. To overexpress them, a copy of each gene has been individually integrated into the chromosome at *ycgH* locus (SEQ ID NO: 20) and an artificial promoter, mRNA stabilizing sequence and ribosome binding site (SEQ ID NO: 6) were added upstream of the translational start of the *asd* or *guaB* or *iscS* or *acnB* gene, by using homologous recombination strategy described by Datsenko & Wanner, 2000 (according to Protocol 1). Briefly, a PCR product carrying, the artificial promoter, mRNA stabilizing sequence and ribosome binding site, *asd or guaB* or *iscS* or *acnB* gene and the chloramphenicol resistance gene together with FRT sites, surrounded by sequences homologous to *ycgH* locus (SEQ ID NO: 16 and SEQ ID NO: 17), was generated and introduced into MG1655 *metA**11 (pKD46) strain. The chloramphenicol resistant transformants MG1655 *metA**11 *DycgH::asd::Cm* or *DycgH::guaB::Cm* or *DycgH::iscS::Cm* or *DycgH::acnB::Cm* were verified by PCR with the appropriate primers.

Overexpression of the genes of the MHA pathway

To overproduced couples of enzymes (A and B) involved in MHA production pathway n°1 in different L-methionine overproducer backgrounds listed above, *aspC* overexpression was first transferred by phage transduction from MG1655 *metA**11 *ybeH::aspC::Km* modified strain to strains 1, 3, 4, 5, 6 and 7, giving rise to strains 8 to 13. Then each overexpression of *asd* or *guaB* or *iscS* or *acnB* gene was individually transferred by phage transduction from MG1655 *metA**11 *ycgH::asd::Cm or ycgH::guaB::Cm* or *ycgH::iscS::Cm* or *ycgH::acnB::Cm* modified strains to different L-methionine overproducer strains carrying already the *aspC* over-expression (strains 8 to 13), giving rise to the strains 14 to 37. The resulting strains are listed in table 14 below.

Table 14: L-methionine overproducer strains overexpressing genes from the MHA pathway N° 1

Genes overexpressed	Reference Strains					
	Genetic Backgrounds					
	A	B	C	D	E	F
No MHA pathway	Strain 1	Strain 3	Strain 4	Strain 5	Strain 6	Strain 7
aspC + asd	Strain 14	Strain 15	Strain 16	Strain 17	Strain 18	Strain 19
aspC + guaB	Strain 20	Strain 21	Strain 22	Strain 23	Strain 24	Strain 25
aspC + iscS	Strain 26	Strain 27	Strain 28	Strain 29	Strain 30	Strain 31
aspC + acnB	Strain 32	Strain 33	Strain 34	Strain 35	Strain 36	Strain 37

EXAMPLE 5: PRODUCTION OF L-METHIONINE HYDROXY ANALOG BY FERMENTATION

The strains described in example 4 carrying the pathway n°1 for the production of L-hydroxymethionine were cultivated in 2.5 L reactors. The MHA producing strains were cultivated in two different conditions to enhance the production of hydroxymethionine; classical fermentation under phosphate limitation and with or without nitrogen limitation. Indeed, to improve hydroxymethionine production of strains containing MHA pathway, the ratio C/N was changed in fedbatch media of 2.5 L cultures. This ratio is to be determined according to the need (growth and production) of the strain and as previously well described in patent application WO2012/090022 which is incorporated herein as reference.

For the backgrounds D, E and F, details of culture conditions are presented in patent WO2012/090022.

For the backgrounds A, B and C, culture conditions are the same as those used in the patent WO2012/090022 except for the feeding media. Feeding medium F2 was used for nitrogen excess condition and feeding medium F5 was used for nitrogen limitation conditions (Table 15).

Table 15: Feeding medium composition for background A, B and C with nitrogen limitation.

Compound	F5 Concentration ($g.L^{-1}$)
$Zn(CH_3COO)_2.2H_2O$	0.0104
$CuCl_2.2H_2O$	0.0012
$MnCl_2.4H_2O$	0.0120
$CoCl_2.6H_2O$	0.0020
H_3BO_3	0.0024
$Na_2MoO_4.2H_2O$	0.0020
Fe(III) citrate H_2O	0.0524
EDTA	0.0067
$MgSO_4$	5.00
$(NH_4)_2S_2O_3$	30.00
Thiamine	0.01
Vitamin B12	0.01
Glucose	500

To produce the L-methionine hydroxy analogue (MHA), enzymes of pathway n°1 described above were overproduced in other recombinant L-methionine producing organisms like for instance *Corynebacterium glutamicum*, described in the following patent applications WO2007/012078, WO2004/050694, WO2009/144270, WO2015/028675 or WO2015/028674. The man skilled in the art knows how to adapt vectors and promoters to over-express genes depending on the recipient microorganism

and knows how to optimize the coding sequence with respect of codon usage of the recipient organism.

Tables 16 to 21: Final hydroxymethionine concentrations are indicated in mM for the reference strains cultivated with the different fedbatch media (with or without nitrogen limitation). For the strains expressing the MHA pathway, production levels are compared to a specific reference strain and indicated with different symbols: "~" indicates that the variation compared to the reference of the serie is closed to zero percent. The symbols "+" and "++" indicate an increase from 1 to 3 % and from 3 to 6 % respectively.

Culture fedbatch media/Strain	Strain 1	Strain 14	Strain 20	Strain 26	Strain 32
F5	2.1	++	+	+	~
F2	0.4	+	~	~	~

Culture fedbatch media/Strain	Strain 3	Strain 15	Strain 21	Strain 27	Strain 33
F5	5.8	++	~	+	+
F2	0.8	+	~	+	~

Culture fedbatch media/Strain	Strain 4	Strain 16	Strain 22	Strain 28	Strain 34
F5	5.7	++	+	+	+
F2	0.8	+	+	~	~

Culture fedbatch media/Strain	Strain 5	Strain 17	Strain 23	Strain 29	Strain 35
F2	10.5	++	+	++	+
F4	1.2	++	+	+	+

Culture fedbatch media/Strain	Strain 6	Strain 18	Strain 24	Strain 30	Strain 36
F2	13.4	++	++	+	+
F4	1.5	++	+	+	+

Culture fedbatch media/Strain	Strain 7	Strain 19	Strain 25	Strain 31	Strain 37
F2	19.8	++	+	+	++
F4	1.4	++	+	+	+

EXAMPLE 6: PRODUCTION OF METHIONINE HYDROXY ANALOG BY BIOCONVERSION

In order to demonstrate the MHA production from methionine by bioconversion, we purified AspC and Asd enzymes. To do so, genes *aspC* and *asd* were cloned into the expression plasmid pET101/D-TOPO (Lifetechnologies®) and then overexpressed in BL21(DE3) *E.coli* strain. The recombinant proteins were purified using the Ni-NTA Purification System (Lifetechnologies®). AspC was showed to be able to convert the methionine into 4-methylthio-2-oxobutanoic acid (KMTB) whereas Asd was showed to convert the KMTB into Methionine Hydoxy Analog (MHA).

The reaction of bioconversion was performed at 37°C in a solution containing 20 mM potassium phosphate buffer pH 7.5, 1mM Pyridoxal 5-phosphate (PLP), 10 mM α-ketoglutarate, 10 mM NAD(P)H, 10mM of hydroxybutyrate neutralized and 10mM Methionine. The reaction was initiated by addition of 0.5mg of each pure enzyme AspC and Asd. The control (blank) reaction was performed in the same conditions without one or both enzymes.

The products of the reactions were analysed by GC-MS after derivatization in two stages (oximation and silylation). A standard of hydroxymethionine prepared in the matrix was used for the quantification.

We identified 5mM of hydroxymethionine only in the reaction sample containing both AspC and Asd enzymes.

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CLAIMS

1. A method for the production of 2-hydroxy-4-(methylthio) butyric acid wherein in a first enzymatic step methionine is converted into 4-methylthio-2-oxobutanoic acid and in a second enzymatic step 4-methylthio-2-oxobutanoic acid is directly or indirectly converted into 2-hydroxy-4-(methylthio) butyric acid.

2. The method according to claim 1 wherein methionine is converted into 4-methylthio-2-oxobutanoic acid by an enzyme having transaminase or oxidoreductase activity.

3. The method according to claim 2 wherein methionine is converted into 4-methylthio-2-oxobutanoic acid by an enzyme having transaminase activity.

4. The method according to claim 3 wherein the enzyme having transaminase activity is an aspartate aminotransferase.

5. The method according to claim 1 wherein 4-methylthio-2-oxobutanoic acid is converted directly or indirectly into 2-hydroxy-4-(methylthio) butyric acid by at least one enzyme having activity chosen among oxidoreductase, transferase, hydrolase, lyase and ligase activity.

6. The method according to claim 5 wherein
   - the enzyme having oxidoreductase activity is an aspartate-semialdehyde dehydrogenase and/or an inosine-5'-monophosphate dehydrogenase,
   - the enzyme having transferase activity is a cysteine desulfurase,
   - the enzyme having lyase activity is an aconitate hydratase B.

7. A genetically modified microorganism for the production of 2-hydroxy-4-(methylthio) butyric acid according to anyone of claims 1 to 6 wherein said microorganism overexpresses (i) at least one gene encoding enzyme having transaminase or oxidoreductase activity, and (ii) at least one gene encoding enzyme having activity chosen among the group consisting of oxidoreductase, transferase, hydrolase, lyase and ligase.

8. The microorganism according to claim 7, wherein in said microorganism *aspC* gene encoding enzyme having transaminase activity for the conversion of methionine into 4-methylthio-2-oxobutanoic acid is overexpressed.

9. The microorganism according to anyone of claims 7 to 8 wherein in said microorganism at least one gene chosen among *asd, guaB, iscS* and *acnB* and catalyzing the conversion of 4-methylthio-2-oxobutanoic acid into 2-hydroxy-4-(methylthio) butyric acid is overexpressed.

10. The microorganism of anyone of claims 7 to 9 wherein in said microorganism *aspC* gene and *asd* gene are overexpressed.

11. The microorganism according to anyone of claims 7 to 10 wherein it further comprises the following genetic modifications:

- Increased expression of at least one the following genes: *pyc, ptsG*, *pntAB*, *cysP*, *cysU*, *cysW*, *cysA*, *cysM*, *cysJ*, *cysI*, *cysH*, *gcvT*, *gcvH*, *gcvP*, *lpd*, *glyA*, *serA*, *serB*, *serC*, *cysE*, *metF*, *metH*, *fldA*, *fpr*, *metN*, *metI*, *metQ*, *metA*, *metA** allele encoding for an enzyme with reduced feed-back sensitivity to S-adenosylmethionine and/or methionine, *thrA*, or a *thrA** allele encoding for an enzyme with reduced feed-back inhibition to threonine, and/or
- Attenuated expression of at least one of the following genes: *metJ, pykA, pykF, purU*, *yncA*, *metE*, *dgsA*, *sgrS*, *sgrT*, *ygaZH* or *udhA*.

12. A method for the production of 2-hydroxy-4-(methylthio) butyric acid comprising:

- culturing the genetically modified microorganism of anyone of claims 7 to 11 in an appropriate culture medium comprising a source of carbon, a source of sulphur and a source of nitrogen,
- recovering 2-hydroxy-4-(methylthio) butyric acid from the culture medium.

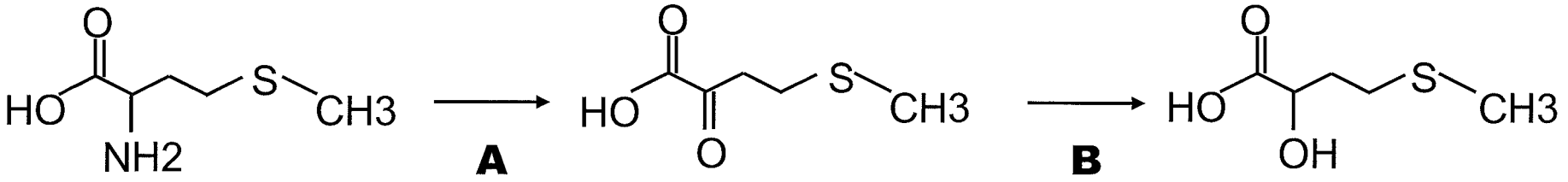
13. The method of claim 12 wherein the genetically modified microorganism is cultivated under conditions of nitrogen limitation.

14. The method of claim 13 wherein the microorganism is cultivated in a bio-reactor system in two successive steps:

- Growth of the microorganisms for about 10h to 20h in an appropriate culture medium comprising a source of carbon, a source of sulphur and a source of nitrogen, preferably for about 15h to 20h,
- Culture of the microorganisms for about 10h to 20h in nitrogen limitation conditions in an appropriate culture medium, preferably for about 10h to 15h.

15. The method of claims 12 to 14 wherein the source of sulfur is sulphate, thiosulfate, hydrogen sulfide, dithionate, dithionite, sulfite, methylmercaptan, dimethylsulfide, dimethyl disulfide and other methyl capped sulfides or a combination of the different sources.

Fig. 1: 2-hydroxy-4-(methylthio) butyric acid production pathway n°1



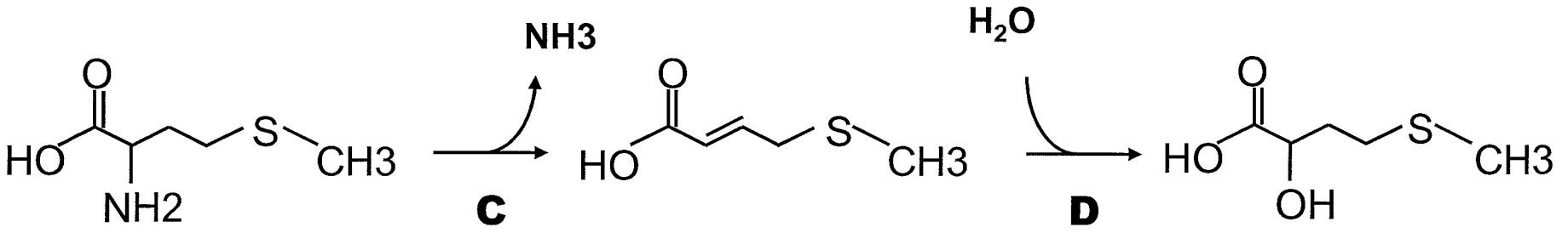
Methionine

4-Methylthio-2-oxobutanoic acid (KMTB)

2-hydroxy-4-(methylthio) butyric acid (HMBA or MHA)

A: Transaminase or oxidoreductase acting on CH-NH2 group of donors
B: Oxidoreductase and/or transferase and/or hydrolase and/or lyase and/or ligase.

Fig. 2: 2-hydroxy-4-(methylthio) butyric acid production pathway n°2



Methionine

4-(Methylsulfanyl)but-2-enoic acid

2-hydrox-4-(methylthio) butyric acid (HMBA or MHA)

C: Ammonia lyase enzyme acting on CH-NH2 group of donors
D: Carbon-oxygen lyase or carbon oxygen dehydratase.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/066305

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P7/42 C12P7/52
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12P

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
12 August 2016	22/08/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Mateo Rosell, A

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	WO 2012/090022 A1 (METABOLIC EXPLORER SA [FR]; FIGGE RAINER [FR]; DISCHERT WANDA [FR]) 5 July 2012 (2012-07-05) cited in the application page 1, lines 5-10 page 2, lines 29-36 page 3, lines 24-29 page 6, line 12 - page 7, line 11 page 9, line 27 - page 11, line 38; examples I,II; tables 1,6	1-15

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Information on patent family members

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