

Enzymatic Method for Producing L-Glufosinate and its Phosphoesters

The present invention relates to an enzymatically catalyzed method for producing L-glufosinate
5 (“L-GA” or “LGA”) or a phosphoester thereof. The method comprises a step in which an activated
L-homoserine H_A is reacted with a substrate S selected from methylphosphinic acid and the esters
of methylphosphinic acid. The invention makes accessible new substrates in the enzymatic
production of L-glufosinate and its phosphoesters.

10 1. Background of the Invention

Organic phosphor compounds, i.e. chemical agents comprising a carbon-phosphor bond, are
widely applied as herbicides in the area of plant protection. Agents such as the herbicides
glyphosate (Roundup®, Touchdown®) and glufosinate (Basta®, Liberty®) as well as the growth
15 regulator glyphosine (Polaris®) are used for this purpose (as described for example by G. Hörlein,
Rev. Environ. Contam. Toxicol. **1994**, *138*, 73-145).

The esters of *P*-methyl phosphinic acid (for example, *P*-methyl phosphinic acid butyl ester;
“MPBE”; CAS-No: 6172-80-1) have a key role as synthetic building blocks in the synthesis of the
20 non-selective herbicide glufosinate. These esters are accessible via two fundamental synthetic
pathways (summarized in Figures 3 a and 3 b, p. 130, of the article of K. Haack, Chem. Unserer
Zeit **2003**, *37*, 128-138):

a. Reacting diethyl chlorophosphite $[CIP(OC_2H_5)_2]$ with CH_3MgCl provides methyl diethoxy
phosphine $[H_3CP(OC_2H_5)_2]$; “DEMP”; CAS-No. 15715-41-0], which is partially hydrolyzed to give the
25 corresponding methylphosphinic acid ethyl ester (MPEE; CAS-Nr: 16391-07-4).

b. Alternatively, methane can be reacted with phosphor trichloride at 500 °C to give methyl dichloro
phosphane H_3CPCl_2 . The latter can then be solvolyzed in alcohols to give the corresponding
methyl phosphinic acid esters.

The esters of *P*-methyl phosphinic acid add to carbon-carbon double bonds regioselectively. This
30 property is used in the synthesis of glufosinate for the formation of the second phosphor-carbon
bond. For example, $H_3CPH(O)OR$ ($R = \text{Alkyl}$) reacts with 1-cyano allyl acetate in an addition
reaction to provide an intermediate. Subsequent exchange of the acetate substituent with ammonia
and hydrolysis of the cyano group and the ester group of the phosphinic acid moiety give
glufosinate.

35 Acrylic acid ester is a cheaper alternative starting material. It can react with the ester of *P*-methyl
phosphinic acid to 3-[alkoxy(methyl)phosphinyl]propionic acid alkyl ester. Claisen reaction of this
diester with diethyl oxalate, hydrolysis and decarboxylation provide the corresponding α -keto acid,
which can be reductively aminated to give glufosinate.

These and further synthetic routes towards L-glufosinate are also described in the art, e.g. in WO 1999/009039 A1, EP 0 508 296 A1.

5 WO 2020/145513 A1 and WO 2020/145514 A1 describe a chemical route to L-glufosinate. In this route, a homoserine derivative such as O-acetyl homoserine or O-succinyl homoserine is used as starting material and L-glufosinate is obtained by a sequence of reactions including lactonization and halogenation.

10 WO 2020/145627 A1 describes a similar route, wherein, during halogenation, a bromine derivative is obtained.

The route disclosed by CN 106083922 A is similar but starts off from L-methionine.

15 EP 2402453 A2 describes an enzymatic method for producing methionine by enzymatically reacting a mixture of methyl mercaptan and dimethyl sulfide with O-acetyl homoserine or O-succinyl homoserine.

20 CN 108516991 A describes another synthetic pathway to L-glufosinate, starting with the azeotropic dehydration of L-homoserine to give L-3,6-bis(2-haloethyl)-2,5-diketopiperazine, followed by the introduction of a methylphosphinate diester group and hydrolysis.

A general disadvantage of all synthetic routes to glufosinate is that the obtained glufosinate is a racemic mixture. However, as there is no herbicidal activity of the D-enantiomer, L-glufosinate is the enantiomer of economical interest.

25 For the enantioselective syntheses of L-glufosinate, enzymatic pathways are described in the art.

30 WO 2017/151573 A1 discloses a two-step enzymatic synthesis of L-glufosinate from D-glufosinate. In the first step, D-glufosinate is oxidatively deaminated to give 2-oxo-4-[hydroxy(methyl)phosphinoyl]butyric acid ("PPO"), followed by the specific amination of PPO to L-glufosinate as the second step. The first step is carried out by the catalysis of a D-amino acid oxidase, the second step is catalyzed by a transaminase.

35 WO 2020/051188 A1 discloses a similar method of converting racemic glufosinate to the L-glufosinate enantiomer. In addition, it discloses a step in which the α -ketoacid or ketone byproduct formed during amination of PPO with an amine donor is converted by ketoglutarate decarboxylase to further shift the equilibrium to L-glufosinate.

40 WO 2019/018406 A1 discloses a method of purifying L-glufosinate from a mixture comprising L-glufosinate and glutamate. Glutamate is converted to pyroglutamate enzymatically by glutaminyl-

peptidyl cyclotransferase, and L-glufosinate is then purified from the resulting mixture by ion-exchange.

- The object of the present invention is to provide a further enzymatic process for producing
- 5 L-glufosinate in high enantiomeric excess. In particular, such process should allow to use new substrates which heretofore were not used in the enzymatic synthesis of L-glufosinate.

2. Summary of the Invention

- 10 The present invention solves the problems mentioned above by providing a method for producing L-glufosinate from a substrate that has not been used in the enzymatic production of L-glufosinate before. In particular, the present invention provides a method for producing L-glufosinate or a phosphoester of L-glufosinate from methylphosphinic acid and its esters using an enzymatically catalyzed pathway. These phosphor compounds thus serve as alternative substrates in the
- 15 production of L-glufosinate, allowing for flexibility of production where there is no reliance on the known substrates that are currently being used for L-glufosinate production.

In particular, this object is achieved by the present invention which relates to an enzymatically catalyzed method for producing L-glufosinate or a phosphoester thereof, comprising a step (a) in which an activated L-homoserine H_A is reacted with a substrate **S** to produce these compounds.

20

3. Brief Description of the Drawings

Figure 1 shows the pET-26b(+)_metY-Cg plasmid map.

Figure 2 shows the pET-26b(+)_metY_P2T plasmid map.

- 25 Figure 3 shows the pET-26b(+)_metZ-Cv plasmid map.

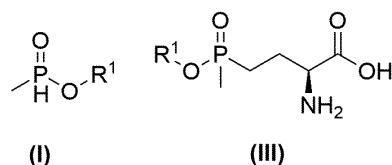
Figure 4 shows the pET-26b(+)_metZ-Hn plasmid map.

4. Detailed Description of the Invention

- 30 It was surprisingly found that certain phosphor-containing compounds, namely methylphosphinic acid and esters of methylphosphinic acid, can react under enzymatic catalysis with activated L-homoserine, thus opening a new synthetic pathway to L-glufosinate and L-glufosinate phosphoesters. This was especially surprising because similar compounds such as DEMP did not react in the analogous reaction with activated L-homoserine.

35

The present invention thus relates to an enzymatically catalyzed method for producing L-glufosinate or a phosphoester thereof, comprising a step (a) in which an activated L-homoserine H_A is reacted with a substrate **S** of the following structure (I) to produce a compound of the following structure (III),



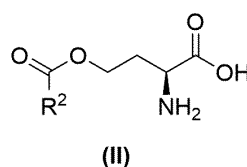
wherein R^1 is selected from hydrogen, alkyl, alkenyl, alkynyl, hydroxyalkyl, aryl.

- 5 The compounds denoted as “L-glufosinate or a phosphoester thereof” according to the invention are represented by structure (III). When R^1 = hydrogen in structure (III), the compound is L-GA.

When R^1 is selected from alkyl, alkenyl, alkynyl, hydroxyalkyl, aryl in structure (III), the compound is a phosphoester of L-GA.

10

The activated L-homoserine H_A has the following structure (II):



- 15 wherein R^2 is a hydrocarbon group with 1 to 15 carbon atoms which optionally comprises at least one functional group selected from OH, COOH, NH.

The reaction in step (a) is catalyzed by at least one enzyme selected from the group consisting of a sulfhydrylase E_1 , a cystathionine γ -synthase E_2 .

20 4.1 Substrate S

The substrate **S** according to the invention is selected from the group consisting of methylphosphinic acid and the esters of methylphosphinic acid.

- 25 The substrate **S** has the structure (I). In structure (I), R^1 is selected from hydrogen, alkyl, alkenyl, alkynyl, hydroxyalkyl, aryl,
preferably selected from hydrogen, alkyl,
preferably selected from hydrogen, alkyl with 1 to 6, preferably 1 to 4 carbon atoms,
more preferably selected from hydrogen, methyl, ethyl, *iso*-propyl, *n*-propyl, *n*-butyl, *sec*-butyl, *iso*-
30 butyl, *tert*-butyl, even more preferably selected from hydrogen, methyl, ethyl, *n*-butyl, even more
preferably selected from hydrogen, methyl, *n*-butyl, preferably selected methyl, *n*-butyl. Most
preferably, R^1 is *n*-butyl.

When R^1 = hydrogen in structure (I), the compound is methylphosphinic acid.

When R^1 is selected from alkyl, alkenyl, alkynyl, hydroxyalkyl, aryl in structure (I), the compound is an ester of methylphosphinic acid.

R^1 in structure (I) and structure (III) is the same.

5

4.2 Activated L-homoserine H_A

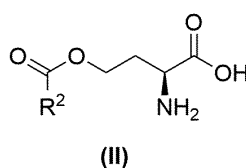
The other reaction partner in the reaction according to the present invention is activated L-homoserine H_A .

10

The skilled person is aware of activated L-homoserine H_A (sometimes also denoted as "L-methionine precursor", e.g. in WO 2008/013432 A1), which in particular means O-acyl L-homoserine.

4.2.1 Activated L-homoserine H_A

The activated L-homoserine has a chemical structure (II) as follows:



20 wherein R^2 is a hydrocarbon group with 1 to 15 carbon atoms which optionally comprises at least one functional group selected from OH, COOH, NH.

More preferably, the activated L-homoserine is selected from the group consisting of

O-acetyl-L-homoserine [structure (II-A)], O-succinyl-L-homoserine [structure (II-B)],

25 O-propionyl-L-homoserine [structure (II-C)], O-acetoacetyl-L-homoserine [structure (II-D)],

O-coumaroyl-L-homoserine [structure (II-E)], O-malonyl-L-homoserine [structure (II-F)],

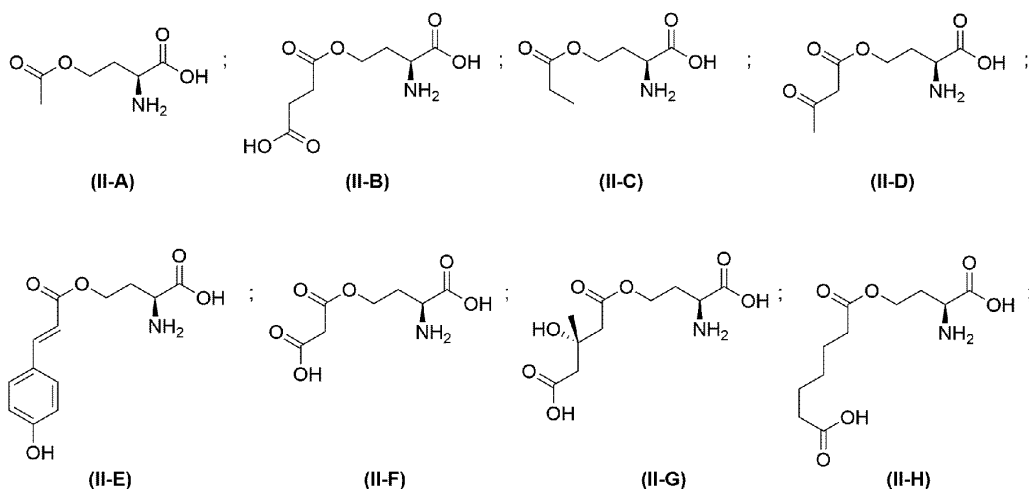
O-hydroxymethylglutaryl-L-homoserine [structure (II-G)], and O-pimelyl-L-homoserine [structure (II-H)].

30 Even more preferably, the activated L-homoserines is selected from the group consisting of

O-acetyl-L-homoserine [structure (II-A)], O-succinyl-L-homoserine [structure (II-B)].

Most preferably, the activated L-homoserine is O-acetyl-L-homoserine [structure (II-A)].

35



4.2.2 Chemical synthesis of activated L-homoserine **H_A**

5

The activated L-homoserine **H_A** used in the method of the present invention can be obtained by organochemical synthesis routes known to the skilled person. For example, the synthesis of O-succinyl homoserine is described in M. Flavin, C. Slaughter, *Biochemistry* **1965**, 4, 1370-1375.

10 The synthesis of O-acetyl-homoserine is described by S. Nagai, M. Flavin, *Methods in Enzymology, Metabolism of Amino Acids and Amines Part B* **1971**, 17(Part B), 423-424.

The chemical synthesis of the potential precursor is described for example by M.D. Armstrong, *J. Am. Chem. Soc.* **1948**, 70, 1756- 1759.

15 4.2.3 Biotechnological synthesis of activated L-homoserine **H_A**

Alternatively, and preferably, the activated L-homoserine **H_A** used in the present invention is obtained by biotechnological means. For example, this is described in WO 2008/013432 A1 or by H. Kase, K. Nakayama, *Agr. Biol. Chem.* **1974**, 38, 2021-2030.

20 The strain producing activated L-homoserine **H_A** is preferably selected from the group consisting of *Escherichia sp.*, *Erwinia sp.*, *Serratia sp.*, *Providencia sp.*, *Corynebacterium sp.*, *Pseudomonas sp.*, *Leptospira sp.*, *Salmonella sp.*, *Brevibacterium sp.*, *Hypomononas sp.*, *Chromobacterium sp.*, *Norcardia sp.*, fungi, which are in particular yeasts.

25 Biotechnological processes for obtaining L-homoserine are also described in the art, e.g. in US 3,598,701, US 6,303,348 B1, EP 0 994 190 A2, EP 1 149 911 A2, WO 2004/067757 A1.

4.3 Enzyme

The method according to the present invention is enzymatically catalyzed.

- 5 The term “enzyme” means any substance composed wholly or largely of protein or polypeptides that catalyzes or promotes, more or less specifically, one or more chemical or biochemical reactions.

- Any of the enzymes used according to any aspect of the present invention may be an isolated
10 enzyme. In particular, the enzymes used according to any aspect of the present invention may be used in an active state and in the presence of all cofactors, substrates, auxiliary and/or activating polypeptides or factors essential for its activity.

- In particular, this also means that the terms “cystathionine γ -synthase” and “sulfhydrylase”, in
15 particular “O-acetyl homoserine sulfhydrylase” or “O-succinyl homoserine sulfhydrylase” comprise the respective enzymes in combination with all the cofactors necessary for their function. In particular, this cofactor is pyridoxal 5'-phosphate mono-hydrate (“PMP”).

- A “polypeptide” is a chain of chemical building blocks called amino acids that are linked together by
20 chemical bonds called peptide bonds. A protein or polypeptide, including an enzyme, may be “native” or “wild-type”, meaning that it occurs in nature or has the amino acid sequence of a native protein, respectively. These terms are sometimes used interchangeably. A polypeptide may or may not be glycosylated.

- 25 The enzyme used according to any aspect of the present invention may be recombinant. The term “recombinant” as used herein, refers to a molecule or is encoded by such a molecule, particularly a polypeptide or nucleic acid that, as such, does not occur naturally but is the result of genetic engineering or refers to a cell that comprises a recombinant molecule. For example, a nucleic acid molecule is recombinant if it comprises a promoter functionally linked to a sequence encoding a
30 catalytically active polypeptide and the promoter has been engineered such that the catalytically active polypeptide is overexpressed relative to the level of the polypeptide in the corresponding wild type cell that comprises the original unaltered nucleic acid molecule. As a further example, a polypeptide is recombinant if it is identical to a polypeptide sequence occurring in nature but has been engineered to contain one or more point mutations that distinguish it from any polypeptide
35 sequence occurring in nature.

- The term “overexpressed”, as used herein, means that the respective polypeptide encoded or expressed is expressed at a level higher or at higher activity than would normally be found in the cell under identical conditions in the absence of genetic modifications carried out to increase the
40 expression, for example in the respective wild type cell.

The term "isolated", as used herein, means that the enzyme of interest is enriched compared to the cell in which it occurs naturally. The enzyme may be enriched by SDS polyacrylamide electrophoresis and/or activity assays. For example, the enzyme of interest may constitute more than 5, 10, 20, 50, 75, 80, 85, 90, 95 or 99 percent of all the polypeptides present in the preparation as judged by visual inspection of a polyacrylamide gel following staining with Coomassie blue dye.

4.3.1 Enzymes E_1 and E_2

Step (a) of the method according to the invention is catalysed by at least one enzyme selected from the group consisting of a sulfhydrylase E_1 and a cystathionine γ -synthase E_2 .

A sulfhydrylase is known to the skilled person as an enzyme that catalyzes at least one of the following reactions <1A>, <1B>:

<1A>: O-acetyl-L-homoserine + methanethiol $\rightarrow$ L-methionine + acetate.

<1B>: O-succinyl-L-homoserine + methanethiol $\rightarrow$ L-methionine + succinate.

A sulfhydrylase that has a higher catalytic activity for reaction <1A> than reaction <1B> may be denoted as an "O-acetyl-L-homoserine sulfhydrylase".

A sulfhydrylase that has a higher catalytic activity for reaction <1B> than reaction <1A> may be denoted as an "O-succinyl-L-homoserine sulfhydrylase".

A cystathionine γ -synthase is known to the skilled person as an enzyme that catalyzes at least the following reaction <2>:

<2>: O-succinyl-L-homoserine + L-cysteine $\rightarrow$ L-cystathionine + succinate

In a preferred embodiment, step (a) of the method according to the invention is catalysed by a sulfhydrylase E_1 , which is even more preferably an O-acetyl homoserine sulfhydrylase or an O-succinyl homoserine sulfhydrylase, most preferably an O-acetyl homoserine sulfhydrylase.

The sulfhydrylase or the cystathionine γ -synthase that may be used in step (a) of the method according to the invention may be derived from *Bacillus* sp., in particular *Bacillus subtilis*; *Bradyrhizobium* sp., in particular *Bradyrhizobium japonicum*; *Brevibacterium* sp.; *Colwellia* sp., in particular *Colwellia psychrerythraea*; *Corynebacterium* sp., in particular *Corynebacterium glutamicum*, *Corynebacterium humireducens*; *Chromobacterium* sp., in particular *Chromobacterium violaceum*; *Erwinia* sp.; *Escherichia* sp., in particular *Escherichia coli*; *Hyphomonas* sp., in particular *Hyphomonas neptunium*; *Klebsiella* sp., in particular *Klebsiella pneumoniae*; *Leptospira* sp., in particular *Leptospira interrogans*; *Methylobacillus* sp., in particular *Methylobacillus*

flagellates; *Methylococcus* sp., in particular *Methylococcus capsulatus*; *Nitrosomonas* sp., in particular *Nitrosomonas europaea*; *Nocardia* sp., in particular *Nocardia farcinica*; *Providencia* sp.; *Pseudomonas* sp., in particular *Pseudomonas aeruginosa*; *Rhodobacter* sp., in particular *Rhodobacter sphaeroides*; *Salmonella* sp., in particular *Salmonella enterica*; *Serratia* sp.; *Shigella* sp., in particular *Shigella flexneri*; fungi, which are preferably yeast, more preferably *Saccharomyces* sp., even more preferably *Saccharomyces cerevisiae*.

The sulfhydrylase enzyme that may be used in the method according to the present invention may be an O-acetyl-L-homoserine sulfhydrylase categorized in the EC class EC 2.5.1.49 or an

O-succinyl-L-homoserine sulfhydrylase categorized in the EC class EC 2.5.1.-.

These enzymes are part of the direct sulfurylation pathway for methionine biosynthesis and PMP-dependent. They are described e.g. by M. P. Ferla and W. M. Patrick, Microbiology **2014**, 160, 1571-1584.

WO 02/18613 A1, WO 2007/024933 A2, EP 2 657 345 A1, EP 2 657 250 A2, WO 2015/165746 A1 and WO 2008/013432 A1 disclose examples of enzymes having O-acetyl-L-homoserine sulfhydrylase and O-succinyl-L-homoserine sulfhydrylase activity according to the invention.

An O-acetyl-L-homoserine sulfhydrylase suitable for the method according to the present invention may originate from *Corynebacterium* sp., in particular *Corynebacterium glutamicum* or *Corynebacterium humireducens*; *Leptospira* sp., in particular *Leptospira interrogans*; *Pseudomonas* sp., in particular *Pseudomonas aeruginosa*; yeast, in particular *Saccharomyces* sp., preferably *Saccharomyces cerevisiae*. A preferable O-acetyl-L-homoserine sulfhydrylase suitable for the method according to the present invention may originate from *Corynebacterium* sp., more preferably *Corynebacterium glutamicum* or *Corynebacterium humireducens*, even more preferably *Corynebacterium glutamicum*, even more preferably *Corynebacterium glutamicum* ATCC 13032.

An O-succinyl-L-homoserine sulfhydrylase suitable for the method according to the present invention may originate from *Bradyrhizobium* sp., in particular *Bradyrhizobium japonicum*; *Chromobacterium* sp., in particular *Chromobacterium violaceum*; *Hyphomonas* sp., in particular *Hyphomonas neptunium*; *Methylobacillus* sp., in particular *Methylobacillus flagellatus*; *Methylococcus* sp., in particular *Methylococcus capsulatus*; *Nitrosomas* sp., in particular *Nitrosomas europaea*; *Nocardia* sp., *Nocardia farcinica*; *Pseudomonas* sp., in particular *Pseudomonas aeruginosa*, *Pseudomonas putida*; *Rhodobacter* sp., in particular *Rhodobacter sphaeroides*.

A preferable O-succinyl-L-homoserine sulfhydrylase suitable for the method according to the present invention may originate from *Chromobacterium* sp., in particular *Chromobacterium*

violaceum; *Hyphomonas* sp., in particular *Hyphomonas neptunium*. Even more preferably, it may originate from *Chromobacterium violaceum* ATCC 12472, *Hyphomonas neptunium* ATCC 15444.

The cystathionine γ -synthase that may be used in the method according to the present invention
5 may be a cystathionine γ -synthase categorized in the EC class EC 2.5.1.48.

These enzymes are PMP-dependent and are described e.g. by T. Clausen, R. Huber, L. Prade, M.C.Wahl and A. Messerschmidt, The EMBO Journal **1998**, 17, 6827-6838.

10 A cystathionine γ -synthase suitable for the method according to the present invention may originate from *Escherichia coli*, in particular *Escherichia coli*; *Corynebacterium* sp., in particular *Corynebacterium glutamicum*; *Klebsiella* sp., in particular *Klebsiella pneumoniae*; *Bacillus* sp., in particular *Bacillus subtilis*; *Shigella* sp., in particular *Shigella flexneri*; *Colwellia* sp., in particular *Colwellia psychrerythraea*; *Salmonella* sp., in particular *Salmonella enterica*.

15 The respective sequences can be derived from databases such as the Braunschweig Enzyme Database (BRENDA, Germany, available under www.brenda-enzymes.org/index.php), the National Center for Biotechnological Information (NCBI, available under <https://www.ncbi.nlm.nih.gov/>) or the Kyoto Encyclopedia of Genes and Genomes (KEGG, Japan, available under
20 www.genome.jp/kegg/).

The following table 1 gives preferred examples for sulfhydrylases and cystathionine γ -synthases that may be used in step (a) of the method according to the invention. The genes encoding sulfhydrylase are indicated as "*metY*", "*met17*", and "*Met17*" for O-acetyl-L-homoserine
25 sulfhydrylase ("AHS") and "*metZ*" for O-succinyl-L-homoserine sulfhydrylase ("SHS"). The gene encoding cystathionine γ -synthase ("CGS") is indicated as "*metB*" and "*metI*".

Table 1

Strain	Gene name	Gene ID / locus tag	NCBI accession	SEQ ID NO: of the polypeptide
<i>Corynebacterium glutamicum</i> ATCC 13032	metY	NCgl0625	NC_003450	SEQ ID NO: 2
<i>Corynebacterium glutamicum</i> ATCC 13032	metY_P2T	-	-	SEQ ID NO: 6
<i>Chromobacterium violaceum</i> ATCC 12472	metZ	CV_2725	AE016825	SEQ ID NO: 10
<i>Hyphomonas neptunium</i> ATCC 15444	metZ	HNE_2672	CP000158	SEQ ID NO: 14
<i>Escherichia coli</i> K12 MG1655	metB	b3939	U00096	SEQ ID NO: 17
<i>Pseudomonas aeruginosa</i> PAO1	metZ	880476/PA3107	AE004091	SEQ ID NO: 18
<i>Pseudomonas aeruginosa</i> PAO1	metY	881205/PA5025	AE004091	SEQ ID NO: 19
<i>Pseudomonas putida</i> KT2440	metZ	PP_RS10380	NC_002947	SEQ ID NO: 20
<i>Corynebacterium glutamicum</i> ATCC 13032	metB	NCgl2360/CGL_RS12110	NC_003450	SEQ ID NO: 21
<i>Leptospira interrogans</i> serovar Lai str. 56601	met17	LA_2062	AE010300	SEQ ID NO: 22
<i>Saccharomyces cerevisiae</i> S288C	Met17	Met17	NC_001144	SEQ ID NO: 23
<i>Nocardia farcinica</i> IFM 10152	metZ	NFA_53590	AP006618	SEQ ID NO: 24
<i>Bradyrhizobium japonicum</i> USDA 6	metZ	BJ6T_RS05520	NC_017249	SEQ ID NO: 25
<i>Methylococcus capsulatus</i> str. Bath	metZ	MCA2488	AE017282	SEQ ID NO: 26
<i>Methylobacillus flagellatus</i> KT	"metZ"	MFLA_RS08660	NC_007947	SEQ ID NO: 27
<i>Nitrosomonas europaea</i> ATCC 19718	metZ	ALW85_RS03655	NC_004757	SEQ ID NO: 28
<i>Klebsiella pneumoniae</i> subsp. <i>pneumoniae</i> MGH 78578	metB	KPN_RS22820	NC_009648	SEQ ID NO: 29
<i>Bacillus subtilis</i> subsp. <i>subtilis</i> str. 168	metI	BSU_11870	NC_000964	SEQ ID NO: 30
<i>Shigella flexneri</i> 2a 2457T ATCC 700930	metB	S3730	AE014073	SEQ ID NO: 31
<i>Colwellia psychrerythraea</i> 34H ATCC BAA-681	metB	CPS_0455	CP000083	SEQ ID NO: 32
<i>Salmonella enterica</i> serovar <i>Paratyphi A</i> ATCC 9150	metB	SPA3943	CP000026	SEQ ID NO: 33
<i>Rhodobacter sphaeroides</i> ATCC 17029	metZ	RSPH17029_RS02535	NC_009049	SEQ ID NO: 34

In a preferred embodiment of the method of the present invention, the reaction in step (a) is catalyzed by at least one enzyme selected from the group consisting of a sulfhydrylase **E**₁, a cystathionine γ -synthase **E**₂, wherein the polypeptide sequence of the sulfhydrylase enzyme **E**₁ is selected from the group consisting of:

- 5 - O-acetyl homoserine sulfhydrylases selected from the group consisting of SEQ ID NO: 2 and variants of SEQ ID NO: 2, SEQ ID NO: 6 and variants of SEQ ID NO: 6, SEQ ID NO: 19 and variants of SEQ ID NO: 19, SEQ ID NO: 22 and variants of SEQ ID NO: 22, SEQ ID NO: 23 and variants of SEQ ID NO: 23, and
- 10 - O-succinyl homoserine sulfhydrylases selected from the group consisting of SEQ ID NO: 10 and variants of SEQ ID NO: 10, SEQ ID NO: 14 and variants of SEQ ID NO: 14, SEQ ID NO: 18 and variants of SEQ ID NO: 18, SEQ ID NO: 20 and variants of SEQ ID NO: 20, SEQ ID NO: 24 and variants of SEQ ID NO: 24, SEQ ID NO: 25 and variants of SEQ ID NO: 25, SEQ ID NO: 26 and variants of SEQ ID NO: 26, SEQ ID NO: 27 and variants of SEQ ID NO: 27, SEQ ID NO: 28 and variants of SEQ ID NO: 28, SEQ ID NO: 34 and variants of SEQ ID NO: 34,
- 15 - and wherein the polypeptide sequence of the cystathionine γ -synthase **E**₂ is selected from the group consisting of SEQ ID NO: 17 and variants thereof, SEQ ID NO: 21 and variants thereof, SEQ ID NO: 29 and variants thereof, SEQ ID NO: 30 and variants thereof, SEQ ID NO: 31 and variants thereof, SEQ ID NO: 32 and variants thereof, SEQ ID NO: 33 and variants thereof.
- 20

In a more preferred embodiment of the method of the present invention, the reaction in step (a) is catalyzed by a sulfhydrylase **E**₁ selected from the group consisting of

- 25 O-acetyl homoserine sulfhydrylases selected from the group consisting of SEQ ID NO: 2 and variants of SEQ ID NO: 2, SEQ ID NO: 6 and variants of SEQ ID NO: 6, SEQ ID NO: 19 and variants of SEQ ID NO: 19, SEQ ID NO: 22 and variants of SEQ ID NO: 22, SEQ ID NO: 23 and variants of SEQ ID NO: 23, and
- 30 O-succinyl homoserine sulfhydrylases selected from the group consisting of SEQ ID NO: 10 and variants of SEQ ID NO: 10, SEQ ID NO: 14 and variants of SEQ ID NO: 14, SEQ ID NO: 18 and variants of SEQ ID NO: 18, SEQ ID NO: 20 and variants of SEQ ID NO: 20, SEQ ID NO: 24 and variants of SEQ ID NO: 24, SEQ ID NO: 25 and variants of SEQ ID NO: 25, SEQ ID NO: 26 and variants of SEQ ID NO: 26, SEQ ID NO: 27 and variants of SEQ ID NO: 27, SEQ ID NO: 28 and variants of SEQ ID NO: 28, SEQ ID NO: 34 and variants of SEQ ID NO: 34.

- 35 In an even more preferred embodiment of the method of the present invention, the reaction in step (a) is catalyzed by a sulfhydrylase **E**₁ selected from the group consisting of O-acetyl homoserine sulfhydrylases selected from the group consisting of SEQ ID NO: 2 and variants of SEQ ID NO: 2, SEQ ID NO: 6 and variants of SEQ ID NO: 6 and O-succinyl homoserine sulfhydrylases selected from the group consisting of SEQ ID NO: 10 and variants of SEQ ID NO: 10, SEQ ID NO: 14 and variants of SEQ ID NO: 14.
- 40

In an even more preferred embodiment of the method of the present invention, the reaction in step (a) is catalyzed by a sulfhydrylase E₁ selected from the group consisting of O-acetyl homoserine sulfhydrylases selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 6, and O-succinyl homoserine sulfhydrylases selected from the group consisting of SEQ ID NO: 10, SEQ ID NO: 14.

The term "variant" is further explained below (item 4.3.3.1). In the context of the present application, it is understood to mean a polypeptide sequences with at least 80 % sequence identity to the respective polypeptide sequence.

10

4.3.2 Methods for obtaining enzymes

The enzymes that can be used in the method according to the present invention can be synthesized by methods that are known to the skilled person.

15

One approach is to express the enzyme(s) in microorganism(s) such as *Escherichia coli* (= "*E. coli*"), *Saccharomyces cerevisiae*, *Pichia pastoris*, and others, and to add the whole cells to the reactions as whole cell biocatalysts. Another approach is to express the enzyme(s), lyse the microorganisms, and add the cell lysate. Yet another approach is to purify, or partially purify, the enzyme(s) from a lysate and add pure or partially pure enzyme(s) to the reaction. If multiple enzymes are required for a reaction, the enzymes can be expressed in one or several microorganisms, including expressing all enzymes within a single microorganism.

For example, the skilled person can obtain the enzymes according to the invention by expression, in particular, overexpression, (hereinafter, "expression, in particular overexpression" is abbreviated as (over)expression", and "express, in particular overexpress" is abbreviated as "(over)express") of these enzymes in a cell and subsequent isolation thereof, e.g. as described in DE 100 31 999 A1. Episomal plasmids, for example, are employed for increasing the expression of the respective genes. In such plasmids, the nucleic acid molecule to be (over)expressed or encoding the polypeptide or enzyme to be (over)expressed may be placed under the control of a strong inducible promoter such as the *lac* promoter, located upstream of the gene. A promoter is a DNA sequence consisting of about 40 to 50 base pairs which constitutes the binding site for an RNA polymerase holoenzyme and the transcriptional start point (M. Pátek, J. Holátko, T. Busche, J. Kalinowski, J. Nešvera, Microbial Biotechnology **2013**, 6, 103-117), whereby the strength of expression of the controlled polynucleotide or gene can be influenced. A "functional linkage" is obtained by the sequential arrangement of a promoter with a gene, which leads to a transcription of the gene.

Suitable strong promoters or methods of producing such promoters for increasing expression are known from the literature (e.g. S. Lisser & H. Margalit, Nucleic Acid Research **1993**, 21, 1507-1516; M. Patěk and J. Nesvera in H. Yukawa and M Inui (eds.), Corynebacterium glutamicum,

40

Microbiology Monographs 23, Springer Verlag Berlin Heidelberg 2013, 51-88; B. J. Eikmanns, E. Kleinertz, W. Liebl, H. Sahm, *Gene* **1991**, 102, 93-98). For instance, native promoters may be optimized by altering the promoter sequence in the direction of known consensus sequences with respect to increasing the expression of the genes functionally linked to these promoters (M. Pátek, 5 B. J. Eikmanns, J. Pátek, H. Sahm, *Microbiology* **1996**, 142, 1297-1309; M. Pátek, J. Holátko, T. Busche, J. Kalinowski, J. Nešvera, *Microbial Biotechnology* **2013**, 6, 103-117).

Constitutive promoters are also suitable for the (over)expression, in which the gene encoding the enzyme activity is expressed continuously under the control of the promoter such as, for example, 10 the glucose dependent *deo* promoter. Chemically induced promoters are also suitable, such as *tac*, *lac* or *trp*. The most widespread system for the induction of promoters is the *lac* operon of *E. coli*. In this case, either lactose or isopropyl β -D-thiogalactopyranoside (IPTG) is used as inducer. Also, systems using arabinose (e.g. the pBAD system) or rhamnose (e.g. *E. coli* KRX) are common as inducers. A system for physical induction is, for example, the temperature-induced cold shock 15 promoter system based on the *E. coli* *cspA* promoter from Takara or Lambda PL and also osmotically inducible promoters, for example, *osmB* (e.g. WO 95/25785 A1).

Suitable plasmids or vectors are in principle all embodiments available for this purpose to the person skilled in the art. The state of the art describes standard plasmids that may be used for this 20 purpose, for example the pET system of vectors exemplified by pET-3a or pET-26b(+) (commercially available from Novagen). Further plasmids and vectors can be taken, for example, from the brochures of the companies Novagen, Promega, New England Biolabs, Clontech or Gibco BRL. Further preferred plasmids and vectors can be found in: Glover, D.M. (1985) DNA cloning: a practical approach, Vol. I-III, IRL Press Ltd. , Oxford; Rodriguez, R.L. and Denhardt, D. T (eds) 25 (1988) Vectors : a survey of molecular cloning vectors and their uses, 179-204, Butterworth, Stoneham; Goeddel, D. V. (1990) Systems for heterologous gene expression, Methods Enzymol. 185, 3-7; Sambrook, J.; Fritsch, E. F. and Maniatis, T. (1989), Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York.

30 The plasmid vector, which contains the gene to be amplified, is then converted to the desired strain, e.g. by conjugation or transformation. The method of conjugation is described, for example, by A. Schäfer, J. Kalinowski, A. Pühler, *Applied and Environmental Microbiology* **1994**, 60, 756-759. Methods for transformation are described, for example, in G. Thierbach, A. Schwarzer, A. Pühler, *Applied Microbiology and Biotechnology* **1988**, 29, 356-362, L. K. Dunican & E. Shivan, 35 *Bio/Technology* **1989**, 7, 1067-1070 and A. Tauch, O. Kirchner, L. Wehmeier, J. Kalinowski, A. Pühler, *FEMS Microbiology Letters* **1994**, 123, 343-347. After homologous recombination by means of a "cross-over" event, the resulting strain contains at least two copies of the gene concerned.

The desired enzyme can be isolated by disrupting cells which contain the desired activity in a manner known to the person skilled in the art, for example with the aid of a ball mill, a French press or of an ultrasonic disintegrator and subsequently separating off cells, cell debris and disruption aids, such as, for example, glass beads, by centrifugation for 10 minutes at 13000 rpm and 4 °C.

- 5 Using the resulting cell-free crude extract, enzyme assays with subsequent LC-ESI-MS detection of the products can then be carried out. Alternatively, the enzyme can be enriched in the manner known to the person skilled in the art by chromatographic methods (such as nickel-nitrilotriacetic acid affinity chromatography, streptavidin affinity chromatography, gel filtration chromatography or ion-exchange chromatography) or else purified to homogeneity.

10

Whether or not a nucleic acid or polypeptide is (over)expressed, may be determined by way of quantitative PCR reaction in the case of a nucleic acid molecule, SDS polyacrylamide electrophoreses, Western blotting or comparative activity assays in the case of a polypeptide.

- 15 Genetic modifications may be directed to transcriptional, translational, and/or post-translational modifications that result in a change of enzyme activity and/or selectivity under selected and/or identified culture conditions.

4.3.3 Definitions

20 4.3.3.1 "Variants"

- In the context of the present invention, the term "variant" with respect to polypeptide sequences refers to a polypeptide sequence with a degree of identity to the reference sequence of at least 80%, more preferably at least 81%, more preferably at least 82%, more preferably at least 83%,
25 more preferably at least 84%, more preferably at least 85%, more preferably at least 86%, more preferably at least 87%, more preferably at least 88%, more preferably at least 89%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, more preferably at least 93%, more preferably at least 94%, more preferably at least 95%, more preferably at least 96%, more preferably at least 97%, more preferably at least 98%, more
30 preferably at least 99%. In still further particular embodiments, the degree of identity is at least 98.0%, more preferably at least 98.2%, more preferably at least 98.4%, more preferably at least 98.6%, more preferably at least 98.8%, more preferably at least 99.0%, more preferably at least 99.1%, more preferably at least 99.2%, more preferably at least 99.3%, more preferably at least 99.4%, more preferably at least 99.5%, more preferably at least 99.6%, more preferably at least
35 99.7%, more preferably at least 99.8%, or at least more preferably at least 99.9%. It goes without saying that a "variant" of a certain polypeptide sequence is not identical to the polypeptide sequence.

- Such variants may be prepared by introducing deletions, insertions, substitutions, or combinations thereof, in particular in amino acid sequences, as well as fusions comprising such macromolecules
40 or variants thereof.

Modifications of amino acid residues of a given polypeptide sequence which lead to no significant modifications of the properties and function of the given polypeptide are known to those skilled in the art. Thus for example many amino acids can often be exchanged for one another without
 5 problems; examples of such suitable amino acid substitutions are: Ala by Ser; Arg by Lys; Asn by Gln or His; Asp by Glu; Cys by Ser; Gln by Asn; Glu by Asp; Gly by Pro; His by Asn or Gln; Ile by Leu or Val; Leu by Met or Val; Lys by Arg or Gln or Glu; Met by Leu or Ile; Phe by Met or Leu or Tyr; Ser by Thr; Thr by Ser; Trp by Tyr; Tyr by Trp or Phe; Val by Ile or Leu. It is also known that modifications, particularly at the N- or C-terminus of a polypeptide in the form of for example amino
 10 acid insertions or deletions, often exert no significant influence on the function of the polypeptide.

In line with this, preferable variants according to the invention of any of SEQ ID NO: 2, SEQ ID NO: 6, SEQ ID NO: 19, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 10, SEQ ID NO: 14, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID
 15 NO: 28, SEQ ID NO: 34, SEQ ID NO: 17, SEQ ID NO: 21, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, and SEQ ID NO: 33, respectively, have a polypeptide sequence that comprises the amino acids of the respective sequence that are essential for the function, for example the catalytic activity of a protein, or the fold or structure of the protein. The other amino acids may be deleted, substituted or replaced by insertions or essential amino acids are replaced in
 20 a conservative manner to the effect that the activity of the enzyme, in particular the sulfhydrylase or the cystathionine γ -synthase, is preserved.

4.3.3.2 "Sequence Identity"

25 The person skilled in the art is aware that various computer programs are available for the calculation of similarity or identity between two nucleotide or amino acid sequences.

Preferred methods for determining the identity initially generate the greatest alignment between the sequences to be compared. Computer programs for determining the identity include, but are not
 30 limited to, the GCG program package including

- GAP [J. Deveroy *et al.*, Nucleic Acid Research **1984**, 12, page 387, Genetics Computer Group University of Wisconsin, Medicine (WI)], and
- BLASTP, BLASTN and FASTA (S. Altschul *et al.*, Journal of Molecular Biology **1990**, 215, 403-410). The BLAST program can be obtained from the National Center for Biotechnology
 35 Information (NCBI) and from other sources (BLAST Handbook, S. Altschul *et al.*, NCBI NLM NIH Bethesda ND 22894; S. Altschul *et al.*, above).

For instance, the percentage identity between two amino acid sequences can be determined by the algorithm developed by S. B. Needleman and C. D. Wunsch, J. Mol. Biol. **1970**, 48, 443-453, which
 40 has been integrated into the GAP program in the GCG software package, using either a

BLOSUM62 matrix or a PAM250 matrix, a gap weight of 16, 14, 12, 10, 8, 6 or 4 and a length weight of 1, 2, 3, 4, 5 or 6. The person skilled in the art will recognize that the use of different parameters will lead to slightly different results, but that the percentage identity between two amino acid sequences overall will not be significantly different. The BLOSUM62 matrix is typically used

5 applying the default settings (gap weight: 12, length weight: 1).

In the context of the present invention, a sequence identity of 80% according to the above algorithm means 80% homology. The same applies to higher identities.

Most preferably, the degree of identity between sequences is determined in the context of the

10 present invention by the programme "Needle" using the substitution matrix BLOSUM62, the gap opening penalty of 10, and the gap extension penalty of 0.5. The Needle program implements the global alignment algorithm described in S. B. Needleman and C. D. Wunsch, J. Mol. Biol. **1970**, *48*, 443-453. The substitution matrix used according to the present invention is BLOSUM62, gap opening penalty is 10, and gap extension penalty is 0.5. The preferred version used in the context

15 of this invention is the one presented by F. Madeira, Y.M. Park, J. Lee, N. Buso, T. Gur, N. Madhusoodanan, P. Basutkar, A.R.N. Tivey, S.C. Potter, R.D. Finn, Nucleic Acids Research **2019**, *47*, W636–W641, Web Server issue (preferred version accessible online on March 31, 2021 via https://www.ebi.ac.uk/Tools/psa/emboss_needle/).

20 In a particular embodiment, the percentage of identity of an amino acid sequence of a polypeptide with, or to, a reference polypeptide sequence is determined by i) aligning the two amino acid sequences using the Needle program, with the BLOSUM62 substitution matrix, a gap opening penalty of 10, and a gap extension penalty of 0.5; ii) counting the number of exact matches in the alignment; iii) dividing the number of exact matches by the length of the longest of the two amino

25 acid sequences, and iv) converting the result of the division of iii) into percentage.

4.3.3.3 "Preferred assay to identify particularly active variants"

4.3.3.3.1 Assay A

30 Especially preferable polypeptide variants of any of SEQ ID NO: 2, SEQ ID NO: 6, SEQ ID NO: 19, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 10, SEQ ID NO: 14, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 34, SEQ ID NO: 17, SEQ ID NO: 21, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ

35 ID NO: 32, and SEQ ID NO: 33, respectively, in the context of the present invention may be identified by the skilled person as those displaying activity in the following assay ("**Assay A**").

Assay A is carried out by the following steps:

A1) First, the activity of the variant to be tested is determined by the following steps A1.1), A1.2), and A1.3) as follows:

5 A1.1) 990 µl of a reaction solution containing phosphate buffer (0.1 M, pH 7.5), 3 mM O-acetyl L-homoserine-HCl, 10 µM pyridoxal 5'-phosphate mono-hydrate and 1.0 nmol of the polypeptide to be tested are prepared and heated to 50 °C.

A1.2) The reaction is started by adding 10 µl of a 200 mM solution of butyl-*P*-methylphosphinate (MPBE, CAS#6172-80-1). The concentration of MPBE in the reaction solution is 2 mM.

10 A1.3) After addition of the MPBE, the reaction is conducted for 120 minutes at 50 °C. Then, the reaction is stopped by adding 10 µl 1 % formate solution and cooling on ice.

A2) Then, a blank test is carried out by the following steps A2.1), A2.2), and A2.3) as follows:

15 A2.1) 990 µl of a reaction solution containing phosphate buffer (0.1 M, pH 7.5), 3 mM O-acetyl L-homoserine-HCl, 10 µM pyridoxal 5'-phosphate mono-hydrate are prepared and heated to 50 °C.

A2.2) The reaction is started by adding 10 µl of a 200 mM solution of butyl-*P*-methylphosphinate (MPBE, CAS#6172-80-1).

20 A2.3) After addition of the MPBE, the reaction is conducted for 120 minutes at 50 °C. Then, the reaction is stopped by adding 10 µl 1 % formate solution and cooling on ice.

A3) Finally, the amount (in mole) of the butyl-phosphoester of L-GA (i.e. the compound according to formula (III) with R¹ = *n*-butyl) obtained in the reaction solution in A1.3) and A2.3), respectively, is determined and compared, preferably by LC-MS analysis described under item 5.7.

25 A4) If the amount of butyl-phosphoester of L-GA determined for A1.3) is more than the amount determined for A2.3), then the variant to be tested displays activity in **Assay A**.

If the amount of butyl-phosphoester of L-GA determined for A1.3) is the same or less than the amount determined for A2.3), then the variant to be tested does not display activity in **Assay A**.

30 Preferable formate solutions in steps A1.3) and A2.3) are ammonium formate or sodium formate solutions. Alternatively, the reaction in steps A 1.3) and A2.3) can also be stopped by adding methanol, preferably 1 ml of methanol.

35 4.3.3.3.2 Assay B

In a further assay ("**Assay B**"), the activity of a polypeptide variants of any of SEQ ID NO: 2, SEQ ID NO: 6, SEQ ID NO: 19, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 10, SEQ ID NO: 14, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, 40 SEQ ID NO: 28, SEQ ID NO: 34, SEQ ID NO: 17, SEQ ID NO: 21, SEQ ID NO: 29, SEQ ID NO:

30, SEQ ID NO: 31, SEQ ID NO: 32, and SEQ ID NO: 33, respectively, with respect to the polypeptide may be determined.

Assay B is carried out by the following steps:

5

B1) First, the activity of the "standard" polypeptide standard (i.e., one polypeptide sequence selected from SEQ ID NO: 2, SEQ ID NO: 6, SEQ ID NO: 19, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 10, SEQ ID NO: 14, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 34, SEQ ID NO: 17, SEQ ID NO: 21, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, and SEQ ID NO: 33) is determined by the following steps B1.1), B1.2), and B1.3) as follows:

15 B1.1) 990 µl of a reaction solution containing phosphate buffer (0.1 M, pH 7.5), 3 mM O-acetyl L-homoserine-HCl, 10 µM pyridoxal 5'-phosphate mono-hydrate and 1.0 nmol of the "standard" polypeptide to be tested are prepared and heated to 50 °C.

B1.2) The reaction is started by adding 10 µl of a 200 mM solution of butyl-*P*-methylphosphinate (MPBE, CAS#6172-80-1). The concentration of MPBE in the reaction solution is 2 mM.

20 B1.3) After addition of the MPBE, the reaction is conducted for 120 minutes at 50 °C. Then, the reaction is stopped by adding 10 µl 1 % formate solution and cooling on ice.

B2) Then, steps B1.1), B1.2), and B1.3) are repeated with the variant:

25 B2.1) 990 µl of a reaction solution containing phosphate buffer (0.1 M, pH 7.5), 3 mM O-acetyl L-homoserine-HCl, 10 µM pyridoxal 5'-phosphate mono-hydrate and 1.0 nmol of the "variant" polypeptide to be tested are prepared and heated to 50 °C.

30 B2.2) The reaction is started by adding 10 µl of a 200 mM solution of butyl-*P*-methylphosphinate (MPBE, CAS#6172-80-1). The concentration of MPBE in the reaction solution is 2 mM.

B2.3) After addition of the MPBE, the reaction is conducted for 120 minutes at 50 °C. Then, the reaction is stopped by adding 10 µl 1 % formate solution and cooling on ice.

35 B3) Finally, the amount of the butyl-phosphoester of L-GA (i.e. the compound according to formula (III) with R¹ = *n*-butyl) obtained in the reaction solution in B1.3) and B2.3) is determined and compared, preferably by LC-MS analysis described under item 5.7.

40 B4) Then, the ratio of the amount (in mole) of butyl-phosphoester of L-GA obtained in B2.3) is divided by the amount (in mole) of butyl-phosphoester of L-GA obtained in B1.3). This ratio is then

multiplied with a factor of 100, giving the relative activity of the variant polypeptide with respect to the "standard" polypeptide in percent.

- Preferable formate solutions in steps B1.3) and B2.3) are ammonium formate or sodium formate solutions. Alternatively, the reaction in steps B1.3) and B2.3) can also be stopped by adding methanol, preferably 1 ml of methanol.

4.3.3.4 "Preferred variants"

10 **4.3.3.4.1 "Variants of SEQ ID NO: 2"**

- In particular, a variant of SEQ ID NO: 2 is a polypeptide with sequence identity of $\geq 80\%$, more preferably $\geq 85\%$, more preferably $\geq 90\%$, more preferably $\geq 91\%$, more preferably $\geq 92\%$, more preferably $\geq 93\%$, more preferably $\geq 94\%$, more preferably $\geq 95\%$, more preferably $\geq 96\%$, more preferably $\geq 97\%$, more preferably $\geq 98\%$, more preferably $\geq 99\%$, more preferably $\geq 99.9\%$ sequence identity to polypeptide sequence SEQ ID NO: 2.

Preferred variants of SEQ ID NO: 2 display activity in **Assay A** under item 4.3.3.3.1.

- Even more preferably, the activity of the respective variant of SEQ ID NO: 2 is at least 1% , preferably at least 10% , more preferably at least 20% , more preferably of at least 30% , more preferably of at least 40% , more preferably of at least 50% , more preferably of at least 60% , more preferably of at least 70% , more preferably of at least 80% , more preferably of at least 90% , more preferably of at least 99% relative to the activity of SEQ ID NO: 2 as determined in **Assay B** under item 4.3.3.3.2.

- Even more preferably, the activity of the respective variant of SEQ ID NO: 2 is in the range of 1 to 1000% , preferably in the range of 5 to 500% , more preferably in the range of 10 to 400% , more preferably in the range of 40 to 200% , more preferably in the range of 50 to 150% , more preferably in the range of 60 to 140% , more preferably in the range of 70 to 130% , more preferably in the range of 80 to 120% , more preferably in the range of 90 to 110% , more preferably 100% relative to the activity of SEQ ID NO: 2 as determined in **Assay B** under item 4.3.3.3.2.

35 **4.3.3.4.2 "Variants of SEQ ID NO: 6"**

- In particular, a variant of SEQ ID NO: 6 is a polypeptide with sequence identity of $\geq 80\%$, more preferably $\geq 85\%$, more preferably $\geq 90\%$, more preferably $\geq 91\%$, more preferably $\geq 92\%$, more preferably $\geq 93\%$, more preferably $\geq 94\%$, more preferably $\geq 95\%$, more preferably $\geq 96\%$, more preferably $\geq 97\%$, more preferably $\geq 98\%$, more preferably $\geq 99\%$, more preferably $\geq 99.9\%$ sequence identity to polypeptide sequence SEQ ID NO: 6.

Preferred variants of SEQ ID NO: 6 display activity in **Assay A** under item 4.3.3.3.1.

Even more preferably, the activity of the respective variant of SEQ ID NO: 6 is at least 1 %, preferably at least 10 %, more preferably at least 20 %, more preferably of at least 30 %, more preferably of at least 40 %, more preferably of at least 50 %, more preferably of at least 60 %, more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 % relative to the activity of SEQ ID NO: 6 as determined in **Assay B** under item 4.3.3.3.2.

10

Even more preferably, the activity of the respective variant of SEQ ID NO: 6 is in the range of 1 to 1000 %, preferably in the range of 5 to 500 %, more preferably in the range of 10 to 400 %, more preferably in the range of 40 to 200 %, more preferably in the range of 50 to 150 %, more preferably in the range of 60 to 140 %, more preferably in the range of 70 to 130 %, more preferably in the range of 80 to 120 %, more preferably in the range of 90 to 110 %, more preferably 100 % relative to the activity of SEQ ID NO: 6 as determined in **Assay B** under item 4.3.3.3.2.

15

4.3.3.4.3 “Variants of SEQ ID NO: 10”

20

In particular, a variant of SEQ ID NO: 10 is a polypeptide with sequence identity of ≥ 80 %, more preferably ≥ 85 %, more preferably ≥ 90 %, more preferably ≥ 91 %, more preferably ≥ 92 %, more preferably ≥ 93 %, more preferably ≥ 94 %, more preferably ≥ 95 %, more preferably ≥ 96 %, more preferably ≥ 97 %, more preferably ≥ 98 %, more preferably ≥ 99 %, more preferably ≥ 99.9 % sequence identity to polypeptide sequence SEQ ID NO: 10.

25

Preferred variants of SEQ ID NO: 10 show activity in **Assay A** under item 4.3.3.3.1.

Even more preferably, the activity of the respective variant of SEQ ID NO: 10 is at least 1 %, preferably at least 10 %, more preferably at least 20 %, more preferably of at least 30 %, more preferably of at least 40 %, more preferably of at least 50 %, more preferably of at least 60 %, more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 % relative to the activity of SEQ ID NO: 10 as determined in **Assay B** under item 4.3.3.3.2.

35

Even more preferably, the activity of the respective variant of SEQ ID NO: 10 is in the range of 1 to 1000 %, preferably in the range of 5 to 500 %, more preferably in the range of 10 to 400 %, more preferably in the range of 40 to 200 %, more preferably in the range of 50 to 150 %, more preferably in the range of 60 to 140 %, more preferably in the range of 70 to 130 %, more preferably in the range of 80 to 120 %, more preferably in the range of 90 to 110 %, more

40

preferably 100 % relative to the activity of SEQ ID NO: 10 as determined in **Assay B** under item 4.3.3.3.2.

4.3.3.4.4 "Variants of SEQ ID NO: 14"

5

In particular, a variant of SEQ ID NO: 14 is a polypeptide with sequence identity of ≥ 80 %, more preferably ≥ 85 %, more preferably ≥ 90 %, more preferably ≥ 91 %, more preferably ≥ 92 %, more preferably ≥ 93 %, more preferably ≥ 94 %, more preferably ≥ 95 %, more preferably ≥ 96 %, more preferably ≥ 97 %, more preferably ≥ 98 %, more preferably ≥ 99 %, more preferably ≥ 99.9 %

10 sequence identity to polypeptide sequence SEQ ID NO: 14.

Preferred variants of SEQ ID NO: 14 display activity in **Assay A** under item 4.3.3.3.1.

Even more preferably, the activity of the respective variant of SEQ ID NO: 14 is at least 1 %, preferably at least 10 %, more preferably at least 20 %, more preferably of at least 30 %, more preferably of at least 40 %, more preferably of at least 50 %, more preferably of at least 60 %, more preferably of at least 70 %, more preferably of at least 80 %, more preferably of at least 90 %, more preferably of at least 99 % relative to the activity of SEQ ID NO: 14 as determined in **Assay B** under item 4.3.3.3.2.

20

Even more preferably, the activity of the respective variant of SEQ ID NO: 14 is in the range of 1 to 1000 %, preferably in the range of 5 to 500 %, more preferably in the range of 10 to 400 %, more preferably in the range of 40 to 200 %, more preferably in the range of 50 to 150 %, more preferably in the range of 60 to 140 %, more preferably in the range of 70 to 130 %, more preferably in the range of 80 to 120 %, more preferably in the range of 90 to 110 %, more preferably 100 % relative to the activity of SEQ ID NO: 14 as determined in **Assay B** under item 4.3.3.3.2.

25

4.3.3.4.5 Further variants

30

Preferred variants of any of SEQ ID NO: 19, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 34, SEQ ID NO: 17, SEQ ID NO: 21, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, respectively, can be determined mutatis mutandis as described for SEQ ID NO: 2 as described under item 4.3.3.4.1.

35

Preferred variants of SEQ ID NO: 19 can be determined by the procedure that results when in the description under item 4.3.3.4.1, the word "SEQ ID NO: 2" is substituted by the word "SEQ ID NO: 19".

40

Preferred variants of SEQ ID NO: 22 can be determined by the procedure that results when in the description under item 4.3.3.4.1, the word "SEQ ID NO: 2" is substituted by the word "SEQ ID NO: 22".

- 5 Preferred variants of SEQ ID NO: 23 can be determined by the procedure that results when in the description under item 4.3.3.4.1, the word "SEQ ID NO: 2" is substituted by the word "SEQ ID NO: 23".

- 10 Preferred variants of SEQ ID NO: 18 can be determined by the procedure that results when in the description under item 4.3.3.4.1, the word "SEQ ID NO: 2" is substituted by the word "SEQ ID NO: 18".

- 15 Preferred variants of SEQ ID NO: 20 can be determined by the procedure that results when in the description under item 4.3.3.4.1, the word "SEQ ID NO: 2" is substituted by the word "SEQ ID NO: 20".

- 20 Preferred variants of SEQ ID NO: 24 can be determined by the procedure that results when in the description under item 4.3.3.4.1, the word "SEQ ID NO: 2" is substituted by the word "SEQ ID NO: 24".

- Preferred variants of SEQ ID NO: 25 can be determined by the procedure that results when in the description under item 4.3.3.4.1, the word "SEQ ID NO: 2" is substituted by the word "SEQ ID NO: 25".

- 25 Preferred variants of SEQ ID NO: 26 can be determined by the procedure that results when in the description under item 4.3.3.4.1, the word "SEQ ID NO: 2" is substituted by the word "SEQ ID NO: 26".

- 30 Preferred variants of SEQ ID NO: 27 can be determined by the procedure that results when in the description under item 4.3.3.4.1, the word "SEQ ID NO: 2" is substituted by the word "SEQ ID NO: 27".

- 35 Preferred variants of SEQ ID NO: 28 can be determined by the procedure that results when in the description under item 4.3.3.4.1, the word "SEQ ID NO: 2" is substituted by the word "SEQ ID NO: 28".

- Preferred variants of SEQ ID NO: 34 can be determined by the procedure that results when in the description under item 4.3.3.4.1, the word "SEQ ID NO: 2" is substituted by the word "SEQ ID NO: 34".

Preferred variants of SEQ ID NO: 17 can be determined by the procedure that results when in the description under item 4.3.3.4.1, the word "SEQ ID NO: 2" is substituted by the word "SEQ ID NO: 17".

- 5 Preferred variants of SEQ ID NO: 21 can be determined by the procedure that results when in the description under item 4.3.3.4.1, the word "SEQ ID NO: 2" is substituted by the word "SEQ ID NO: 21".

- 10 Preferred variants of SEQ ID NO: 29 can be determined by the procedure that results when in the description under item 4.3.3.4.1, the word "SEQ ID NO: 2" is substituted by the word "SEQ ID NO: 29".

- 15 Preferred variants of SEQ ID NO: 30 can be determined by the procedure that results when in the description under item 4.3.3.4.1, the word "SEQ ID NO: 2" is substituted by the word "SEQ ID NO: 30".

- 20 Preferred variants of SEQ ID NO: 31 can be determined by the procedure that results when in the description under item 4.3.3.4.1, the word "SEQ ID NO: 2" is substituted by the word "SEQ ID NO: 31".

- Preferred variants of SEQ ID NO: 32 can be determined by the procedure that results when in the description under item 4.3.3.4.1, the word "SEQ ID NO: 2" is substituted by the word "SEQ ID NO: 32".

- 25 Preferred variants of SEQ ID NO: 33 can be determined by the procedure that results when in the description under item 4.3.3.4.1, the word "SEQ ID NO: 2" is substituted by the word "SEQ ID NO: 33".

30 4.4 Method conditions

The reaction in step a) of the method according to the present invention may be carried out under conditions known to the skilled person.

- 35 The reaction medium in which activated L-homoserine **H_A** is reacted with the substrate **S** is preferably aqueous, more preferably an aqueous buffer.

- Exemplary buffers commonly used in biotransformation reactions and advantageously used herein include Tris, phosphate, or any of Good's buffers, such as 2-(*N*-morpholino)ethanesulfonic acid ("MES"), *N*-(2-acetamido)iminodiacetic acid ("ADA"), piperazine-*N,N'*-bis(2-ethanesulfonic acid) ("PIPES"), *N*-(2-acetamido)-2-aminoethanesulfonic acid ("ACES"), *P*-hydroxy-4-
- 40

morpholinepropanesulfonic acid ("MOPSO"), cholamine chloride, 3-(*N*-morpholino)propanesulfonic acid ("MOPS"), *N,N*-Bis(2-hydroxyethyl)- 2-aminoethanesulfonic acid ("BES"), 2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid ("TES"), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid ("HEPES"), 3-(Bis(2-hydroxyethyl)amino)-2-hydroxypropane-1-sulfonic acid ("DIPSO"), acetamidoglycine, 3-(*N*-Tris(hydroxymethyl)methylamino(-2-hydroxypropane)sulfonic acid ("TAPSO"), piperazine-*N,N'*-bis(2-hydroxypropanesulfonic acid) ("POPSO"), 4-(2-Hydroxyethyl)piperazine-1-(2-hydroxypropanesulfonic acid) ("HEPPSO"), 3-[4-(2-Hydroxyethyl)-1-piperazinyl]propanesulfonic acid ("HEPPS"), tricine, glycineamide, bicine, or 3-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]propane-1-sulfonic acid ("TAPS").

In some embodiments, ammonium can act as a buffer. One or more organic solvents can also be added to the reaction.

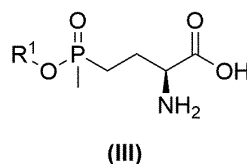
Preferably, step a) of the method according to the invention is carried out in a phosphate buffer.

The pH of the reaction medium in step a) of the method is preferably in the range of from 2 to 10, more preferably in the range of from 5 to 8, most preferably 7.5.

The method according to the invention is preferably carried out at a temperature in the range of from 20 °C to 70 °C, more preferably in the range of from 30 °C to 55 °C, most preferably 50 °C.

4.5 L-glufosinate phosphoester

The product of the method according to the invention, is a compound of the following structure (III):



The compound according to structure (III) is L-glufosinate (for R¹ = H) or L-glufosinate phosphoester (for R¹ = alkyl, alkenyl, alkynyl, hydroxyalkyl or aryl).

The compound according to structure (III), wherein R¹ = *n*-butyl, is abbreviated as "L-GA-Bu" or "LGA-Bu".

The skilled person understands that the identity of residue R¹ in the compound according to structure (III) depends on the identity of residue R¹ in the substrate structure (I).

If R¹ is hydrogen, L-glufosinate is directly obtained in the method according to the invention.

In the preferred embodiment, in which R¹ is selected from alkyl, alkenyl, alkynyl, hydroxyalkyl, aryl, preferably alkyl, more preferably methyl, ethyl, *n*-butyl, the compound (III) is a L-glufosinate phosphoester.

5

In those embodiments, it is further preferable that the method according to the invention contains a further step b) wherein the compound of the structure (III) which is obtained in step (a), and in which R¹ is selected from alkyl, alkenyl, alkynyl, hydroxyalkyl, aryl, preferably alkyl, more preferably methyl, ethyl, *n*-butyl, is saponified to give L-glufosinate.

10

This can be carried out by methods known to the skilled person.

Preferably, such saponification is carried out under acidic conditions, more preferably by mixing 1 Volume of the reaction medium containing the compound of the structure (III) which is obtained in step (a) and 4 Volumes of 6N HCl for 2 h and incubating the resulting mixture at a temperature of 50 °C to 150 °C, preferably at 100 °C.

15

Alternatively, an enzymatic saponification can be carried out.

5. Examples

- Genes of different origin encoding sulfhydrylase (EC 2.5.1.- and EC 2.5.1.49) were tested for their ability to react with an activated homoserine derivate and different substrates according to structure (I) to form a glufosinate derivate.

5.1 Example 1 Identification of suitable enzymes and construction of plasmids

5.1.1 Examined sulfhydrylase

Bibliographic details of the genes of O-acetylhomoserine sulfhydrylases and O-succinylhomoserine sulfhydrylases that were used in the examples are summarized in table 2.

Table 2

Strain	comment	Gene name	Gene ID	ReferenceNCBI accession	SEQ ID NO:
<i>Corynebacterium glutamicum</i> ATCC 13032	wild type	<i>metY</i>	NCgl0625	NC_003450	1
<i>Corynebacterium glutamicum</i> ATCC 13032	<i>metY</i> variant	<i>metY_P2T</i>			5
<i>Chromobacterium violaceum</i> ATCC 12472	wild type	<i>metZ</i>	CV_2725	AE016825	9
<i>Hyphomonas neptunium</i> ATCC 15444	wild type	<i>metZ</i>	HNE_2672	CP000158	13

5.1.2 Preparation of the gene expression plasmids

20

5.1.2.1 O-acetylhomoserine sulfhydrylase *metY* derived from *Corynebacterium glutamicum*

- The *metY* gene derived from *Corynebacterium glutamicum* ATCC 13032 codes for an O-acetylhomoserine sulfhydrylase and can be found at the NCBI under locus tag NCgl0625 of the genome sequence accessible under NC_003450.

To realize the expression of the enzyme, the expression vector pET-26b(+) (Novagen EMD Millipore) was used. Therefore, the *metY*_Cg polynucleotide according to SEQ ID NO: 3 was synthesized by GeneArt (ThermoFisher Scientific (Waltham, USA)).

- To assemble the expression vector pET-26b(+)_*metY*-Cg, the vector pET-26b(+) and the *metY*_Cg polynucleotide were both treated with *NdeI* and *NotI*, ligated, and the ligation mixture was used to transform *E. coli*. The resulting vector, pET-26b(+)_*metY*-Cg, is shown schematically in Figure 1. DNA of the expression vector pET-26b(+)_*metY*-Cg was isolated from a transformant.

5.1.2.2 O-acetylhomoserine sulfhydrylase *metY* P2T derived from *Corynebacterium glutamicum*

The O-acetylhomoserine sulfhydrylase variant *metY*_P2T is encoded by a polynucleotide according to SEQ ID NO: 5, which is identical to the polynucleotide of SEQ ID NO: 1, except that the nucleobases from position 4 to 6 are aca.

The polynucleotide of SEQ ID NO: 5 encodes a polypeptide comprising the amino acid sequence of SEQ ID NO: 6, which is identical to the polynucleotide of SEQ ID NO: 1, except that the amino acid proline at position 2 is substituted by threonine.

To realize the expression of the enzyme variant *metY*_P2T, the expression vector pET-26b(+) (Novagen EMD Millipore) was used. Therefore, the *metY*_P2T polynucleotide according to SEQ ID NO: 7 was synthesized by GeneArt (ThermoFisher Scientific (Waltham, USA)).

To assemble the expression vector pET-26b(+)_*metY*_P2T, the vector pET-26b(+) and the *metY*_P2T polynucleotide were both treated with *NdeI* and *NotI*, ligated, and the ligation mixture was used to transform *E. coli*. The resulting vector, pET-26b(+)_*metY*_P2T, is shown schematically in Figure 2.

DNA of the expression vector pET-26b(+)_*metY*_P2T was isolated from a transformant.

5.1.2.3 O-succinylhomoserine sulfhydrylase *metZ* derived from *Chromobacterium violaceum*

The *metZ* gene derived from *Chromobacterium violaceum* ATCC 12472 codes for an O-succinylhomoserine sulfhydrylase and can be found at the NCBI under locus tag CV_2725 of the genome sequence accessible under AE016825.

To realize the expression of the enzyme, the expression vector pET-26b(+) (Novagen EMD Millipore) was used. Therefore, the *metZ*_Cv polynucleotide according to SEQ ID NO: 11 was synthesized by GeneArt (ThermoFisher Scientific (Waltham, USA)).

To assemble the expression vector pET-26b(+)_*metZ*-Cv the vector pET-26b(+) and the *metZ*_Cv polynucleotide were both treated with *NdeI* and *XhoI*, ligated, and the ligation mixture was used to transform *E. coli*. The resulting vector, pET-26b(+)_*metZ*-Cv, is shown schematically in Figure 3.

DNA of the expression vector pET-26b(+)_*metZ*-Cv was isolated from a transformant.

5.1.2.4 O-succinylhomoserine sulfhydrylase *metZ* derived from *Hyphomonas neptunium*

The *metZ* gene derived from *Hyphomonas neptunium* ATCC 15444 codes for an O-succinylhomoserine sulfhydrylase and can be found at the NCBI under locus tag HNE_2672 of the genome sequence accessible under CP000158.

To realize the expression of the enzyme, the expression vector pET-26b(+) (Novagen EMD Millipore) was used. Therefore, the *metZ*_Hn polynucleotide according to SEQ ID NO: 15 was synthesized by GeneArt (ThermoFisher Scientific (Waltham, USA)).

To assemble the expression vector pET-26b(+)_metZ-Hn, the vector pET-26b(+) and the metZ_Hn polynucleotide were both treated with *Xba*I and *Xho*I, ligated, and the ligation mixture was used to transform *E. coli*. The resulting vector, pET-26b(+)_metZ-Hn, is shown schematically in Figure 4. DNA of the expression vector pET-26b(+)_metZ-Hn was isolated from a transformant.

5

5.2 Transforming and expression of the sulfhydrylase

These vectors carrying the sulfhydrylases genes were each transformed in *Escherichia coli* BL21 (DE3) (New England Biolabs), which were subsequently cultured on LB medium agar plates with 50 mg/l kanamycin at 37 °C for 16 h. Strain BL21 carrying the vector pET26b(+) without any insert was used as negative control.

The resulting strain were named Ec BL21 pET-26b(+)_metY-Cg, Ec BL21 pET-26b(+)_metY-Cg_P2T, Ec BL21 pET-26b(+)_metZ-Cv, Ec BL21 pET-26b(+)_metZ-Hn and Ec BL21 pET-26b(+)_metZ-Hn respectively.

15 In each case a colony has been selected which was inoculated into 10 ml of LB medium with 50 mg/l kanamycin and cultured at 37°C, 250 rpm for 6 hours. 50 µl of LB medium were subsequently treated with 50 mg/l kanamycin and inoculated with 50 µl of the growth cell culture and incubated at 28 °C, 250 rpm for 16 h. This cell culture was diluted with 200 ml of fresh LB medium containing 50 µg/l kanamycin in a 2 l flask to an OD of 0.15 and was further cultured under identical conditions until an OD of 0.5 was attained (circa 4 h). The start point of the induction of gene expression was then affected by adding 200 µl of a 300 mM IPTG stock solution (final concentration 300 µM isopropyl-β-D-thiogalactopyranoside (IPTG), Sigma-Aldrich, Germany). The induction was carried out at 28 °C, 250 rpm for 4 h. The culture was then harvested (8 ml normalised to an OD = 1), the supernatant removed by centrifugation (20 min, 4000 rpm, 4 °C) and the pelleted cells were washed twice with 800 µl of 0.1 M potassium phosphate buffer (pH 7.5) and taken up in 1 ml of buffer. The mechanical cell digestion was carried out in a FastPrep FP120 instrument (QBiogene, Heidelberg), wherein the cells were shaken four times for 30 s at 6.5 m/s in digestion vessels with 300 mg of glass beads (Ø 0.2-0.3 mm). The crude extract was then centrifuged at 12000 rpm, 4 °C, 20 min, in order to remove undigested cells and cell debris. The supernatant was used for the enzyme assay. The concentration of the polypeptide in the supernatant was determined by SDS page and analysis of the respective bands via the software GelQuant® (BiochemLabSolutions).

5.3 Inventive Examples I1 to I4

35 The following assay was carried out to determine whether the respective polypetide catalyzed the reaction of the respective phosphor-containing substrate and activated L-homoserine. O-acetyl L-homoserine was used as activated L-homoserine substrate.

To 880 µl phosphate buffer (0.1 M, pH 7.5) containing 1 nmol of the polypeptide to be tested were added 100 µl of a 30 mM aqueous solution of *O*-acetyl homoserine-HCl, 10 µl of a 1 mM aqueous solution of pyridoxal 5'-phosphate mono-hydrate (1 mM), and 10 µl of a 200 mM aqueous solution of Butyl *P*-methylphosphinate (CAS-No. 6172-80-1; "MPBE"). The reaction was conducted for 120 min at 50 °C. Then, 100 µl batch solution was diluted in 100 µl methanol and applied on the LC-MS QQQ (item 5.7) to analyse the LGA-Bu.

5.4 Inventive Example I5

Catalysis of the polypeptide isolated from pET-26b(+)_metY-Cg_P2T was further tested in a reaction with methyl *P*-methylphosphinate ("MPME"; CAS-No. 16391-06-3).

To 880 µl phosphate buffer (0.1 M, pH 7.5) containing 1 nmol of this polypeptide were added 100 µl of a 30 mM aqueous solution of *O*-acetyl homoserine-HCl, 10 µl of a 1 mM aqueous solution of pyridoxal 5'-phosphate mono-hydrate (1 mM), and 10 µl of a 200 mM aqueous solution of MPME. The reaction was conducted for 120 min at 50 °C. Then, 100 µl batch solution was diluted in 100 µl methanol and applied on the LC-MS QQQ (item 5.7) to analyse the LGA-Bu.

5.4 Comparative Examples C1 to C4

It was further investigated whether other phosphor compounds would serve as substrates for the above reaction. To this end, diethyl methylphosphonite („DEMP“; CAS-No. 15715-41-0) was tested.

To 880 µl phosphate buffer (0.1 M, pH 7.5) containing 1 nmol of the polypeptide to be tested were added 100 µl of a 30 mM aqueous solution of *O*-acetyl homoserine-HCl, 10 µl of a 1 mM aqueous solution of pyridoxal 5'-phosphate mono-hydrate (1 mM), and 10 µl of a 200 mM aqueous solution of DEMP. The reaction was conducted for 120 min at 50 °C. Then, 100 µl batch solution was diluted in 100 µl methanol and applied on the LC-MS QQQ (item 5.7) to analyse the LGA-Bu.

5.5 Comparative Examples C5 to C8

It was further investigated whether the resectove polypeptide would catalyze the wild type reaction of *O*-acetylhomoserine with methylmercaptane ("MM"; CAS-No. 74-93-1) according to the above reaction <1A>.

To 880 µl phosphate buffer (0.1 M, pH 7.5) containing 1 nmol of the polypeptide to be tested were added 100 µl of a 30 mM aqueous solution of *O*-acetyl homoserine-HCl, 10 µl of a 1 mM aqueous solution of pyridoxal 5'-phosphate mono-hydrate (1 mM), and 10 µl of a 200 mM aqueous solution of MM. The reaction was conducted for 120 min at 50 °C. Then, 100 µl batch solution was diluted in 100 µl methanol and applied on the LC-MS QQQ (item 5.7) to analyse for the respective product.

In case of inventive examples **I1** to **I4**, the product is the compound according to structure (III), wherein $R^1 = n$ -butyl ("LGA-Bu").

In case of inventive example **I5**, the product is the compound according to structure (III), wherein $R^1 =$ methyl.

- 5 In case of comparative examples **C1** to **C4**, the product is the compound according to structure (III), wherein $R^1 =$ ethyl ("LGA-Et").

In case of comparative examples **C5** to **C6**, the product is methionine.

The results are summarized in the following table 3. The abbreviations used are

- 10 "DEMP" = methyl diethoxy phosphine (CAS-No. 15715-41-0);
 "MPBE" = butyl P-methylphosphinate (CAS-No. 6172-80-1);
 "MPME" = methyl P-methylphosphinate (CAS-No. 16391-06-3);
 "MM" = methyl mercaptane (CAS-No: 74-93-1).

15 **Table 3**

Example	Enzyme	Polypeptide	Substrate	Product
I1	Cg_MetY	SEQ ID NO: 2	MPBE	yes
I2	Cg_MetY_P2T	SEQ ID NO: 6	MPBE	yes
I3	Cv_MetZ	SEQ ID NO: 10	MPBE	yes
I4	Hn_MetZ	SEQ ID NO: 14	MPBE	yes
I5	Cg_MetY_P2T	SEQ ID NO: 6	MPME	yes
C1	Cg_MetY	SEQ ID NO: 2	DEMP	no
C2	Cg_MetY_P2T	SEQ ID NO: 6	DEMP	no
C3	Cv_MetZ	SEQ ID NO: 10	DEMP	no
C4	Hn_MetZ	SEQ ID NO: 14	DEMP	no
C5	Cg_MetY	SEQ ID NO: 2	MM	yes
C6	Cg_MetY_P2T	SEQ ID NO: 6	MM	yes
C7	Cv_MetZ	SEQ ID NO: 10	MM	yes
C8	Hn_MetZ	SEQ ID NO: 14	MM	yes

5.6 Results

- 20 The results summarized in table 3 surprisingly show that the tested polypeptides accept other substrates methylphosphinic acid compounds such as MPBE and MPME, and catalyze their reaction with activated L-homoserine to the respective *n*-butyl or methyl phosphate ester of LGA. This finding was even more surprising, as the skilled person would not have expected that these enzyme would catalyze these reactions, as other phosphate compounds such as DEMP do not
- 25 work as substrates.

This finding opens new enzymatic pathways to LGA and its derivatives.

5.7 Analytical Methods

All analytical measurements for the experiments have been carried out via a scan on a LC-MS QQQ system. Samples were diluted in in methanol ($v : v = 1 : 2$).

5

The applicated HPLC belongs to the 1260 Infinity-series from Agilent connected to a mass spectrometer 6420 triple quadrupole with electrospray ionization. Peak identification was carried out via retention time and molecular mass in a positive detection mode.

10 Data evaluation was carried out via peak area and a quadratic calibration without zero.

Aquisition Method Details

Ion Source ESI (electrospray ionization)

15 **Time Parameters**

Mass Range $m/z = 50-300$

Scans per second 400

Fragmentation 40 V

Polarity Positive (Scan-Mode)

20

Source Parameters

Gas temperature 350 °C

Gas flow 12 l/min

Nebulizer 50 psi

25 **Capillary** 4000 V

Binary Pump

Injection Volume 2.00 μL

30 **Flow** 0.60 mL/min

Solvent Composition

Solvent A 100 mM Ammonium acetate plus 0.1% (v/v) formic acid in H_2O

Solvent B 0.1% formic acid in acetonitrile

Gradient see table 4.

Table 4

Time (min)	Solvent A (%)	Solvent B (%)
0.5	5	95
1.2	45	55
4.0	45	55
4.1	95	5
7.0	95	5
7.1	5	95
10.0	5	95

5

Column

Type Luna HILIC; 100 x 2mm; 3 µm; Phenomenex 00D-4449-BO
Temperature 30.0 °C

10

6. Sequence Overview

The following table provides an overview of the DNA and protein sequences referred to in the context of this present application:

5 Abbreviations used are: "AHS" = O-acetylhomoserine sulphydrylase; "SHS" = O-succinylhomoserine sulphydrylase; "CGS" = cystathionine γ -synthase.

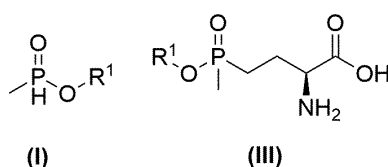
SEQ ID NO:	
1	DNA sequence of <i>metY</i> gene (nucleotide sequence comprising locus_tag NCgl0625 disclosed in NC_003450) from <i>Corynebacterium glutamicum ATCC 13032</i> , encoding AHS.
2	Protein sequence translated from SEQ ID NO: 1.
3	DNA sequence: nucleotides 7 to 1320 are identical with SEQ ID NO: 1, further containing six additional nucleotides (aaacat) at position 1 to 6, thus forming a NdeI restriction site (nucleotides 4 to 9), and ten additional nucleotides at 1321-1330 (gcggccgcaaa), forming a NotI restriction site (nucleotides 1321-1328).
4	Protein sequence translated from nucleotides 7 to 1320 of SEQ ID NO: 3; identical with SEQ ID NO: 2.
5	DNA sequence identical to SEQ ID NO: 1, except for one allelic variation [cca (for Pro) $\rightarrow$ aca (for Thr)] at nucleotides 4-6. "metY_P27"-variant of AHS
6	Protein sequence translated from SEQ ID NO: 5 (99.8 % sequence identity with SEQ ID NO: 2).
7	DNA sequence: nucleotides 7 to 1320 are identical with SEQ ID NO: 5, further containing six additional nucleotides (aaacat) at position 1 to 6, thus forming a NdeI restriction site (nucleotides 4 to 9), and ten additional nucleotides at 1321-1330 (gcggccgcaaa), forming a NotI restriction site.
8	Protein sequence translated from nucleotides 7 to 1320 of SEQ ID NO: 7; identical with SEQ ID NO: 6.
9	DNA sequence of <i>metZ</i> gene (nucleotide sequence comprising locus_tag CV_2725 disclosed in AE016825) from <i>Chromobacterium violaceum ATCC 12472</i> , encoding SHS.

10	Protein sequence translated from SEQ ID NO: 9.
11	DNA sequence: nucleotides 7 to 1191 are identical with SEQ ID NO: 9, further containing six additional nucleotides (aaacat) at position 1 to 6, thus forming a NdeI restriction site (nucleotides 4 to 9), and nine additional nucleotides at positions 1192-1200 (ctcgagaaa), forming a XhoI restriction site (nucleotides 1192-1197).
12	Protein sequence translated from nucleotides 7 to 1191 of SEQ ID NO: 9; identical with SEQ ID NO: 10.
13	DNA sequence of <i>metZ</i> gene (nucleotide sequence comprising locus_tag HNE_2672 disclosed in CP000158) from <i>Hyphomonas neptunium</i> ATCC 15444, encoding SHS.
14	Protein sequence translated from SEQ ID NO: 13.
15	DNA sequence: nucleotides 46 to 1245 are identical with SEQ ID NO: 13, further containing 45 additional nucleotides (aaatctagaa ataatttgt ttaactttaa gaaggagata tacat) at position 1 to 45, thus forming a XbaI restriction site (nucleotides 4 to 9), and nine additional nucleotides at positions 1246-1254 (ctcgagaaa), forming a XhoI restriction site (nucleotides 1246-1251).
16	Protein sequence translated from nucleotides 46 to 1245 of SEQ ID NO: 13; identical with SEQ ID NO: 14.
17	Protein sequence of <i>metB</i> -gene from <i>Escherichia coli</i> K-12, encoding CGS.
18	Protein sequence of <i>metZ</i> -gene from <i>Pseudomonas aeruginosa</i> PAO1, encoding SHS.
19	Protein sequence of <i>metY</i> -gene from <i>Pseudomonas aeruginosa</i> PAO1, encoding AHS.
20	Protein sequence of <i>metZ</i> -gene from <i>Pseudomonas putida</i> KT2440, encoding SHS.
21	Protein sequence of <i>metB</i> -gene from <i>Corynebacterium glutamicum</i> ATCC 13032, encoding CGS.
22	Protein sequence of <i>met17</i> gene from <i>Leptospira interrogans</i> serovar <i>Lai str. 56601</i> , encoding AHS.
23	Protein sequence of <i>Met17</i> gene from <i>Saccharomyces cerevisiae</i> S288c, encoding AHS.
24	Protein sequence of <i>metZ</i> -gene from <i>Nocardia farcinica</i> IFM 10152, encoding SHS.
25	Protein sequence of <i>metZ</i> -gene from <i>Bradyrhizobium japonicum</i> USDA 6, encoding SHS.
26	Protein sequence of <i>metZ</i> -gene from <i>Methylococcus capsulatus str. Bath</i> , encoding SHS.
27	Protein sequence from <i>Methylobacillus flagellatus</i> KT, encoding an SHS, therefore abbreviated herein as "metZ".

28	Protein sequence of <i>metZ</i> -gene from <i>Nitrosomonas europaea</i> ATCC 19718, encoding SHS.
29	Protein sequence of <i>metB</i> -gene from <i>Klebsiella pneumoniae</i> subsp. <i>pneumoniae</i> MGH 78578, encoding CGS.
30	Protein sequence of <i>metI</i> -gene from <i>Bacillus subtilis</i> subsp. <i>subtilis</i> str. 168, encoding CGS.
31	Protein sequence of <i>metB</i> -gene from <i>Shigella flexneri</i> 2a str. 2457T, encoding CGS.
32	Protein sequence of <i>metB</i> -gene from <i>Colwellia psychrerythraea</i> 34H, encoding CGS.
33	Protein sequence of <i>metB</i> -gene from <i>Salmonella enterica</i> serovar <i>Paratyphi A</i> ATCC 9150, encoding CGS.
34	Protein sequence of <i>metZ</i> -gene from <i>Rhodobacter sphaeroides</i> ATCC 17029, encoding SHS.

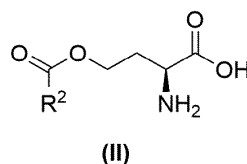
Claims

1. An enzymatically catalyzed method for producing L-glufosinate or a phosphoester thereof, comprising a step (a) in which an activated L-homoserine **H_A** is reacted with a substrate **S** of the following structure (I) to produce a compound of the following structure (III),



wherein R¹ is selected from hydrogen, alkyl, alkenyl, alkynyl, hydroxyalkyl, aryl,

- 10 and wherein the activated L-homoserine **H_A** has the following structure (II):



wherein R² is a hydrocarbon group with 1 to 15 carbon atoms which optionally comprises at least one functional group selected from OH, COOH, NH,

- 15 and wherein the reaction in step (a) is catalyzed by at least one enzyme selected from the group consisting of a sulfhydrylase **E₁**, a cystathionine γ-synthase **E₂**.

2. The method according to claim 1, wherein the activated L-homoserine **H_A** is selected from O-succinyl-L-homoserine, O-acetyl-L-homoserine.

3. The method according to claim 2, wherein the activated L-homoserine **H_A** is O-acetyl-L-homoserine.

- 25 4. The method according to one of claims 1 to 3, wherein R¹ is selected from hydrogen, alkyl.

5. The method according to claim 4, wherein the alkyl group is selected from methyl, ethyl, *n*-butyl.

6. The method according to one of claims 1 to 3, wherein R¹ is selected from alkyl, alkenyl, alkynyl, hydroxyalkyl, aryl, further comprising a step (b) wherein the compound of the structure (III) which is obtained in step (a) is saponified to give L-glufosinate.

7. The method according to one of claims 1 to 6, wherein the reaction in step (a) is catalyzed by a sulfhydrylase **E**₁.
- 5 8. The method according to claim 7, wherein the sulfhydrylase **E**₁ is an *O*-acetyl homoserine sulfhydrylase or an *O*-succinyl homoserine sulfhydrylase.
9. The method according to one of claims 1 to 8, wherein the polypeptide sequence of the sulfhydrylase enzyme **E**₁ is selected from the group consisting of
- 10 SEQ ID NO: 2 and variants of SEQ ID NO: 2, SEQ ID NO: 6 and variants of SEQ ID NO: 6, SEQ ID NO: 19 and variants of SEQ ID NO: 19, SEQ ID NO: 22 and variants of SEQ ID NO: 22, SEQ ID NO: 23 and variants of SEQ ID NO: 23, SEQ ID NO: 10 and variants of SEQ ID NO: 10, SEQ ID NO: 14 and variants of SEQ ID NO: 14, SEQ ID NO: 18 and variants of SEQ ID NO: 18, SEQ ID NO: 20 and variants of SEQ ID NO: 20,
- 15 SEQ ID NO: 24 and variants of SEQ ID NO: 24, SEQ ID NO: 25 and variants of SEQ ID NO: 25, SEQ ID NO: 26 and variants of SEQ ID NO: 26, SEQ ID NO: 27 and variants of SEQ ID NO: 27, SEQ ID NO: 28 and variants of SEQ ID NO: 28, SEQ ID NO: 34 and variants of SEQ ID NO: 34, and wherein the polypeptide sequence of the cystathionine γ -synthase **E**₂ is selected from the group consisting of SEQ ID NO: 17 and variants of SEQ ID NO: 17, SEQ ID NO: 21 and variants of
- 20 SEQ ID NO: 21, SEQ ID NO: 29 and variants of SEQ ID NO: 29, SEQ ID NO: 30 and variants of SEQ ID NO: 30, SEQ ID NO: 31 and variants of SEQ ID NO: 31, SEQ ID NO: 32 and variants of SEQ ID NO: 32, SEQ ID NO: 33 and variants of SEQ ID NO: 33, wherein variants are defined as polypeptide sequences with at least 80 % sequence identity to the respective polypeptide sequence.
- 25 10. The method according to one of claims 1 to 9, wherein the activated L-homoserine **H**_A is prepared by fermentation of a strain producing activated L-homoserine **H**_A.
11. The method according to claim 10, wherein the strain producing activated L-homoserine **H**_A is
- 30 selected from the group consisting of *Escherichia sp.*, *Erwinia sp.*, *Serratia sp.*, *Providencia sp.*, *Corynebacterium sp.*, *Pseudomonas sp.*, *Leptospira sp.*, *Salmonella sp.*, *Brevibacterium sp.*, *Hypomononas sp.*, *Chromobacterium sp.* and *Norcardia sp.*, fungi.