

METHOD FOR PRODUCING L-METHIONINE

5 **[0001]** The present invention relates to a process for the production of L-Methionine by enzymatic reaction between an L-Methionine precursor, dimethyl disulfide (DMDS) and an organic reducing compound. It also relates to a two-step process for the production of L-Methionine by enzymatic reaction between an L-Methionine precursor and methyl mercaptan, the latter being obtained by enzymatic
10 hydrogenolysis of DMDS.

[0002] Methionine is one of the human body's essential amino acids and is widely used as an additive for animal feed. It is also used as a starting material for pharmaceutical products. Methionine acts as a precursor for compounds such as choline (lecithin) and creatine. It is also a synthesis starting material for cysteine and
15 taurine.

[0003] S-Adenosyl-L-Methionine (SAM) is a derivative of L-Methionine and is involved in the synthesis of various neurotransmitters in the brain. L-Methionine and/or SAM inhibit(s) the accumulation of lipids in the body and improves blood circulation in the brain, heart and kidneys. L-Methionine may also be used to aid
20 digestion, detoxification and excretion of toxic substances or heavy metals such as lead. It has an anti-inflammatory effect on bones and joint diseases and is also an essential nutrient for the hair, thereby preventing the premature undesired loss thereof.

[0004] Methionine is already known to be prepared industrially by chemical routes
25 from petrochemical-derived starting materials, as described for example in the documents FR2903690, WO2008006977, US2009318715, US5990349, JP19660043158 and WO9408957. Aside from the fact that these preparation processes do not fall within a process of sustainable development, these chemical routes have the drawback of producing an equal mixture of the two L and D
30 enantiomers.

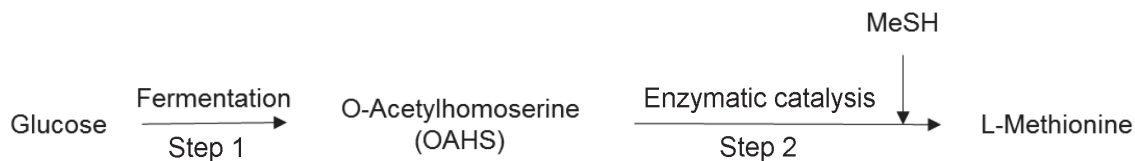
[0005] Completely biological syntheses by bacterial fermentation have been proposed in the literature, with the advantage of only producing the L enantiomer of

methionine, as described for example in international applications WO07077041, WO09043372, WO10020290 and WO10020681. Nonetheless, the absence of large-scale industrial implementation to date leads to the assumption that the performance and/or cost price of these processes remain unsatisfactory.

5 **[0006]** Mixed chemical/biological processes have recently been successfully industrialized jointly by the company CJ Cheil-Jedang and the applicant, in which an L-methionine precursor is produced by bacterial fermentation and then reacts enzymatically with methyl mercaptan to produce L-methionine exclusively (cf. WO2008013432 and/or WO2013029690). While these processes have high levels of
10 performance, they require the on-site synthesis of methyl mercaptan, which in turn requires the synthesis of hydrogen by steam methane reforming, the synthesis of hydrogen sulfide by hydrogenation of sulfur and the synthesis thereof from methanol and hydrogen sulfide; that is to say, very large equipment which is not very compatible with industrial extrapolation on a more modest scale in terms of annual
15 production than that which already exists.

[0007] There therefore remains a need to produce L-methionine by a mixed process in which the equipment required for the synthesis of methyl mercaptan is smaller than for a synthesis starting from hydrogen, hydrogen sulfide and methanol. It is within this perspective that the present invention comes.

20 **[0008]** Indeed, the present invention proposes replacing methyl mercaptan in the process summarized below (WO2008013432 and/or WO2013029690) with dimethyl disulfide (DMDS):



25 **[0009]** Here, methyl mercaptan (MeSH) is used directly in the second step. The present invention proposes substituting methyl mercaptan with the product of the enzymatic hydrogenolysis of dimethyl disulfide in a prior step or combining everything in a "one pot" reaction, in which glucose and DMDS produce L-méthionine.

[0010] The following elements can be found in the prior art in relation to the synthesis of methyl mercaptan from dimethyl disulfide.

[0011] Patent application EP0649837 proposes a process for the synthesis of methyl mercaptan by catalytic hydrogenolysis, with transition metal sulfides, of dimethyl disulfide with hydrogen. Although this process is efficient, it requires relatively high temperatures of the order of 200°C to obtain industrially advantageous levels of productivity.

[0012] Those skilled in the art also know that it is possible to prepare methyl mercaptan by acidification of an aqueous solution of sodium methyl mercaptide (CH_3SNa). This method has the major drawback of producing large amounts of salts, such as sodium chloride or sodium sulfate, depending on whether hydrochloric acid or sulfuric acid is used. These saline aqueous solutions are often very difficult to treat and the traces of foul-smelling products which remain mean that this method cannot be readily envisaged on the industrial scale.

[0013] It has now been found that it is possible to prepare methyl mercaptan by enzymatic reduction of dimethyl disulfide (DMDS) during a step prior to the synthesis of the L-methionine and it has also been found, surprisingly, that it is possible to carry out this enzymatic reduction of DMDS during the synthesis of the L-methionine.

[0014] Thus, a subject-matter of the present invention is a process for the preparation of L-methionine similar to that proposed in international applications WO2008013432 and/or WO2013029690 and which makes it possible to dispense with, or at the very least to reduce, the handling of methyl mercaptan, by generating said methyl mercaptan in a reaction for the enzymatic catalysis of DMDS, just before the use of said methyl mercaptan in the synthesis of methionine, or by generating said methyl mercaptan in a reaction for the enzymatic catalysis of DMDS in situ in the reactor for the synthesis of L-methionine.

[0015] More particularly, a first subject-matter of the present invention is the process for the preparation of L-methionine, comprising at least the steps of:

a) preparation of a mixture comprising:

1) dimethyl disulfide (DMDS),

2) a catalytic amount of amino acid bearing a thiol group or of a thiol-

group-containing peptide,

3) a catalytic amount of enzyme catalyzing the reduction reaction of the disulfide bridge of said amino acid bearing a thiol group or of said thiol-group-containing peptide,

4) an organic reducing compound in a stoichiometric amount relative to the disulfide, in particular the DMDS,

5) a catalytic amount of enzyme catalyzing the reaction for the dehydrogenation of the organic reducing compound in question,

6) a catalytic amount of a cofactor common to the two enzymes of the catalytic system (dehydrogenase and reductase),

b) carrying out the enzymatic reaction to form the methyl mercaptan ($\text{CH}_3\text{-SH}$),

c) addition of a precursor of L-methionine and conversion of said precursor with the methyl mercaptan formed in step b), and

d) recovery and optional purification of the L methionine formed.

[0016] The components of step a) above may be added in different orders (the order of addition in step a) is not restrictive). In one embodiment of the invention, the amino acid bearing a thiol group and/or the peptide bearing a thiol group may be in the form of the disulfide of said amino acid and/or of said peptide, respectively, for example glutathione in the form of glutathione disulfide.

[0017] Generally speaking, the enzyme catalyzing the reduction of the disulfide bridge created between two equivalents of said amino acid bearing a thiol group or of said thiol-group-containing peptide is a reductase enzyme. The term “reductase” is used in the remainder of the description for the explanation of the present invention. Similarly, the enzyme catalyzing the dehydrogenation of the organic reducing compound involved in step b) is generally referred to as a dehydrogenase enzyme, the term “dehydrogenase” being chosen in the remainder of the description for the explanation of the present invention.

[0018] Among the cofactors common to the two enzymes catalyzing the reduction and the dehydrogenation (reductase and dehydrogenase), mention may be made, by way of non-limiting examples, of flavinic cofactors and nicotinic cofactors. Preference is given to using nicotinic cofactors and more particularly nicotinamide adenine dinucleotide (NAD), or better still nicotinamide adenine dinucleotide phosphate

(NADPH). The cofactors listed above are advantageously used in their reduced forms (for example NADPH, H⁺) and/or their oxidized forms (for example NADP⁺), that is to say that they may be added in the reduced and/or oxidized forms into the reaction medium.

5 **[0019]** The organisation and the order of the additions of the components 1) to 6) in step a) may be carried out in different ways. The enzymatic reaction of step b) is triggered by the addition of one of the components of the catalytic system of the mixture of step a): either an enzyme, or one of the compounds added in a stoichiometric amount (disulfide or organic reducing compound), or one of the
10 compounds added in a catalytic amount (amino acid bearing a thiol group or thiol-group-containing peptide or disulfide corresponding to said thiol or to said peptide or else the cofactor).

[0020] Thus, and according to one embodiment of the present invention, the process for the preparation of L-methionine comprises at least the steps of:

15 a') preparation of a mixture comprising:

- dimethyl disulfide (DMDs),
- a catalytic amount of amino acid bearing a thiol group or of a thiol-group-containing peptide,
- a catalytic amount of reductase enzyme corresponding to said amino acid
20 bearing a thiol group or to said thiol-group-containing peptide,
- a catalytic amount of NADPH,

b') addition of an organic reducing compound in a stoichiometric amount relative to the dimethyl disulfide) with a catalytic amount of the corresponding dehydrogenase enzyme,

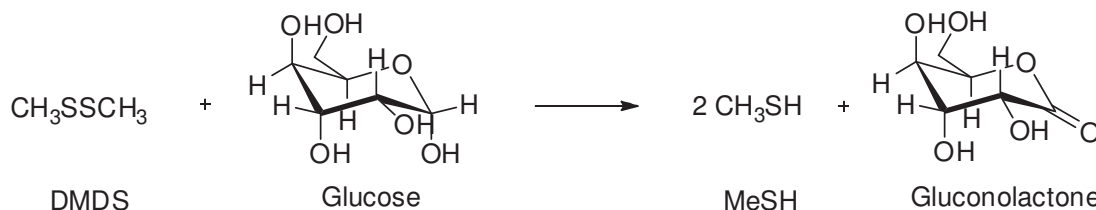
25 c') carrying out the enzymatic reaction to form methyl mercaptan (CH₃-SH),

d') conversion of an L-methionine precursor with the methyl mercaptan formed in step c'), and

e') recovery and optionally purification of the L-methionine formed.

[0021] According to the process of the invention, the methyl mercaptan, generally
30 formed in the gaseous state, is then directly placed in contact with a methionine precursor as described below.

[0022] The process for the synthesis of L-methionine according to the invention is first and foremost based on the enzymatic reduction of dimethyl disulfide with an organic reducing compound, which is a hydrogen donor as will be defined below, according to the following reaction, using glucose as organic reducing compound (hydrogen donor):



[0023] It has now been discovered that this reaction is readily catalyzed by the enzymatic system employing a thiol-group-containing amino acid or a thiol-group-containing peptide, for example glutathione, in the form of an (amino acid or peptide)/corresponding reductase enzyme complex, regenerated by the hydrogen-donating organic compound, as described in the appended figure 1.

[0024] Thus, according to the illustration in figure 1, the peptide (“glutathione” represented) reduces the disulfide (“DMDS” represented) to mercaptan (“methyl mercaptan” represented) by converting into a peptide with a disulfide bridge (“glutathione disulfide” represented). The reductase enzyme (“glutathione reductase” represented, EC 1.8.1.7 or EC 1.6.4.2) regenerates the peptide (glutathione) while oxidizing the cofactor (“NADPH, H⁺” represented). The oxidized form (“NADP⁺” represented) is then reduced by means of a “recycling” redox enzymatic complex well known to those skilled in the art and comprising the dehydrogenase enzyme involved (“glucose dehydrogenase” represented with the example of enzyme classification number EC 1.1.1.47) and the organic reducing molecule (“glucose” represented). The oxidized form of the organic reducing compound is then obtained (“gluconolactone” represented).

[0025] More particularly, the peptide (the example represented being glutathione) reduces the dimethyl disulfide to methyl mercaptan by converting into a peptide with a disulfide bridge (glutathione disulfide represented). The reductase enzyme (glutathione reductase represented, EC 1.8.1.7 or EC 1.6.4.2) regenerates the peptide (glutathione) and this same enzyme is regenerated by a redox enzymatic complex well known to those skilled in the art, for example the NADPH/NADP⁺

(nicotinamide adenine dinucleotide phosphate (reduced form and oxidized form)) complex. NADP⁺ is in turn regenerated to NADPH by means of the dehydrogenase enzyme corresponding to the organic reducing compound used (here, glucose dehydrogenase, EC 1.1.1.47) by virtue of said organic reducing compound (glucose represented) which provides hydrogen (hydrogen donor) by converting to its oxidized form (here, gluconolactone).

[0026] According to a most particularly suited embodiment, the glutathione/glutathione disulfide system combined with the glutathione reductase enzyme makes it possible according to the present invention to reduced the DMDS to methyl mercaptan.

[0027] Glutathione is a tripeptide widely used in biology. In reduced form (glutathione) or oxidized form (glutathione disulfide), this species forms an important redox couple in cells. Thus, glutathione is vital for eliminating heavy metals from organisms. Thus, for example, application WO05107723 describes a formulation in which glutathione is used to form a chelating preparation and patent US4657856 teaches that glutathione also makes it possible to break down peroxides such as H₂O₂ into H₂O via glutathione peroxidase. Finally, glutathione also makes it possible to reduce disulfide bridges present in proteins (Rona Chandrawati, "Triggered Cargo Release by Encapsulated Enzymatic Catalysis in Capsosomes", *Nano Lett.*, (2011), vol. 11, 4958-4963).

[0028] According to the process of the invention, a catalytic amount of amino acid bearing a thiol group or of a thiol-group-containing peptide is used to produce methyl mercaptan from dimethyl disulfide.

[0029] Among the amino acids bearing a thiol group which may be used in the process of the present invention, mention may be made by way of nonlimiting examples of cysteine and homocysteine. In these cases, the redox enzymatic systems used which can regenerate the catalytic cycle in the same way as the system cysteine/cystine reductase EC 1.8.1.6 and homocysteine/homocysteine reductase.

[0030] It may be advantageous to use homocysteine since this amino acid can be prepared from OAHS (L-methionine precursor), hydrogen sulfide (H₂S) and the methionine enzyme, that is to say the enzyme catalyzing the reaction leading to

methionine. Thus, a very small amount of H₂S in the reaction medium creates, *in situ*, the equivalent cycle to that of glutathione.

5 **[0031]** Among the peptides bearing a thiol group which may be used in the process of the present invention, mention may be made by way of nonlimiting examples of glutathione and thioredoxin. The glutathione/glutathione reductase system described above may thus be replaced by the thioredoxin (CAS No. 52500-60-4)/thioredoxin reductase (EC 1.8.1.9 or EC 1.6.4.5) system.

10 **[0032]** Glutathione and the glutathione/glutathione reductase system are most particularly preferred for the present invention, due to the costs of these compounds and the ease with which they are procured.

15 **[0033]** Among the organic reducing compounds which may be used within the context of the present invention, hydrogen-donating compounds are most particularly preferred, and among these, the entirely suitable compounds are hydrogen-donating organic reducing compounds bearing a hydroxyl function, such as alcohols, polyols, sugars, etc.

20 **[0034]** The enzyme used is an enzyme able to dehydrogenate the hydrogen-bearing compound, for example an alcohol dehydrogenase. Glucose is a most particularly well-suited sugar to be used in the process of the present invention with glucose dehydrogenase to give gluconolactone.

25 **[0035]** In the process according to the invention, in the case in which the enzymatic reduction of the DMDS is carried out in a separate reactor to the synthesis of the L-Methionine, only the glucose is used in a stoichiometric amount and all the other components (glutathione, cofactor (for example NADPH) and the two enzymes) are used in catalytic amounts. In the case in which the enzymatic reduction reaction of the DMDS is carried out with the synthesis of the L-methionine in a single reactor ("one pot"), the L-methionine precursor is also added in a stoichiometric amount, while the supplemental reagents for this synthesis such as pyridoxal phosphate (PLP) and the enzyme specific to this reaction are added in catalytic amounts.

30 **[0036]** The preferred concentrations of pyridoxal phosphate and of enzyme specific to the precursor are those that can be found in international applications WO2008013432 and/or WO2013029690.

[0037] The advantages brought about by the synthesis of methyl mercaptan from dimethyl disulfide by enzymatic catalysis are numerous, whether in the case of two successive steps or of the "one pot" process. Among these advantages, mention may be made of the possibility of working in aqueous or aqueous-organic solution, under
5 very mild temperature and pressure conditions and under pH conditions close to neutrality. All these conditions are typical of a "green" or "sustainable" process, and are entirely compatible with the preparation of L-methionine as described in international applications WO2008013432 and/or WO2013029690.

[0038] Another advantage when the process uses dimethyl disulfide is that the
10 methyl mercaptan produced, which is in the gaseous state under the reaction conditions, leaves the reaction medium as it is formed. The methyl mercaptan may therefore be directly used, upon leaving the reactor, in the synthesis of the L-methionine, as described for example in WO2008013432 and/or WO2013029690, that is to say for example from O-acetylhomoserine or O-succinylhomoserine and
15 enzymes such as O-acetylhomoserine sulfhydrylase or O-succinylhomoserine sulfhydrylase, respectively.

[0039] The methyl mercaptan can also be readily liquefied cryogenically for example, if it is desired to isolate it. It is optionally possible to accelerate its departure from the reaction medium by introducing a low flow rate of inert gas, advantageously
20 nitrogen, by bubbling.

[0040] The outlet gases containing nitrogen and methyl mercaptan may, if desired and if necessary, be recycled into the first reactor (enzymatic reduction of DMDS) after passing into the second reactor (L-methionine synthesis) if the methyl mercaptan has not been completely converted to L-methionine. The process
25 according to the invention therefore describes a process for the synthesis of L-methionine in two successive enzymatic steps from an L-methionine precursor and DMDS.

[0041] It is also possible to carry out the synthesis of L-methionine in one and the same reactor. In this case, all the reagents necessary for the synthesis of the L-
30 methionine are added to the system for the enzymatic reduction of the DMDS (step a) above) and the reactor is closed to avoid loss of the methyl mercaptan formed by *in situ* enzymatic reduction of the DMDS. The methyl mercaptan then reacts with the

L-methionine precursor to give L-methionine. The process according to the present invention therefore describes a process for the direct synthesis of L-methionine from an L-methionine precursor and DMDS, as illustrated by the appended figure 2, or the synthesis from OAHS, DMDS and glucose.

5 **[0042]** The dimethyl disulfide (DMDS) may be produced at another site from methyl mercaptan and an oxidizer such as oxygen, sulfur or aqueous hydrogen peroxide solution, for example, or else from dimethyl sulfate and sodium disulfide. The DMDS may also originate from a source of disulfide oils (DSO), purified for example by reactive distillation as described in application WO2014033399.

10 **[0043]** The reduction of DMDS by enzymatic catalysis may be considered as a process which makes it possible to avoid transporting methyl mercaptan from its site of production by existing industrial routes, to its site of use, if they are different. Indeed, methyl mercaptan is a toxic and extremely foul-smelling gas at room temperature, which significantly complicates its transportation, which is already
15 heavily regulated unlike DMDS. Thus, DMDS can therefore be used to produce methyl mercaptan directly on the site of use of the latter in the synthesis of L-methionine, thereby further reducing the drawbacks linked to the toxicity and odour of this product, and also the industrial risks linked to it.

20 **[0044]** In the case of the synthesis process in two successive steps, since the DMDS is consumed in the reaction and the methyl mercaptan leaves the reaction medium as it is formed, only the product of the dehydrogenation of the organic reducing compound, for example gluconolactone, accumulates in the reaction medium, if it is assumed that glucose and DMDS are fed continuously. When the gluconolactone concentration exceeds the saturation point under the reaction
25 conditions, it will precipitate out and may then be isolated from the reaction medium by any means known to those skilled in the art.

30 **[0045]** Gluconolactone may have several uses. It is for example used as a food additive, known by the reference E575. Gluconolactone is hydrolysed in acidic aqueous media to form gluconic acid, also used as a food additive (E574). Gluconolactone is also used for the production of tofu (cf. CN103053703) for the food industry.

[0046] Especially and advantageously, in the sense that it represents the "waste" from the process according to the present invention, gluconolactone may replace glucose in a possible fermentation reaction to produce either bioethanol or any other molecule originating from the fermentation of sugar or starch.

5 **[0047]** Indeed, certain bacteria may use gluconolactone as carbon source in fermentation, as described by J. P. van Dijken, "*Novel pathway for alcoholic fermentation of gluconolactone in the yeast *Saccharomyces bulderi**", *J. Bacteriol.*, (2002), Vol. 184(3), 672-678.

[0048] An obvious benefit of gluconolactone in the process according to the
10 invention is to recycle it into the synthesis of the L-methionine precursor. Indeed, since this synthesis is a bacterial fermentation using glucose, gluconolactone could easily replace a portion of this glucose. Under these conditions, this recycling may represent a very significant economic advantage.

[0049] Even in the case in which the reaction is carried out under the "one pot"
15 conditions defined above, and with gluconolactone being much more soluble than L-methionine, it is easy to separate it from the reaction medium using conventional techniques well known to those skilled in the art.

[0050] Yet other sugars may be used in the process of the invention, and for example it is possible to replace the glucose/gluconolactone/glucose dehydrogenase
20 system with the following system: glucose 6-phosphate/6-phosphoglucono- δ -lactone/glucose 6-phosphate dehydrogenase (EC 1.1.1.49).

[0051] It is also possible, in the process of the invention, to use an alcohol in place of the sugar, and thus to use the following general system instead of the glucose/gluconolactone/glucose dehydrogenase system: alcohol/ketone or
25 aldehyde/alcohol dehydrogenase (EC 1.1.1) and more particularly the isopropanol/acetone/isopropanol dehydrogenase system (EC 1.1.1.80).

[0052] Indeed, this system makes it possible to obtain, from DMDS and isopropanol, a mixture consisting of methyl mercaptan (MeSH) and acetone which leaves the reaction medium (therefore no accumulation of any product). The MeSH and the
30 acetone may be easily separated by simple distillation if desired.

[0053] According to one embodiment, the process according to the invention comprises the preparation by enzymatic reduction of DMDS, then reaction of the

methyl mercaptan formed with an L-methionine precursor to give L-methionine. In this case, the process according to the invention comprises at least the following steps:

Step 1: preparation of an L-methionine precursor, for example by bacterial fermentation of glucose (cf. WO2008013432 and/or WO2013029690),

5 Step 2: enzymatic reduction of DMDS in a reactor R1 with formation of methyl mercaptan leaving said reactor R1 (corresponding to steps a') to c') above),

Step 3: enzymatic synthesis of L-methionine in a reactor R2 with the precursor from step 1 and the methyl mercaptan from step 2 (corresponding to step d') above),

Step 4 (optional): recycling of the gluconolactone formed in step 3 into step 1,

10 Step 5: recovery and optionally purification of the L-methionine formed (corresponding to step e') above).

[0054] For step 1, the range of conditions which can be used will be found in the following patents (cf. WO2008013432 and/or WO2013029690).

15 **[0055]** For step 2, the reaction temperature is within a range extending from 10°C to 50°C, preferably between 15°C and 45°C, more preferably between 20°C and 40°C.

[0056] The pH of the reaction may be between 6 and 8, preferably between 6.5 and 7.5. The pH of the reaction medium may be adjusted by means of a buffer. Entirely preferably, for example, the pH of the 0.1 mol.l⁻¹ phosphate buffer will be chosen to be 7.3.

20 **[0057]** The pressure used for the reaction may range from a reduced pressure compared to atmospheric pressure to several bar (several hundred kPa), depending on the reagents and equipment used. A reduced pressure may indeed enable quicker degassing of the methyl mercaptan formed, but has the drawback of increasing the saturated vapour pressures of the water and the DMDS, polluting the
25 methyl mercaptan formed slightly more. Preferably, use will be made of a pressure ranging from atmospheric pressure to 20 bar (2MPa) and even more preferably the process will be carried out under a pressure ranging from atmospheric pressure to 3 bar (300 kPa).

30 **[0058]** For step 3, reference will be made to international application WO2013029690 for the ideal conditions, with the possible difference of introducing a flow of nitrogen into the reactor R1 to pass into the reactor R2 and recycling these

gases from the reactor R2 to the reactor R1 at the desired pressure if the methyl mercaptan has not entirely reacted in the reactor R2.

[0059] According to another embodiment (another variant), the process according to the present invention is carried out in one and the same reactor ("one pot") and in this case comprises at least the following steps:

Step 1': preparation of an L-methionine precursor by bacterial fermentation of glucose for example (similar to step 1 above),

Step 2': enzymatic reduction of DMDS in a reactor R1 with *in situ* formation of methyl mercaptan and concomitant enzymatic synthesis of L-methionine in the same reactor with the precursor obtained in step 1',

Step 3' (optional): recycling of the gluconolactone formed in step 2' into step 1', and

Step 4': recovery and optionally purification of the L-methionine formed.

[0060] For step 1', the range of conditions which can be used will be found in the international applications WO2008013432 and/or WO2013029690.

[0061] For step 2', the operating conditions are as follows.

[0062] The reaction temperature is within a range extending from 10°C to 50°C, preferably from 15°C to 45°C, more preferably from 20°C to 40°C.

[0063] The pH of the reaction is advantageously between 6 and 8, preferably between 6.2 and 7.5. Entirely preferably, the reaction is carried out at the pH of the 0.2 mol.l⁻¹ phosphate buffer and equal to 7.0.

[0064] Preferably, the process is carried out at a pressure ranging from atmospheric pressure to 20 bar (2 MPa) and even more preferably from atmospheric pressure to 3 bar (300 kPa).

[0065] The DMDS/L-methionine precursor molar ratio is between 0.1 and 10, generally between 0.5 and 5, and preferably the molar ratio is stoichiometry (molar ratio = 0.5) but may be higher if this proves beneficial to the reaction kinetics.

[0066] In one or the other of the variants of the process according to the invention, the process can be carried out batchwise or continuously, in a glass or metal reactor depending on the operating conditions selected and the reagents used.

[0067] In one or the other of the variants of the process according to the invention, the ideal organic reducing compound/DMDS molar ratio is stoichiometry (molar ratio = 1) but may vary from 0.01 to 100, if those skilled in the art find any benefit therein,

such as continuous addition of DMDS while the reducing compound is introduced from the start into the reactor. Preferably, this molar ratio is chosen between 0.5 and 5 overall, over the whole of the reaction.

5 **[0068]** The elements present in catalytic amounts in the mixture prepared in step a) above (amino acid bearing a thiol group or a thiol-group-containing peptide or else the disulfide corresponding to said amino acid or the disulfide corresponding to said peptide, reductase enzyme, dehydrogenase enzyme, cofactor, for example NADPH) are easily available commercially or can be prepared according to techniques well known to those skilled in the art. These different elements may be in solid or liquid
10 form and may very advantageously be dissolved in water to be used in the process of the invention. The enzymes used may also be grafted onto a support (in the case of supported enzymes).

[0069] The aqueous solution of enzymatic complex comprising the amino acid or the peptide may also be reconstituted by methods known to those skilled in the art, for
15 example by permeabilization of cells which contain these elements. This aqueous solution, a composition of which is given in the following Example 1, may be used in contents by weight of between 0.01% and 20% relative to the total weight of the reaction medium. Preferably, a content of between 0.5% and 10% will be used.

[0070] The invention will be better understood with the following examples
20 nonlimiting relative to the scope of the invention.

EXAMPLE 1: Process in 2 successive steps

[0071] 10 ml of glutathione enzymatic complex (Aldrich) and 19.2 g (0.1 mol) of glucose are introduced into a reactor R1 containing 150 ml of 0.1 mol/l phosphate buffer at pH 7.30. The solution of enzymatic complex contains: 185 mg (0.6 mmol) of
25 glutathione, 200 U of glutathione reductase, 50 mg (0.06 mmol) of NADPH and 200 U of glucose dehydrogenase. The reaction medium is brought to 25°C with mechanical stirring. A first sample is taken at $t = 0$. Subsequently, the dimethyl disulfide (9.4 g, 0.1 mol) is placed in a burette and added dropwise to the reactor; the reaction begins. A stream of nitrogen is placed in the reactor.

30 **[0072]** Gas chromatography analysis of the gases leaving the reactor shows virtually essentially the presence of nitrogen and methyl mercaptan (some traces of water). These outlet gases are sent into the reactor R2. The DMDS is introduced in 6 hours

into the reactor R1. A final gas chromatography analysis of the reaction medium of the reactor R1 confirms the absence of DMDS, and an analysis by UPLC/mass spectrometry shows traces of glucose and the virtually exclusive presence of gluconolactone (traces of gluconic acid).

5 **[0073]** In parallel, 5 g of O-acetyl-L-homoserine (OAHS) (the O-acetylhomoserine was synthesized from L-homoserine and acetic anhydride as per Sadamu Nagai, "*Synthesis of O-acetyl-L-homoserine*", Academic Press, (1971), vol.17, pp. 423-424) are introduced into the second reactor R2 containing 75 ml of 0.1 mol.l⁻¹ phosphate buffer at pH 6.60. The solution is brought to 35°C with mechanical stirring.

10 **[0074]** Before the reaction starts, a sample (t = 0) of 1 ml of the reaction medium is taken. A solution of pyridoxal phosphate (1.6 mmol, 0.4 g) and the enzyme O-acetyl-L-homoserine sulfhydrylase (0.6 g) are dissolved in 10 ml of water then added to the reactor.

[0075] The methyl mercaptan is introduced via the reaction of the reactor R1 and propelled by a nitrogen stream. The reaction then begins. The formation of L-methionine and the disappearance of OAHS are monitored by HPLC. The outlet gases from the reactor R2 are trapped in a 20% aqueous sodium hydroxide solution. The analyses show that the OAHS has been converted to a degree of 52% into L-methionine and that the excess DMDS has been converted into methyl mercaptan found in the sodium hydroxide trap.

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EXAMPLE 2: "One pot" process

[0076] 10 ml of the enzymatic complex, 6 g (33 mmol) of glucose and 5 g (31 mmol) of O-acetyl-L-homoserine (OAHS - the O-acetyl-L-homoserine was synthesized from L-homoserine and acetic anhydride as per Sadamu Nagai, "*Synthesis of O-acetyl-L-homoserine*", Academic Press, (1971), vol.17, pp. 423-424) are introduced into a reactor containing 150 ml of 0.2 mol.l⁻¹ phosphate buffer at pH 7. The solution of the enzymatic complex contains: 185 mg (0.6 mmol) of glutathione, 200 U of glutathione reductase, 50 mg (0.06 mmol) of NADPH, 200 U of glucose dehydrogenase, 0.4 g (1.6 mmol) of pyridoxal phosphate and 0.6 g of O-acetyl-L-homoserine sulfhydrylase.

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[0077] The reaction medium is brought to 27°C with mechanical stirring. A first sample at t = 0 is taken. Subsequently, the dimethyl disulfide (3 g, 32 mmol) is placed

in a burette and added dropwise to the reactor which has been closed to avoid any release of methyl mercaptan; the reaction begins. The reaction is monitored by HPLC to see the disappearance of the OAHS and the formation of the L-methionine. After 6 hours, 21% of the OAHS has been converted into L-methionine, demonstrating the possibility of producing L-methionine by a "one pot" process from an L-methionine precursor, DMDS and an organic reducing compound.

CLAIMS

- 5 **1.** Process for the preparation of L-methionine, comprising at least the steps of:
- a) preparation of a mixture comprising:
- 1) dimethyl disulfide (DMDS),
- 2) a catalytic amount of amino acid bearing a thiol group or of a thiol-group-
- containing peptide,
- 10 3) a catalytic amount of enzyme catalyzing the reduction reaction of the
- disulfide bridge of said amino acid bearing a thiol group or of said thiol-group-
- containing peptide,
- 4) an organic reducing compound in a stoichiometric amount relative to the
- disulfide, in particular the DMDS,
- 15 5) a catalytic amount of enzyme catalyzing the reaction for the
- dehydrogenation of an organic reducing compound in question,
- 6) a catalytic amount of a cofactor common to the two enzymes of the catalytic
- system (dehydrogenase and reductase),
- b) carrying out the enzymatic reaction to form the methyl mercaptan ($\text{CH}_3\text{-SH}$),
- 20 c) addition of a precursor of L-methionine and conversion of said precursor with
- the methyl mercaptan formed in step b), and
- d) recovery and optional purification of the L-methionine formed.
- 2.** Process according to Claim 1, comprising at least the steps of:
- 25 a') preparation of a mixture comprising:
- dimethyl disulfide (DMDS),
 - a catalytic amount of amino acid bearing a thiol group or of a thiol-group-
 - containing peptide,
 - a catalytic amount of reductase enzyme corresponding to said amino acid
 - 30 bearing a thiol group or to said thiol-group-containing peptide,
 - a catalytic amount of NADPH,

- b') addition of an organic reducing compound in a stoichiometric amount relative to the dimethyl disulfide) with a catalytic amount of the corresponding dehydrogenase enzyme,
- c') carrying out the enzymatic reaction to form methyl mercaptan ($\text{CH}_3\text{-SH}$),
- 5 d') conversion of an L-methionine precursor with the methyl mercaptan formed in step c'), and
- e') recovery and optionally purification of the L-methionine formed.
3. Process according to Claim 1 or Claim 2, wherein the methyl mercaptan is
- 10 directly placed in contact with a methionine precursor.
4. Process according to any one of the preceding claims, wherein the organic reducing compound is a hydrogen-donating organic reducing compound bearing a hydroxyl function, chosen from alcohols, polyols, sugars, etc.
- 15 5. Process according to any one of the preceding claims, wherein the organic reducing compound is chosen from glucose, glucose 6-phosphate and isopropanol.
6. Process according to any one of the preceding claims, wherein the amino
- 20 acid bearing a thiol group or the peptide bearing a thiol group is chosen from cysteine, homocysteine, glutathione and thioredoxin.
7. Process according to any one of the preceding claims, wherein the L-methionine precursor is chosen from O-acetyl-L-homoserine and O-succinyl-L-
- 25 homoserine.
8. Process according to any one of the preceding claims, wherein the methyl mercaptan is directly used, upon leaving the reactor, in the synthesis of the L-methionine.
- 30 9. Process according to Claim 8, comprising at least the following steps:

Step 1: preparation of an L-methionine precursor, for example by bacterial fermentation of glucose,

Step 2: enzymatic reduction of DMDS in a reactor R1 with formation of methyl mercaptan leaving said reactor R1,

5 Step 3: enzymatic synthesis of L-methionine in a reactor R2 with the precursor from step 1 and the methyl mercaptan from step 2,

Step 4 (optional): recycling of the gluconolactone formed in step 3 into step 1,

Step 5: recovery and optionally purification of the L-methionine formed.

10 **10.** Process according to any one of Claims 1 to 7, wherein the synthesis of methyl mercaptan from DMDS and the synthesis of L-methionine from said methyl mercaptan are carried out in one and the same reactor.

11. Process according to Claim 10, comprising at least the following steps:

15 Step 1': preparation of an L-methionine precursor by bacterial fermentation of glucose,
Step 2': enzymatic reduction of DMDS in a reactor R1 with *in situ* formation of methyl mercaptan and concomitant enzymatic synthesis of L-methionine in the same reactor with the precursor obtained in step 1',

Step 3' (optional): recycling of the gluconolactone formed in step 2' into step 1', and

20 Step 4': recovery and optionally purification of the L-methionine formed.

12. Process according to any one of the preceding claims, carried out batchwise or continuously.

25 **13.** Process according to any one of the preceding claims, wherein the ideal organic reducing compound/DMDS molar ratio varies from 0.01 to 100, preferably said molar ratio is chosen between 0.5 and 5, and entirely preferably the molar ratio is equal to 1.

30 **14.** Process according to any one of the preceding claims, wherein the DMDS/L-methionine precursor molar ratio is between 0.1 and 10, generally between 0.5 and 5, and preferably the molar ratio is stoichiometry (molar ratio = 0.5).

15. Process according to any one of the preceding claims, wherein the reaction temperature is within a range extending from 10°C to 50°C, preferably from 15°C to 45°C, more preferably from 20°C to 40°C.