The present invention is related to a recombinant microorganism optimised for the fermentative production of methionine, wherein the activity of the cobalamin-independent methionine synthase MetE is attenuated in said microorganism. The invention is also related to a method for producing methionine by fermentation.
Recombinant microorganism for the fermentative production of methionine

FIELD OF THE INVENTION

The present invention relates to a recombinant microorganism for the production of methionine and to a method for producing methionine, by culturing the recombinant microorganism in an appropriate culture medium comprising a source of carbon and a source of sulphur. The microorganism is modified in a way that the methionine/carbon source yield is increased by attenuating the activity of the cobalamin-independent methionine synthase. In particular, the gene *metE* is deleted in the recombinant microorganism.

PRIOR ART

Sulphur-containing compounds such as cysteine, homocysteine, methionine or S-adenosylmethionine are critical to cellular metabolism and are produced industrially to be used as food or feed additives and pharmaceuticals. In particular methionine, an essential amino acid, which cannot be synthesized by animals, plays an important role in many body functions. Aside from its role in protein biosynthesis, methionine is involved in transmethylation and in the bioavailability of selenium and zinc. Methionine is also directly used as a treatment for disorders like allergy and rheumatic fever. Nevertheless, most of the methionine that is produced is added to animal feed. With the decreased use of animal-derived proteins as a result of BSE and chicken flu, the demand for pure methionine has increased. Commonly, D,L-methionine is produced chemically from acrolein, methyl mercaptan and hydrogen cyanide. However, the racemic mixture does not perform as well as pure L-methionine (Saunderson, 1985). Additionally, although pure L-methionine can be produced from racemic methionine, for example, through the acylase treatment of N-acetyl-D,L-methionine, this dramatically increases production costs. Accordingly, the increasing demand for pure L-methionine coupled with environmental concerns render microbial production of methionine an attractive prospect. Optimising the production of a chemical from a microorganism typically involves overexpressing proteins involved in the biosynthesis pathway, attenuating proteins involved in repression of the biosynthesis pathway or attenuating proteins involved in the production of undesirable by-products. All these approaches for the optimisation of L-methionine production in microorganisms have been described previously (see, for example, Patents or patent applications US 7,790,424, US 7,611,873, WO 2002/010209, WO 2005/059093 and WO 2006/008097); however, industrial production of L-methionine from microorganisms requires further improvements.
In *Escherichia coli* and in other microorganisms like *Corynebacterium glutamicum*, two distinct enzymes catalyze the terminal step in the *de novo* biosynthesis of methionine (Foster et al., 1961; Gonzalez et al., 1992). The cobalamin-dependent methionine synthase (MetH, EC 2.1.1.13) is encoded by the *metH* gene and contains a prosthetic group that is required for activity. The cobalamin-independent methionine synthase (MetE, EC 2.1.1.14) is encoded by the *metE* gene and has no known requirement for a vitamin-derived prosthetic group.

Numerous patents applications are related to the over-production of MetH and MetE enzymes to enhance the last step of methionine biosynthesis, as for example:

- WO2007/012078 and WO2007/1 35188 from BASF describe genetic alterations leading to overexpression of the genes *metH* and/or *metE*.
- WO2009/1 44270 from EVONIK describes a method for producing methionine with a microorganism that displays an increased amount and/or activity of a cob(I)alamin-dependent MetH reactivation system.

Inventors have found, surprisingly and unexpectedly, that an attenuation of the amount and/or activity of the cobalamin-independent methionine synthase (MetE) leads to an improved production of methionine. This is the first time that the loss of activity of one of the enzymes belonging to the methionine biosynthesis pathway is proposed as being beneficial for the methionine production.

**SUMMARY OF THE INVENTION**

The invention relates to a recombinant microorganism optimised for the production of methionine, wherein the activity of the cobalamin-independent methionine synthase MetE is attenuated. Preferably, the gene *metE* encoding the MetE enzyme is deleted or mutated. The recombinant microorganism may also comprise other genetic modifications such as:

- an increased expression of at least one of the following genes: *ptsG*, *pyc*, *pntAB*, *cysP*, *cysL*, *cysW*, *cysA*, *cysM*, *cysJ*, *cysL*, *cysH*, *gcvT*, *gcvH*, *gcvP*, *ipd*, *serA*, *serB*, *serC*, *cysE*, *metF*, *metH*, *thra*, *meta* allele encoding for an enzyme with reduced feed-back sensitivity to S-adenosylmethionine and/or methionine (*meta*), *thra*, or a *thra* allele encoding for an enzyme with reduced feed-back inhibition to threonine (*thra*) and/or
- an attenuated expression of one of the following genes: *meta*, *pykA*, *pykF*, *purU*, *ybdL* or *yncA*.

In a particular embodiment, the present invention is related to a microorganism wherein: a) the gene *metE* is deleted, and b) the expression of the genes *meta* (*meta*), *metH*, *cysPUWAM*, *cysJIH*, *gcvTHP*, *metF*, *serA*, *serB*, *serC*, *cysE*, *thra* and/or *pyc* are enhanced; and c) the expression of the genes *meta*, *pykA*, *pykF*, *purU* and *yncA* are attenuated.
The invention also relates to a method for the production of methionine or methionine derivatives in a fermentative process comprising the steps of: a) culturing the recombinant microorganism according to the invention in an appropriate culture medium comprising a fermentable source of carbon containing glucose and a source of sulphur and b) recovering methionine or methionine derivatives from the culture medium.

**DETAILED DESCRIPTION OF THE INVENTION**

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

All publications, patents and patent applications cited herein, whether supra or infra, are hereby incorporated by reference in their entirety. However, publications mentioned herein are cited for the purpose of describing and disclosing the protocols, reagents and vectors that are reported in the publications and that might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

Furthermore, the practice of the present invention employs, unless otherwise indicated, conventional microbiological and molecular biological techniques within the skill of the art. Such techniques are well known to the skilled worker, and are explained fully in the literature. See, for example, Prescott et al. (1999) and Sambrook et al. (1989) (2001).

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

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

The term "methionine" designates the essential sulphur-containing amino-acid with chemical formula H\(_2\)CCH(NH\(_2\))CH\(_2\)CH\(_2\)SCH\(_3\) and CAS number 59-51-8 or 63-68-3 for the specific L-isomer.

"Derivatives of methionine" refers to molecules analogs to methionine which present the same chemical backbone but differ from methionine with at least one chemical group. In this invention, preferred methionine derivatives are N-acetyl methionine (NAM), S-adenosyl methionine (SAM) and hydroxy-methionine.

The term "microorganism", as used herein, refers to a bacterium, yeast or fungus which is not modified artificially. Preferentially, the microorganism is selected among Enterobacteriaceae, Bacillaceae, Streptomycetaceae and Corynebacteriaceae. More preferentially the microorganism is a species of Escherichia, Klebsiella, Pantoea, Salmonella, or Corynebacterium. Even more preferentially the microorganism is either the species Escherichia coli or Corynebacterium glutamicum.

The term "recombinant microorganism" or "genetically modified microorganism", as used herein, refers to a bacterium, yeast or fungus that is not found in nature and is genetically different from its equivalent found in nature. It means, it is modified either by introduction or by deletion or by modification of genetic elements. It can also be transformed by forcing the development and evolution of new metabolic pathways by combining directed mutagenesis and evolution under specific selection pressure (see, for example, WO 2004/076659).

A microorganism may be modified to express exogenous genes if these genes are introduced into the microorganism with all the elements allowing their expression in the host microorganism. The modification or "transformation" of microorganisms with exogenous DNA is a routine task for those skilled in the art.

A microorganism may be modified to modulate the expression level of an endogenous gene.

The term "endogenous gene" means that the gene was present in the microorganism before any genetic modification, in the wild-type strain. Endogenous genes may be overexpressed by introducing heterologous sequences in addition to, or to replace endogenous regulatory elements, or by introducing one or more supplementary copies of the gene into the chromosome or a plasmid. Endogenous genes may also be modified to modulate their expression and/or activity. For example, mutations may be introduced into the coding sequence to modify the gene product or heterologous sequences may be introduced in addition to or to replace endogenous regulatory elements. Modulation of an endogenous gene may result in the up-regulation and/or enhancement of the activity of the gene product, or alternatively, down regulate and/or lower the activity of the endogenous gene product.
Another way to modulate their expression is to exchange the endogenous promoter of a gene (e.g., wild type promoter) with a stronger or weaker promoter to up or down regulate expression of the endogenous gene. These promoters may be homologous or heterologous. It is well within the ability of the person skilled in the art to select appropriate promoters.

The term "exogenous gene" means that the gene was introduced into a microorganism, by means well known by the man skilled in the art whereas this gene is not naturally occurring in the microorganism. Exogenous genes may be integrated into the host chromosome, or be expressed extra-chromosomally by plasmids or vectors. A variety of plasmids, which differ with respect to their origin of replication and their copy number in the cell, are well known in the art. These genes may be heterologous or homologous.

The term "heterologous gene" means that the gene is derived from a species of microorganism different from the recipient microorganism that expresses it. It refers to a gene which is not naturally occurring in the microorganism.

In the present application, all genes are referenced with their common names from *E. coli*. Their nucleotidic sequences are available on the websites [http://www.ncbi.nlm.nih.gov/gene](http://www.ncbi.nlm.nih.gov/gene) or [http://www.ebi.ac.uk/emby](http://www.ebi.ac.uk/emby).

Using the references given in Genbank for known genes, those skilled in the art are able to determine the equivalent genes in other organisms, bacterial strains, yeast, fungi, mammals, plants, etc. This routine work is advantageously done using consensus sequences that can be determined by carrying out sequence alignments with genes derived from other microorganisms and designing degenerate probes to clone the corresponding gene in another organism. These routine methods of molecular biology are well known to those skilled in the art, and are claimed, for example, in Sambrook *et al.*, (1989) and (2001).

The terms "improved methionine production", "improve methionine production" and grammatical equivalents thereof, as used herein, refer to an increased methionine/carbon source yield (ratio of gram/mol methionine produced per gram/mol carbon source consumed that it can be expressed in percent). Methods for determining the amount of carbon source consumed and of methionine produced are well known to those in the art. The yield is higher in the recombinant microorganism compared to the corresponding unmodified microorganism.

The terms "microorganism optimised for the fermentative production of methionine" refers to microorganisms evolved and/or genetically modified to present an improved methionine production in comparison with the endogenous production of the corresponding wild-type microorganisms. Such microorganisms "optimised" for methionine production are well known in the art, and have been disclosed in particular in patent applications WO2005/111202, WO2007/077041 and WO2009/043803.
According to the invention the terms "fermentative production", "culture" or "fermentation" are used to denote the growth of bacteria. This growth is generally conducted in fermenters with an appropriate culture medium adapted to the microorganism being used and containing at least one simple carbon source, and if necessary co-substrates.

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

The term "carbon source" or "carbon substrate" or "source of carbon" according to the present invention denotes any source of carbon that can be used by those skilled in the art to support the normal growth of a microorganism, including monosaccharides (such as glucose, galactose, xylose, fructose or lactose), oligosaccharides, disaccharides (such as sucrose, cellobiose or maltose), molasses, starch or its derivatives, hemicelluloses and combinations thereof. An especially preferred simple carbon source is glucose. Another preferred simple carbon source is sucrose. The carbon source can be derived from renewable feed-stock. Renewable feed-stock is defined as raw material required for certain industrial processes that can be regenerated within a brief delay and in sufficient amount to permit its transformation into the desired product. Vegetal biomass, treated or not, is an interesting renewable carbon source.

The term "source of sulphur" according to the invention refers to sulphate, thiosulfate, hydrogen sulphide, dithionate, dithionite, sulphite, methylmercaptan, dimethylsulfide and other methyl capped sulphides or a combination of the different sources. More preferentially, the sulphur source in the culture medium is sulphate or thiosulfate or a mixture thereof.

The terms "source of nitrogen" corresponds to either an ammonium salt or ammoniac gas. The nitrogen source is supplied in the form of ammonium or ammoniac.

The terms "attenuation" or "expression attenuated" mean in this context that the expression of a gene or an enzyme is decreased or suppressed compared to a non modified microorganism. Decrease or suppression of the expression of an enzyme is obtained by the attenuation of the expression of gene encoding said enzyme.
Attenuation of genes may be achieved by means and methods known to the man skilled in the art. Generally, attenuation of gene expression may be achieved by:

- Mutating the coding region or the promoter region or,
- Deleting of all or a part of the promoter region necessary for the gene expression or,
- Deleting the coding region of the gene by homologous recombination or
- Inserting an external element into coding region or into promoter region or
- Expressing the gene under control of a weak promoter.

The man skilled in the art knows a variety of promoters which exhibit different strength and which promoter to use for a weak genetic expression.

The term "activity" of an enzyme is used interchangeably with the term "function" and designates, in the context of the invention, the reaction that is catalyzed by the enzyme. The man skilled in the art knows how to measure the enzymatic activity of said enzyme. In particular, for measuring the activity of the protein MetE, see example 5.

The terms "attenuated activity" or "reduced activity" of an enzyme mean either a reduced specific catalytic activity of the protein obtained by mutation in the aminoacids sequence and/or decreased concentrations of the protein in the cell obtained by mutation of the nucleotidic sequence or by deletion of the coding region of the gene.

The terms "enhanced activity" or "increased activity" of an enzyme designates either an increased specific catalytic activity of the enzyme, and/or an increased quantity/availability of the enzyme in the cell, obtained for example by overexpressing the gene encoding the enzyme.

The terms "increased expression", "enhanced expression" or "overexpression" and grammatical equivalents thereof, are used interchangeably in the text and have a similar meaning. These terms mean that the expression of a gene or an enzyme is increased compared to a non modified microorganism. Increase expression of an enzyme is obtained by increasing expression of the gene encoding said enzyme.

To increase the expression of a gene, the man skilled in the art knows different techniques:

- Increasing the number of copies of the gene in the microorganism. The gene is encoded chromosomally or extrachromosomally. When the gene is located on the chromosome, several copies of the gene can be introduced on the chromosome by methods of recombination, known to the expert in the field (including gene replacement). When the gene is located extra-chromosomally, it may be carried by different types of plasmids that differ with respect to their origin of replication and thus their copy number in the cell. These plasmids are present in the microorganism in 1 to 5 copies, or about 20 copies, or up to 500 copies, depending on the nature of the plasmid : low copy number plasmids with
tight replication (pSC101, RK2), low copy number plasmids (pACYC, pRSF1010) or high copy number plasmids (pSK bluescript II).

- Using a promoter inducing a high level of expression of the gene. The man skilled in the art knows which promoters are the most convenient, for example promoters Ftrc, Ploc, P/ac, or the lambda promoter cl are widely used. These promoters can be "inducible" by a particular compound or by specific external condition like temperature or light. These promoters may be homologous or heterologous.

- Attenuating the activity or the expression of a transcription repressor, specific or non-specific of the gene.

- Using elements stabilizing the corresponding messenger RNA (Carrier and Keasling, 1998) or elements stabilizing the protein (e.g., GST tags, GE Healthcare).

The terms "encoding" or "coding" refer to the process by which a polynucleotide, through the mechanisms of transcription and translation, produces an amino-acid sequence. The gene(s) encoding the enzyme(s) can be exogenous or endogenous.

The terms "feed-back sensitivity" or "feed-back inhibition" refer to a cellular mechanism control in which an or several enzyme that catalyse the production of a particular substance in the cell are inhibited or less active when that substance has accumulated to a certain level. So the terms "reduced feed-back sensitivity" or "reduced feed-back inhibition" mean that the activity of such a mechanism is decreased or suppressed compared to a non modified microorganism. The man skilled in the art knows how to modify the enzyme to obtain this result. Such modifications have been described in the patent application WO 2005/11202 or in the patent US 7,611,873.

The invention relates to a recombinant microorganism optimised for the fermentative production of methionine, wherein the activity of the cobalamin-independent methionine synthase MetE is attenuated.

The man skilled in the art knows many means and methods to attenuate enzymatic activity like protein mutation, gene mutation or attenuation of gene expression. Protein mutation may be achieved by replacing specific amino-acids present in the catalytic site of the enzyme, or introducing additional amino-acids, or deleting certain amino-acids.

In a first aspect of the invention, the expression of the metE gene, encoding the cobalamin-independent methionine synthase MetE, is attenuated. The nucleotide sequence of the E. coli metE gene is shown in SEQ ID NO 20.

Gene attenuation may be achieved by introducing foreign DNA into the gene to inactivate it or by expressing the gene under control of a weak promoter or an inducible promoter. The man skilled in the art knows a wide variety of promoters exhibiting different expression strength and/or different induction parameters and how to modify a promoter to
decrease its expression strength by modifying the wild type promoter, for instance, in its consensus sequence, Ribosome Binding Site or start codon … Thus, the man skilled in the art is able to chose a promoter which lead to an attenuate expression of metE.

In a preferred embodiment of the invention, at least a portion of the metE gene is deleted. Preferably this deleted portion represents at least 10% of the coding sequence, more preferably at least 20%, 30%, 40%, or 50% of the coding sequence. More preferably, at least 80% of the coding sequence is deleted. In a specific embodiment of the invention, the metE gene is completely deleted. The man skilled in the art knows many techniques to delete gene portions such as homologous recombination.

In a second aspect of the invention, the metE gene is mutated in order to encode a modified protein exhibiting attenuated activity. In a preferred embodiment of the invention, the mutation in the gene metE leads to the translation of a truncated MetE protein which is inactive. More preferably the mutation is a deletion of a portion of 13 base pairs (bp) : from the 417th to the 429th base of the E. coli gene whose nucleotide sequence is shown in SEQ ID NO 20, leading to a frame shift mutation. Consequently, the translation of the protein is shortened (a stop codon is introduced by the frame shift) and gives rise to a truncated protein of 152 amino acids as shown in SEQ ID NO22) instead of 753 amino acids in the wild-type sequence, as shown in SEQ ID NO21. Any equivalent mutation allowing the introduction of a STOP codon in a metE gene from any microorganism species is also part of the invention.

**Optimisation of methionine biosynthesis pathway.**

The recombinant microorganism according to the invention is modified for improving the production of methionine. Genes involved in methionine production are well known in the art, and comprise genes involved in the methionine specific biosynthesis pathway as well as genes involved in precursor-providing pathways and genes involved in methionine consuming pathways.

Efficient production of methionine requires the optimisation of the methionine specific pathway and several precursor-providing pathways. Methionine producing strains have already been described, in particular in patent applications WO2005/111202, WO2007/077041 and WO2009/043803. These applications are incorporated as reference into this application.

In a specific embodiment of the invention, the recombinant microorganism is modified as described below: the expression of at least one of the following genes is increased: ptsG, pyc, pntAB, cysP, cysU, cysW, cysA, cysM, cysJ, cysL, cysH, gcvT, gcvH, gcvP, lpd, serA, serB, serC, cysE, metF, metH, meta, thrA allele encoding for an enzyme with reduced
feed-back sensitivity to S-adenosylmethionine and/or methionine (MetA*), thrA, and thrA allele encoding for an enzyme with reduced feed-back inhibition to threonine (thrA *).

- **ptsG** encodes the PTS enzyme IICB\textsuperscript{Glc} as described in patent application EP1 1305829.
- **pyc** encodes a pyruvate carboxylase as described in patent application EP1 1305829. In a preferred embodiment, the pyc gene is heterologous and is chosen from pyc genes from *Rhizobium etli*, *Bacillus subtilis*, *Lactococcus lactis*, *Pseudomonas fluorescens* or *Corynebacterium* species,
- **pntAB** encode subunits of a membrane-bound transhydrogenase, such as described in patent application WO2012/055798,
- **cysP** encodes a periplasmic sulphate binding protein, as described in WO2007/077041 and in WO2009/043803,
- **cysU** encodes a component of sulphate ABC transporter, as described in WO2007/077041 and in WO2009/043803,
- **cysW** encodes a membrane bound sulphate transport protein, as described in WO2007/077041 and in WO2009/043803,
- **cysA** encodes a sulphate permease, as described in WO2007/077041 and in WO2009/043803,
- **cysM** encodes an O-acetyl serine sulfhydrylase, as described in WO2007/077041 and in WO2009/043803,
- **cysl** and **cysJ** encode respectively the alpha and beta subunits of a sulfite reductase as described in WO2007/077041 and in WO2009/043803. Preferably cysl and cysJ are overexpressed together,
- **cysH** encodes an adenylylsulfate reductase, as described in WO2007/077041 and in WO2009/043803.

Increasing C1 metabolism is also a modification that leads to improved methionine production. It relates to the increase of the activity of at least one enzyme involved in the CI metabolism chosen among GcvTHP, Lpd, MetF or MetH. In a preferred embodiment of the invention, the one carbon metabolism is increased by enhancing the expression and/or the activity of at least one of the following:

- **gcvT**, **gcvH**, **gcvP**, and **lpd**, coding for the glycine cleavage complex, as described in patent application WO 2007/077041. The glycine-cleavage complex (GCV) is a multienzyme complex that catalyzes the oxidation of glycine, yielding carbon dioxide, ammonia, methylene-THF and a reduced pyridine nucleotide. The GCV complex consists of four protein components, the glycine dehydrogenase said P-protein (GcvP), the lipoyl-GcvH-protein said H-protein (GcvH), the aminomethyltransferase said T-protein (GcvT), and the dihydrolipoamide dehydrogenase said L-protein (GcvL or Lpd). P-protein
catalyzes the pyridoxal phosphate-dependent liberation of CO2 from glycine, leaving a methylamine moiety. The methylamine moiety is transferred to the lipoic acid group of the H-protein, which is bound to the P-protein prior to decarboxylation of glycine. The T-protein catalyzes the release of NH3 from the methylamine group and transfers the remaining CI unit to THF, forming methylene-THF. The L protein then oxidizes the lipoic acid component of the H-protein and transfers the electrons to NAD+, forming NADH;

- *MetF* encoding a methylenetetrahydrofolate reductase, as described in patent application WO 2007/077041;
- *MetH* (B12-dependent homocysteine-N5-methyltetrahydrofolate transmethylase) encoding methyltransferases.

The overexpression of at least one of the following genes involved in serine biosynthesis also reduces the production of the by-product isoleucine:

- *serA* which encodes a phosphoglycerate dehydrogenase, as described in WO2007/077041 and in WO2009/043803,
- *serB* which encodes a phosphoserine phosphatase, as described in WO2007/077041 and in WO2009/043803,
- *serC* which encodes a phosphoserine aminotransferase, as described in WO2007/077041 and in WO2009/043803.

The overexpression of the following genes has already been shown to improve the production of methionine:

- *cysE* encodes a serine acyltransferase; its overexpression allows an increase in methionine production, as described in WO 2007/077041;
- *metA* encodes a homoserine succinyltransferase. The allele MetA* codes for an enzyme with reduced feed-back sensitivity to S-adenosylmethionine and/or methionine. Preferentially, the allele MetA* described in the patent application WO 2005/1 11202 is used;
- *thrA* encodes an aspartokinase/homoserine dehydrogenase; the thrA * allele codes for an enzyme with reduced feed-back inhibition to threonine, as described in WO 2005/1 11202.

In a specific embodiment of the invention, genes may be under control of an inducible promoter. In a preferred embodiment of the invention, at least one of these genes is under the control of a temperature inducible promoter. Preferably, the expression of at least one of the genes: *thrA, cysE, metA*, is under the control of an inducible promoter, directly or indirectly. More preferably, the genes *thrA, cysE* and *metA* are under control of an inducible promoter, directly or indirectly. In a preferred embodiment of the invention, expression of *thrA* gene is under direct control of an inducible promoter and expression of *cysE* gene is under polar effect of inducible expression of *thrA* gene. In another preferred
embodiment of the invention, expression of \textit{thrA} gene is under direct control of an inducible promoter and expressions of \textit{cysE} and \textit{metA} genes are under polar effect of inducible expression of \textit{thrA} gene.

In a most preferred embodiment, the temperature inducible promoter belongs to the family of \textit{pR} promoters. A methionine producing strain having genes under control of inducible promoters is described in patent application WO2011/073122.

In another specific embodiment of the invention, the microorganism has been further modified, and the expression of at least one of the following genes is attenuated: \textit{metJ}, \textit{pykA}, \textit{pykF}, \textit{purU}, \textit{ybdL} or\textit{yncA}.

- the gene \textit{metJ} codes for the repressor protein MetJ (GenBank 1790373), responsible for the down-regulation of the methionine regulon as was suggested in patent application JP 2000/157267,

  - The genes \textit{pykA} and \textit{pykF} code for the enzymes 'pyruvate kinase'. The attenuation of the expression of at least one or both of the pyruvate kinases decrease the consumption of phosphoenol pyruvate (PEP). Increased availability of PEP can increase the production of oxaloacetate, an important precursor of aspartate, which in turn is a precursor of methionine, as described in WO2007/077041 and in WO2009/043803,

  - \textit{purU} codes for a formyltetrahydrofolate deformylase, an enzyme that catalyzes the formyl-THF deformylase reaction. The attenuation of the deformylase activity increases the production of methyl-THF that is required for methylation of homocysteine.

Loss of CI metabolites by deformylation leads to an increased production of homocysteine that cannot be transformed into methionine. Homocysteine can then be a substrate for the enzyme cystathionine gamma synthase (MetB) that can catalyze the reaction between O-succinylhomoserine and homocysteine resulting in the production of homolanthionine, as described in WO2007/077041 and in WO2009/043803,

- \textit{ybdL} encodes an aminotransferase as described in patent application PCT/FR2010/052937,

- \textit{yncA} encodes a N-acyltransferase, as described in patent application WO 2010/020681.

In a more preferred embodiment of the invention, the fermentative production of methionine by a recombinant microorganism, wherein the activity of the cobalamin-independent methionine synthase MetE is attenuated, from glucose as a main carbon source, may be achieved through a combination of the above discussed modifications in said microorganism, for example:
the expression of the gene metJ is attenuated and the expression of a metA allele encoding for an enzyme with reduced feed-back sensitivity to S-adenosylmethionine and/or methionine (MetA*) is enhanced;

- the expression of the gene metJ is attenuated; the expression of a metA allele encoding for an enzyme with reduced feed-back sensitivity to S-adenosylmethionine and/or methionine (MetA*) is enhanced; and the expression of a thrA allele encoding for an enzyme with reduced feed-back inhibition to threonine (thrA*) is enhanced;

- the expression of the gene metJ is attenuated; the expression of a metA allele encoding for an enzyme with reduced feed-back sensitivity to S-adenosylmethionine and/or methionine (MetA*) is enhanced; the expression of a thrA allele encoding for an enzyme with reduced feed-back inhibition to threonine (thrA*) is enhanced; and the expression of the gene cysE is enhanced;

- the expression of the gene metJ is attenuated; the expression of a metA allele encoding for an enzyme with reduced feed-back sensitivity to S-adenosylmethionine and/or methionine (MetA*) is enhanced; the expression of a thrA allele encoding for an enzyme with reduced feed-back inhibition to threonine (thrA*) is enhanced; the expression of the gene cysE is enhanced; and the expression of the genes metF and/or metH is enhanced.

In a particular aspect of the invention, the recombinant microorganism comprises the following genetic modifications:

- the gene metE is deleted,

- the expression of the genes metA*, metH, cysPUWAM, cysJIIH, gcvTHP, metF, serA, serB, serC, cysE, thrA* and pyc are enhanced, and

- the genes metJ, pykA, pykF, purU and yncA are attenuated.

In a particular embodiment of the invention, the microorganism is from the bacterial family Enterobacteriaceae or Corynebacteriaceae.

Preferentially, the microorganism is Escherichia coli or Corynebacterium glutamicum.

Culture conditions

The invention is also related to a method of production of methionine comprising the followings steps:

- Culturing a recombinant microorganism in an appropriate culture medium comprising a fermentable source of carbon and a source of sulphur, and,

- Recovering methionine or its derivatives from the culture medium.
Those skilled in the art are able to define the culture conditions for the microorganisms according to the invention. In particular the bacteria are fermented at a temperature between 20°C and 55°C, preferentially between 25°C and 40°C, and more specifically about 30°C for \textit{C. glutamicum} and about 37°C for \textit{E. coli}.

For \textit{E. coli}, the culture medium can be of identical or similar composition to an M9 medium (Anderson, 1946), an M63 medium (Miller, 1992); or a medium such as defined by Schaefer \textit{et al.}, (1999).

For \textit{C. glutamicum}, the culture medium can be of identical or similar composition to BMCG medium (Liebl \textit{et al.}, 1989) or to a medium such as described by Riedel \textit{et al.}, (2001).

In some embodiment of the invention, the culture is subjected to a limitation or starvation for one or several inorganic substrate. It refers to condition under which growth of the microorganisms is governed by the quantity of an inorganic chemical supplied that still permits weak growth. Such limitation in microorganism growth has been described in the patent application WO 2009/043372. In a preferred embodiment of the invention, the culture is subjected to phosphate limitation.

The action of "recovering methionine or its derivatives from the culture medium" designates the action of recovering L-methionine and/or one of its derivatives, in particular N-acetyl methionine (NAM) and S-adenosyl methionine (SAM) and all other derivatives that may be useful. The methods for the recovery and purification of the produced compounds are well known to those skilled in the art (see in particular WO 2005/007862, WO 2005/059155).

The amount of product in the fermentation medium can be determined using a number of methods known in the art, for example, high performance liquid chromatography (HPLC) or gas chromatography (GC). For example the quantity of methionine obtained in the medium is measured by HPLC after OPA/Fmoc derivatization using L-methionine (Fluka, Ref 64319) as a standard. The amount of NAM is determined using refractometric HPLC using NAM (Sigma, Ref 013 10) as a standard.
EXEMPLARY

The present invention is further defined in the following examples. It should be understood that these examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From above disclosure and these examples, the man skilled in the art can make various changes of the invention to adapt it to various uses and conditions without modify the essentials means of the invention.

In particular, examples show modified *Escherichia coli* (*E. coli*) strains, but these modifications can easily be performed in other microorganisms of the same family.

*Escherichia coli* belongs to the *Enterobacteriaceae* family, which comprises members that are Gram-negative, rod-shaped, non-spore forming and are typically 1-5 μm in length. Most members have flagella used to move about, but a few genera are non-motile. Many members of this family are a normal part of the gut flora found in the intestines of humans and other animals, while others are found in water or soil, or are parasites on a variety of different animals and plants. *E. coli* is one of the most important model organisms, but other important members of the *Enterobacteriaceae* family include *Klebsiella*, in particular *Klebsiella terrigena*, *Klebsiella planticola* or *Klebsiella oxytoca*, and *Salmonella*.

Moreover, several patent applications point out that optimisation for methionine production can easily be applied in *E. coli* and in *Corynebacterium glutamicum* without undue experimentation.

EXAMPLE 1: PROTOCOLES

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

**Protocol 1:** Chromosomal modifications by homologous recombination and selection of recombinants (Datsenko, & Wanner, (2000)).

Allelic replacement or gene insertion in specified chromosomal locus was carried out by homologous recombination as described by Datsenko & Wanner (2000). The kanamycin (Km) resistance kan, flanked by Flp recognition sites was amplified by PCR by using pKD4 plasmid as template. The resulting PCR products were used to transform the recipient *E. coli* strain harbouring plasmid pKD46 that expresses the λ Red (γ, β, exo) recombinase. Antibiotic-resistant transformants were then selected and the chromosomal structure of the modified locus was verified by PCR analysis with the appropriate primers listed in Table 3.

The kan resistance gene can be excised by using plasmid pCP20 that carries the gene coding Flp recombinase as described by Datsenko & Wanner (2000). The pCP20 plasmid was introduced into the appropriated strain and the transformants were spread on LB
supplemented with ampicillin at 30°C. In order to express the \textit{flp} gene and to remove the kanamycin cassette, the transformants were cultivated at 37°C. Then after isolation, the antibiotic sensible clones were verified by PCR using oligonucleotides listed in Table 3.

5 \textbf{Protocol 2:} Transduction of phage P1

Chromosomal modifications were transferred to a given \textit{E. coli} recipient strain by P1 transduction. The protocol includes 2 steps: (i) preparation of the phage lysate on a donor strain containing the resistance associated chromosomal modification and (ii) infection of the recipient strain by this phage lysate.

10 \textbf{Preparation of the phage lysate}

- Inoculate 100 µl of an overnight culture of the strain MG1655 with the chromosomal modification of interest in 10 ml of Km 50µg/ml + glucose 0.2% + CaCl$_2$ 5 mM.
- Incubate 30 min at 37°C with shaking.
- Add 100 µl of P1 phage lysate prepared on the donor strain MG1655 (approx. 1 x 10$^9$ phage/ml).
- Shake at 37°C for 3 hours until the complete lysis of the cells.
- Add 200 µl of chloroform, and vortex
- Centrifuge 10 min at 4500 g to eliminate cell debris.
- Transfer of supernatant to a sterile tube.
- Store the lysate at 4°C.

15 \textbf{Transduction}

- Centrifuge 10 min at 1500 g 5 ml of an overnight culture of the \textit{E. coli} recipient strain cultivated in LB medium.
- Suspend the cell pellet in 2.5 ml of MgSO$_4$ 10 mM, CaCl$_2$ 5 mM.
- Infect 100 µl cells with 100 µl P1 phage of strain MG1655 with the modification on the chromosome (test tube) and as a control tubes 100 µl cells without P1 phage and 100 µl P1 phage without cells.
- Incubate 30 min at 30°C without shaking.
- Add 100 µl sodium citrate 1 M in each tube, and vortex.
- Add 1 ml of LB.
- Incubate 1 hour at 37°C with shaking
- Centrifuge 3 min at 7000 rpm.
- Plate on LB + Km 50 µg/ml
- Incubate at 37°C overnight.
<table>
<thead>
<tr>
<th>Strain number</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>MG1655 metA*11 metE:Km Ptc01<em>2/RBS08</em>1-metH Ptc01-cysPUWAM Ptc01-cysJHH Ptc01/RBS01-gcvTHP Ptc01/ARN01/RBS01-metF Ptc94-serB ΔmetJ ΔpykF ΔpykA ΔpurU ΔyncA ΔmalS:RN/PRM-C1857-TTadcca-PR01/RBS01<em>4-thrA</em>1-cysE ΔpgaaBCD:RN/PR01/RBS01-thrA<em>1-cysE-Pgapa-metA</em>11 ΔCP4-6:RN/PR01/RBS01-thrA<em>1-cysE-Pgapa-metA</em>11 ΔwcaM:RN/PR01/RBS01-thrA<em>1-cysE-Pgapa-metA</em>11 ΔtreBC::RN/serA-serC Δyjbl::RN/Ptc01/RBS01-gcvTHP-TT07</td>
</tr>
<tr>
<td>7</td>
<td>MG1655 metA*11 metE:Km Ptc01<em>2/RBS08</em>1-metH Ptc01-cysPUWAM Ptc01-cysJHH Ptc01/RBS01-gcvTHP Ptc01/ARN01/RBS01-metF Ptc94-serB ΔmetJ ΔpykF ΔpykA ΔpurU ΔyncA ΔmalS:RN/PRM-C1857-TTadcca-PR01/RBS01<em>4-thrA</em>1-cysE ΔpgaaBCD:RN/PR01/RBS01-thrA<em>1-cysE-Pgapa-metA</em>11 ΔCP4-6:RN/PR01/RBS01-thrA<em>1-cysE-Pgapa-metA</em>11 ΔwcaM:RN/PR01/RBS01-thrA<em>1-cysE-Pgapa-metA</em>11 ΔtreBC::RN/serA-serC Δyjbl::RN/Ptc01/RBS01-gcvTHP-TT07 (pC1920-Pgapa-pycre-TT07) (pCC1BAC-TT02-Ptc30/RBS01-serC-TT07*2-Ptc30/RBS01-serA-TTadcca)</td>
</tr>
</tbody>
</table>
Table 2: Correspondence between the previous and the current nomenclature for the genotype of strain 1 described in patent application EP10306164.

<table>
<thead>
<tr>
<th>Previous nomenclature</th>
<th>Current nomenclature</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG1655 meta*11</td>
<td>MG1655 meta*11</td>
</tr>
<tr>
<td>Ptre-metH</td>
<td>Ptre01<em>2/RBS08</em>1-metH</td>
</tr>
<tr>
<td>PtreF-cysPUWAM</td>
<td>Ptre01-cysPUWAM</td>
</tr>
<tr>
<td>PtreF-cysIHH</td>
<td>Ptre01-cysIHH</td>
</tr>
<tr>
<td>Ptre09-gcvTHP</td>
<td>Ptre01/RBS01-gcvTHP</td>
</tr>
<tr>
<td>Ptre36-ARNmst17-metF</td>
<td>Ptre01//ARN01/RBS01-metF</td>
</tr>
<tr>
<td>Ptre07-serB</td>
<td>Ptre94-serB</td>
</tr>
<tr>
<td>ΔmetaI ΔpykF ΔpykA ΔpurU ΔyncA</td>
<td>ΔmetaI ΔpykF ΔpykA ΔpurU ΔyncA</td>
</tr>
</tbody>
</table>
| ΔmalS::TTade-C857-PlambdaR*(-35)-thrA*1-cysE | ΔmalS::RN/PRM-C857-TTadecca-
PR01/RBS01*4-thrA*1-cysE |
| ΔpgaABCD::TT02-TTade-PlambdaR*(-35)-RBS01-thrA*1-cysE-PgapA-metA*11 | ΔpgaABCD::RN/PR01/RBS01-thrA*1-
cysE-PgapA-metA*11 |
| ΔwcaA::TT07-TTade-PlambdaR*(-35)-RBS01-thrA*1-cysE-PgapA-metA*11 | ΔwcaA::RN/PR01/RBS01-thrA*1-cysE-
PgapA-metA*11 |
| ΔCP4-6::TT02-TTade-PlambdaR*(-35)-RBS01-thrA*1-cysE-PgapA-metA*11 | ΔCP4-6::RN/PR01/RBS01-thrA*1-cysE-
PgapA-metA*11 |
| ΔwcaM::TT02-TTade-PlambdaR*(-35)-RBS01-thrA*1-cysE-PgapA-metA*11 | ΔwcaM::RN/PR01/RBS01-thrA*1-cysE-
PgapA-metA*11 |
| ΔtreBC::TT02-serA-serC | ΔtreBC::RN/serA-serC |

Table 3: Oligonucleotides used in the following examples.

<table>
<thead>
<tr>
<th>Oligonucleotide name</th>
<th>SEQ ID N°</th>
<th>Sequence 5’ → 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yjbipu-F</td>
<td>1</td>
<td>cgtagggcgggtacgtagctgaagctgggaagggc</td>
</tr>
<tr>
<td>Yjbipu-R</td>
<td>2</td>
<td>gcttgatataacagataaaagaaagccagtttcgactcagtctttctttt</td>
</tr>
<tr>
<td>Yjbdown-F</td>
<td>3</td>
<td>agactggccctttgatattcatgtgtgataacaagttacctaggggtatataaatagatagatagtacttgctgtcgactgg</td>
</tr>
<tr>
<td>Yjbdown-R</td>
<td>4</td>
<td>cgtagggccgggtacgtagctgaagctgggaagggc</td>
</tr>
<tr>
<td>Km-F</td>
<td>5</td>
<td>tccccccgggtatatcaatatatatctctcttag</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td><strong>Km-R</strong></td>
<td>6</td>
<td>gcccaagcttttgtaggctggagctgcttcg</td>
</tr>
<tr>
<td><strong>PtrcOl/RBSOl-GcvTHP-F</strong></td>
<td>7</td>
<td>cgtaggcctggcccgagctgttgacaattaatcatccg</td>
</tr>
<tr>
<td><strong>GcvTHP-TT07-R</strong></td>
<td>8</td>
<td>cgaaggccttttaattaagcagaaagggcaccgaaggctggcagcgtatgctggaatggtttaagcagtatggtgggaaga</td>
</tr>
<tr>
<td><strong>yjbl-gcvTHP-F</strong></td>
<td>9</td>
<td>cagacccaacaactggcgacc</td>
</tr>
<tr>
<td><strong>yjbl-gcvTHP-R</strong></td>
<td>10</td>
<td>gccattggaatcgaccagcc</td>
</tr>
<tr>
<td><strong>Ptrc30/RBS01-F</strong></td>
<td>11</td>
<td>ttcgttttatctgtttacgtagagctgttgacgattaatcatccgctcgtatactgtgtggaataaagagtagtttgagccataaatatacctcttattccacacagttgacagac</td>
</tr>
<tr>
<td><strong>Ptrc30/RBS01-serC-R</strong></td>
<td>12</td>
<td>ccagaactaaatggagaggtatattgagccataaatatacctcttattccacacagttgacagac</td>
</tr>
<tr>
<td><strong>serC-TT07*2-R</strong></td>
<td>13</td>
<td>cccagaagttgcagcgtgctggcagcttgagaagggccaccggaaggtagcga ggttaaccgtgacggcggttcg</td>
</tr>
<tr>
<td><strong>Ptrc30/RBS01-serA-F</strong></td>
<td>14</td>
<td>tacgttagctagcagctgttgacgattaatcctccgctgatatactgtgtggaataaagggataattatatggcaaga ggtagatattatggcacaaggtatctgcggagaag</td>
</tr>
<tr>
<td><strong>serA-TTadcca-R</strong></td>
<td>15</td>
<td>cccagaagttgcagcgtgctggcagcttgagaagggccaccggaaggtagcga ggttaaccgtgacggcggttcg</td>
</tr>
<tr>
<td><strong>metE-Km-F</strong></td>
<td>16</td>
<td>agaaaccgcggcggcagcctttgcagctggcagcttgagaagggccaccggaaggtagcga ggttaaccgtgacggcggttcg</td>
</tr>
<tr>
<td><strong>metE-Km-R</strong></td>
<td>17</td>
<td>gcagaagttgcagcgtgctggcagcttgagaagggccaccggaaggtagcga ggttaaccgtgacggcggttcg</td>
</tr>
<tr>
<td><strong>metE-F</strong></td>
<td>18</td>
<td>cgtttgggactggatgtgctgg</td>
</tr>
</tbody>
</table>
| **metE-R** | 19 | gcgtggtaggcaacaagtacg
EXAMPLE 2: Construction of strain 5, MG1655 metA*\n\nPr01*2/RBS08*1-metH\nVtrcOl-cysPUWAM VtrcOl-cysJIIH Prc01l/RBS01-l-gcvTHP Prc01/ARN01/RBS01-metF\nVtrc94-serB AmetJ ApykF ApykA ApyrU AyncA AmalS::KN/VKM-C1857-TTadcca-pr01/RBS01*4-i/ii^\nVtrcOl-cysPUWAM VtrcOl-cysJIH tr... restriction sites, named SMC). This last region was PCR
amplified from genomic DNA using the following oligonucleotides:

HindlU, the pUC18-Ajy:/w::TT02-SMC the pUC18 but rrnB
For This TT07::Km. This homologous T
Ayjbl:
T
2
cysE-VgapA-metA*
metA* PR01/RBS01*4-i/ii^ *l-cysE-rgapA-metA*\nVtrc94-serB

EXAMPLE amplified associated artificial chromosome encoded methionine producing strain 1 (genotype in table 1) has been described in patent application EP10306164 which is incorporated as reference into this application.

2. Construction of the strain 2
To increase the methylene-tetrahydrofolate pool into the cell, the glycine cleavage complex encoded by gcvTHP operon was overproduced by adding one copy of this operon on the chromosome at the yjbl locus. This additional copy of gcvTHP was expressed using an artificial inducible trc promoter and an optimised ribosome binding site, giving the Ayjbl:RN/Pirc01l/RBS01-gcvTHP-1101::Km chromosomal integration.

To delete the yjbl gene and replace it by the VtrcOl1/RBS01-gcv7HP-TT07 region, the homologous recombination strategy described by Datsenko & Wanner (2000) was used. This strategy allows the insertion of a chloramphenicol or a kanamycin resistance cassette but also an additional DNA, while deleting most of the genes concerned. For this purpose, the following plasmid was constructed, pUC 18-Ayjbl:TT02-PircO1/RBS01-gcvTHP-TT07::Km.
This pUC18-Aj7W::TT02-Pirc01/RBS01-gcv7HP-TT07::Km plasmid is derived from the pUC18 vector (Norlander et al., 1983) and harbors the kanamycin resistance cassette associated to Pirc01/RBS01-gcvZHP-TT07 region, both cloned between the upstream and the downstream regions of yjbl.

For the construction of pUC18-Ay7W::TT02-Pirc01/RBS01-gcv7HP-TT07::Km, first the pUC18-Ajy/W::TT02-SMC plasmid was constructed. This plasmid carries the upstream and the downstream regions of yjbl which are separated by a transcriptional terminator (Ti of rnbB gene of E. coli, named TT02) and a multiple cloning site (composed of BstZII, HindlU, Avrll, Apal and PacI restriction sites, named SMC). This last region was PCR amplified from genomic DNA using the following oligonucleotides:
Yjblup-F (SEQ ID NO 1)
CGTAGGCGCCGTACCgagtgcagatcggctggaaggcg
with
- a region (lower case) homologous to the sequence (4247987-4248009) of the yjbl region (reference sequence on the website http://www.ecogene.org/),
- a region (upper case) for Sfol and Kpnl restriction site and extra-bases.

Yjblup-R (SEQ ID NO 2)
GCTTGTTTACAACAGATAAAAAGCAAAGGC CCAGTC TTTC GACTGAGC CTTT
CGTTTTATTGTGATcatttctgtagaattttacacttatagtatcattactgattgagacttca
with
- a region (lower case) homologous to the sequence (4248931-4248980) of the yjbl region (reference sequence on the website http://www.ecogene.org/),
- a region (upper bold case) for transcription terminator Ti of rrnB gene of E. coli (Orosz et al, 1991),
- a region (upper case) for BstZI restriction site and part of the HindIII restriction site of the multiple cloning site.

Yjbldown-F (SEQ ID NO 3)
AGACTGGGCGCTTTCTGTTTATCTGTTGATACAAAGCTTTACCTAGGGCCCTT
AATTAAataagtaaaaaggttttaagtaaaagaaaaacacgtctctggcat
with
- a region (lower case) homologous to the sequence (4250286-4250335) of the yjbl region (reference sequence on the website http://www.ecogene.org/),
- a region (upper bold case) for part of the transcription terminator Ti of rrnB gene of E. coli (Orosz et al, 1991),
- a region (upper case) for the entire multiple cloning site.

Yjbldown-R (SEQ ID NO 4)
CGTAGGCGCCGTACCcagcataatcaccacacatccg
with
- a region (lower case) homologous to the sequence (4251224-4251249) of the yjbl region (reference sequence on the website http://www.ecogene.org/),
- a region (upper case) for Sfol and Kpnl restriction site and extra-bases.

First, the "up Yjbl" and "down Yjbl" fragments were PCR amplified from MG1655 genomic DNA using Yjblup-F / Yjblup-R and Yjbldown-F / Yjbldown-R oligonucleotides, respectively. Secondly, "upYjbl-downYjbl" fragment was amplified from "upYjbl" and "downYjbl" PCR fragments (that possess an overlapping region
including a part of the transcription terminator Ti of \textit{rrnB} gene of \textit{E. coli} and a part of the multiple cloning site) using Yjblup-F / Ybldown-R oligonucleotides. The "upYjbl-downYjbl" PCR fragment was cut with the restriction enzyme \textit{SfoI} and cloned into the blunted \textit{EcoRI / HindIII} sites of the pUC18 vector, giving the pUC18-AyyW::TT02-SMC plasmid.

Then, the kanamycin resistance cassette was PCR amplified from pKD4 vector using the following oligonucleotides:

Km-F (SEQ ID NO 5)
TCCCCCGGGGTATAACatatgaatctctcttag
with
- a region (lower case) for the amplification of the kanamycin resistance cassette (reference sequence in Datsenko & Wanner, 2000),
- a region (upper case) for \textit{Smal} and \textit{BstZII} restriction sites and extra-bases.

Km-R (SEQ ID NO 6)
GCCCAAGCTTTgaagcttgagctgttgac
with
- a region (lower case) for the amplification of the kanamycin resistance cassette (reference sequence in Datsenko & Wanner, 2000)
- a region (upper case) for \textit{HindIII} restriction site and extra-bases.

The PCR fragment was cut with the restriction enzymes \textit{BstZII} and \textit{HindIII} and cloned into the \textit{BstZII 1 HindIII} sites of the pUC18-AyyW::TT02-SMC plasmid, giving the pUC18-AyyW::TT02-SMC::Km plasmid.

Finally, the Pircl/RBS01-gcv7HP-TT07 fragment was PCR amplified from the genomic DNA of strain 1 using Pircl/RBS01-GcvTHP-F / GcvTHP-TT07-R oligonucleotides (described below). The PCR fragment was cut with the restriction enzymes \textit{Apal} and \textit{Pad} and cloned into the \textit{Apal I PacI} sites of the pUC18-Aj^W::TT07-SMC::Km plasmid, giving the pUC18-Aj^W::TT07-Pircl/RBS01-gcv7HP-TT07::Km plasmid. Recombinant plasmids were verified by DNA sequencing.

Ptrcl/RBS01-GcvTHP-F (SEQ ID NO 7)
CGTAGGCCTGGGCCCgagctgttgacaattaatcatccg
with
- a region (lower case) homologous to a part of the artificial inducible trc promoter located upstream of gcvTHP operon in the strain 1,
- a region (upper case) for Stul and Apal restriction sites and extra-bases.

GcvTHP-TT07-R (SEQ ID NO 8)
CGAAGGCCTTT AATTAGC AGAAGGC CCACCCGAAGGTG AGC CAGGC GGC
CGCtactggatatgtactggtacg
with
- a region (lower case) homologous to the sequence (3044190-3044214) of the gcvP gene (reference sequence on the website http://www.ecogene.org/),
- a region (upper bold case) for T7te transcriptional terminator sequence, named TT07 (Harrington et al., 2001),
- a region for Pad, Stul restriction sites and extra-bases.

Finally, the AjyW::TT02-P/rc01/RBS01-gcvmP-TT07::Km fragment was obtained by cutting the pUC18-AjyW::TT02-P/rc01/RBS01-gcvmP-TT07::Km plasmid with Kpnl restriction enzyme and was then introduced by electroporation, according Protocol 1, into a MG1655 metA*11 pKD46 strain. The kanamycin resistant transformants were then selected, and the insertion of the AjyW::TT02-P/rc01/RBS01-gcvmP-TT07::Km fragment was verified by a PCR analysis with the oligonucleotides yjbl-gcvTHP-F and yjbl-gcvTHP-R. The verified and selected strain was called MG1655 metA*ll pKD46 Ajybl::RN/Ptrc01/RBSO1-gcvTHP-YY07::Km.

yjbl-gcvTHP-F (SEQ ID NO 9)
cagaccaccacccaactggcgacc
homologous to the sequence (4247754-4247774) of yjbl region (reference sequence on the website http://www.ecogene.org/)

yjbl-gcvTHP-R (SEQ ID NO 10)
gccattggaatcgaccagcc
homologous to the sequence (4251489-4251508) of the yjbl region (reference sequence on the website http://www.ecogene.org/)

The Ajy bl::RN/Vtrc01/RBS0l-gcvTHP-TT07::Km chromosomal modification was then transduced into the strain 1, according to Protocol 2. Kanamycin resistant transductants were selected and the presence of Ajy bl::RN/Vtrc01/RBS01-gcvTHP-TT01::Km chromosomal modification was verified by PCR with primers yjbl-gcvTHP-F and yjbl-gcvTHP-R.
The resulting strain MG1655 \textit{metaA*} \textit{\textbackslash} P{	extsubscript{rc01}}*2/RBS08*1-\textit{wei H P}trcOl-cysPUWAM PtrcOl-cysJIIH PtrcOl/RBS01-gcvTHP PircOl/\textit{ARNO} 1/RBS01-metF Ptrc94-serB A\textit{m}etJ \textit{ApykF ApykA ApurU AyncA $\Delta$maS::RN/PRM-\textit{CI857-7-TTa} icca-\textit{PR01}/RBS01 *4-thrA*l-cysE ApgaABC\textit{D}:RN/PR0 1/RBS0 1-thra *1-cysE-PgapA-metA *11 AuxCA::\textit{KN/PRO1/RBS01-thra}*-cysE-PgapA-metA *11 3. \textit{Construction of the strain 3}

For construction of strain 3, the resistance cassette associated to the chromosomal integration \textit{A}j\textit{W}::RN/Pirc01/RBS01-gcvr \textsl{HP-TT07::Km} of strain 2 was removed according to Protocol 1.

Kanamycin sensible clones were selected and the absence of the kanamycin cassette was verified by PCR with primers \textit{yjbl-gcvrTHP-F} and \textit{yjbl-gcvrTHP-R}.

The resulting strain MG1655 \textit{metaA*} \textit{\textbackslash} P{	extsubscript{rc01}}*2/RBS08*1-\textit{wei H P}trcOl-cysPUWAM PtrcOl-cysJIIH \textit{Ptrc01}/RBS01-gcvTHP PircOl/ARNO 1/RBS01-metF Ptrc94-serB A\textit{m}etJ \textit{ApykF ApykA ApurU AyncA $\Delta$maS::RN/PRM-\textit{CI857-7-TTa} adccca-PR0\textit{V}/RBS01 *4-thrA*l-cysE ApgaABC\textit{D}:RN/PR0 1/RBS0 1-thra *1-cysE-PgapA-metA *11 AuxCA::\textit{RN/PRO1/RBS01-thra}*-cysE-PgapA-metA *11 \textit{ACP4-6}:RN/PRO1/RBS01-thra *1-cysE-PgapA-metA *11 \textit{A}wcaM::RN/PR0 1/RBS0 1-thra *1-cysE-PgapA-metA *11 \textit{A}treBC::\textit{RN/serA-serC Ayjbl:}:RN/Pirc01/RBS01-gcvr \textsl{HP-TT07::Km} (pCL 1920-PgapA-\textit{pycre-TT07}) was named strain 3.

4. \textit{Construction of the strain 4}

The plasmid pCL 1920-PgapA-\textit{pycre-TT07}, described in patent application PCT/FR20 10/052937 (which is incorporated as reference into this application), was introduced into strain 2, giving the following strain MG1655 \textit{metaA*} \textit{\textbackslash} P{	extsubscript{rc01}}*2/RBS08*1-mei H \textit{P}trcOl-cysPUWAM PtrcOl-cysJIIH \textit{Ptrc01}/RBS01-gcvTHP PircOl/\textit{ARNO} 1/RBS01-metF Ptrc94-serB A\textit{m}etJ \textit{ApykF ApykA ApurU AyncA $\Delta$maS::RN/PRM-\textit{CI857-7-TTa} icca-\textit{PR01}/RBS01H-thra *1-cysE ApgaABC\textit{D}:RN/PR0 1/RBS0 1-thra *1-cysE-PgapA-metA *11 AuxCA::\textit{RN/PRO1/RBS01-thra}*-cysE-PgapA-metA *11 \textit{ACP4-6}:RN/PRO1/RBS01-thra *1-cysE-PgapA-metA *11 \textit{A}wcaM::RN/PR0 1/RBS0 1-thra *1-cysE-PgapA-metA *11 \textit{A}treBC::\textit{RN/serA-serC Ayjbl:}:RN/Pirc01/RBS01-gcvr \textsl{HP-TT07::Km} (pCL 1920-PgapA-\textit{pycre-TT07}), named strain 4.

5. \textit{Construction of the strain 5}

To increase the flux into the serine pathway, the \textit{serC} and \textit{serA} genes were overexpressed owing artificial promoters and an optimised ribosome binding sites and using of the
bacterial artificial chromosome pCC1BAC (Epicentre). For this purpose, the following plasmid vCC1BAC-TT02-Vtrc30/RBS01-serC-TT07*2-Vtrc30/RBS01-serA-TTadcca was constructed.

For the construction of pCC1BAC-TT02-Ptrc30/RBS01-serC-TT07*2-Ptrc30/RBS01-serA-TTadcca, the “TT02-Vtrc30/RBS01-serC-TT07*2” and “Vtrc30/RBS01-serA-TTadcca” regions were PCR amplified. For the "TT02-Ptrc30/RBS01-serC-TT07*2" region, at first a megaprimer harbouring the transcriptional terminator (Ti of rrrnB, annotated TT02), the artificial promoter (Ptrc30), the optimised ribosome binding site (RBS01) and the beginning of serC gene was synthesized by a short PCR using the oligonucleotides Ptrc30/RBS01-F and Ptrc30/RBS01-serC-R (described below) without adding matrix. Secondly, the "TT02-Vtrc30/RBS01-serC-TT07*2" fragment was amplified by PCR using E. coli MG1655 genomic DNA as matrix and the synthesized megaprimer and the serC-TT07*2-R oligonucleotide (described below).

Ptrc30/RBS01-F (SEQ ID NO 11)
tcggegccttaataaaC ATCAAAATAAAAAGCAAGGCTCAGTCGAAGACTGGCCTT
TCGTTTTTATCTGTGtacetaGAGCTTGTGACGATTAATCATCCGGCTCGTATACTG
20 TGTGGAA TAA GGA GGTATA TT
with
- a region (upper bold case) for transcription terminator Ti of rrrnB gene of E. coli (Orosz et al, 1991),
- a region (upper underlined case) homologous to the artificial inducible trc promoter,
- a region (upper italic case) homologous to an optimised ribosome binding site,
- a region (lower case) for Narl, Pad restriction sites and extra-bases.

Ptrc30/RBS01-serC-R (SEQ ID NO 12)
ccagaactaaattgagatttgagc44 TA AT X TCC7774TTCCACACAGTATAACGAGC
30 with
- a region (upper underlined case) homologous to the artificial inducible trc promoter,
- a region (upper italic case) homologous to an optimised ribosome binding site,
- a region (lower case) homologous to the sequence (956876-956904) of serC gene (reference sequence on the website http://www.ecogene.org/).

serC-TT07*2-R (SEQ ID NO 13)
CCCAAGCTTGCATGCGCTAGCGAGCTCGAGAAAGGCCACCCGAAGGTGAG
CCAGGttaaectgtaacgtggtcgc
with
- a region (upper bold case) for T7te transcriptional terminator sequence (Harrington et al., 2001) which possesses a base deletion at the 29th position (named TT07*2),
- a region (lower case) homologous to the sequence (957946-957964) of serC gene (reference sequence on the website http://www.ecogene.org/),
- a region (upper case) for HindIII, SphI, SacI and Nhel restriction sites and extra-bases.

In the same manner, the "Vtrc30/RBS01-serA-\text{TTadcca}" fragment was amplify by PCR using \textit{E. coli} MG1655 genomic DNA as matrix and the Prtc30/RBS01-serA-F and serA-\text{TTadcca}-R oligonucleotides (described below).

Prtc30/RBS01-serA-F (SEQ ID NO 14)
TACGTAGCTAGCGAGCTGTTGACGATTAATCATCCGGCTCGTATACTGTGTGAATAA GGA GGTA 
ATATTTTTTTTT
with
- a region (upper underlined case) homologous to the artificial inducible \textit{trc} promoter,
- a region (upper italic case) homologous to an optimised ribosome binding site,
- a region (lower case) homologous to the sequence (3056408-3056432) of serA gene (reference sequence on the website http://www.ecogene.org/),
- a region (upper case) for Nhel restriction site and extra-bases.

serA-\text{TTadcca}-R (SEQ ID NO 15)
CCCAAGCTTGATGCCTAGTAAAATAAGAGTTACCATTTAAGGTAACCTTGAGGCTGGGATATGATATTTTTTTATAGTACAGCAGACGCGC
- a region (upper bold case) for \textit{TTadcc} transcriptional terminator sequence (transcription terminator of the \textit{adc} gene from \textit{Clostridium acetobutylicum}, homologous from 179847 to 179807 of the pSLOI megaplasmid),
- a region (lower case) homologous to the sequence (3055200-3055220) of serA gene (reference sequence on the website http://www.ecogene.org/),
- a region (upper case) for \textit{^vrll}, SphI, HindIII restriction sites and extra-bases.

The PCR fragments, "TI02-Vtrc30/RBS01-serC-Tim*2" and the "Vtrc30/RBS01-serA-\text{TTadcca}" were cut with the restriction enzymes NarI Nhel, and Nhel SphI, respectively, and both cloned into the NarI SphI sites of the pCCIBAC plasmid, giving the pCCIBAC-JIQ2-?trc30/RBS01-serC-IIQ)*2-?trc30/RBS01-serA-\text{lladcca} plasmid.
The recombinant plasmid was verified by DNA sequencing.
Finally, the plasmid pCCIBAC-TT02-Ptrc30/RBS01-serC-TT07*2-Ptrc30/RBS01-serA-TTadcca was introduced into strain 4, giving the following strain MG1655 metA*\ Prc01*2/RBS08*1/-wei H PtrlOl-cysPUWAM PtrlOl-cysJH PtrlOl/RBS01-gcvTHP PtrlOl/ARNO 1/RBSO 1-metF Vtrc94-serB AmetJ ApykF ApykA ApurU AyncA AmalS: :RN/PRM- CI85 7-TTadcca-PR0 1/RBS01H-thrA *-cysE ApgaABCD:::RN/PR0 1/RBS0 1-thra*1-cysE-PgapA-metA *11 AuxaCA:::RN/PR0 1/RBS0 1-thra*1-cysE-VgapA-metA *11 Acp4-6:::RN/PR0 1/RBS0 1-thra*1-cysE-VgapA-metA *11 AwcaM:::RN/PR0 1/RBS0 1-thra*1-cysE-VgapA-metA *11 AtreBC:::KN/serA-serC Ayjbl:::RN/Pirc0 1/RBSO 1-gcvTHP-TYW ::Km (pCL 1920-PgapA-pycE-TT07) (pCCIBAC-TT02-Pirc30/RBS01-5erC-TT07 *2-Pirc30/RBS01-5er^-TTaJcca), n a m e d
strain 5.

6. Identification of the metE mutation in strain 5

By measuring the methionine synthase activity (METE) of the strain 5, we identified that the metE gene was not functional, because of some mutations giving a truncated MetE protein.

The mutation is a deletion of 13 bp (the 417th to the 429th base of the gene) of the metE gene leading to a frame shift mutation. Consequently, the translation of the protein is shortened (stop codon introduced by the frame shift) and gives rise to a truncated protein of 152 amino acids instead of 753.

Here is the sequence of the WT MetE protein (SEP_ID NO 21):

MTILNHTLGFPRVGLRRELKKAAQESYWAGNSTREELAVGRELARHWDQQKQA GIDLLPVGDFAWYDHVLTTSSLGLGNVPARHQNKGDSVIDTFLIRGRAPRGTGEP AAMEMTKWFNHYMVPEFVKQGKFLTWTQLLDVEDEALALGHKVKPVLLG PVTLWLGKVGEQFDRSLNLNDILPYQQVVLAEAKRGIEWVQIDEPALVLELP QAQLDAYKPAYDALGQVFQKLTTTTYFEGVTNPNLDTITALPVQGLVHDVLVHGGD VAEHHRPL SDWLLSAQLINGRN VWRADLTEKY AQIKDIVKGRLD Waltonw SSCLLH SPIDLSVETRLDAEVKSWFAFLQKCHELALLRDALNSGDTAALEWSAPIQARR HSTRVHNPAVEKRLAIAITAQDSQRANVYEVRAEAAQRARFKPAPWPTTTIGSFQPQ TEIRTIRLDFKKGNLADNYRTGIAEHKQAIVEKERGLDLVHLVGEAERNDME YFGEHLDGFVFTQONGWQVSYSRCVPKPIVIGISRPAITVEWASYAQSLTDKPV KGMLTPVITCWSFPREDVSRETIKAQIALLRDEVADLEAAGIIGIQIDEPALREG LPLRRSDWDAYLQWGVAFRINTAAXAKDTQIHTHMCYCEFNDIDSIAALDAD VITIETSRSDMELLESFEFEYPNEIGPGVYDHSPVNPVSVEWIEALLKAAKRIPAE

RLWVPNDCGLKTRGWEPETPvAALANMVQAAQNLRG*
Here is the sequence of the truncated MetE* protein (SEQ ID NO 22):
MTILNHTLGPFRVGLRELKKAQESYWAGNSTREELAVGRELARHWDQKQA
GIDLLPVGDFAWYDHVLTTLLGNVPARHQNKDGSDLTDLTEGRAPTEPAA
AAAEMTKWFNTNYHYMVPEFVKQQFKLTFKMTKWRTRWRWATR*

EXAMPLE 3: Construction of strain 7, MG1655 metA*\ metE r. Km
Ptrc01*2/RBS08*I-metH VtrcOl-cysPUWAM VtrcOl-cysJIH Ptrc01/RBS01-l-gcvTHP
Vtrc01/ARN01/RBS01-metF Vtrc94-serB AmetJ ApykF ApykA ApurU AyncA
AmalS::RN/VRM-Cl857-\TTadca -Vmi/RBS01*4-thra*l-cysE
ApgaABCD::RN/Ymi/KBS01-thra*l-cysE-YgapA-metA*ll
AuxaCA::RN/Vmi/KBS01-thra*l-cysE-VgapA-metA* 11 ACPY-6::RN/PR01/RBS01-
\thrA*l-cysE-\gapA-metA* 11 AucaM::KN/VR01/KBS01-thra*l-cysE-VgapA-metA*ll
AtreBcr.KN/serA-serC Ajy bl::RN/Vtrc01/RBS01-l-gcvTHP-\TT07  (pCL1920-PgapA-
pyre-\TY07)  (pCCIBAC-TTO2-Pii-c30/RBS01-serC-\TT07*2-Pii-c30/RBS01-s^-
\TTadcd)

I. Construction of the strain 6
To study if restoration of a functional MetE protein could modify the methionine
production of the strain 5, we replaced the truncated metE gene by a wild-type one using
the homologous recombination strategy described by Datsenko & Wanner (2000).

For this purpose, the kanamycin cassette flanked with fragments homologous to the metE
region, "meiE::Km" fragment was PCR amplified using oligonucleotides metE-Km-F and
metE-Km-R (described below). The "meiE::Km" fragment was introduced into a MG1655
metA* 11 pKD46 strain which possesses a functional version of the metE gene.

metE-Km-F (SEQ ID NO 16)
agaaaccggccggcactgggcaacatgtgcggcggcgagtcggcgggggtaaaatccaaccgggtggtataa
ccacccggttttctcaTGTAGGCTGGAGCTGCCTCCG
with
- a region (lower case) homologous to the sequence (4013277-4013376) of metE region
(reference sequence on the website http://www.ecogene.org/),
- a region (upper case) for the amplification of the kanamycin resistance cassette (reference
sequence in Datsenko & Wanner, 2000).
metE-Km-R (SEQ ID NO 17)
gcagaagatggctggcagcgtatgctggaatggtttaagcagtatggtgggaagaagtcgctgtaaGCAGAAAGGCC
CACCCGAAGGTGAGCCAGTGTGACATATGAATATCCTCCTTAG

with
- a region (lower case) homologous to the sequence (4013377-4013442) of metE region (reference sequence on the website http://www.ecogene.org/),
- a region (upper bold case) for T7e transcriptional terminator sequence (Harrington et al, 2001),
- a region (upper case) for the amplification of the kanamycin resistance cassette (reference sequence in Datsenko & Wanner, 2000).

Kanamycin resistant recombinants were selected and the presence of the Km cassette downstream of the metE gene was verified by PCR with oligonucleotides metE-F and metE-R (described below). The verified and selected strain was called MG1655 metA*ll

pKD46 metE::Km.

metE-F (SEQ ID NO 18)
ctttgggactggatgtgctgg
homologous to the sequence (4012495-4012516) of the metE region (reference sequence on the website http://www.ecogene.org/)

metE-R (SEQ ID NO 19)
gctttggtacggcaaactgac
homologous to the sequence (4013672-4013691) of the metE region (reference sequence on the website http://www.ecogene.org/)

The metE::K1H chromosomal modification was then transduced into the strain 3, according to Protocol 2.
Kanamycin resistant recombinants were selected and the presence of the Km cassette downstream of the metE gene was verified by PCR with oligonucleotides metE-F and metE-R (described above). The presence of metE gene with the wild type sequence was verified by DNA seqencing.

The resulting strain MG1655 metA*\ metE ::Km P/pc01*2/RBS08*1/-wei H Ptrc01-
cysPUWAM PtrcOl-cysJIIH PptrcOl/RBS01-gcvTHP PircOl/ARNO 1/RBS0 1-metF Ptrc94-
serB AimetJ ApykF ApykA ApurU AyncA AmalS::KN/PRM-C1857-CTadcca-
PR01/RBS01H-thrA *\cysE ApgaABCD::RN/PR0 1/RBS0 1-thrA *1-cysE-PgapA-metA *11
AuxaCA ::RN/PR0 1/RBS0 1-thrA *\cysE-PgapA-metA *11 ACP4-6::RN/PR0 1/RBS0 1-
AtreBC::KN/serA-serC  Δ\textit{bjI}::RN/Ptrc01/RBS01-l-gcvTHP-TT07 was named strain 6.

2. Construction of strain 7

The plasmid \textit{pCL}1920-\textit{PgapA-pycr}-1 T07 (described in patent application PCT/FR20 10/052937) and the plasmid \textit{pCC}IBAC-TT02-Pirc30/RBS01-serC-TT07*2-Pirc30/RBS01-serA-TTadcca (described above) were introduced into the strain 6, giving the strain MG1655 \textit{metA}^-\textit{metE}::\textit{Km}  \textit{Ptrc01}^*2/RBS08*l-metH  \textit{PtrcOl-cysPUWAM}
\textit{PtrcOl-cysJH}  \textit{Ptrc01}/RBS01-l-gcv\textit{THP}  \textit{PircOl/ARNO 1/RBS01-metF}  \textit{Pirc94-serB}  \textit{AmetJ}
\textit{ApykF}  \textit{ApykA}  \textit{ApurU}  \textit{AyncA}  \Δ\textit{halS}::\textit{RN/PRM-Cl857-TTadcca}^*01^*801^*4^*1-cysE
\textit{ApykA}  \textit{ApurU}  \textit{AyncA}  \Δ\textit{halS}::\textit{RN/PRM-Cl857-TTadcca}^*01^*801^*4^*1-cysE
\textit{ApykA}  \textit{ApurU}  \textit{AyncA}  \Δ\textit{halS}::\textit{RN/PRM-Cl857-TTadcca}^*01^*801^*4^*1-cysE
\textit{ApykA}  \textit{ApurU}  \textit{AyncA}  \Δ\textit{halS}::\textit{RN/PRM-Cl857-TTadcca}^*01^*801^*4^*1-cysE
\textit{ApykA}  \textit{ApurU}  \textit{AyncA}  \Δ\textit{halS}::\textit{RN/PRM-Cl857-TTadcca}^*01^*801^*4^*1-cysE

Measurement of the cobalamin-independent Methionine Synthase (MS, MetE) activity of
the strain 6 confirmed that the MetE protein is functional.

EXAMPLE 4: PRODUCTION OF L-METHIONINE BY FERMENTATION IN
BIO-REACTOR

Strains that produced substantial amounts of methionine were subsequently tested
under production conditions in 0.5 L fermentors (GX, GPC) using a fedbatch strategy.

Briefly, a 24 hours culture grown in 10 mL LB medium with 2.5 g.L^{-1} glucose was
used to inoculate a 24 hours preculture in minimal medium (Bla). These incubations were
carried out in 500 mL baffled flasks containing 40 mL of minimal medium (Bla) in a
rotary shaker (200 RPM). The first preculture was realized at a temperature of 30\degree C, the
second one at a temperature of 34\degree C.

A third preculture step was carried out in bio-reactors (Sixfors) filled with 200 mL
of minimal medium (Bib) inoculated to a biomass concentration of 1.2 g.L^{-1} with 5 mL of
concentrated preculture. The preculture temperature was maintained constant at 34\degree C and
the pH was automatically adjusted to a value of 6.8 using a 10 % NH_4OH solution. The
dissolved oxygen concentration was continuously adjusted to a value of 30 % of the partial
air pressure saturation with air supply and/or agitation. After glucose exhaustion from the batch medium, the fedbatch was started with an initial flow rate of 0.7 mL.h⁻¹ and increased exponentially for 24 hours with a growth rate of 0.13 h⁻¹ in order to obtain a final cellular concentration of about 20 g.L⁻¹.

Table 4: Preculture batch mineral medium composition (Bla and Bib).

<table>
<thead>
<tr>
<th>Compound</th>
<th>B1a Concentration (g.L⁻¹)</th>
<th>B1b Concentration (g.L⁻¹)</th>
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</thead>
<tbody>
<tr>
<td>Zn(CH₂COO)₂₂H₂O</td>
<td>0.0130</td>
<td>0.0130</td>
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<tr>
<td>CuCl₂·2H₂O</td>
<td>0.0015</td>
<td>0.0015</td>
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<tr>
<td>MnCl₂·4H₂O</td>
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<td>CoCl₂·6H₂O</td>
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<td>Na₂MoO₄·2H₂O</td>
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<td>Fe(III) citrate H₂O</td>
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<td>0.1064</td>
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<td>EDTA</td>
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<td>1.00</td>
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<tr>
<td>CaCl₂·2H₂O</td>
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<td>Vitamin B12</td>
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<tr>
<td>Glucose</td>
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<td>MOPS</td>
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<tr>
<td>NH₂OH 28% Adjusted to pH 6.8</td>
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<td>Adjusted to pH 6.8</td>
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Table 5: Preculture fedbatch mineral medium composition (Fl)

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<thead>
<tr>
<th>Compound</th>
<th>Concentration (g.L⁻¹)</th>
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<tr>
<td>Zn(CH₂COO)₂₂H₂O</td>
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<td>CuCl₂·2H₂O</td>
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<td>MnCl₂·4H₂O</td>
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Table 6: Culture batch mineral medium composition (B2).

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</thead>
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<td>Zn(CH₃COO)₂·2H₂O</td>
<td>0.0130</td>
</tr>
<tr>
<td>CuCl₂·2H₂O</td>
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</tr>
<tr>
<td>MnCl₂·4H₂O</td>
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<td>CoCl₂·6H₂O</td>
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<tr>
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<tr>
<td>Na₂MoO₄·2H₂O</td>
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<tr>
<td>Fe(III) citrate H₂O</td>
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<tr>
<td>EDTA</td>
<td>0.0084</td>
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<tr>
<td>MgSO₄·7H₂O</td>
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<td>K₂HPO₄·3H₂O</td>
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<td>0.72</td>
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<tr>
<td>(NH₄)₂S₂O₃</td>
<td>3.74</td>
</tr>
<tr>
<td>Thiamine</td>
<td>0.01</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>0.01</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.10</td>
</tr>
<tr>
<td>Glucose</td>
<td>10.00</td>
</tr>
<tr>
<td>NH₂OH 28%</td>
<td>Adjusted to pH 6.8</td>
</tr>
</tbody>
</table>

Table 7: Culture fedbatch medium composition (F2).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (g.L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn(CH₃COO)₂·2H₂O</td>
<td>0.0104</td>
</tr>
<tr>
<td>CuCl₂·2H₂O</td>
<td>0.0012</td>
</tr>
<tr>
<td>MnCl₂·4H₂O</td>
<td>0.0120</td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>0.0020</td>
</tr>
<tr>
<td>H₂B₄O₃</td>
<td>0.0024</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>0.0020</td>
</tr>
<tr>
<td>Fe(III) citrate H₂O</td>
<td>0.0524</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.0067</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>5.00</td>
</tr>
<tr>
<td>(NH₄)₂S₂O₃</td>
<td>55.50</td>
</tr>
<tr>
<td>Thiamine</td>
<td>0.01</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>0.01</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.10</td>
</tr>
<tr>
<td>Glucose</td>
<td>500.00</td>
</tr>
</tbody>
</table>
Subsequently, GX 0.5 L fermentors (GPC) were filled with 220 mL of minimal medium (B2) and were inoculated to a biomass concentration of 2.1 g.L⁻¹ with a preculture volume ranging between 20 to 30 mL.

The culture temperature was maintained constant at 37 °C and pH was maintained to the working value (6.8) by automatic addition of NH₄OH solutions (NH₄OH 10 %). The initial agitation rate was set at 200 RPM during the batch phase and was increased up to 1000 RPM during the fedbatch phase. The initial airflow rate was set at 0.3 L.min⁻¹ during the batch phase and was increased up to 0.7 L.min⁻¹ at the beginning of the fedbatch phase. The dissolved oxygen concentration was maintained at values between 20 and 40%, preferentially 30% saturation by increasing the agitation.

When the cell mass reached a concentration close to 5 g.L⁻¹, the fedbatch was started with an initial flow rate of 1.9 mL.h⁻¹. Feeding solution was injected with a sigmoid profile with an increasing flow rate that reached 8.8 mL.h⁻¹ after 26 hours. The precise feeding conditions were calculated by the equation: 

\[ Q(t) = p1 + \frac{p2}{1 + e^{-p3(t-p4)}} \]

where Q(t) is the feeding flow rate in mL.h⁻¹ for a batch volume of 600 mL with p1 = 0.66, p2 = 8.21, p3 = 0.27, p4 = 6.50.

After 26 hours of fedbatch, the feeding solution pump was stopped and the culture was finished after glucose complete exhaustion.

Extracellular amino acids were quantified by HPLC after OPA/Fmoc derivatization and other relevant metabolites were analyzed using HPLC with refractometric detection (organic acids and glucose) and GC-MS after silylation.

**Table 8**: Final methionine yield \( y_{\text{met final}} \) in % g of methionine per g of glucose produced in fedbatch culture by the different strains. For the definition of methionine/glucose yield see below. Each strain was evaluated once.

<table>
<thead>
<tr>
<th>Strain</th>
<th>( y_{\text{met final}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain 5</td>
<td>0.225</td>
</tr>
<tr>
<td>Strain 7</td>
<td>0.186</td>
</tr>
</tbody>
</table>

As can be seen in table 8 above, the yield of methionine production increased significantly upon metE gene mutation. The strain 5, containing the mutated metE gene has a yield higher of 4 points compared to strain 7 which contains a functional MetE protein.
The fermentor volume was calculated by adding to the initial volume of the reactor, the amount of the solutions added to regulate the pH and to feed the culture and by subtracting the volume used for sampling and lost by evaporation.

The fedbatch volume was followed continuously by weighing the feeding stock.

The amount of injected glucose was then calculated on the basis of the injected weight, the density of the solution and the glucose concentration determined by the method of Brix ([Glucose]). The methionine yield was expressed as followed:

\[
Y_{\text{met}} = \frac{\text{Methionine}_t \times V_t - \text{Methionine}_o \times V_o \times 100}{\text{Consumed glucose}}
\]

The final yield obtained during the culture was presented here for each strain. With Methionineo and Methionine_t respectively the initial and final methionine concentrations and V_o and V_t the initial and the final volumes.

The consumed glucose was calculated as follows:

\[
\text{fed' volume}_t = \frac{\text{fed weight}_t - \text{fed weight}}{\text{density fed solution}}
\]

\[
\text{Injected Glucose}_t = \text{fed volume}_t \times [\text{Glucose}]
\]

\[
\text{Consumed glucose}_t = [\text{Glucose}]_o \times V_o + \text{Injected Glucose} - [\text{Glucose}]_{t, \text{iduial}} \times V_t
\]

With [Glucose]_o, [Glucose], [Glucose]_{t, \text{iduial}} respectively the initial, the fed and the residual glucose concentrations.

**EXAMPLE 5: MEASUREMENT OF THE COBALAMIN-INDEPENDENT METHIONINE SYNTHASE (MetE) ACTIVITY.**

For the *in vitro* determination of the cobalamin-independent Methionine Synthase (MS, MetE) activity, *E. coli* strains 7 and 5 carrying wild-type or mutated metE gene respectively were cultured in minimal medium as described in example 3 above and harvested at the end of the log phase by centrifugation. Pellets were resuspended in cold 20mM potassium phosphate buffer pH 7.2 containing a cocktail of protease inhibitors with EDTA. Then, the cells were broken by bead beating with a Precellys system (Bertin Technologies; 2x10s at 6500rpm) followed by centrifugation at 12000g at 4°C for 30 minutes. Supernatants were desalted and used for enzymatic analyses. Protein concentrations were determined using Bradford assay reagent (Bradford, 1976).
For the determination of MS activity, 40µg of crude cell extracts were incubated for 15 minutes at 37°C with 1mM DL-homocysteine and 0.25mM methyl-tetrahydropteroyl-triglutamate in 100mM potassium phosphate buffer pH7.2, 5mM MgSO₄. The methionine produced by cobalamin-independent Methionine Synthase enzyme was quantified by GC-MS after derivatization with tert-butyldimethylsilyltrifluoroacetamide (TBDMSTFA). Aspartate and Norleucine were included as internal standards.

Results of cobalamin-independent Methionine Synthase activities are presented in table 9 below.

**Table 9**: Cobalamin-independent Methionine Synthase activities (in mUI/mg proteins) of *E. coli* strains carrying wild-type or mutated enzymes. Each strain was evaluated once.

<table>
<thead>
<tr>
<th>Strain</th>
<th>MS (mUI/mg proteins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain 5</td>
<td>0</td>
</tr>
<tr>
<td>Strain 7</td>
<td>12.7</td>
</tr>
</tbody>
</table>

As can be seen in table 9, strain 5 (*AmetE*) has completely lost its MS activity whereas strain 7 kept a significant one. This loss of activity was correlated to a significant improvement of methionine production.

**EXAMPLE 6: EFFECT OF DELETION OF METE GENE ON PRODUCTION OF L-METHIONINE**

To evaluate the effect of a complete deletion of the *metE* gene on the production of L-methionine, we deleted the mutated *metE* gene of the strain 5. We introduced a clean deletion of *metE* gene in that strain using the homologous recombination as described previously and using the strategy provided by Datsenko & Wanner (2000).

After replacement of the mutated *metE* gene by the kanamycin cassette, the kanamycin resistant recombinants are selected and verified by DNA sequencing.

One of them is cultured as described in example 4 and the produced L-methionine is quantified by HPLC.

The strain with the clean deletion of *metE* produces more methionine than strain 7 which possesses a functional MetE protein: the deletion of *metE* results in increased yield of methionine of more than 15%.
REFERENCES

CLAIMS

1) A recombinant microorganism optimised for the fermentative production of methionine, wherein the activity of the cobalamin-independent methionine synthase MetE is attenuated in said microorganism.

2) The microorganism of claim 1, wherein the cobalamin-independent methionine synthase MetE is encoded by the metE gene whose expression is attenuated.

3) The microorganism of claim 1 or 2, wherein at least a portion of the metE gene is deleted.

4) The microorganism of claim 1, wherein the MetE protein is encoded by a mutated metE gene.

5) The microorganism of claim 4 wherein the mutation of metE gene is a deletion of the bases comprised between the 417th and 429th positions.

6) The microorganism of anyone of claims 1 to 5, wherein the expression of at least one of the following genes is increased: ptsG, pyc, pntAB, cysP, cysll, cysW, cysA, cysM, cysJ, cysl, cysH, gcvT, gcvH, gcvP, lpd, serA, serB, serC, cysE, metF, metH, thrA, metA allele encoding for an enzyme with reduced feed-back sensitivity to S-adenosylmethionine and/or methionine (metA*), thrA, or a thrA allele encoding for an enzyme with reduced feed-back inhibition to threonine (thrA *).

7) The microorganism of claim 6, wherein at least one gene is under the control of an inducible promoter.

8) The microorganism of anyone of claims 1 to 7, wherein the expression of at least one of the following genes is attenuated: metJ, pykA, pykF, purU, ybdL orncA.

9) The microorganism of anyone of claims 1 to 8, wherein:
   a. the gene metE is deleted
   b. the expression of the genes metA*, metH, cysPUWAM, cysJIH, gcvTHP, metF, serA, serB, serC, cysE, thrA* and pyc are enhanced; and
   c. the expression of the genes metJ, pykA, pykF, purU and yncA are attenuated.
10) The microorganism of anyone of claims 1 to 9, wherein said microorganism is from the bacterial family *Enterobacteriaceae* or *Corynebacteriaceae*.

11) The microorganism of anyone of claims 1 to 10, wherein said microorganism is *Escherichia coli*.

12) A method for the fermentative production of methionine comprising the steps of:
   a. culturing a recombinant microorganism according to anyone of claims 1 to 11 in an appropriate culture medium comprising a fermentable source of carbon and a source of sulphur, and
   b. recovering methionine or its derivatives from the culture medium.

13) The method of claim 12 wherein growth of the recombinant microorganism is subjected to limitation or deficiency for one or several inorganic substrate(s), in particular phosphate and/or potassium, in the culture medium.
INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2012/001336

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/10 C12P13/12

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C12P

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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Further documents are listed in the continuation of Box C.

X See patent family annex.

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Date of the actual completion of the international search
24 April 2013

Date of mailing of the international search report
07/05/2013

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

Authorized officer
Strobel, Andreas
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<td>wo 2012/055798 AI (METABOLIC EXPLORER SA [FR]; DISCHERT WANDA [FR]; VASSEUR PERRINE [FR];) 3 May 2012 (2012-05-03) cited in the application claims 1,6</td>
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