The present invention generally relates to a modified microbial cell capable of producing high levels of spermidine and/or spermidine derivatives. The genetically modified microbial cell comprises at least one modification to native spermidine biosynthetic pathways via putrescine together with genes involved in the S-adenosylmethionine biosynthetic pathway.
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MICROBIAL CELLS FOR SPERMIDINE PRODUCTION

TECHNICAL FIELD

The present invention relates generally to the development of genetically engineered microorganisms. More specifically the invention relates to microbial cells able to produce the pro-longevity compound spermidine in an economic fashion.

BACKGROUND

Spermidine is a low molecular weight aliphatic nitrogen compound that is found ubiquitously in microorganisms, plants, and animals. Intracellularly, it is derived from L-ornithine, an intermediate in L-arginine biosynthesis. Recent years have shown this compound to promote longevity in a variety of organisms ranging from microbes to mammals. In addition, spermidine uptake has also been shown to alleviate or protect against a variety of age-associated conditions including cancer, multiple sclerosis, osteoporosis, cardiovascular disease, memory impairment, skin ageing and hair loss. Therefore, growing interest exists obtaining an economic, reliable and sustainable supply of this compound.

Several inventions have described the chemical synthesis of spermidine. However, these methods either suffer from issues of expensive substrates, poor enantiopure purity or are environmental unfriendly.

Spermidine can also be derived from biological sources, such as wheat germ. However, even the highest spermidine-producing natural sources only produce spermidine at levels of approximately 200 μg/g, making it very expensive to obtain a pure product.

Production of spermidine from microbial sources could overcome these issues. However, microbial sources naturally produce only low levels of spermidine and therefore additional engineering is required to obtain economically feasible production levels. To date, only a few studies have described engineering of microbial cells to over-produce spermidine.

Qin et al [1] have described engineering of the ornithine metabolism in a yeast cell in combination with over-expression of spermidine-producing genes to over-produce spermidine, resulting in 35 mg/L of spermidine.

Kim et al [2] and Kim et al [3] reported engineering yeast cells for enhanced production of intracellular spermidine. In these cells OAZ1, encoding ornithine decarboxylase (ODC) antizyme, a protein involved in feedback inhibition of the spermidine biosynthesis pathway, and TPOI coding for the polyamine transport protein were disrupted to increase intracellular spermidine levels through alleviation of feedback inhibition on ODC and prevention of
spermidine excretion, respectively. Combined with spermidine synthesis genes, this led to increase in the cellular spermidine contents, with content of 1.1 mg spermidine per gram dry cell weight (DCW).

Kim et al [4] have reported overexpression of spermidine synthesis genes in a yeast, combined with disruption of OAZ1, expression of the transporter TPO1 and fermentation optimization, resulting in 63.6 mg/L of spermidine in batch, and up to 224 mg/L in fed-batch (2.2 mg/g sugars).

There is, however, still room for improvements in the field of spermidine production in microbial cells.

SUMMARY

A primary object of the present invention is to provide an improved microbial cell for spermidine production. Such cell can be used for fermentation-based production of spermidine. The microbial cell and the method disclosed herein combine the over-expression of spermidine pathway genes with modifications in the pathway related to synthesis and regeneration of cofactors or co-substrates involved in spermidine production, in particular S-adenosylmethionine (SAM). SAM can be subsequently converted to S-adenosylmethioninamine (dSAM) and used in spermidine synthesis.

An aspect of the embodiments relates to a microbial cell capable of producing spermidine.

The microbial cell is genetically modified for overexpression of S-adenosylmethionine decarboxylase and/or spermidine synthase. The microbial cell is also genetically modified for enhanced SAM biosynthesis.

Another aspect of the embodiments relates to a method for producing spermidine. The method comprises culturing a microbial cell according to the embodiments in a culture medium and in culture conditions suitable for production of spermidine from the microbial cell. The method also comprises collecting spermidine from the culture medium and/or the microbial cell.

A further aspect of the embodiments relates to use of a microbial cell according to the embodiments as a food additive.

Previous efforts to increase spermidine production in microbial cells have combined overexpression of genes that convert putrescine to spermidine with overexpression of SAM decarboxylase. While it was shown that increased levels of dSAM are beneficial for spermidine production, no studies have reported that SAM itself is a limiting factor for spermidine production. We have surprisingly found that SAM itself is indeed limiting for spermidine
production. This was surprising because unlike dSAM, which is mainly involved in production of spermidine and spermine, SAM is a common co-substrate in the cell. By increasing the precursor pools of this co-substrate, and/or increasing recycling of SAM, we could significantly increase spermidine production. By combining this strategy with the strategies previously reported, we were able to produce a record >1 g/L and> 30 mg/g DCW of spermidine, representing a significant improvement over previous processes.

**BRIEF DESCRIPTION OF THE DRAWINGS**

The embodiments, together with further objects and advantages thereof, may best be understood by making reference to the following description taken together with the accompanying drawings, in which:

**Figure 1:** Overview of the native pathway leading to production of spermidine from glucose in eukaryotic microbial cells, such as *Saccharomyces cerevisiae*. Similar reactions also occur in prokaryotic microbes, albeit without the compartmentalization. EC numbers are shown for important reactions that can be targeted to increase spermidine production via the ornithine route. Upward grey arrows next to EC numbers represent reactions that are increased via genetic modification, while downward arrows represent reactions that are decreased.

**Figure 2:** Overview of reactions targeted in the present invention to increase spermidine production via the SAM route. EC numbers are shown for important reactions. Upward grey arrows next to EC numbers represent reactions that are increased via genetic modification, while downward arrows represent reactions that are decreased. Abbreviations: SAM: S-Adenosylmethionine, dSAM: S-Adenosylmethioninamine, MTA: 5'-Methylthioadenosine, MRP: S-Methyl-5-thio-D-ribose 1-phosphate, MOB: 4-Methylthio-2-oxobutanoate, THF: methylenetetrahydrofolate, H2S: Hydrogen sulfide.

**Figure 3:** Production of spermidine in a microbial cell by overexpression of heterologous pathway genes. In this example, spermidine is produced from the condensation of putrescine and L-aspartate-4-semialdehyde. The heterologous enzymes carboxy(nor)spermidine dehydrogenase (CASDH/CANSDH) [EC 1.5.1.43] and carboxy(nor)spermidine decarboxylase (CASDC/CANSDC) [EC 4.1.1.96] are over-expressed in a yeast cell. Enzymes are taken from either *Campylobacter jejuni* (plasmid YP1) or *Vibrio cholera* (plasmid YP2). Overexpression of these enzymes in yeast results in increased spermidine production.

**Figure 4:** Production of spermidine in a microbial cell by combining overexpression of spermidine synthetic genes and upregulation of the pathway leading to L-aspartate-4-semialdehyde formation. Increasing the flux towards L-aspartate-4-semialdehyde was achieved
by overexpression of heterologous Aspartate kinase (\textit{Cglyc}) \textit{Aspartate-semialdehyde dehydrogenase (Cgasd) and Aspartate aminotransferase (CgaspB). In addition, flux towards the competitive Homoserine dehydrogenase reaction was decreased by inserting a weak Kex2p promoter in front of the endogenous gene encoding Homoserine dehydrogenase (\textit{HOM6}). All modifications were performed in the yeast \textit{S. cerevisiae}. The figure shows that increasing the flux towards L-aspartate-4-semialdehyde and decreasing flux to competitive reactions resulted in increased production of spermidine compared to overexpression of the spermidine biosynthetic genes alone.

**Figure 5:** Production of spermidine in a microbial cell by combining overexpression of spermidine synthetic genes including S-adenosylmethionine decarboxylase (EC 4.1.1.50) and spermidine synthase (EC 2.5.1.16), as well as putrescine biosynthetic pathway ("increased SPD") and upregulation of the methionine salvage pathway. Increasing the activity of either methylthioadenosine phosphorylase (MTAP) [EC 2.4.2.28] through overexpression of the endogenous \textit{MEW} gene, or branched-chain amino acid transaminase (BAT) [EC 2.6.1.42] through overexpression of the endogenous \textit{BAT2} gene in \textit{S. cerevisiae} allowed for increased flux through the methionine salvage pathway, and increased spermidine production.

**Figure 6:** Production of spermidine in a microbial cell via fed-batch fermentation. The best-producing strain (SPDC10) was grown under glucose-limited fed-batch conditions, resulting in a production of up to 1.4 g/L of spermidine.

**Figure 7:** Map of the constructs used for plasmid expression in examples 1-4.

**Figure 8:** Production of spermidine in a microbial cell by combining overexpression of spermidine synthetic genes including S-adenosylmethionine decarboxylase (EC 4.1.1.50) and spermidine synthase (EC 2.5.1.16), as well as putrescine biosynthetic pathway ("increased SPD") and overexpression of methionine adenosyltransferase (MAT) [EC 2.5.1.6]. Increasing the activity of MAT through overexpression of either mutated MAT from \textit{Streptomyces spectabilis (SsMAT)} or MAT from \textit{Leishmania infantum JPCM5 (SiMAT)} in \textit{S. cerevisiae} resulted in increased spermidine production compared to a strain not overexpressing MAT.

**Figure 9:** Production of spermidine in a microbial cell by combining overexpression of spermidine synthetic genes including S-adenosylmethionine decarboxylase (EC 4.1.1.50) and spermidine synthase (EC 2.5.1.16), as well as putrescine biosynthetic pathway ("increased SPD") and overexpression of methylenetetrahydrofolate reductase (MTHFR) [EC 1.5.1.20]. Increasing the activity of MTHFR through overexpression of a chimeric MTHFR comprised of the yeast Metl3p N-terminal catalytic domain and the \textit{Arabidopsis thaliana} MTHFR (AtMTHFR-1) C-terminal regulatory domain (ScAtMTHFR) in \textit{S. cerevisiae} resulted in
increased spermidine production compared to a strain not overexpressing MTHFR.

Figure 10: Production of spermidine in a microbial cell by combining overexpression of spermidine synthetic genes including S-adenosylmethionine decarboxylase (EC 4.1.1.50) and spermidine synthase (EC 2.5.1.16), as well as putrescine biosynthetic pathway ("increased SPD") and overexpression of transcription factors involved in the pleiotropic drug response (S. cerevisiae PDR1 and PDR2). Overexpression of PDR1 or PDR2 in S. cerevisiae resulted in increased spermidine production compared to a strain not overexpressing any PDR.

Figure 11: Production of spermidine in a microbial cell by combining overexpression of spermidine synthetic genes including S-adenosylmethionine decarboxylase (EC 4.1.1.50) and spermidine synthase (EC 2.5.1.16), as well as putrescine biosynthetic pathway ("increased SPD") and overexpression of subunits of the RNA Polyimerase II mediator complex, in particularly S. cerevisiae GAL11. Overexpression of GAL11 under the control of either the TEF1 or CYC1 promoter in S. cerevisiae resulted in increased spermidine production compared to a strain not overexpressing GAL11.

DETAILED DESCRIPTION

The present invention now will be described hereinafter with reference to the accompanying drawings and examples, in which embodiments of the invention are shown. This description is not intended to be a detailed catalogue of all the different ways in which the invention may be implemented, or all the features that may be added to the instant invention. For example, features illustrated with respect to one embodiment may be incorporated into other embodiments, and features illustrated with respect to a particular embodiment may be deleted from that embodiment. Thus, the invention contemplates that in some embodiments of the invention, any feature or combination of features set forth herein can be excluded or omitted.

In addition, numerous variations and additions to the various embodiments suggested herein will be apparent to those skilled in the art in light of the instant disclosure, which do not depart from the instant invention. Hence, the following descriptions are intended to illustrate some particular embodiments of the invention, and not to exhaustively specify all permutations, combinations and variations thereof.

Unless otherwise defined herein, scientific and technical terms used herein will have the meanings that are commonly understood by those of ordinary skill in the art.

Generally, nomenclatures used in connection with techniques of biochemistry, enzymology, molecular and cellular biology, microbiology, genetics and protein and nucleic
acid chemistry and hybridization, described herein, are those well-known and commonly used in the art.

Conventional methods and techniques mentioned herein are explained in more detail, for example, in Molecular Cloning, a laboratory manual [second edition] Sambrook et al. Cold Spring Harbor Laboratory, 1989, for example in Sections 1.21 "Extraction And Purification Of Plasmid DNA", 1.53 "Strategies For Cloning In Plasmid Vectors", 1.85 "Identification Of Bacterial Colonies That Contain Recombinant Plasmids", 6 "Gel Electrophoresis Of DNA", 14 "In vitro Amplification Of DNA By The Polymerase Chain Reaction", and 17 "Expression Of Cloned Genes In Escherichia coli" thereof.

Enzyme Commission (EC) numbers (also called "classes" herein), referred to throughout this specification, are according to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) in its resource "Enzyme Nomenclature" (1992, including Supplements 6-17) available, for example, as "Enzyme nomenclature 1992: recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology on the nomenclature and classification of enzymes", Webb, E. C. (1992), San Diego: Published for the International Union of Biochemistry and Molecular Biology by Academic Press (ISBN 0-12-227164-5). This is a numerical classification scheme based on the chemical reactions catalyzed by each enzyme class.

The terminology used in the description of the invention herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention.

All publications, patent applications, patents and other references cited herein are incorporated by reference in their entireties for the teachings relevant to the sentence and/or paragraph in which the reference is presented.

Unless the context indicates otherwise, it is specifically intended that the various features of the invention described herein can be used in any combination. Moreover, the present invention also contemplates that in some embodiments of the invention, any feature or combination of features set forth herein can be excluded or omitted. To illustrate, if the specification states that a composition comprises components A, B and C, it is specifically intended that any of A, B or C, or a combination thereof, can be omitted and disclaimed singularly or in any combination.

As used in the description of the invention and the appended claims, the singular forms "a," "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise.
Also as used herein, "and/or" refers to and encompasses any and all possible combinations of one or more of the associated listed items, as well as the lack of combinations when interpreted in the alternative ("or").

Throughout the description and claims of this specification, the words "comprise" and "contain" and variations of the words, for example "comprising" and "comprises", mean "including but not limited to" and do not exclude other moieties, additives, components, integers or steps. Throughout the description and claims of this specification, the singular encompasses the plural unless the context otherwise requires. In particular, where the indefinite article is used, the specification is to be understood as contemplating plurality as well as singularity, unless the context requires otherwise.

As used herein, the transitional phrase "consisting" essentially of means that the scope of a claim is to be interpreted to encompass the specified materials or steps recited in the claim and those that do not materially affect the basic and novel characteristic(s) of the claimed invention. Thus, the term "consisting essentially of when used in a claim of this invention is not intended to be interpreted to be equivalent to "comprising."

To facilitate understanding of the invention, a number of terms are defined below.

As used herein, the term "polyamine" refers to an organic compound having two or more primary amino groups. Examples for polyamines include putrescine, cadaverine, spermidine and spermine.

Also as used herein, the terms "nucleotide sequence" "nucleic acid," "nucleic acid molecule," "oligonucleotide" and "polynucleotide" refer to RNA or DNA, including cDNA, a DNA fragment or portion, genomic DNA, synthetic DNA, plasmid DNA, mRNA, and antisense RNA, any of which can be single stranded or double stranded, linear or branched, or a hybrid thereof. Nucleic acid molecules and/or nucleotide sequences provided herein are presented herein in the 5' to 3' direction, from left to right and are represented using the standard code for representing the nucleotide characters as set forth in the U.S. sequence rules, 37 CFR §§1.821 - 1.825 and the World Intellectual Property Organization (WIPO) Standard ST.25. When dsRNA is produced synthetically, less common bases, such as inosine, 5-methylcytosine, 6- methyladenine, hypoxanthine and others can also be used for antisense, dsRNA, and ribozyme pairing. For example, polynucleotides that contain C-5 propyne analogues of uridine and cytidine have been shown to bind RNA with high affinity and to be potent antisense inhibitors of gene expression. Other modifications, such as modification to the phosphodiester backbone, or the 2'- hydroxy in the ribose sugar group of the RNA can also be made.
As used herein the term "recombinant" when used means that a particular nucleic acid (DNA or RNA) is the product of various combinations of cloning, restriction, and/or ligation steps resulting in a construct having a structural coding or non-coding sequence distinguishable from endogenous nucleic acids found in natural systems.

As used herein, the term "gene" refers to a nucleic acid molecule capable of being used to produce mRNA, antisense RNA, miRNA, anti-microRNA antisense oligodeoxyribonucleotide (AMO) and the like. Genes may or may not be capable of being used to produce a functional protein or gene product. Genes can include both coding and non-coding regions, e.g. introns, regulatory elements, promoters, enhancers, termination sequences and/or 5' and 3' untranslated regions. A gene may be "isolated" by which is meant a nucleic acid that is substantially or essentially free from components normally found in association with the nucleic acid in its natural state. Such components include other cellular material, culture medium from recombinant production, and/or various chemicals used in chemically synthesizing the nucleic acid.

A "disrupted gene" as defined herein involves any mutation or modification to a gene resulting in a partial or fully non-functional gene and gene product. Such a mutation or modification includes, but is not limited to, a missense mutation, a nonsense mutation, a deletion, a substitution, an insertion, addition of a targeting sequence and the like. Furthermore, a disruption of a gene can be achieved also, or alternatively, by mutation or modification of control elements controlling the transcription of the gene, such as mutation or modification in a promoter, terminator and/or enhancement elements. In such a case, such a mutation or modification results in partially or fully loss of transcription of the gene, i.e. a lower or reduced transcription as compared to native and non-modified control elements. As a result a reduced, if any, amount of the gene product will be available following transcription and translation.

Furthermore, disruption of a gene could also entail adding or removing a localization signal from the gene, resulting in decreased presence of the gene product in its native subcellular compartment.

The objective of gene disruption is to reduce the available amount of the gene product, including fully preventing any production of the gene product, or to express a gene product that lacks or having lower enzymatic activity as compared to the native or wild type gene product.

As used herein the term "deletion" or "knock-out" refers to a gene that is inoperative or knocked out.

The term "attenuated activity" when related to an enzyme refers to a decrease in the activity of the enzyme in its native compartment compared to a control or wildtype state.
Manipulations that result in attenuated activity of an enzyme include, but are not limited to, a missense mutation, a nonsense mutation, a deletion, a substitution, an insertion, addition of a targeting sequence, removal of a targeting sequence, or the like. Furthermore, attenuation of enzyme activity can be achieved also, or alternatively, by mutation or modification of control elements controlling the transcription of the gene encoding the enzyme, such as mutation or modification in a promoter, terminator and/or enhancement elements. A cell that contains modifications that result in attenuated enzyme activity will have a lower activity of the enzyme compared to a cell that does not contain such modifications. Attenuated activity of an enzyme may be achieved by encoding a nonfunctional gene product, e.g., a polypeptide having essentially no activity, e.g., less than about 10% or even 5% as compared to the activity of the wild type polypeptide.

A codon optimized version of a gene refers to an exogenous gene introduced into a cell and where the codons of the gene have been optimized with regard to the particular cell. Generally, not all tRNAs are expressed equally or at the same level across species. Codon optimization of a gene sequence thereby involves changing codons to match the most prevalent tRNAs, i.e. to change a codon recognized by a low prevalent tRNA with a synonymous codon recognized by a tRNA that is comparatively more prevalent in the given cell. This way the mRNA from the codon optimized gene will be more efficiently translated. The codon and the synonymous codon preferably encode the same amino acid.

As used herein, the term "allele" refers to a variant form of a given gene. This can include a mutated form of a gene where one or more of the amino acids encoded by the gene have been removed or substituted by a different amino acid.

As used herein, the terms "peptide", "polypeptide", and "protein" are used interchangeably to indicate to a polymer of amino acid residues. The terms "peptide", "polypeptide" and "protein" also includes modifications including, but not limited to, lipid attachment, glycosylation, glycosylation, sulfation, hydroxylation, \( \gamma \)-carboxylation of L-glutamic acid residues and ADP-ribosylation.

As used herein, the term "enzyme" is defined as a protein which catalyses a chemical or a biochemical reaction in a cell. Usually, according to the present invention, the nucleotide sequence encoding an enzyme is operably linked to a nucleotide sequence (promoter) that causes sufficient expression of the corresponding gene in the cell to confer to the cell the ability to produce spermidine.

As used herein, the term "open reading frame (ORF)" refers to a region of RNA or DNA encoding polypeptide, a peptide, or protein.
As used herein, the term "genome" encompasses both the plasmids and chromosomes in a host cell. For instance, encoding nucleic acids of the present disclosure which are introduced into host cells can be portion of the genome whether they are chromosomally integrated or plasmids-localized.

As used herein, the term "promoter" refers to a nucleic acid sequence which has functions to control the transcription of one or more genes, which is located upstream with respect to the direction of transcription of the transcription initiation site of the gene. Suitable promoters in this context include both constitutive and inducible natural promoters as well as engineered promoters, which are well known to the person skilled in the art.

Suitable promoters for use in eukaryotic host cells, such as yeast cells, may be the promoters of PDC, GPD1, TEF1, PGK1 and TDH. Other suitable promoters include the promoters of GAL1, GAL2, GAL10, GAL7, CUP1, HIS3, CYC1, ADH1, PGL, GAPDH, ADC1, URA3, TRP1, LEU2, TPI, AOX1 and ENO1.

Suitable promoters for the use in prokaryotic host cells include a bacteriophage T7 RNA polymerase promoter, a trp promoter, a lac operon promoter, the trc promoter, the lambda promoter and the like. Non limiting example of suitable strong promoter for the use in prokaryotic cells include lacUV5 promoter, T5, T7, Trc, Tac and the like. When Bacillus subtilis is chosen as the host cell, exemplary promoters include Pr promoter, Spol promoter, Tac promoter, and Lad promoter.

As used herein, the term "terminator" refers to a "transcription termination signal" if not otherwise noted. Terminators are sequences that hinder or stop transcription of a polymerase. As used herein, "recombinant eukaryotic cells" according to the present disclose is defined as cells which contain additional copies or copy of an endogenous nucleic acid sequence or are transformed or genetically modified with polypeptide or a nucleotide sequence that does not naturally occur in the eukaryotic cells. The wildtype eukaryotic cells are defined as the parental cells of the recombinant eukaryotic cells, as used herein.

As used herein, "recombinant prokaryotic cells" according to the present disclose is defined as cells which contain additional copies or copy of an endogenous nucleic acid sequence or are transformed or genetically modified with polypeptide or a nucleotide sequence that does not naturally occur in the prokaryotic cells. The wildtype prokaryotic cells are defined as the parental cells of the recombinant prokaryotic cells, as used herein.

As used herein, the terms "increase," "increases," "increased," "increasing," "enhance," "enhanced," "enhancing," and "enhancement" (and grammatical variations thereof) indicate an elevation of at least about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%,
35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, 150%, 200%, 300%, 400%, 500% or more, or any range therein, as compared to a control.

As used herein, the terms "reduce," "reduces," "reduced," "reduction," "diminish," "suppress," and "decrease" and similar terms mean a decrease of at least about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, 150%, 200%, 300%, 400%, 500% or more, or any range therein, as compared to a control.

A reduced expression of a gene as used herein involves a genetic modification that reduces the transcription of the gene, reduces the translation of the mRNA transcribed from the gene and/or reduces post-translational processing of the protein translated from the mRNA. Such genetic modification includes insertion(s), deletion(s), replacement(s) or mutation(s) applied to the control sequence, such as a promoter and enhancer, of the gene. For instance, the promoter of the gene could be replaced by a less active or inducible promoter to thereby result in a reduced transcription of the gene. Also a knock-out of the promoter would result in reduced, typically zero, expression of the gene.

As used herein, the term "portion" or "fragment" of a nucleotide sequence of the invention will be understood to mean a nucleotide sequence of reduced length relative to a reference nucleic acid or nucleotide sequence and comprising, consisting essentially of and/or consisting of a nucleotide sequence of contiguous nucleotides identical or almost identical, e.g. 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 98%, 99% identical, to the reference nucleic acid or nucleotide sequence. Such a nucleic acid fragment or portion according to the invention may be, where appropriate, included in a larger polynucleotide of which it is a constituent.

Different nucleic acids or proteins having homology are referred to herein as "homologues." The term homologue includes homologous sequences from the same and other species and orthologous sequences from the same and other species. "Homology" refers to the level of similarity between two or more nucleic acid and/or amino acid sequences in terms of percent of positional identity, i.e. sequence similarity or identity. Homology also refers to the concept of similar functional properties among different nucleic acids or proteins. Thus, the compositions and methods of the invention further comprise homologues to the nucleotide sequences and polypeptide sequences of this invention. "Orthologous," as used herein, refers to homologous nucleotide sequences and/or amino acid sequences in different species that arose from a common ancestral gene during speciation. A homologue of a nucleotide sequence of this invention has a substantial sequence identity, e.g. at least about 70%, 75%, 80%, 81%, 82%,
83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, and/or 100%, to said nucleotide sequence.

The term "overexpress," "overexpresses" or "overexpression" as used herein refers to higher levels of activity of a gene, e.g. transcription of the gene; higher levels of translation of mRNA into protein; and/or higher levels of production of a gene product, e.g. polypeptide, than would be in the cell in its native or control, e.g. not transformed with the particular heterologous or recombinant polypeptides being overexpressed, state. A typical example of an overexpressed gene is a gene under transcription control of another promoter as compared to the native promoter of the gene. Also, or alternatively, other changes in the control elements of a gene, such as enhancers, could be used to overexpress the particular gene. Furthermore, modifications that affect, i.e. increase, the translation of the mRNA transcribed from the gene could, alternatively or in addition, be used to achieve an overexpressed gene as used herein. These terms can also refer to an increase in the number of copies of a gene and/or an increase in the amount of mRNA and/or gene product in the cell. Overexpression can result in levels that are 25%, 50%, 100%, 200%, 500%, 1000%, 2000% or higher in the cell, or any range therein, as compared to control levels.

As used herein, the terms "exogenous" or "heterologous" when used with respect to a nucleic acid (RNA or DNA), protein or gene refer to a nucleic acid, protein or gene which occurs non-naturally as part of the cell, organism, genome, RNA or DNA sequence into which it is introduced, including non-naturally occurring multiple copies of a naturally occurring nucleotide sequence. Such an exogenous gene could be a gene from another species or strain, a modified, mutated or evolved version of a gene naturally occurring in the host cell or a chimeric version of a gene naturally occurring in the host cell or fusion genes. In these former cases, the modification, mutation or evolution causes a change in the nucleotide sequence of the gene to thereby obtain a modified, mutated or evolved gene with another nucleotide sequence as compared to the gene naturally occurring in the host cell. Evolved gene refers to genes encoding evolved genes and obtained by genetic modification, such as mutation or exposure to an evolutionary pressure, to derive a new gene with a different nucleotide sequence as compared to the wild type or native gene. A chimeric gene is formed through the combination of portions of one or more coding sequences to produce a new gene. These modifications are distinct from a fusion gene, which merges whole gene sequences into a single reading frame and often retain their original functions.

An "endogenous", "native" or "wild type" nucleic acid, nucleotide sequence, polypeptide or amino acid sequence refers to a naturally occurring or endogenous nucleic acid, nucleotide
sequence, polypeptide or amino acid sequence. Thus, for example, a "wild type mRNA" is an mRNA that is naturally occurring in or endogenous to the organism. A "homologous" nucleic acid sequence is a nucleotide sequence naturally associated with a host cell into which it is introduced.

As used herein, the term "modified", when it is used with respect to an organism, refers to a host organism that has been modified to increase production of spermidine, as compared with an otherwise identical host organism that has not been so modified. In principle, such "modification" in accordance with the present disclosure may comprise any physiological, genetic, chemical, or other modification that appropriately alters production of spermidine in a host organism as compared with such production in an otherwise identical organism which is not subject to the said modification. In most of the embodiments, however, the modification will comprise a genetic modification. In certain embodiments, as described herein, the modification comprises introducing genes into a host cell. In some embodiments, a modification comprises at least one physiological, chemical, genetic, or other modification; in other embodiments, a modification comprises more than one chemical, genetic, physiological, or other modification. In certain aspects where more than one modification is made use of, such modifications can include any combinations of physiological, genetic, chemical, or other modification (e.g., one or more genetic, chemical and/or physiological modification(s)). Genetic modifications which boost the activity of a polypeptide include, but are not limited to: introducing one or more copies of a gene encoding the polypeptide (which may distinguish from any gene already present in the host cell encoding a polypeptide having the same activity); altering a gene present in the cell to increase transcription or translation of the gene (e.g., altering, adding additional sequence to, replacement of one or more nucleotides, deleting sequence from, or swapping for example, regulatory, a promoter or other sequence); and altering the sequence (e.g. non-coding or coding) of a gene encoding the polypeptide to boost activity (e.g., by increasing enzyme activity, decrease feedback inhibition, targeting a specific subcellular location, boost mRNA stability, boost protein stability). Genetic modifications that reduce activity of a polypeptide include, but are not limited to: deleting a portion or all of a gene encoding the polypeptide; inserting a nucleic acid sequence which disrupts a gene encoding the polypeptide; changing a gene present in the cell to reduce transcription or translation of the gene or stability of the mRNA or polypeptide encoded by the gene (for example, by adding additional sequence to, altering, deleting sequence from, replacement of one or more nucleotides, or swapping for example, replacement of one or more nucleotides, a promoter, regulatory or other sequence).
The term "overproducing" is used herein in reference to the production of spermidine in a host cell and indicates that the host cell is producing more of spermidine by virtue of the introduction of nucleic acid sequences which encode different polypeptides involved in the host cell’s metabolic pathways or as a result of other modifications as compared with the unmodified host cell or wild-type cell.

As used herein, the term "flux", "metabolic flux" or "carbon flux" refers to the rate of turnover of molecules through a given reaction or a set of reactions. Flux in a metabolic pathway is regulated by the enzymes involved in the pathway. Pathways or reactions characterized by a state of increased flux compared to a control have an increased rate of generation of products from given substrates. Pathways or reactions characterized by a state of decreased flux compared to a control have a decreased rate of generation of products from given substrates. Flux towards products of interest can be increased by removing or decreasing competitive reactions or by increasing the activities of enzymes involved in generation of said products.

As used herein the term "putrescine biosynthetic pathway", "putrescine biosynthesis pathway" or "putrescine pathway" refers to enzymatic reactions that drive flux towards synthesis of putrescine from a carbon source, such as glucose, as well as competitive reactions and inhibitive reactions that reduce putrescine formation. Enzymatic reactions that drive flux towards the synthesis of putrescine include, but are not limited to, reactions that convert pyruvate to acetyl-CoA, acetyl-CoA to a-ketoglutarate, a-ketoglutarate to ornithine and ornithine to putrescine, see Figure 1. Competitive reactions that drain intermediates from this pathway include enzymatic reactions catalyzed by ornithine carbamoyltransferase [EC 2.1.3.3] and L-ornithine transaminase [EC 2.6.1.13]. Furthermore, inhibitive reactions include the production of ornithine decarboxylase antizyme (OAZ), which is an inhibitor of ornithine decarboxylase that otherwise catalyzes the decarboxylation of ornithine into putrescine. Increase in putrescine biosynthesis can be achieved by overexpression of any of the enzymatic steps that drive flux towards putrescine and/or downregulation of competitive reactions and inhibitive reactions.

As used herein the term "spermidine biosynthetic pathway", "spermidine biosynthesis pathway" or "spermidine pathway" refers to the combination of reactions involved in the putrescine biosynthetic pathway with enzymes that convert putrescine into spermidine. Non-limiting examples of enzymes involved in these reactions are spermidine synthase (SPDS) [EC 2.5.1.16], S-adenosylmethionine decarboxylase [EC 4.1.1.50], carboxy(nor)spermidine dehydrogenase (CASDH/CANSDH) [EC 1.5.1.43] and carboxy(nor)spermidine decarboxylase
(CASDC/CANSDC) [EC 4.1.1.96]. This term does not encompass reactions involved in S-adenosyl methionine (SAM) formation.

As used herein the term "enzymes involved in the synthesis of S-adenosylmethionine (SAM)", "S-adenosylmethionine pathway" or "S-adenosylmethionine biosynthetic pathway" refers to enzymatic reactions required for the synthesis of S-adenosylmethionine from a carbon source, such as glucose. This includes but is not limited to reactions involved in the conversion of pyruvate to oxaloacetate, oxaloacetate to aspartate, aspartate to L-Aspartate-4-semialdehyde, L-Aspartate-4-semialdehyde to methionine and methionine to S-adenosylmethionine. In addition, this includes reactions involved in the methionine salvage pathway, reactions involved in folate synthesis and conversion to 5-methyltetrahydrofolate, and reactions involved in sulfate assimilation (Figure 2). This term does not encompass reactions involved in decarboxylation of SAM to S-adenosylmethioninamine (dSAM).

As used herein the term "vector" is defined as a linear or circular DNA molecule comprising a polynucleotide encoding a polypeptide of the invention, and which is operably linked to additional nucleotides that ensure its expression.

"Introducing" in the context of a yeast cell means contacting a nucleic acid molecule with the cell in such a manner that the nucleic acid molecule gains access to the interior of the cell. Accordingly, polynucleotides and/or nucleic acid molecules can be introduced yeast cells in a single transformation event, in separate transformation events. Thus, the term "transformation" as used herein refers to the introduction of a heterologous nucleic acid into a cell. Transformation of a yeast cell can be stable or transient.

"Transient transformation" in the context of a polynucleotide means that a polynucleotide is introduced into the cell and does not integrate into the genome of the cell.

By "stably introducing" or "stably introduced" in the context of a polynucleotide introduced into a cell, it is intended that the introduced polynucleotide is stably incorporated into the genome of the cell, and thus the cell is stably transformed with the polynucleotide.

"Stable transformation" or "stably transformed" as used herein means that a nucleic acid molecule is introduced into a cell and integrates into the genome of the cell. As such, the integrated nucleic acid molecule is capable of being inherited by the progeny thereof, more particularly, by the progeny of multiple successive generations. Stable transformation as used herein can also refer to a nucleic acid molecule that is maintained extrachromosomally, for example, as a minichromosome.

Transient transformation may be detected by, for example, an enzyme-linked immunosorbent assay (ELISA) or Western blot, which can detect the presence of a peptide or
polypeptide encoded by one or more nucleic acid molecules introduced into an organism. Stable transformation of a cell can be detected by, for example, a Southern blot hybridization assay of genomic DNA of the cell with nucleic acid sequences which specifically hybridize with a nucleotide sequence of a nucleic acid molecule introduced into an organism (e.g., a yeast).

Stable transformation of a cell can be detected by, for example, a Northern blot hybridization assay of RNA of the cell with nucleic acid sequences which specifically hybridize with a nucleotide sequence of a nucleic acid molecule introduced into a yeast or other organism. Stable transformation of a cell can also be detected by, e.g., a polymerase chain reaction (PCR) or other amplification reaction as are well known in the art, employing specific primer sequences that hybridize with target sequence(s) of a nucleic acid molecule, resulting in amplification of the target sequence(s), which can be detected according to standard methods. Transformation can also be detected by direct sequencing and/or hybridization protocols well known in the art.

Embodiments of the present invention also encompass variants of the polypeptides as defined herein. As used herein, a "variant" means a polypeptide in which the amino acid sequence differs from the base sequence from which it is derived in that one or more amino acids within the sequence are substituted for other amino acids. For example, a variant of SEQ ID NO: 1 may have an amino acid sequence at least about 50% identical to SEQ ID NO: 1, for example, at least about 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or about 100% identical. The variants and/or fragments are functional variants/fragments in that the variant sequence has similar or identical functional enzyme activity characteristics to the enzyme having the non-variant amino acid sequence specified herein (and this is the meaning of the term "functional variant" as used throughout this specification).

A "functional variant" or "functional fragment" of any of the presented amino acid sequences, therefore, is any amino acid sequence which remains within the same enzyme category (i.e., has the same EC number) as the non-variant sequences. Methods of determining whether an enzyme falls within a particular category are well known to the skilled person, who can determine the enzyme category without use of inventive skill. Suitable methods may, for example, be obtained from the International Union of Biochemistry and Molecular Biology.

Amino acid substitutions may be regarded as "conservative" where an amino acid is replaced with a different amino acid with broadly similar properties. Non-conservative substitutions are where amino acids are replaced with amino acids of a different type.

By "conservative substitution" is meant the substitution of an amino acid by another amino acid of the same class, in which the classes are defined as follows:
Class Amino Acid Examples

Uncharged polar: G, S, T, C, Y, N, Q
Acidic: D, E
Basic: K, R, H.

As it is well known to those skilled in the art, altering the primary structure of a polypeptide by a conservative substitution may not significantly alter the activity of that polypeptide because the side-chain of the amino acid which is inserted into the sequence may be able to form similar bonds and contacts as the side chain of the amino acid which has been substituted out. This is so even when the substitution is in a region which is critical in determining the polypeptide's conformation.

In embodiments of the present invention, non-conservative substitutions are possible provided that these do not interrupt the enzyme activities of the polypeptides, as defined elsewhere herein. The substituted versions of the enzymes must retain characteristics such that they remain in the same enzyme class as the non-substituted enzyme, as determined using the NC-IUBMB nomenclature discussed above.

Broadly speaking, fewer non-conservative substitutions than conservative substitutions will be possible without altering the biological activity of the polypeptides. Determination of the effect of any substitution (and, indeed, of any amino acid deletion or insertion) is wholly within the routine capabilities of the skilled person, who can readily determine whether a variant polypeptide retains the enzyme activity according to aspects of the invention. For example, when determining whether a variant of the polypeptide falls within the scope of the invention (i.e., is a "functional variant or fragment" as defined above), the skilled person will determine whether the variant or fragment retains the substrate converting enzyme activity as defined with reference to the NC-IUBMB nomenclature mentioned elsewhere herein. All such variants are within the scope of the invention.

Using the standard genetic code, further nucleic acid sequences encoding the polypeptides may readily be conceived and manufactured by the skilled person, in addition to those disclosed herein. The nucleic acid sequence may be DNA or RNA, and where it is a DNA molecule, it may for example comprise a cDNA or genomic DNA. The nucleic acid may be contained within an expression vector, as described elsewhere herein.

Embodiments of the invention, therefore, encompass variant nucleic acid sequences encoding the polypeptides contemplated by embodiments of the invention. The term "variant" in relation to a nucleic acid sequence means any substitution of, variation of, modification of,
replacement of, deletion of, or addition of one or more nucleotide(s) from or to a polynucleotide sequence, providing the resultant polypeptide sequence encoded by the polynucleotide exhibits at least the same or similar enzymatic properties as the polypeptide encoded by the basic sequence. The term includes allelic variants and also includes a polynucleotide ("probe sequence") which substantially hybridizes to the polynucleotide sequence of embodiments of the present invention. Such hybridization may occur at or between low and high stringency conditions. In general terms, low stringency conditions can be defined as hybridization in which the washing step takes place in a 0.330-0.825 M NaCl buffer solution at a temperature of about 40-48° C below the calculated or actual melting temperature (Tm) of the probe sequence (for example, about ambient laboratory temperature to about 55° C), while high stringency conditions involve a wash in a 0.0165-0.0330 M NaCl buffer solution at a temperature of about 5-10° C below the calculated or actual Tm of the probe sequence (for example, about 65° C).

The buffer solution may, for example, be SSC buffer (0.15M NaCl and 0.015M tri-sodium citrate), with the low stringency wash taking place in 3×SSC buffer and the high stringency wash taking place in 0.1×SSC buffer. Steps involved in hybridization of nucleic acid sequences have been described for example in Molecular Cloning, a laboratory manual [second edition] Sambrook et al. Cold Spring Harbor Laboratory, 1989, for example in Section 11 "Synthetic Oligonucleotide Probes" thereof (herein incorporated by reference).

Preferably, nucleic acid sequence variants have about 55% or more of the nucleotides in common with the nucleic acid sequence of embodiments of the present invention, more preferably at least 60%, 65%, 70%, 80%, 85%, or even 90%, 95%, 98% or 99% or greater sequence identity.

Variant nucleic acids of the invention may be codon-optimized for expression in a particular host cell.

As used herein, "sequence identity" refers to sequence similarity between two nucleotide sequences or two peptide or protein sequences. The similarity is determined by sequence alignment to determine the structural and/or functional relationships between the sequences.

Sequence identity between amino acid sequences can be determined by comparing an alignment of the sequences using the Needleman-Wunsch Global Sequence Alignment Tool available from the National Center for Biotechnology Information (NCBI), Bethesda, Md., USA, for example via http://blast.ncbi.nlm.nih.gov/Blast.cgi, using default parameter settings (for protein alignment, Gap costs Existence: 11 Extension: 1). Sequence comparisons and percentage identities mentioned in this specification have been determined using this software. When comparing the level of sequence identity to, for example, SEQ ID NO:1, this, preferably
should be done relative to the whole length of SEQ ID NO:1 (i.e., a global alignment method is used), to avoid short regions of high identity overlap resulting in a high overall assessment of identity. For example, a short polypeptide fragment having, for example, five amino acids might have a 100% identical sequence to a five amino acid region within the whole of SEQ ID NO:1, but this does not provide a 100% amino acid identity unless the fragment forms part of a longer sequence which also has identical amino acids at other positions equivalent to positions in SEQ ID NO:1. When an equivalent position in the compared sequences is occupied by the same amino acid, then the molecules are identical at that position. Scoring an alignment as a percentage of identity is a function of the number of identical amino acids at positions shared by the compared sequences. When comparing sequences, optimal alignments may require gaps to be introduced into one or more of the sequences, to take into consideration possible insertions and deletions in the sequences. Sequence comparison methods may employ gap penalties so that, for the same number of identical molecules in sequences being compared, a sequence alignment with as few gaps as possible, reflecting higher relatedness between the two compared sequences, will achieve a higher score than one with many gaps. Calculation of maximum percent identity involves the production of an optimal alignment, taking into consideration gap penalties. As mentioned above, the percentage sequence identity may be determined using the Needleman-Wunsch Global Sequence Alignment tool, using default parameter settings. The Needleman-Wunsch algorithm was published in J. Mol. Biol. (1970) vol. 48:443-53.

An aspect of the embodiments relates to a microbial cell capable of producing spermidine. The microbial cell is genetically modified for enhanced putrescine biosynthesis. The microbial cell is also genetically modified for enhanced S-adenosylmethionine (SAM) biosynthesis.

Thus, an aspect of the embodiments relates to a microbial cell capable of producing spermidine. The microbial cell is genetically modified for overexpression of at least one enzyme selected from a group consisting of S-adenosylmethionine decarboxylase [EC 4.1.1.50] and spermidine synthase [EC 2.5.1.16]. The microbial cell is also genetically modified for enhanced SAM biosynthesis.

The present embodiments relates to a microbial cell having capacity of producing high levels of spermidine. In a general embodiment, the microbial cell is genetically modified for enhanced conversion of putrescine into spermidine and for enhanced SAM biosynthesis. Further gene modifications according to various embodiments as disclosed herein include down-regulation or attenuation of specially selected genes, wherein the genes encode enzymes involved in the spermidine consumption and/or degradation pathways. Further spermidine production ability is improved by down-regulation, attenuation, deletion or over-expression of
specially selected genes, wherein the genes encode enzymes and/or proteins involved in the spermidine synthesis pathway, 5’-methylthioadenosine (MTA) cycle, L-ornithine synthesis and/or L-glutamate synthesis pathways. In further embodiments, spermidine overproduction is obtained by combining the above modifications with modifications in the pathway leading to biosynthesis of the propylamine donor acting as a cofactor for spermidine formation. Such modifications can include overexpression of endogenous or heterologous genes encoding enzymatic steps responsible for production of the propylamine donor and down-regulation of competing and/or inhibitory reactions. Further spermidine production ability is improved by overexpression of genes encoding polyamine export proteins, down-regulation of genes encoding polyamine uptake proteins and modification of expression of various proteins associated with polyamine toxicity, giving a very effective overall process.

In the following, various embodiments of the present invention will be described in more detail.

In an embodiment, the microbial cell is an eukaryotic cell selected from a group consisting of Saccharomyces, Kluyveromyces, Zygosaccharomyces, Candida, Hansenula, Torulopsis, Kloekerca, Pichia, Schizosaccharomyces, Trigonopsis, Brettanomyces, Debaromyces, Nadsonia, Lipomyces, Cryptococcus, Aureobasidium, Trichosporon, Lipomyces, Rhodotorula, Yarrowia, Rhodosporidium, Phaffia, Schwanniomyces, Aspergillus and Ashbya. In a particular embodiment, the fungal cell can be Saccharomyces cerevisiae, Saccharomyces boulardii, Zygosaccharomyces bailii, Kluyveromyces lactis, Rhodosporidium toruloides, Yarrowia lipolytica, Schizosaccharomyces pombe, Pichia pastoris, Hansenula anomala, Candida sphaerica, or Schizosaccharomyces malidevorans. Saccharomyces cerevisiae is a preferred yeast species.

In certain embodiments the microbial cell is a prokaryotic cell, such as a bacterial cell or archaeal cell. The bacterial cell could be gram positive or gram negative bacteria. The bacteria may also be photosynthetic bacteria, e.g. cyanobacteria.

In an embodiment, the microbial cell is a prokaryotic cell selected from a group consisting of Neisseria, Spirillum, Pasteurella, Brucella, Yersinia, Francisella, Haemophilus, Bordetella, Escherichia, Salmonella, Shigella, Klebsiella, Proteus, Vibrio, Pseudomonas, Bacteroides, Acetobacter, Aerobacter, Agrobacterium, Azotobacter, Spirilla, Serratia, Vibrio, Rhizobium, Chlamydia, Rickettsia, Treponema, Fusobacterium, Actinomyces, Bacillus, Clostridium, Corynebacterium, Erysipelothrix, Lactobacillus, Listeria, Mycobacterium, Myxococcus, Nocardia, Staphylococcus, Streptococcus, and Streptomyces. Examples of prokaryotic cells that can be used include Escherichia coli, Bacillus subtilis and Corynebacterium glutamicum.
The objective of the invention disclosed is to provide for microbial cells engineered for increased spermidine production. Previous efforts to increase spermidine production in microbial cells have reported that modifications to the cell's ornithine and putrescine production pathways resulted in improved spermidine production, suggesting that those precursors were limiting. While limitation in putrescine might be strain specific, to ensure that putrescine was not limiting when evaluating our invention and to achieve high titers of spermidine, we chose to test SAM biosynthesis pathways in a strain that has previously been engineered to produce higher levels of putrescine. This strain included modifications to increase flux toward ornithine, a precursor to putrescine and putrescine, including downregulation of ornithine carbamoyltransferase, deletion of ornithine aminotransferase, overexpression of the mitochondrial amino acid transporter AGCI, overexpression of the mitochondrial ornithine importer ORT1, overexpression of the NADP+-dependent glutamate dehydrogenase, deletion of the gene encoding ornithine decarboxylase (ODC) antizyme and overexpression of ornithine decarboxylase. While in previous studies we have found that these modifications improved ornithine and putrescine production in the used background strain background, other modifications could instead or also be used to achieve similar effects (as has been shown by [5] and others). Therefore, the inventive modifications presented herein improve spermidine production even in a strain lacking the modifications above, for example microbial strains that have different modifications to increase flux towards putrescine, strains that can naturally produce high levels of putrescine, or strains that are fed with putrescine.

In an embodiment, the ability of the microbial cell to convert putrescine to spermidine is increased. This can be achieved by increasing the activity of spermidine synthase (SPDS) [EC 2.5.1.16]. For instance, the increased activity of SPDS could be achieved by overexpressing the encoding gene of SPDS. The SPDS encoding gene can be from any known species, for instance *Triticum aestivum*. In an embodiment, the SPDS from *Triticum aestivum* (SEQ ID NO: 19) is introduced into *S. cerevisiae* or *E. coli*. Other heterologous SPDS, e.g. from *Streptomyces spectabilis, Oryza sativa, Glycine max, Citrus sinensis, Homo sapiens, Rattus norvegicus, Thermotoga maritima, Caenorhabditis elegans*, could also be overexpressed in a eukaryotic or prokaryotic cell. Alternatively, or in addition, the endogenous SPDS could be overexpressed. The increased activity of spermidine synthase could also be combined with increased activity of S-adenosylmethionine decarboxylase [EC 4.1.1.50]. This could be achieved by overexpressing the gene encoding S-adenosylmethionine decarboxylase. Alternatively, such overexpression of the gene encoding S-adenosylmethionine decarboxylase could be used instead of increased activity of spermidine synthase. For example, the
endogenous S-adenosylmethionine decarboxylase gene (SPE2; SEQ ID NO: 21) could be overexpressed in *S. cerevisiae*. Alternatively, the endogenous speD gene could be overexpressed in *E. coli*. Other heterologous S-adenosylmethionine decarboxylase, e.g. from *Streptomyces griseochromogenes*, *Zea mays*, *Streptomyces spectabilis*, *Oryza sativa*, *Glycine max*, *Citrus sinensis*, *Homo sapiens*, *Rattus norvegicus*, *Mus musculus*, *Thermotoga maritima*, *Trypanosoma cruzi*, *Trypanosoma brucei*, *Caenorhabditis elegans*, could also be overexpressed in a eukaryotic or prokaryotic cell.

Thus, in an embodiment, the microbial cell is genetically modified for overexpression of at least one enzyme selected from a group consisting of S-adenosylmethionine decarboxylase [EC 4.1.1.50] and spermidine synthase [EC 2.5.1.16]. As is shown in Figure 1, S-adenosylmethionine decarboxylase catalyzes the synthesis of S-adenosylmethionamine from SAM. Spermidine synthase uses this S-adenosylmethionamine together with putrescine to produce the desired product spermidine.

In an embodiment, conversion of L-ornithine into putrescine is increased. This can be achieved by increasing the activity of Ornithine decarboxylase (ODC) [EC 4.1.1.17]. In an embodiment, the activity of ODC can be increased by overexpression of the native gene encoding ODC. For example, *SPE1* (SEQ ID NO: 20) could be overexpressed in *S. cerevisiae*, or *speC* (Genbank accession: NP_417440) could be overexpressed in *E. coli*. Alternatively, heterologous ODC could be expressed, e.g. from *Escherichia coli*, *Streptomyces spectabilis*, *Triticum aestivum*, *Oryza sativa*, *Glycine max*, *Citrus sinensis*, *Homo sapiens*, *Rattus norvegicus*, *Nicotiana glutinosa*, *Trypanosoma brucei*, *Neurospora crassa*, *Entamoeba histolytica*, *Physarum polycephalum*, *Mus musculus*, *Plasmodium falciparum*.

In addition, or alternatively, conversion of ornithine to putrescine could also be increased by reducing the activity of the ornithine decarboxylase antizyme (OAZ). OAZ is a regulator of ODC which binds to ODC to stimulate ubiquitin-independent degradation by the proteasome. Therefore, reduction of the activity of OAZ could facilitate increased ODC levels. Reduction of OAZ activity could be achieved, for example by disrupting or downregulating the native gene encoding OAZ. For example, in an embodiment, the native gene encoding OAZ (*OAZ1*; SEQ ID NO: 23) is disrupted in *S. cerevisiae*.

Furthermore, spermidine levels could be increased by increasing the L-ornithine concentration in the cell. This can be achieved, for example by decreasing the activity of ornithine-utilizing reactions in the cell. For example, the activity of Ornithine carbamoyltransferase [EC 2.1.3.3], which converts ornithine to citrulline can be decreased. This can be achieved by down-regulating the expression of the native gene encoding Ornithine
carbamoyltransferase. For example, in an embodiment, the native ARG3 gene encoding Ornithine carbamoyltransferase (SEQ ID NO: 24) could be down-regulated in S. cerevisiae. In another embodiment, the activity of L-ornithine transaminase [EC 2.6.1.13], which catalyzes the conversion of ornithine to L-glutamate γ-semialdehyde, is decreased. This can be achieved, for example, by disrupting the endogenous gene encoding L-ornithine transaminase activity, e.g. CAR2 in S. cerevisiae (SEQ ID NO: 117).

Other strategies for increased production of cytosolic L-ornithine, could include increase in the flux through the L-ornithine production pathway from glutamate, for example by increasing the activities of Acetylglutamate synthase [EC 2.3.1.1], Acetylglutamate kinase [EC 2.7.2.8] and N-acetyl-gamma-glutamyl-phosphate reductase [EC 1.2.1.38], Acetylmithine aminotransferase [EC 2.6.1.1], acetylmithine deacetylase [EC 3.5.1.16] and/or ornithine acetyltransferase [EC 2.3.1.35]. In a preferred embodiment, the activities of these enzymes are increased in the cytosol. This could be achieved, for example, by expression/overexpression of bacterial L-ornithine biosynthetic genes encoding these activities, e.g. argA (SEQ ID NO: 103-104), argB (SEQ ID NO: 105-106), argC (SEQ ID NO: 107-108), argD (SEQ ID NO: 109-110) argJ/argE (SEQ ID NO: 111-112), from i.e. coli or c. glutamicum, in a cell, or the targeting of the endogenous S. cerevisiae ornithine biosynthetic genes, e.g. ARG2 (SEQ ID NO: 113), ARG5, 6 (SEQ ID NO: 114), ARG8 (SEQ ID NO: 115) and/or ARG7 (SEQ ID NO: 116), to the cytosol.

Thus, in an embodiment, the microbial cell is genetically modified for overexpression of at least one enzyme selected from a group consisting of ornithine decarboxylase (ODC) [EC 4.1.1.17]; N-acetylglutamate synthase (NAGS) [EC 2.3.1.1], also referred to as amino-acid-N-acetyltransferase; acetylglutamate kinase [EC 2.7.2.8]; N-acetyl-gamma-glutamyl-phosphate reductase [EC 1.2.1.38]; acetylmithine aminotransferase [EC 2.6.1.1], also referred to as acetylmithine transaminase; acetylmithine deacetylase [EC 3.5.1.16] and ornithine acetyltransferase [EC 2.3.1.35], also referred to as glutamate N-acetyltransferase.

As is more clearly shown in Figure 1, the above mentioned enzymes are involved in the putrescine biosynthesis pathway involving synthesis of putrescine from a carbon source, such as glucose. In more detail, the above mentioned enzymes are involved in the synthesis of putrescine from a-ketoglutarate, which in turn is the output of the tricarboxylic acid (TCA) cycle.

In an embodiment, the microbial cell is genetically modified for attenuated activity of ornithine decarboxylase antizyme (OAZ); L-ornithine transaminase [EC 2.6.1.13], also referred to as ornithine aminotransferase; and/or ornithine carbamoyltransferase (OTC) [EC 2.1.3.3] or
deletion or disruption of at least one gene selected from a group consisting of a gene encoding OAZ, a gene encoding L-ornithine transaminase and a gene encoding OTC.

OTC and L-ornithine transaminase are enzymes involved in the conversion of ornithine into citrulline and glutamate γ-semialdehyde, see Figure 1. Hence, these two enzymes are involved in pathways draining the intermediate ornithine and thereby competes with ODC in using ornithine as substrate. Attenuated activity of these enzymes or deletion or disruption of the genes encoding these enzymes would thereby result in higher amounts of ornithine for ODC and thereby enhanced production of putrescine in the microbial cell.

OAZ is an inhibitor of ODC and thereby reduces the activity of ODC in synthesizing putrescine from ornithine.

In an embodiment, any of the above modifications could be further combined with a pathway leading to increased levels of SAM, or this pathway may be used independent of the above modifications. SAM can then be decarboxylated by S-adenosylmethionine decarboxylase to dSAM, which serves as the propylamine donor for spermidine formation. For example, in a preferred embodiment, increased biosynthesis of SAM can be achieved by increasing the activity of S-adenosylmethionine synthetase (MAT) [EC 2.5.1.6], which catalyzes the transfer of the adenosyl group of ATP to the sulfur atom of methionine to form SAM. For instance, the increased activity of MAT could be achieved by overexpressing the encoding gene of MAT. The MAT encoding gene can be from any known species, for instance S. cerevisiae. In one preferred embodiment the overexpression of the native SAM2 gene which encodes MAT is achieved in S. cerevisiae. The native SAM1 gene could also be overexpressed. Alternatively, the endogenous E. coli metK gene which encodes MAT could be overexpressed in E. coli or S. cerevisiae. In addition, heterologous MAT from other eukaryotic or prokaryotic sources can be expressed in a cell, e.g. from Streptomyces spectabilis, Triticum aestivum, Oryza sativa, Glycine max, Citrus sinensis, Homo sapiens, Rattus norvegicus, Cryptosporidium parvum, Leishmania donovani, Leishmania infantum, Sus scrofa. For example, in an embodiment, heterologous MAT from Leishmania infantum (SEQ ID NO: 25) is overexpressed in a microbial cell. In another embodiment, a mutated allele of MAT is expressed. For example, MAT from Streptomyces spectabilis wherein lysine 18, leucine 31, isoleucine 65 and/or aspartic acid 341 are mutated into arginine, proline, valine and/or glycine, respectively (K18R, L31P, 165V, D341G) (SEQ ID NO: 1) can be expressed in a microbial cell. Similarly, MAT from S. cerevisiae with a mutation in lysine 21, preferably into arginine (K21R) (SEQ ID NO: 2), could also be expressed.
Thus, in an embodiment, the microbial cell is genetically modified for overexpression of S-adenosylmethionine synthetase (MAT) [EC 2.5.1.6], also referred to as methionine adenosyltransferase.

MAT is involved in the synthesis of SAM from methionine as shown in Figure 2. In an embodiment increased S-adenosylmethionine (SAM) levels are increased by increasing the activity of Methylene tetrahydrofolate reductase (MTHFR) [EC 1.5.1.20], which catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate used to methylate homocysteine in methionine biosynthesis. For instance, the increased activity of MTHFR could be achieved by overexpressing the encoding gene of MTHFR. The MTHFR encoding gene can be from any known species, for instance *S. cerevisiae*. In one preferred embodiment the overexpression of the native *MET13* (SEQ ID NO: 26) and/or *MET12* genes which encode MTHFR is achieved in the modified *S. cerevisiae*. In another embodiment, a chimeric MTHFR which is not subject to repression due to accumulation of high levels of SAM can be expressed in a microbial cell. Such a chimeric MTHFR could, for example be comprised of the yeast Metl3p N-terminal catalytic domain and the *Arabidopsis thaliana* MTHFR (AtMTHFR-1) C-terminal regulatory domain (SEQ ID NO: 3). In addition, heterologous MTHFR from other eukaryotic or prokaryotic sources can be expressed/overexpressed in a microbial cell, e.g. from *Escherichia coli*, *Streptomyces spectabilis*, *Triticum aestivum*, *Oryza sativa*, *Glycine max*, *Citrus sinesis*, *Arabidopsis sp.*, *Homo sapiens*, *Sus scrofa*, *Rattus norvegicus* or *Clostridium formicaceticum*.

Thus, in an embodiment, the microbial cell is genetically modified for overexpression of methylenetetrahydrofolate reductase (MTHFR) [EC 1.5.1.20].

5,10-Methylenetetrahydrofolate (N5,N10-methylenetetrahydrofolate; 5,10-CH$_2$-THF) is the substrate used by MTHFR to generate 5-methyltetrahydrofolate (5-MTHF, or levomefolinic acid), which is in turn a substrate in the production of methionine, see Figure 2.

In a particular embodiment, the microbial cell is genetically modified for overexpression of a chimeric MTHFR comprising the yeast Metl3p N-terminal catalytic domain and the *Arabidopsis thaliana* MTHFR C-terminal regulatory domain.

In another embodiment, levels of S-adenosylmethionine (SAM) are increased by increasing folate levels. This can be achieved, for example, by folate supplementation. This can also be realized by increasing flux through the folate synthesis pathway, for example by increasing the activities of enzymes in this pathway. For example, the activities of 3-deoxyarabino-heptulosonate-7-phosphate synthase [DAHPS] [EC 2.5.1.54], Pentafunctional AROM
protein [EC 4.2.3.4, 4.2.1.10, 1.1.1.25, 2.7.1.71, 2.5.1.19], Chorismate synthase [EC 4.2.3.5], Para-aminobenzoate (PABA) synthase [EC 6.3.5.8], Aminodeoxy chorismate lyase [EC 4.1.3.38], Folic acid synthesis protein [EC 4.1.2.25, 2.7.6.3, 2.5.1.15], GTP-cyclohydrolase I [EC 3.5.4.16], Dihydrofolate synthetase [EC 6.3.2.12], Dihydrofolate reductase [EC 1.5.1.3], Serine hydroxymethyltransferase [EC 2.1.2.1], or Glycine cleavage complex. This can be achieved by overexpressing the endogenous genes encoding for these activities. For example, the endogenous genes AR03, AR04, ARO1, AR02, ABZ1, ABZ2, FOL1, FOL2, FOL3, DFR1, SHM2, SHM1, LPD1, GCV2, GCV1 or GCV3 could be overexpressed in a S. cerevisiae cell.

In another embodiment, S-adenosylmethionine (SAM) levels are increased by increasing the flux through the purine biosynthesis pathway, which can supply cofactors, such as ATP and GTP. This can be achieved by increasing the activities of enzymes in the pathways. For example, the activity of PRPP amidotransferase [EC 2.4.2.14], which catalyses the first committed step in purine biosynthesis can be increased. This can be achieved by overexpression of the endogenous gene encoding for this enzyme. For example, the endogenous ADE4 gene (SEQ ID NO: 27) can be overexpressed in S. cerevisiae. Alternatively, the endogenous purF gene (SEQ ID NO: 28) could be overexpressed in E.coli. Alternatively, a mutated allele of the gene encoding PRPP amidotransferase that is not subject to feedback regulation can be expressed. For example, ADE4 from Ashbya gossypii where aspartic acid310, lysine333 and/or alanine417 are replaced, preferably by valine, glutamine and tryptophan, respectively (D310V, K333Q, A417W, SEQ ID NO: 4) could be expressed in a eukaryotic or prokaryotic cell. Alternatively, mutated PurF from Bacillus subtilis, where aspartic acid 293, lysine 316 and/or Serine 400 are replaced, preferably by valine, glutamine and tryptophan (D293, K316, S400W, SEQ ID NO: 5), can be expressed in a microbial cell.

Thus, in an embodiment, the microbial cell is genetically modified for overexpression of phosphoribosylpyrophosphate (PRPP) 5-amidotransferase [EC 2.4.2.14], also referred to as amidophosphoribosyltransferase. The overexpression of this enzyme would generally lead to increase in ATP and/or GTP levels in the microbial cell. The increased levels of ATP and/or GRP can in turn be used, for instance, in the SAM biosynthesis in the microbial cell.

In a particular embodiment, the microbial cell comprises the Ashbya gossypii ADE4 gene with at least one mutation in an amino acid residue selected from a group consisting of D310V, K333Q and A417W. In another particular embodiment, the microbial cell comprises the Bacillus subtilis PurF gene with at least one mutation selected from a group consisting of D293V, K316Q and S400W.
In another embodiment, S-adenosylmethionine (SAM) levels are increased by recycling of the 5'-methylthioadenosine formed from polyamine synthesis via the methionine salvage pathway. For example, the flux through the methionine salvage pathway can be increased. This can be done by increasing the activities of enzymes in this pathway, such as methylthioadenosine phosphorylase (MTAP) [EC 2.4.2.28], 5'-methylthioribose-1-phosphate isomerase (MRI) [EC 5.3.1.23], 5'-methylthioribulose-1-phosphate dehydratase (MDE) [EC 4.2.1.109], 2,3-dioxomethiopentane-1-phosphate enolase/phosphatase [EC 3.1.3.77], acireductone dioxygenase [EC 1.13.1.54] or branched-chain amino acid transaminase (BAT) [EC 2.6.1.5, EC 2.6.1.57, EC 2.6.1.42]. For instance, the increased activity of MTAP could be achieved by overexpressing the encoding gene of MTAP. The MTAP encoding gene can be from any known species, for instance *S. cerevisiae*. In one preferred embodiment the overexpression of the native MEW gene (SEQ ID NO: 17) which encodes MTAP is achieved in the modified *S. cerevisiae*. Alternatively, the *S. cerevisiae* MEU1 gene could also be expressed in *E. coli*. In another preferred embodiment, the overexpression of the native BAT2 gene (SEQ ID NO: 18), which encodes Branched-chain Amino acid Transaminase could be achieved in the modified *S. cerevisiae*. In addition, the genes MRU (SEQ ID NO: 118), MDE1 (SEQ ID NO: 119), UTR4 (SEQ ID NO: 120), ADII (SEQ ID NO: 121), AR08 (SEQ ID NO: 122), AR09 (SEQ ID NO: 123) or BAT1 (SEQ ID NO: 124), which encode 5-methylthioribose-1-phosphate isomerase, methylthioribulose-1-phosphate dehydratase, 2,3-dioxomethiopentane-1-phosphate enolase/phosphatase, acireductone dioxygenase, aromatic aminotransferase I, aromatic aminotransferase II and mitochondrial branched-chain amino acid (BCAA) aminotransferase (respectively), could also be overexpressed. This pathway could also be overexpressed in a prokaryotic host. For example, the enzymatic steps in the pathway could be introduced into *E. coli*. This could potentially be combined with the endogenous tyrB gene, which encodes BAT in *E. coli*. In addition, heterologous genes from other eukaryotic or prokaryotic sources can be expressed, e.g. from *Streptomyces spectabilis, Triticum aestivum, Oryza sativa, Glycine max, Citrus sinesis, Homo sapiens, Arabidopsis thaliana, Bos taurus, Pyrococcus furiosus, Sulfolobus solfataricus*.

Thus, in an embodiment, the microbial cell is genetically modified for overexpression of at least one enzyme in the methionine salvage pathway selected from a group consisting of methylthioadenosine phosphorylase (MTAP) [EC 2.4.2.28], also referred to as S-methyl-5'-thioadenosine phosphorylase; 5-methylthioribose-1-phosphate isomerase (MRI) [EC 5.3.1.23], also referred to as S-methyl-5-thioribose-1-phosphate isomerase; methylthioribulose-1-phosphate dehydratase (MDE) [EC 4.2.1.109]; 2,3-dioxomethiopentane-1-phosphate
enolase/phosphatase [EC 3.1.3.77], also referred to as acireductone synthase; acireductone dioxygenase [EC 1.13.1.54]; and branched-chain amino acid transaminase (BAT) [EC 2.6.1.42], also referred to as tyrosine transaminase [EC 2.6.1.5], and aromatic-amino-acid transaminase [EC 2.6.1.57]. In a particular embodiment, the microbial cell is genetically modified for overexpression of MTAP and/or BAT.

As is shown in Figure 2, the methionine salvage pathway comprises the synthesis of methionine from 5'-methylthioadenosine (MTA) via 5'-methylthioribose (MRP) and 4-methylthio-2-oxobutyrate (MOB). The salvaged methionine can then be used as substrate for production of SAM.

In another embodiment, S-adenosylmethionine (SAM) levels are increased by increasing intracellular methionine availability for SAM production. This can be achieved, for example, by methionine supplementation. Alternatively, intracellular methionine can be derived from the intermediate L-Aspartate-4-semialdehyde. For example, the flux through the pathway responsible for methionine formation from L-Aspartate-4-semialdehyde can be increased. This can be done by increasing the activities of the enzymes in the pathway, such as Homoserine dehydrogenase [EC 1.1.1.3], homoserine O-acetyltransferase [EC 2.3.1.31], O-acetylhomoserine (thiol)-lyase [EC 2.5.1.49] and N5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (METE) [EC 2.1.1.14]. For instance, in a preferred embodiment, the activity of O-acetylhomoserine (thiol)-lyase can be increased by overexpressing the endogenous genes encoding O-acetylhomoserine (thiol)-lyase (MET17 or STR2) in *S. cerevisiae*. In another preferred embodiment, heterologous genes encoding O-acetylhomoserine (thiol)-lyase from other organisms, such as *Corynebacterium acetylphilum*, *Geobacillus stearothermophilus*, *Brevibacterium flavum*, *Thermus thermophiles* (oah1), *Pseudomonas sp.* or *Corynebacterium glutamicum* (metY), could also be overexpressed in a yeast or bacterial cell. In another preferred embodiment, the activity of N5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase can also be increased. This can be done, for example, by overexpressing the endogenous gene encoding METE (MET6) in *S. cerevisiae*. In another preferred embodiment, heterologous genes encoding METE from other organisms, such as *Catharanthus roseus* (MetE), *Neurospora crassa* (me-8) or *E. coli* (metE) can be overexpressed in a microbial cell. In another embodiment, the activities of Homoserine dehydrogenase and homoserine O-acetyltransferase [EC 2.3.1.31] can also be increased by overexpressing the endogenous genes HOM6 (SEQ ID NO: 127) and/or MET2 in *S. cerevisiae*. In another embodiment, the activities of these enzymes can be increased by overexpressing heterologous genes encoding these enzymes from other sources, such as
Haemophilus influenzae, Paenibacillus polymyxa, Bacillus cereus, Thermotoga maritima, Bacillus cereus, Schizosaccharomyces pombe or Cryptococcus neoformans in a microbial cell. In a preferred embodiment, Homoserine dehydrogenase from Zea mays is overexpressed in S. cerevisiae or E. coli. In addition, genes from other eukaryotic or prokaryotic sources can be expressed, e.g. from Escherichia coli, Streptomyces spectabilis, Triticum aestivum, Oryza sativa, Glycine max, Citrus sinensis, Homo sapiens.

Thus, in an embodiment, the microbial cell is genetically modified for enhanced conversion of L-aspartate-4-semialdehyde to methionine.

In a particular embodiment, the microbial cell is genetically modified for overexpression of at least one enzyme selected from a group consisting of: homoserine dehydrogenase [EC 1.1.1.3]; homoserine O-acetyltransferase [EC 2.3.1.31]; O-acetylhomoserine (thiol)-lyase [EC 2.5.1.49], also referred to as O-acetylhomoserine aminocarboxypropyltransferase; and 5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase (METE) [EC 2.1.1.14].

This conversion of L-aspartate-4-semialdehyde into methionine is shown in Figure 2 and involves the above-mentioned enzymes. This synthesis pathway thereby increased the amount of the substrate methionine in the microbial cell to be used in the production of SAM.

In another embodiment, the methionine needed for SAM synthesis can be derived from cysteine. This can be done, for example, by increasing the activity of cystathionine gamma-synthase (CGS) [EC 2.5.1.48], which converts cysteine into cystathionine. This can be achieved by the overexpression of the endogenous gene encoding CGS in S. cerevisiae (STR2), in E. coli (metB), or in C. glutamicum (Cgl2446). The activity of Cystathione beta lyase [EC 4.4.1.8] (CBL), which converts Cystathione to Homocysteine could also, or alternatively, be increased. This can be done by overexpressing the endogenous genes encoding for Cystathione beta lyase activity, such as IRC7 or STR3 in S. cerevisiae, malY or metC in E. coli, or Cgl2309 in C. glutamicum. Genes encoding CGS and CBL from other sources, e.g. Triticum aestivum, Oryza sativa, Glycine max, Citrus sinensis, Homo sapiens, Arabidopsis thaliana or Lysinibacillus sphaericus, could also be expressed.

Thus, in an embodiment, the microbial cell is genetically modified for enhanced conversion of cysteine to methionine by overexpression of at least one enzyme selected from a group consisting of cystathionine gamma-synthase [EC 2.5.1.48] and cystathione beta-lyase (CBL) [EC 4.4.1.8]. This synthesis pathway thereby increased the amount of the substrate methionine in the microbial cell to be used in the production of SAM.
The above modifications could also be combined with enzymes that convert Serine to Cysteine. For example, through combination of the activities of Serine acetyltransferase [EC 2.3.1.30] and Cysteine synthase A [EC 2.5.1.47]. This can be done by expressing/overexpressing genes encoding these activities. For example, the genes cysE and cysK from E. coli could be overexpressed in a prokaryotic or eukaryotic cell.

In another embodiment S-adenosylmethionine (SAM) levels can be increased by reducing the activity of endogenous Cystathionine beta-synthase (CBS) [EC 4.2.1.22], which catalyzes the synthesis of cystathionine from serine and homocysteine. This can be done, for example, by downregulating the native gene encoding Cystathionine beta-synthase (CYS4) in S. cerevisiae. Alternatively, the activity of Cystathionine gamma-lyase (CGS) [EC 4.4.1.1], which catalyzes the conversion of cystathione to cysteine could be reduced. This can be done, for example, by downregulating the native gene encoding Cystathionine gamma-lyase (CYS3) in S. cerevisiae. Thus, in an embodiment, the microbial cell is genetically modified for attenuated activity of cystathionine beta-synthase (CBS) [EC 4.2.1.22] or deletion or disruption of a gene encoding CBS.

This enzyme catalyzes the conversion of homocysteine into cystathionine, i.e., the opposite reaction path as compared to CBS. Accordingly, CBS depletes homocysteine, which in turn decreases the levels of methionine in the microbial cell.

In another embodiment, S-adenosylmethionine (SAM) levels can be increased by increasing the conversion of pyruvate to L-Aspartate-4-semialdehyde. This can be done by increasing the activities of Pyruvate carboxylase [EC 6.4.1.1], aspartate aminotransferase [EC 2.6.1.1], aspartate kinase (AK) [EC 2.7.2.4] and/or aspartic beta semi-aldehyde dehydrogenase [EC 1.2.1.1]. For instance, in a preferred embodiment, the activity of AK can be increased by overexpressing the endogenous HOM3 gene encoding AK in S. cerevisiae (SEQ ID NO: 29) or the metL/LysC/thrA genes in E. coli. Alternatively, a mutated version of AK can be introduced. For example, a mutated allele of HOM3, where Ser399 has been exchanged (to e.g. phenylalanine; S399F; SEQ ID NO: 6) can be expressed. This allele is not subject to feedback regulation and could therefore result in accumulation of higher levels of L-4-Aspartyl-phosphate. Furthermore, feedback-resistant alleles from other organisms can be expressed. For example, metL from Xenorhabdus bovienii with mutation in glutamic acid 257 (to e.g. lysine; E257K; SEQ ID NO: 7) or the lysine-sensitive aspartokinase 3 from the same species with mutation in either threonine 359 (to e.g. isoleucine; T359I) or glutamic acid 257 (to e.g. lysine; E257K), or both (SEQ ID NO: 8), could be introduced into a microbial cell. In addition, a feedback resistant AK from Corynebacterium glutamicum (encoded by the lysC gene) where
threonine 311 is exchanged (to e.g. Isoleucine; T311I; SEQ ID NO: 22) could also be expressed. Furthermore, genes encoding AK from other organisms, e.g. Arabidopsis thaliana, Panicum miliaceum, Escherichia coli, Streptomyces spectabilis, Triticum aestivum, Oryza sativa, Glycine max, Citrus sinesis, Homo sapiens, could also be expressed in a microbial cell.

The activities of other enzymes involved in the conversion of pyruvate to L-Aspartate-4-semialdehyde could be increased. For example, the activity of pyruvate carboxylase [EC 6.4.1.1], which converts pyruvate to oxaloacetate, could be increased by overexpressing the endogenous S. cerevisiae gene encoding pyruvate carboxylase (PYC1 and/or PYC2; SEQ ID NO: 125 and SEQ ID NO: 126, respectively) or mutated versions, such as C. glutamicum pyc where proline 458 has been replaced with serine (P458C). Genes encoding Pyruvate carboxylase from other organisms, e.g. Gallus gallus, Mycobacterium smegmatis, Escherichia coli, Streptomyces spectabilis, Triticum aestivum, Oryza sativa, Glycine max, Citrus sinesis, Homo sapiens, could also be expressed.

Alternatively, or in addition, the intermediate oxaloacetate could be generated by increasing the activity of Phosphoenolpyruvate (PEP) carboxylase [EC 4.1.1.31]. This could be achieved by overexpressing a gene encoding PEP carboxylase. For example, the S. cerevisiae PCK1 gene or the E. coli Ppc gene could be overexpressed in a microbial cell. Alternatively, a mutant version of Ppc, where leucine 620 is replaced (by e.g. serine, L620S) could be expressed in a microbial cell. In another embodiment, the activity of aspartate aminotransferase (AAT) [EC 2.6.1.1] could be increased. This could be done by overexpressing the endogenous gene encoding aspartate aminotransferase, for example AAT1 and/or AAT2 in S. cerevisiae. Genes encoding Aspartate aminotransferase from other organisms, e.g. Arabidopsis thaliana, Panicum miliaceum, Escherichia coli, Streptomyces spectabilis, Triticum aestivum, Oryza sativa, Glycine max, Citrus sinesis, Homo sapiens, could also be introduced. In another embodiment, the activity of aspartic beta semi-aldehyde dehydrogenase [EC 1.2.1.1] could be increased by overexpressing the endogenous gene encoding aspartic beta semi-aldehyde dehydrogenase (HOM2) in S. cerevisiae. Genes encoding aspartic beta semi-aldehyde dehydrogenase from other organisms, e.g. Gallus gallus, Mycobacterium smegmatis, Escherichia coli, Streptomyces spectabilis, Triticum aestivum, Oryza sativa, Glycine max, Citrus sinesis, Homo sapiens, could also be expressed.

Thus, in an embodiment, the microbial cell is genetically modified for enhanced conversion of pyruvate to L-Aspartate-4-semialdehyde.

In a particular embodiment, the microbial cell is genetically modified for overexpression of at least one enzyme selected from a group consisting of pyruvate carboxylase [EC 6.4.1.1],
aspartate aminotransferase [EC 2.6.1.1], aspartate kinase (AK) [EC 2.7.2.4] and aspartic beta semialdehyde dehydrogenase [EC 1.2.1.1], also referred to as aspartate-semialdehyde dehydrogenase.

As is shown in Figure 2, these enzymes catalyze the conversion of pyruvate into L-aspartate-4-semialdehyde, which may be further converted, as described above, into methionine and then into SAM.

In a particular embodiment, the microbial cell comprises at least one gene encoding AK selected from a group consisting of Saccharomyces cerevisiae HOM3 with a mutation in amino acid serine 399, Xenorhabdus bovienii AK with a mutation in amino acid threonine 359 and/or glutamic acid 257 and Corynebacterium glutamicum AK with a mutation in threonine 311.

In another embodiment, spermidine can be formed by first condensing putrescine with L-Aspartate-4-semialdehyde to form carboxyspermidine, followed by decarboxylation to form spermidine. For example, the modifications described above to enhance levels of L-Aspartate-4-semialdehyde in a microbial cell can be combined with expression of heterologous genes encoding enzymes able to condense L-Aspartate-4-semialdehyde and putrescine to form carboxyspermidine, such as carboxy(nor)spermidine dehydrogenase (CASDH/CANSDH) [EC 1.5.1.43]. Carboxyspermidine can then be converted to spermidine via carboxy(nor)spermidine decarboxylase (CASDC/CANSDC) [EC 4.1.1.96]. For example, CASDH/CANSDH and CASDC/CANSDC from Campylobacter jejuni (nucleotide sequences are SEQ ID NO: 10-11, respectively; protein sequences are SEQ ID NO: 149-150, respectively), Vibrio cholera, Vibrio vulnificus, or Vibrio alginolyticus can be expressed in a S. cerevisiae or E. coli cell. This can also be combined with reduction of flux to competitive reactions. For example, in another embodiment, conversion of L-Aspartate-4-semialdehyde to homoserine is reduced. This can be achieved by downregulating the endogenous enzyme encoding homoserine dehydrogenase [EC 1.1.1.3]. For example, the endogenous HOM6 (SEQ ID NO: 127) gene in S. cerevisiae can be down-regulated.

In another embodiment, the above modifications are combined with a reduced flux to the threonine production pathway, or this pathway may be used independent of the above modifications. This can be achieved by decreasing the activities of homoserine kinase [EC 2.7.1.39] and/or threonine synthase [EC 4.2.3.1; 4.2.99.2]. For example, the endogenous THR1 gene encoding homoserine kinase or the endogenous THR4 gene encoding threonine synthase could be down-regulated or disrupted in S. cerevisiae. Alternatively, the endogenous thrB or thrC genes (encoding these activities) could be down-regulated or disrupted in E. coli. In
addition, the activity of Diaminopimelate decarboxylase [EC 4.1.1.20] could be reduced, for example, by downregulating the endogenous lysA gene in E. coli.

Thus, in an embodiment, the microbial cell is genetically modified for attenuated activity of endogenous homoserine kinase [EC 2.7.1.39] and/or endogenous threonine synthase [EC 4.2.3.1 and previously also known as EC 4.2.99.2] or by deletion or disruption of an endogenous gene encoding homoserine kinase and/or an endogenous gene encoding threonine synthase.

These enzymes are involved in the threonine biosynthesis pathway, which uses homoserine as substrate as indicated in Figure 2. Thus, by attenuating activity of these enzymes or deletion or disruption of the genes encoding these enzymes, homoserine is not depleted in the threonine biosynthesis pathway but can rather be further converted into o-acetylhomoserine.

In another embodiment, conversion of SAM back to methionine can be reduced. This can be done by reducing the activity of S-adenosylmethionine-homocysteine methyltransferase [EC 2.1.1.10]. For example, the endogenous gene encoding S-adenosylmethionine-homocysteine methyltransferase (SAM4 or MHT1) could be down-regulated or disrupted in S. cerevisiae.

In another embodiment, SAM levels are increased by reducing the conversion of oxaloacetate to citrate. This can be achieved by reducing the activity of endogenous citrate synthase [EC 2.3.3.1]. This can be done, for example, by downregulating the endogenous gene encoding citrate synthase, such as CIT1, CIT2 or CITS in S. cerevisiae.

In another embodiment the uptake of oxaloacetate into the mitochondria is increased. This can be done by overexpression of the transporter protein that transports oxaloacetate into the mitochondria. For example, the s. cerevisiae gene OAC1 encoding a mitochondrial inner membrane transporter for oxaloacetate could be overexpressed in a eukaryotic cell.

In another embodiment, transport of aspartate from the mitochondria into the cytosol is increased. This can be achieved by increasing the activity of aspartate transporters. For example, the endogenous AGC1, which encodes a mitochondrial amino acid transporter, could be overexpressed in S. cerevisiae. Alternatively, synthesis of aspartate in the cytosol could be promoted by expressing cytosolic versions of AAT, e.g. s. cerevisiae AAT2 or prokaryotic AAT. This could also be combined with increasing oxaloacetate in the cytosol by reducing expression of mitochondrial oxaloacetate transporters (e.g. OAC1 in s. cerevisiae). In addition, transport of pyruvate into the mitochondria could be decreased by decreasing the expression of mitochondrial pyruvate transport proteins, e.g. MPC1, MPC2 or MPSCS in s. cerevisiae.

In another embodiment, flux can be increased from aspartate to L-aspartate-4-phosphate by reducing other endogenous reactions that utilize aspartate. Examples for such reactions...
include arginosuccinate synthetase [EC 6.3.4.5], Asparagine synthetase [EC 6.3.5.4], phosphoribosyl amino imidazolesuccinocarbozamide synthetase [EC 6.3.2.6], adenylosuccinate synthetase [EC 6.3.4.4] and aspartate transcarbamylase [EC 2.1.3.2]. This can be accomplished by downregulating the endogenous genes encoding the enzymes that catalyse these reactions. For example, the genes encoding arginosuccinate synthetase (ARG1), Asparagine synthetase (ASN1 or ASN2), phosphoribosyl amino imidazolesuccinocarbozamide synthetase (ADE1), adenylosuccinate synthetase (ADE12) and/or aspartate transcarbamylase (URA2) can be downregulated in a \textit{S. cerevisiae} cell.

In another embodiment, SAM levels are increased by increasing the formation of hydrogen sulfide (H2S), a precursor of SAM. This can be achieved by increasing the flux through the sulfate assimilation pathway by increasing the activities of ATP sulfurylase [EC 2.7.7.4], Adenylyl sulfate kinase [EC 2.7.1.25], phosphoadenylyl- sulfate (PAPS) reductase [EC 1.8.4.8] and/or Sulfite reductase [EC 1.8.1.2]. This can be achieved by overexpression endogenous genes encoding for these enzymes activities. For example, \textit{MET3, MET14, MET16, MET5, MET10} and/or \textit{ECM17} could be overexpressed in \textit{S. cerevisiae}. Thioredoxin could also be expressed. Genes encoding these activities from heterologous sources, e.g. \textit{Gallus gallus}, \textit{Mycobacterium smegmatis}, \textit{Escherichia coli}, \textit{Streptomyces spectabilis}, \textit{Triticum aestivum}, \textit{Oryza sativa}, \textit{Glycine max}, \textit{Citrus sinesis}, \textit{Homo sapiens}, \textit{Salmonella enterica subsp. enterica serovar Typhimurium}, \textit{Saccharomyces bayanus}, \textit{Rattus norvegicus}, \textit{Penicillium chrysogenum}, \textit{Synechococcus sp.}, \textit{Thiobacillus denitrificans}, \textit{Spinacia oleracea}, \textit{Spinacia oleracea}, \textit{Euglena gracilis}, could also be expressed. Formation of H2S could also be increased by increasing the concentration of sulfate in the media. In another embodiment, sulfate uptake from the media can be increased by increasing the expression of sulfate transporters. For example, the endogenous sulfate permeases of \textit{S. cerevisiae} (\textit{SUL1, SUL2} and/or \textit{SUL3}) could be overexpressed. In addition, heterologous sulfate transporters could be introduced. Furthermore, sulphite efflux could be reduced by disruption/downregulation of endogenous efflux transporters (e.g. \textit{SSU1} in \textit{S. cerevisiae}). In another embodiment, transcriptional regulators that activate sulfate assimilation could be overexpressed. For example, the endogenous genes \textit{MET28} and \textit{MET32} could be overexpressed in \textit{S. cerevisiae}.

In another embodiment, SAM availability for spermidine formation is increased by decreasing other cellular reactions that utilize SAM. For example, reactions involved in ergosterol biosynthesis can be downregulated. This can be achieved by disrupting/downregulating the endogenous enzymes involved in these reactions. For example,
the endogenous genes encoding Delta(24)-sterol C-methyltransferase [EC 2.1.1.41; ERG6] and/or C-24(28) sterol reductase [EC 1.3.1.71; ERG4] can be downregulated in S. cerevisiae.

Thus, in an embodiment, the microbial cell is genetically modified for attenuated activity of endogenous delta(24)-sterol C-methyltransferase [EC 2.1.1.41], also referred to as sterol 24-C-methyltransferase, and/or endogenous C-24(28) sterol reductase [EC 1.3.1.71], also referred to as delta(24(24(l)))-sterol reductase or by deletion or disruption of an endogenous gene encoding delta(24)-sterol C-methyltransferase and/or an endogenous gene encoding C-24(28) sterol reductase.

Delta(24)-sterol C-methyltransferase catalyses the reaction: S-adenosyl-L-methionine + 5alpha-cholesta-8,24-dien-3beta-ol → S-adenosyl-L-homocysteine + 24-methylene-5alpha-cholest-8-en-3beta-ol. Thus, this enzyme depletes SAM and thereby it is preferably attenuated or its gene is preferably deleted or disrupted in the microbial cell. Disruption of endogenous C-24(28) sterol reductase has been linked to increased SAM accumulation.

In further embodiments, the activity of the endogenous glycogen-branching enzyme [EC. 2.4.1.18] could be reduced. For example, the endogenous GLC3 gene could be downregulated in S. cerevisiae.

In an embodiment, the microbial cell is genetically modified for attenuated activity of endogenous glycogen-branching enzyme or by deletion or disruption of an endogenous gene encoding glycogen-branching enzyme.

In another embodiment, SAM levels can be increasing by introducing haemoglobin in order to promote better oxygen availability, cell growth and protein expression. For example, haemoglobin from Vitreoscilla (VHb) (SEQ ID NO: 9) could be expressed in S. cerevisiae.

Thus, in an embodiment, the microbial cell is genetically modified for overexpression of haemoglobin, preferably Vitreoscilla haemoglobin.

Any of the above described embodiments of increasing SAM levels can be combined in the microbial cell.

In another embodiment, any of the modifications described above could be used to produce other polyamines, such as spermine. This can be achieved, by increasing the activity of spermine synthase [EC 2.5.1.22], which can be done, for example, by overexpressing the S. cerevisiae gene encoding spermine synthase (SPE4, SEQ ID NO: 128) in S. cerevisiae or E. coli. Alternatively, spermine synthase from other organisms, e.g. Triticum aestivum, Oryza sativa, Glycine max, Citrus sinensis, Homo sapiens, Bos taurus, could also be expressed. In addition, conversion of spermine back to spermidine can be reduced by reducing the activity of
Polyamine oxidase [EC 1.5.3.17], for example by disrupting the native gene encoding this enzyme, e.g. FMS1 in S. cerevisiae.

Thus, in an embodiment, the microbial cell is genetically modified for enhanced spermine biosynthesis. In a particular embodiment, the microbial cell is genetically modified for overexpression of spermine synthase [EC 2.5.1.22].

In another embodiment, export of polyamines to the media can be facilitated. This can be accomplished by overexpression of different export proteins, such as yeast TPO1, TP02, TP03, TP04 and TP05; Escherichia coli MdtJI, Shigella MdtJI, mammalian SLC3A2, Bacillus subtilis Bit transporter and/or mammalian MDR1. In addition, genes associated with polyamine uptake, such as yeast DUR3, SAM3, AGP2 and/or GAP1 can be down-regulated or deleted. Alternatively, increased intracellular presence of polyamines could be achieved by down-regulation or deletion of the polyamine transporters TPO1, TP02, TP03, TP04 or TP05.

Thus, in an embodiment, the microbial cell is genetically modified for enhanced polyamine export by overexpression of at least one polyamine export protein selected from the group consisting of Saccharomyces cerevisiae TPO1, TP02, TP03, TP04 and TP05, Escherichia coli MdtJI, mammalian SLC3A2, Bacillus subtilis Bit transporter and mammalian MDR1.

In another embodiment, the microbial cell is a Saccharomyces cerevisiae cell and is genetically modified for reduced polyamine uptake by downregulation of at least one polyamine uptake protein selected from the group consisting of Saccharomyces cerevisiae DUR3, SAM3, AGP2 and GAP1.

In another embodiment of the invention, the export of polyamines into the media is increased by the overexpression of transcription factors responsible for regulating exporters involved in the pleiotropic drug response. For example, the genes PDR1 (SEQ ID NO: 30) and/or PDR2 (SEQ ID NO: 129) could be overexpressed in S. cerevisiae.

Thus, in an embodiment, the microbial cell is genetically modified for overexpression of Saccharomyces cerevisiae PDR1 and/or PDR2.

In another embodiment, the resistance of the above strains to polyamine toxicity is increased. Downregulation and/or deletion of several genes have been associated with increased resistance to polyamine toxicity in yeast. This includes SR protein kinase (SRPK) (encoded by SKY1), Putative serine/threonine protein kinase (encoded by PTK2), BRP1 and FES1. In addition, overexpression of several native genes has been associated with increased resistance to polyamine toxicity. This includes QDR3 and YAP1. The above genes can be overexpressed
and/or down-regulated in various combinations to allow for optimal resistance to polyamine toxicity in yeast.

Thus, in an embodiment, the microbial cell is a *Saccharomyces cerevisiae* cell and is genetically modified for downregulation of at least one gene selected from a group consisting of SKYL encoding SR protein kinase (SRPK), PTK2 encoding putative serine/threonine protein kinase, BRP1 and FES1.

In another embodiment, the microbial cell is genetically modified for overexpression of *Saccharomyces cerevisiae* QDR3 and/or YAP1.

In another embodiment, the levels of S-adenosylmethionine are increased by overexpression of subunits of the RNA Polymerase II mediator complex. For example, GAL11 (SEQ ID NO: 31) could be overexpressed in *S. cerevisiae*.

Thus, in an embodiment, the microbial cell is genetically modified for overexpression of subunits of the RNA Polymerase II mediator complex, preferably overexpression of *Saccharomyces cerevisiae* GAL11.

In an embodiment, the microbial cell is capable of producing more than 100 mg of spermidine per L of culture medium, and/or more than 10 mg of spermidine per g DCW.

In a particular embodiment, the microbial cell is capable of producing more than 250 mg, preferably more than 500 mg, and more preferably more than 750 mg, such as more than 1 g of spermidine per L of culture medium.

In an alternative or additional particular embodiment, the microbial cell is capable of producing more than 15 mg, preferably more than 25 mg, and more preferably more than 30 mg spermidine per g DCW.

The above described embodiments may be combined.

Another aspect of the embodiments, which optionally may be combined with any of the above described embodiments, relates to a microbial cell capable of producing spermidine. The microbial cell comprises at least one heterologous gene encoding an enzyme selected from a group consisting of carboxy(nor)spermidine dehydrogenase (CASDH/CANSDH) [EC 1.5.1.43], also referred to as carboxynorspermidine synthase; and carboxynorspermidine decarboxylase (CANSDC) [EC 4.1.1.96].
This aspect of the embodiments thereby provides a microbial cell capable of producing spermidine through the condensation of putrescine and L-aspartate-4-semialdehyde. In particular, CASDH catalyzes the following reaction L-aspartate 4-semialdehyde + putrescine + NADPH + H⁺ → carboxyspermidine + H₂O + NADP⁺. CANSDC then catalyzes the conversion of carboxyspermidine into spermidine carboxyspermidine → spermidine + CO₂.

In a particular embodiment of this aspect, the microbial cell is genetically modified for attenuated activity of endogenous homoserine dehydrogenase [EC 1.1.1.3] or by deletion or disruption of an endogenous gene encoding homoserine dehydrogenase.

A further aspect of the embodiments relates to a method for producing spermidine. The method comprises culturing a microbial cell according to any of the embodiments in a culture medium and in culture conditions suitable for production of spermidine from the microbial cell. The method also comprises collecting spermidine from the culture medium and/or the microbial cell.

In an embodiment, the culture medium comprises methionine.

Yet another aspect of the embodiments relates to use of a microbial cell according to any of the embodiments as a food additive.

Spermidine has, as mentioned in the foregoing, positive effects including, for instance, promoting longevity and alleviating or protecting against a variety of age-associated conditions, such as cancer, multiple sclerosis, osteoporosis, cardiovascular disease, memory impairment, skin ageing and hair loss. Accordingly, a spermidine producing microbial cell according to the embodiment can then be used as a food additive. In such a case, the microbial cell is a microbial cell generally recognized as safe (GRAS). The microbial cell could be used as food additive to any food suitable to be enriched by microbial cells. A non-limiting, but illustrative, example is yogurt.

EXAMPLES

Example 1

Increasing spermidine production in a microbial cell by increased flux towards putrescine with expression of heterologous genes able to convert putrescine and L-aspartate-4-semialdehyde to spermidine

The present example demonstrates that introduction of heterologous enzymes able to convert putrescine and L-aspartate-4-semialdehyde into spermidine further increases spermidine production in a microbial cell previously engineered to produce high levels of putrescine. The modifications introduced rely primarily on yeast strains previously engineered
to produce high levels of L-ornithine, a direct precursor to putrescine (Qin et al [5]). These strains were modified to further increase flux towards putrescine, and further by overexpression of heterologous carboxyspermidine dehydrogenase (CASDH, also known as carboxynorspermidine synthase) [EC 1.5.1.43] and carboxyspermidine decarboxylase (CASDC) [EC 4.1.1.96].

Putrescine can be synthesized from ornithine via the actions of ornithine decarboxylase (ODC). In yeast, ODC is regulated via the ODC antizyme (OAZ), which binds to ODC and targets it for degradation. Therefore, we increased flux towards putrescine by simultaneously deleting OAZ (encoded by the gene OAZI) and overexpressing ODC (encoded by the gene SPE1). The experimental procedure for strain construction was as follows: The SPE1 gene was PCR amplified from CEN.PK1 13-1 1C genomic DNA (with primer pairs 9/10) and fused to promoter TEFlp (with primer pairs 7/8) and terminator PRM9t (with primer pairs 11/12). The resulting fragment TEFlp-SPE1-PRM9t was fused to the 3' site of KanMX cassette, which is PCR amplified from plasmid PUC6, resulting in fragment KanMX-TEFlp-SPE1-PRM9t (primer pairs 3/12). To integrate SPE1 in the OAZI locus and simultaneously delete the OAZI gene, the 300 bp of the 5'ORF of OAZI (with primer pairs 1/2) and the 300 bp 3'ORF of OAZI (with primer pairs 13/6) were PCR amplified from CEN.PK1 13-1 1C genomic DNA, resulting in DNA fragments OAZI-UP and OAZI-DOWN respectively. Then, fusion PCR was used to fuse OAZI-UP and OAZI-DOWN to fragment KanMX-TEFlp-SPE1-PRM9t (with primer pairs 1/6), resulting in fragment OAZI-UP-KanMX-TEFlp-SPE1-PRM9t-OAZI-DOWN. The fragment was used for transformation via the LiAc/SS carrier DNA/PEG method (Gietz et al [8]) of ornithine over-producing strain ORN-L (MATa SUC2 MAL2-8c ura3-52 his3-Al ARG3p::KEX2p car2A::LoxP-CTClt-AGCl-tHXT7p-TPlp-ORT1-pYX212t ura3:: LoxP-TEFlp-GDHI-DITlt) (Qin et al [5]). The transformants were selected on G418 plates and were verified by colony PCR, resulting in putrescine over-producing strain PUT-B(KanMX) (MATa SUC2 MAL2-8C ura3-52 his3-Al ARG3p::KEX2p car2A::LoxP-CTClt-AGCl-tHXT7p-TPlp-ORT1-pYX212t ura3:: LoxP-TEFlp-GDHI-DITlt). All primers used in this implementation are listed in Table 4.

To enable over-production of spermidine with the so-called carboxyspermidine spermidine pathway, CASDH and CASDC from either Campylobacter jejuni (CjCASDH and CjCASDC) or Vibrio cholera (VcCASDH and VcCASDC) were evaluated. All genes used were codon-optimized for expression in S. cerevisiae by Genscript (Piscataway, NJ, USA).

The DNA assembler method (Shao et al [6]) was used for construction of plasmids YP1 (expressing CjCASDH and CjCASDC) and YP2 (expressing VcCASDH and VcCASDC). For
construction of plasmid YP1, CjCASDH (nucleotide SEQ ID NO: 10; corresponding protein SEQ ID NO: 149) and CjCASDC (nucleotide SEQ ID NO: 11; corresponding protein SEQ ID NO: 150) were amplified (with primer pairs 132/133 and 137/136, respectively) from pUC57-CjCASDH and pUC57-CjCASDH respectively. Promoters TPIlp (with primer pairs 130/131), PGKlp (with primer pairs 18/19), TEFlp (with primer pairs 20/8) were amplified from CEN.PK1 13-1 C genomic DNA. The terminators FBAlt-CYC It (with primer pairs 134/135) were amplified from plasmid G04 (Qin et al [5]). The terminator pYX212t was amplified from plasmid pYX212 (with primer pairs 9/294). TPIlp, CjCASDH, FBAIt, CYClt which has overhangs to each other were fused by fusion PCR resulting in the fragment TPIlp-CjCASDH-FBAlt-CYC It. Following the same fusion PCR procedure, FBAIt, CYClt, CjCASDC, PGKlp, TEFlp and pYX212t were fused to construct the fragment FBAIt-CyClt-CjCASDC-PGKlp-TEFlp-pYX212t). A yeast 2μ vector pYX212 containing a URA3 selection marker was used and linearized by digestion with restriction enzyme Sph I and Eco R I. The linearized vector accompanied with fragments TPIlp-CjCASDH-FBAlt-CYClt and FBAIt-CYC It-

CjCASDC-PGK I-TEFlp -pYX212 It were transformed into S. cerevisiae BY4741 and selected on the uracil free plates (“SC-URA”) following a LiAc/SS carrier DNA/PEG method. Cell density (OD at 600 nm) was measured by GENESYS 20 spectrophotometer (Thermo Scientific). Plasmids extracted from transformants by Zymoprep Yeast Plasmid Miniprep II kit were transformed into E. coli DH5α and purified after overnight cultivation, yielding plasmid YPI.

For construction of plasmid YP2, VcCASDH (nucleotide SEQ ID NO: 12; corresponding protein SEQ ID NO: 151) and VcCASDC (nucleotide SEQ ID NO: 13; corresponding protein SEQ ID NO: 152) were amplified (with primer pairs 140/141 and 143/142, respectively) from pUC57-VcCASDH and pUC57-VcCASDH respectively. Promoters TPIlp, PGKlp, TEFlp, terminators FBAIt, CYClt were amplified from CEN.PK1 13-1 C genomic DNA with primers listed in Table 4 as described above. The terminator pYX212t was amplified from plasmid pYX212 with primers listed in Table 4 as described above. TPIlp, VcCASDH, FBAIt, CYClt which has overhangs to each other were fused by fusion PCR resulting in the fragment TPIlp-VcCASDH-FB Alt-CYC It. Following the same fusion PCR procedure as for plasmid YPI, CYClt, VcCASDC, PGKlp, TEFlp and pYX212t were fused to construct the fragment CYC It-VcCASDC-PGK Ip-TEFlp-pYX212 It. A yeast 2μ vector pYX212 containing a URA3 selection marker was used and linearized by digestion with restriction enzyme Sphl and EcoRI. The linearized vector accompanied with fragments TPIlp-VcCASDH-FBAlt-CYClt and CYClt- VcCASDC-PGK Ip-TEFlp-pYX212t were transformed into S. cerevisiae BY4741 and
selected on the uracil free plates ("SC-URA") following a LiAc/SS carrier DNA/PEG method. Cell density (OD at 600 nm) was measured by GENESYS 20 spectrophotometer (Thermo Scientific). Plasmids extracted from transformants by Zymoprep Yeast Plasmid Miniprep II kit were transformed into E. coli DH5a and purified after overnight cultivation yielding plasmid YP2. All plasmids were verified by restriction digest and sequencing.

The strain PUT-B(KanMX) (described above) was then transformed with either two empty plasmids (pYX212 + p423GPD) to act as control (resulting in strain SPDC3), YPI and p423GPD to evaluate CjCASDH/CjCASDC (resulting in strain SPD1), or YP2 and p423GPD to evaluate VcCASDH/VcCASDC (resulting in strain SPD12). All strains were verified by PCR.

The performance of the strains was compared in triplicates via shake-flask cultivations. For shake-flask cultivations, Delft medium(H) was used consisting of the following (per liter): (NH₄)₂SO₄, 7.5 g; KH₂PO₄, 14.4 g; MgSO₄•7H₂O, 0.50 g; trace metals, 1 ml and vitamins, 1 ml. The trace metal solution consisted of the following (per liter): Na₂EDTA•5H₂O, 19.0 g; ZnSO₄•7H₂O, 0.45 g; MnCl₂•4H₂O, 1 g; CoCl₂•6H₂O, 0.3 g; CuSO₄•5H₂O, 0.3 g; Na₂MoO₄•2H₂O, 0.4 g; CaCl₂•2H₂O, 0.45 g; FeSO₄•7H₂O, 0.3 g; H3BO3, 1 g and KI, 0.10 g. The pH of the trace metal solution was adjusted to 4.0 with 2 M NaOH prior to heat sterilization. The vitamin solution contained (per liter): d-biotin, 0.05 g; p-amino benzoic acid, 0.2 g; nicotinic acid, 1 g; Ca-pantothenate, 1 g; pyridoxine-HCl, 1 g; thiamine-HCl, 1 g and myo-inositol, 25 g. The pH of the vitamin solution was adjusted to 6.5 with 2 M NaOH. The vitamin solution was filter sterilized and stored at 4°C. This medium was supplemented with 20 g/liter glucose. Single colonies were initially inoculated into 2 ml liquid medium and cultivated for 24 to 36 hours. The cells were then grown in 100 ml shake flasks with 20 ml medium for 5 days (120 hours) with the initial OD 0.05 at 200 rpm, 30°C.

Fermentation sample was prepared by taking 0.1 ml of liquid culture. Fermentation sample was subject to Hot Water (HW) Extraction (Canelas et al [9]). Tubes containing 0.9 ml of Delft medium were preheated in a water bath at 100°C for 10 min. Then, the hot Delft medium was quickly poured over the 0.1 ml of fermentation sample; the mixture was immediately vortexed, and the sample was placed in the water bath. After 30 min, each tube was placed on ice for 5 min. After centrifugation, the supernatant was directly used for derivatization. For derivatization, the procedure was adapted from (Kim et al [2]). In brief, 0.25 ml of saturated NaHCO₃ solution and 0.5 ml of dansyl chloride solution (5 mg/ml in acetone) were added to 0.5 ml of sample. Then the reaction mixture was incubated at 40°C for 1 h in the dark with occasional shaking. The reaction was stopped by adding 0.1 ml 25% ammonium
hydroxide; followed by 0.3 ml of MeOH. Samples filtered through a 25 mm syringe filter (0.45 µm Nylon) were used for HPLC detection which is equipped with a Kinetex® 2.6 µm C18 100 A column (100 × 4.6 mm, Phenomenex, Torrance, USA). The following chromatographic condition are used: excitation wavelength 340 nm, emission wavelength 515 nm, sample injected was 10 µl, column temperature 40°C, detector sensitivity 7, acquisition started at 3.4 min. The mobile phase was water and methanol with the speed of 1 ml /min. The elution program was as follows: from 0 to 5 min, 50% to 65% MeOH; from 5 to 7.5 min, 65% to 75% MeOH; from 7.5 to 9.5 min, 75% to 87.5% MeOH; from 9.5 to 10.5 min, 87.5% to 100% MeOH; from 10.5 to 11.5 min, 100% MeOH; from 11.5-13.5 min, 100% to 50% MeOH; from 13.5 to 16 50% MeOH. The results are shown in Table 6.

The results showed that overexpression of either CjCASDH and CjCASDC or VcCASDH and VcCASDC had a positive effect on spermidine production (Figure 3), with the best-producing strain (SPD12), producing 42 mg/L of spermidine.

Example 2

Increasing spermidine production in a microbial cell by increasing metabolic flux towards L-aspartate-4-semialdehyde

The present example demonstrates that spermidine production in a microbial cell can be further increased by increasing the metabolic flux towards L-aspartate-4-semialdehyde. This is achieved by overexpressing a three-step pathway to convert oxaloacetic acid into L-aspartate-4-semialdehyde, in combination with reduction of flux to a competitive reaction catalysed by Homoserine dehydrogenase, which catalyze the step converting L-aspartate-4-semialdehyde into homoserine.

In order to increase flux from oxaloacetic acid to L-aspartate-4-semialdehyde, Aspartate aminotransferase [EC 2.6.1.1] encoding gene aspB (CgaspB) (SEQ ID NO: 14), aspartate kinase [EC 2.7.2.4] with mutation T31 I encoding gene lysC (Cglycm) (SEQ ID NO: 15) and aspartate-4-semialdehyde dehydrogenase [EC 1.2.1.11] encoding gene asd (Cgasd) (SEQ ID NO: 16) were chosen to be overexpressed in a yeast cell. All genes were codon-optimized and synthesized by Genscript (Piscataway, NJ, USA). The Gibson assembly method (Gibson et al [7]) was used for construction of plasmid GP1 which overexpresses Cglycm, Cgasd and CgaspB. To construct plasmid GP1, the genes Cglycm, Cgasd and CgaspB were amplified from PUC57-Cglycm, PUC57-Cgasd and PUC57-CgaspB respectively (using the primer pairs 180/181, 185/184 and 186/187, respectively). The promoters TDH3p, PGKlp and TEFlp were amplified from yeast CEN.PK1 13-1 1C genomic DNA with the primer pairs 25/26, 18/19 and
20/8, respectively. The terminators TDH2t-ADHl t and FBAlt-TPIl t were amplified from plasmids G04/Y04 (Qin et al [5]) with the primer pairs 182/183 and 134/194, respectively. TDH3p, Cglycm, TDH2t and ADHl It which have overhangs to each other were fused by fusion PCR resulting in the fragment TDH3p-Cglycm-TDH2t-ADHl It. Following the same fusion PCR procedure, Cgasd, PGKlp, TEFlp, CgaspB, FBAlt and TPIlt were fused to construct the fragment Cgasd-PGKlp-TEFlp-CgaspB-FBAlt-TPIlt. A yeast 2μ vector p423GPD containing a HIS3 selection marker was used as the backbone plasmid. These fragment were assembled together to form plasmid GP1 by Gibson Assembly Master Mix (New England BioLabs) according to manufacturer’s instructions. The plasmids extracted from *E. coli* were verified by restriction digestion and sequencing. All primers used in this implementation are listed in Table 4.

In order to decrease flux towards the competitive reaction that converts L-aspartate-4-semialdehyde to homoserine, we set to decrease the activity of the homoserine dehydrogenase enzyme that catalyzes this reaction by inserting a weaker promoter in front of the native gene encoding this reaction (*HOM6* in *S. cerevisiae*). The weak promoter chosen was the KEX2 promoter (KEX2p). Seamless gene insertion was performed by using *Kluyveromyces lactis* URA3 (K1URA3) as a selection marker, which was looped out by homologous recombination of direct repeats, and selection on SC + 5-FOA plates. The promoter insertion cassettes were constructed by fusion PCR. The KEX2p which is amplified from CEN.PK1 13-1 1C genomic DNA (with primer pairs 201/202) was fused to the 3’ of the K1URA3 (with primer pairs 199/200) with fusion PCR resulting in fragment KIURA3-KEX2p. The promoter region, the 5’ part of the HOM6 ORF was amplified from CEN.PK1 13-1 1C genomic DNA. These fragments were fused together by fusion PCR resulting in the promoter insertion fragment HOM6p-Repeat-KIURA3-KEX2p-5’HOM6 (with primer pairs 197/204). The fragment was then used to transform to putrescine producing strain PUT-B(KanMX) (MATα SUC2 MAL2-8c ura3-52 his3-A1 ARG3p::KEX2p car2A::LoxP-CTC It-AGC 1-tHXT7p-TPIl p-ORT 1-pYX2 It t ura3 ::LoxP-TEF Ip-GDH 1-DIT It oaz t l Δ::LoxP-KanMX-LoxP-TEF Ip-SPE 1-PRM9t). Clones were verified by colony PCR. Subsequently, 3-5 clones with correct module integration were cultivated overnight in YPD liquid medium and then plated on SC-5-FOA plates to loop out K1URA3. Once again, the right clones were verified with colony PCR resulting in strain PUT-B(KanMX)-HOM6 (MATα SUC2 MAL2-8c ura3-52 his3-A1 ARG3p::KEX2p car2A::LoxP-CTC It-AGC 1-tHXT7p-TPIl p-ORT 1-pYX2 It t ura3 ::LoxP-TEF Ip-GDH 1-DIT It oaz t l Δ::LoxP-KanMX-LoxP-TEFIp-SPEl-PRM9t HOM6p::KEX2p). Plasmid GP1 together with plasmid YP2 were co-transformed into strain PUT-B(KanMX)-HOM6 resulting in
spermidine producing strain SPD06. All primers used in this implementation are listed in Table 4. The strains were cultivated and analyzed as described in Example 1 above. We found that the strain SPD06 could produce significantly more spermidine than a similar strain without engineering to increase flux towards L-aspartate-4-semialdehyde (Figure 4).

Example 3
Increasing spermidine production in a microbial cell by increasing metabolic flux in the methionine salvage pathway

The present example demonstrates that spermidine production in a spermidine overproducer can be significantly improved by increasing the flux in the methionine salvage pathway. Specifically, this was done by increasing the activities of the rate-controlling enzymes in this pathway, Methylthioadenosine phosphorylase (MTAP) and Branched-chain Amino acid Transaminase (BAT). Increasing the flux in the methionine salvage pathway allowed for higher levels of SAM, resulting in increased amounts of decarboxylated S-adenosylmethionine (dSAM), which acts as an aminopropyl donor for spermidine synthesis. We have tested the effects of enhanced methionine salvage pathway on spermidine production in yeast strains previously engineered for spermidine accumulation.

To increase MTAP [EC 2.4.2.28] activity, the endogenous MEUl gene (nucleotide SEQ ID NO: 17; corresponding protein SEQ ID NO: 147) was chosen for overexpression in yeast via the plasmid YP6. The plasmid was constructed by the DNA assembler method. First, MEUl and promoter TPIlp were amplified from CEN.PK1 13-1 1C genomic DNA using the primer pairs 157/162 and 156/29, respectively. The terminator pYX212t was amplified from plasmid pYX212 (with primer pairs 15/159). TPIlp, MEUl and pYX212t which has overhangs to each other were fused by fusion PCR, resulting the fragment TPIlp-MEUr-pYX212t (with primer pairs 156/159). A yeast 2µ vector pYX212 containing a URA3 selection marker was used and linearized by digestion with the restriction enzymes Sphl and EcoRI. The linearized vector accompanied with fragments TPIlp-MEUl-pYX212t were transformed into S. cerevisiae BY4741 and selected on the uracil free plates ("SC-URA") following a LiAc/SS carrier DNA/PEG method. Cell density (OD at 600 nm) was measured by GENESYS 20 spectrophotometer (Thermo Scientific). Plasmids extracted from transformants by Zymoprep Yeast Plasmid Miniprep II kit were transformed into E. coli DH5a and purified, yielding plasmid YP6.

To increase BAT [EC 2.6.1.42] activity, the endogenous BAT2 gene (nucleotide SEQ ID NO: 18; corresponding protein SEQ ID NO: 148) was chosen for overexpression in yeast via
the plasmid YP7. The DNA assembler method was used for construction of plasmid YP7. BAT2 and the promoter TPIlp were amplified from CEN.PK1 13-1C genomic DNA with the primer pairs 160/161 and 156/29, respectively. The terminator pYX212t was amplified from plasmid pYX212 (with primer pairs 15/159). TPIlp, BAT2 and pYX212t which have overhangs to each other were fused by fusion PCR, resulting the fragment TPIlp-BAT2-pYX212t. A yeast 2µ vector pYX212 containing a URA3 selection marker was used and linearized by digestion with the restriction enzymes SphI and EcoRI. The linearized vector accompanied with fragments TPIlp-BAT2-pYX212t were transformed into *S. cerevisiae* BY4741 and selected on the uracil free plates ("SC-URA") following a LiAc/SS carrier DNA/PEG method. Cell density (OD at 600 nm) was measured by GENESYS 20 spectrophotometer (Thermo Scientific). Plasmids extracted from transformants by Zymoprep Yeast Plasmid Miniprep II kit were transformed into *E. coli* DH5a and purified, yielding plasmid YP7. All plasmids were verified by digestion and sequencing.

The plasmids YP6 or YP7 were evaluated in the strain SPD-B(KanMX) (MATa SUC2 MAL2-8C ura3-52 his3-A1 ARG3p::KEX2p car2A::LoxP-CTC1t-AGCl-hHT7p-TPIlp-ORT1-pYX212 t ura3::LoxP-TEF lp-GDH1 -DIT1t oaz1Δ::LoxP-KanMX-LoxP-p YX2 12t-SPE3-PGKlp-TEFlp-SPE1-PRM9t-DITlt-SPE2-TDH3p) which was previously engineered for increased putrescine production as well as increased conversion of putrescine to spermidine via overexpression of spermidine synthase and S-adenosylmethionine decarboxylase. This strain was constructed using the same procedure as described in Example 1. The strain SPD-B(KanMX) was transformed with either pYX212 and G04 (an empty plasmid and a plasmid encoding biosynthetic steps for ornithine production, respectively), YP6 and G04, or YP7 and G04. This resulted in the strains SPDC4, SPD07 and SPD08, respectively. All strains were verified by sequencing and evaluation by cultivation and analytics as described in Example 1 above. The results showed that while SPDC4 could already produce high levels of spermidine, spermidine production could be doubled by increasing flux through the methionine salvage pathway via overexpression of either *MEW* or BAT2 (Figure 5).

**Example 4**

**Further increasing spermidine production by overexpression of a heterologous spermidine synthase and fed-batch fermentation**

The present example demonstrates that the modifications described so far can be further combined with overexpression of a heterologous spermidine synthase [EC 2.5.1.16] from *Triticum aestivum* (TaSPDS), which is more efficient than the native yeast SPDS to further
increase spermidine production. Furthermore, subjecting the best-producing strain to fed-batch fermentation allowed for a very strong increase in spermidine production titers.

Thus spermidine synthase encoding gene TaSPDS from Triticum aestivum (wheat) was chosen to be codon-optimized and synthesized by Genscript (Piscataway, NJ, USA). TaSPDS (SEQ ID NO: 19) accompanied with SPE1 (SEQ ID NO: 20) and SPE2 (SEQ ID NO: 21) were subject to co-overexpression based on chromosomal integration in the OAZ1 loci. Fragments contain promoters, terminators, TaSPDS (with primer pairs 283/282), SPE1, SPE2, KanMX cassette, -300 bp of 5’ OAZ ORF and -300 bp of 3’ OAZ ORF were fused together following the method as described in example 1. All the related fragments were co-transformed in to L-ornithine platform strains ORN-L (MATa SUC2 MAL2-8c ura3-52 his3-Al ARG3p::KEX2p car2A::LoxP-CTC ltt-AGC 1-tHXT7p-TPIlp-ORT 1-pYX2 12t ura3::LoxP-TEF lp-GDHl -DITlt) (Qin et al [5]). The transformants were selected on G418 plates. Clones were verified by colony PCR, resulting strain in SPD-D (KanMX) (MATa SUC2 MAL2-8c ura3-52 his3-Al ARG3p::KEX2p car2A::LoxP-CTC ltt-AGC 1-tHXT7p-TPIlp-ORT 1-pYX2 12t ura3::LoxP-TEF lp-GDHl -DITlt oazlA::LoxP-KanMX-LoxP-pYX212t-TaSPDS-PGKlp-TEFlp-SPEl-PRM9t-DITlt-SPE2-TDH3p).

Next, the genes MEUl and BAT2 above were combined into a single plasmid (YP8), which was constructed via the DNA assembler method. MEUl, BAT2, promoter TPIlp, TEF lp and PGKlp, the terminator FBAlt and CYClt were amplified from CEN.PK113-1 IC genomic DNA with primers listed in Table 4 as described in Example 3 above. The terminator pYX212t were amplified from plasmid pYX212 with primers listed in Table 4 as described in Example 3 above. TPIlp, MEUl, FBAlt, CYClt, BAT2, PGKlp, TEF lp and pYX212t which has overhangs to each other were fused by fusion PCR, resulting in the fragment TPIlp-MEUl-FBAAlt-CYClt-BAT2-PGKlp-TEFlp-pYX212t. A yeast 2μ vector pYX212 containing a URA3 selection marker was used and linearized by digestion with restriction enzyme Sphl and EcoRI. The linearized vector accompanied with fragments TPIlp-MEUl-FBAAlt-CYClt-BAT2-PGKlp-TEFlp-pYX212t were transformed into S. cerevisiae BY4741 and selected on the uracil free plates (“SC-URA”) following a LiAc/SS carrier DNA/PEG method. Plasmids extracted from transformants by Zymoprep Yeast Plasmid Miniprep II kit were transformed into E. coli DH5α and purified, yielding plasmid YP8. The plasmid was verified by restriction digest and sequencing.

The plasmid YP8 was transformed into strain SPD-D(KanMX), yielding strain SPD10, able to produce 149 mg/L of spermidine when cultivated as described above.
In order to improve the fermentation efficiency and increase the TYR (titre, yield and rate), a glucose limited fed-batch fermentation was developed. The aerobic fed-batch process was performed in 1 L DasGip bench fermenters with working volume of 0.8 liter. Agitation at 800 rpm was maintained using an integrated stirrer (DasGip, Jiilich, Germany) and the temperature kept at 30°C. The rate of aeration was set to 1 vvm. The pH of the medium was maintained at 4.0 by automatic addition of 2 N KOH during the batch phase and 3 N KOH in the feed phase. The temperature, agitation, gassing, pH and composition of the off-gas were monitored and controlled using the DasGip monitoring and control system. Dissolved oxygen concentration was monitored with an autoclavable polarographic oxygen electrode (Mettler Toledo, Columbus, OH, USA) and kept above 30% via stirrer speed and gas flow rate using the DasGip control system. The effluent gas from the fermentation was analyzed for real-time determination of oxygen and CO₂ concentration by DasGip fedbatch pro® gas analysis systems with the off gas analyzer GA4 based on zirconium dioxide and two-beam infrared sensor.

The fed-batch cultures were initiated as batch cultures using 10 g/liter glucose. Feeding with fresh medium commenced only after residual ethanol produced from the glucose consumption phase was completely depleted. A feed strategy was designed keeping the volumetric growth rate constant. An exponential feed rate \( v(t) \) (liter/h) was calculated according to [10]:

\[
v(t) = \frac{Y_{so} \mu}{s_t - s_0} x_o V_0 \exp(\mu t)
\]

where \( x_o, s_o \) and \( V_0 \) were the biomass density (g DCW/liter), the substrate concentration (g/liter) and the reactor volume (liter) at the start of the fed-batch process, \( Y_{so} \) was the respiratory yield coefficient (g glucose/g DCW); \( s_t \) was the concentration of the growth limiting substrate (g glucose/liter) in the reservoir; \( \mu \) the was the specific growth rate (h-1) during the feed phase and \( t \) the feeding time. According to the equation above the feed was increased exponentially with a specific feed rate of 0.06 h-1. Correct feed rate addition was obtained programming the fermenter fb-pro software (DasGip) and controlled using the DasGip control system.

For batch cultivations, Delft medium was used consisting of the following (per liter): (NH₄)₂SO₄, 5 g; KH₂PO₄, 3 g; MgSO₄·7H₂O, 0.50 g; 0.05 ml; trace metals, 1 ml and vitamins, 1 ml. The trace metal solution consisted of the following (per liter): Na₂EDTA·5H₂O, 19.0 g; ZnSO₄·7H₂O, 0.45 g; MnCl₂·4H₂O, 1 g; CoCl₂·6H₂O, 0.3 g; CuSO₄·5H₂O, 0.3 g; Na₂MoO₄·2H₂O, 0.4 g; CaCl₂·2H₂O, 0.45 g; FeSO₄·7H₂O, 0.3 g; H₃BO₃, 1 g and KI, 0.10 g. The pH of the trace metal solution was adjusted to 4.0 with 2 M NaOH prior to heat sterilization.
The vitamin solution contained (per liter): d-biotin, 0.05 g; p-amino benzoic acid, 0.2 g; nicotinic acid, 1 g; Ca-pantothenate, 1 g; pyridoxine-HCl, 1 g; thiamine-HCl, 1 g and myo-inositol, 2.5 g. The pH of the vitamin solution was adjusted to 6.5 with 2 M NaOH. The vitamin solution was filter sterilized and stored at 4°C. This medium was supplemented with 10 g/liter glucose. The feed composition used for fed-batch cultivation had the same composition as described above, but the \( (\text{N}_3\text{H}_4\text{O}_6\text{S}_2\text{O}_4) \text{KH}_2\text{PO}_4 \); \( \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \), vitamin solution, and trace metal solution concentrations were increased 3 to 5 times, the glucose concentration was set to 200 to 600 g/liter. Glucose-limited fed-batch fermentation allowed for a strong increase in spermidine production, increasing spermidine titers to as much as 1.4 g/L (Figure 6).

**Example 5**

**Spermidine production is increased by overexpression of S-adenosylmethionine synthetase (MAT)**

The present example demonstrates that spermidine production can be increased by increasing synthesis of S-adenosylmethionine (SAM). Increase in the synthesis of SAM is obtained through overexpression of S-adenosylmethionine synthetase (MAT) [EC 2.5.1.6], which catalyzes the transfer of the adenosyl group of ATP to the sulfur atom of methionine to form SAM. To study the effects of MAT overexpression on spermidine production, two heterologous MATs were expressed in the yeast *Saccharomyces cerevisiae*. The first MAT was MAT from *Streptomyces spectabilis* (SsMAT) wherein lysine 18, leucine 31, isoleucine 65 and/or aspartic acid 341 are mutated into arginine, proline, valine and glycine, respectively (K18R, L31P, I65V, D341G) (protein SEQ ID NO: 1). The second MAT was from *Leishmania infantum* JPCM5 (SiMAT, protein SEQ ID NO: 25).

Each gene was synthesized with codon optimization for expression in *S. cerevisiae*. The nucleotide sequences of the codon-optimized genes (flanked by restriction sites *XbaI*Xhol and including a kozak sequence) are SEQ ID NO: 138 for SsMAT and SEQ ID NO: 139 for SiMAT. Both genes were then amplified by PCR using primers HX-fwd/HX-rev. The purified PCR products were digested using *XbaI*Xhol and each one was cloned into plasmid p416TEF [11] under the control of the TEF1 promoter, resulting in plasmids pBPSPD.ST01 (expressing SsMAT) and pBPSPD.ST02 (expressing SiMAT). Both were confirmed by digestion/agarose gel electrophoresis and sequencing.

The background strain used for testing the constructs was SPD-G(KanMX) which contains modifications in the ornithine and spermidine metabolism to facilitate flux towards spermidine production, but no modifications in the SAM metabolism. This strain was obtained
by overexpressing the polyamine transporter encoded by S. cerevisiae TP04 in strain SPD-G(KanMX). This gene was expressed under the control of TPII promoter and END2 terminator. This strain was constructed using the same procedure as described in Example 1. The resulting genotype of the strain is MATa SUC2 MAL2-8c ura3-52 his3-A1 ARG3p::KEX2p car2A::LoxP-CTCIt-AGCl-thXT7p-TPlp-ORT1-pYX212t ura3::: LoxP-TEFlp-GDHI-DITlt oazlA::LoxP-KanMX-LoxP-pYX212t-TaSPDS-PGKlp-TEFlp-SPEl-PRM9t-DITlt-SPE2-TDH3p-TPlp-TP04-END2t.

To test the effect of MAT overexpression on spermidine production, strain SPD-G(KanMX) was transformed with either plasmid p416TEF (empty plasmid used as a control), plasmid pBPSPD.STOl, or plasmid pBPSPD.ST02, following a LiAc/SS carrier DNA/PEG method and selected on the uracil free plates ("SC-URA"). This resulted in in strains SPD13, SPD14 and SPD15 (respectively).

The performance of the strains was compared in triplicates via shake-flask cultivations. For shake-flask cultivations, Delft medium (H) was used consisting of the following (per liter):

- (NH4)2SO4, 7.5 g; KH2PO4, 14.4 g; MgSO 4•7H2O, 0.50 g; histidine, 100 mg; trace metals, 1 ml and vitamins, 1 ml. The trace metal solution consisted of the following (per liter): Na2EDTA•5H2O, 19.0 g; ZnSO 4•7H2O, 0.45 g; MnCl2•4H2O, 1 g; CoCl2•6H2O, 0.3 g; CuSO 4•5H2O, 0.3 g; Na2MoO 4•2H2O, 0.4 g; CaCl2•2H2O, 0.45 g; FeSO 4•7H2O, 0.3 g; H3BO3, 1 g and KI, 0.10 g. The pH of the trace metal solution was adjusted to 4.0 with 2 M NaOH prior to heat sterilization. The vitamin solution contained (per liter): d-biotin, 0.05 g; p-amino benzoic acid, 0.2 g; nicotinic acid, 1 g; Ca-pantothenate, 1 g; pyridoxine-HCl, 1 g; thiamine-HCl, 1 g and myo-inositol, 25 g. The pH of the vitamin solution was adjusted to 6.5 with 2 M NaOH. The vitamin solution was filter sterilized and stored at 4°C. This medium was supplemented with 20 g/liter glucose and the pH was adjusted to 6 using 6M KOH. Single colonies were initially inoculated into 3 ml liquid medium and cultivated for 24 to 36 hours. The cells were then grown in 100 ml shake flasks with 20 ml medium for 3 days (72 hours) with the initial OD 0.1 at 200rpm, 30°C.

Spermidine production by the strains was analyzed as described in Example 1 and normalized by the OD of the respective culture. The results showed that expression of either MAT resulted in increased spermidine production. Expression of SsMAT (SPD14) increased spermidine production by 64%, while expression of SiMAT (SPD15) increased production by 90% relative to control that only harbored the empty plasmid (SPD13) (Figure 8).

Example 6
Spermidine production is increased by overexpression of a chimeric methylenetetrahydrofolate reductase (MTHFR)

The present example demonstrates that spermidine production can be increased by increasing the activity of Methylenetetrahydrofolate reductase (MTHFR) [EC 1.5.1.20], which catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate used to methylate homocysteine in methionine biosynthesis. In this example, a chimeric MTHFR comprised of the yeast Metl3p N-terminal catalytic domain and the Arabidopsis thaliana MTHFR (AtMTHFR-1) C-terminal regulatory domain (ScAtMTHFR; protein SEQ ID NO: 3) and PDR2 (also known as YRR1; nucleotide SEQ ID NO: 142; corresponding protein SEQ ID NO: 129) was used. The gene was codon-optimized for expression in S. cerevisiae and synthesized. The nucleotide sequence of the codon-optimized gene (flanked by restriction sites Xball/Xhol and including a kozak sequence) is SEQ ID NO: 140.

The gene was PCR amplified using primers HX-fwd/HX-rev. The purified PCR product was digested using Xball/Xhol and cloned into plasmid p416TEF under the control of the TEF1 promoter, resulting in plasmid pBPSPD.ST03. Plasmid pBPSPD.ST03 was confirmed by restriction digestion and sequencing and transformed into strain SPD-G(KanMX) using the LiAc/SS carrier DNA/PEG method, followed by selection on uracil free plates ("SC-URA"), resulting in strain SPD16.

To test the effect of MTHFR overexpression, strain SPD16 and strain SPD13 (control strain not overexpressing MTHFR) were cultivated as described in Example 5 and analyzed as describe in Example 1. Spermidine production was normalized by the OD of the respective culture. The results showed that overexpression of ScAtMTHFR (strain SPD16) resulted in 50% increase in spermidine production compared to a strain not overexpressing MTHFR (strain SPD13, which only harbours an empty plasmid), confirming that increased MTHFR activity has a positive effect on spermidine production (Figure 9).

Example 7

Spermidine production is increased by overexpression of transcription factors responsible for regulating exporters involved in the pleiotropic drug response

The present example demonstrates that overexpression of transcription factors involved in the pleiotropic drug response (S. cerevisiae PDR1 and PDR2) results in increased spermidine production.

PDR1 (nucleotide SEQ ID NO: 141; corresponding protein SEQ ID NO: 30) and PDR2 (also known as YRR1; nucleotide SEQ ID NO: 142; corresponding protein SEQ ID NO: 129)
were each amplified from the *S. cerevisiae* genome by PCR using primers PDR1-fwd/PDRR1-rev and PDR2-fwd/PDRR2-rev, respectively and cloned into plasmid p416TEF using restriction enzymes *XbaI/BamHI* and *XhoI/BamHI*, respectively. This resulted in plasmids pBPSPD.ST04 (expressing PDR1) and pBPSPD.ST05 (expressing PDR2). Both plasmids were confirmed by digestion/agarose gel electrophoresis and sequencing. The plasmids were then separately transformed into strain SPD-G(KanMX) using the LiAc/SS carrier DNA/PEG method and selected on uracil free plates ("SC-URA"), resulting in strains SPD17 (containing pBPSPD.ST04) and SPD18 (containing pBPSPD.ST05).

The strains SPD17 and SPD18 were then cultivated along strain SPD13 (control strain harboring empty p416TEF and not overexpressing any PDR) as described in Example 5 and analyzed as describe in Example 1. Spermidine production was normalized by the OD of the respective culture. The results showed that overexpression of either PDR had a positive effect on spermidine production (Figure 10). While overexpression of PDR1 (strain SPD17) resulted in a modest 9% increase in spermidine production, overexpression of PDR2 (strain SPD18) resulted in a 93% increase in spermidine production compared to control (strain SPD13, not overexpressing PDR1).

**Example 8**

**Spermidine production is increased by overexpression of** subunits of the RNA Polymerase II mediator complex

The present example shows that overexpression of subunits of the RNA Polymerase II mediator complex, in particularly *S. cerevisiae* GAL11 (protein sequence shown in SEQ ID NO: 31) has a positive effect on spermidine production.

The gene *GAL11* (nucleotide SEQ ID NO: 143) was amplified from the genome of *S. cerevisiae* strain CEN.PK1 13-7D using primers Gall lfwd/Gall 1-rev and cloned into plasmids p416TEF and p416CYC [11] using restriction enzymes *XbaI/BamHI*. This resulted in plasmid pBPSPD.ST06 (with *GAL11* cloned under the control of a *S. cerevisiae TEF1* promoter) and plasmid pBPSPD.ST07 (with *GAL11* cloned under the control of a *S. cerevisiae CYC1* promoter). Both plasmids were confirmed by digestion/agarose gel electrophoresis and sequencing.

The plasmids were then separately transformed into strain SPD-G(KanMX) using the LiAc/SS carrier DNA/PEG method and selected on uracil free plates ("SC-URA"), resulting in strains SPD19 (containing pBPSPD.ST06) and SPD20 (containing pBPSPD.ST07). The strains SPD19 and SPD20 were then cultivated along strain SPD13 (control strain harboring empty
p416TEF and not overexpressing GAL1) as described in Example 5 and analyzed as described in Example 1. Spermidine production was normalized by the OD of the respective culture.

The results showed that overexpression of GAL11 had a positive effect on spermidine production (Figure 11). Overexpression of GAL11 under the control of the TEF1 promoter (strain SPD19) resulted in a 3.4-fold increase in spermidine production, while overexpression of GAL11 under the control of the CYC1 promoter (strain SPD20) resulted in a 2.5-fold increase in spermidine production, compared to control (strain SPD13, not overexpressing GAL11).

Example 9

Construction of yeast strains for increased spermine production

The purpose of this example is to describe how to construct strains that have increased spermine production as a result of increased S-adenosylmethionine (SAM) levels.

In order to increase spermidine production by the cell, the gene Spermine Synthase (EC 2.5.1.22), encoded by S. cerevisiae SPE4 (nucleotide SEQ ID NO: 144; corresponding protein SEQ ID NO: 128) can be overexpressed.

To overexpress SPE4 in S. cerevisiae, SPE4 is amplified from the genome of S. cerevisiae and cloned into the vector pLYC04 [12] under the control of the TEF1 promoter, yielding plasmid pLYC04-SPE4. This plasmid is then co-transformed with either p416TEF (empty plasmid) into strain SPD-G(KanMX) or pBPSPD.ST02 (p416TEF expressing SiMAT), resulting in strains SPD21 and SPD22, respectively. Transformation is performed using the LiAc/SS carrier DNA/PEG method and selected on uracil and histidine free plates ("SC-URA-HIS"). The resulting strains are cultivated as described in Example 5, with the exception that no histidine is added to the media, and analyzed for spermine production as described in Example 1.

Example 10

Construction of bacterial cells for increased spermidine/spermine production

The purpose of this example is to describe how to construct bacterial strains that have increased spermine production as a result of increased S-adenosylmethionine (SAM) levels.

To create E. coli strain with increased spermidine production, the strain E. coli WL3110 (orig. K12 W3110 (CGSC, Coli Genetic Stock Center) can be used. This strain is transformed with either plasmids for overexpression of endogenous or heterologous speE (EC : 2.5.1.16; SEQ ID no. 145), speD (EC 4.1.1.50; SEQ ID no. 146) and MAT (EC 2.5.1.6; SEQ ID NO: 139) genes. For example the expression vector pTRC-LIC (Plasmid #62343; Addgene
(Massachusetts, USA)) is used to systematically co-express the mentioned genes under control of the strong \( P_{trc} \) promoter. Cloning is done via amplification of target genes through 30 bp overhang primers to the expression vector \( P_{trc} \) promoter control and carried out via the Gibson cloning approach [13]. For the tailored production of spermine, heterologous spermine synthases (E.C. 2.5.1.22) from eukaryotic sources are expressed e.g. derived from \( S. \) cerevisiae, Triticum aestivum, Oryza sativa, Glycine max, Citrus sinesis, Homo sapiens. For example spermine synthase gene \( SPE4 \) (GI: 3201942; nucleotide SEQ ID NO: 144; corresponding protein SEQ ID NO: 128) is amplified from \( S. \) cerevisiae genomic DNA (CEN.PK1 13-1 1C) using PCR with 30 bp overhang primers and cloned via Gibson cloning (Gibson et al. 2009) into expression vector pTRC.

To create a \( C. \) glutamicum strain with increased spermidine production, the \( C. \) glutamicum strain ATCC 13032 can be used. For increased spermidine production the genes \( speE \) (SEQ ID no. 145), \( speD \) (EC 4.1.1.50; SEQ ID no. 146) and MAT (EC 2.5.1.6; SEQ ID NO: 139) can be overexpressed through a plasmid based expression. For example E. coli - \( C. \) glutamicum shuttle vector pMS2 (ATTC®67189TM) with kanamycin as selective marker can be used. 30 bp overhanging primers are used to amplify fragments and subsequently cloned via Gibson cloning [13] into the shuttle vector pMS2 with \( speE, speD \) and MAT under \( Ptac \) control creating the expression vector pFDAMS2. After transformation into the modified \( C. \) glutamicum strain ATCC 13032 strain (see above) it is cultivated as described in Schneider et al. 2010 [14].

For the production of spermine heterologous spermine synthases (E.C. 2.5.1.22) from eukaryotic sources are expressed via plasmid-based expression (see above). For example spermine synthases can be derived from \( S. \) cerevisiae (\( SPE4 \), (nucleotide SEQ ID NO: 144; corresponding protein SEQ ID NO: 128), Triticum aestivum, Oryza sativa, Glycine max, Citrus sinesis, Homo sapiens.

Table 1. Constructed background strains

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<td>Genotype or characteristics</td>
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Table 2. Plasmid constructs
### Table 3. Polyamine over-producing strains

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Table 4. Primers
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| 12 | PRM9t-R1  | 43 | ATTTTCAACATCGTATTTCCGAAGC |
| 13 | OAZ1-R-F2(PRM9)  | 44 | GCTTCGGAATAATACGATGTTTTGAAATCCTGAAACCA AAACTTCAAATTCGAATAC |
| 14 | pyx212t-Ri(kan)  | 45 | GTGATATGACATCCACTAGTGCGCTGGCGTAAACC ACTAAATCGGAACCC |
| 15 | pyx212t-F1  | 46 | TAGGGCCCACAAGCTTACGCGTCGAC |
| 16 | SPE3-Ri(pyx21 2t)  | 47 | GTGCAGCGTGTAAGCTTTGTGGCCACTAATTTTAAT TCTTGGCCTGCCG |
| 17 | SPE3-Fi(PGK)  | 48 | CTACTTTTTAACAATAATAACAAATGGGCACAA GAAATCCTCAACCAAC |
| 18 | PGKlp-R1  | 49 | TTTGTTATATTTAGTGTAAAGAAGTAG |
| 19 | PGKlp-Fi  | 50 | ACGCAGATATTTATAACATCTGCAC |
| 20 | TEFlp-F2(PGKlp)  | 51 | GTGCGAGATGTTATATAATATCTGTGCGTATAGCTTCAA AATGTTTCTACTCC |
| 21 | DITlt-Ri(PRM9t  )  | 52 | CTTCGGAATAACGATGTTAAATGTACTCCGC AACGCTTTTCTGAAAC |
| 22 | DITlt-Fi  | 53 | TAAAGTAAGAGCGCTACATTTGCTTACC |
| 23 | SPE2-Rl(DITlt)  | 54 | GTTAGACAAATGTTGCTCTTTATATCATATTT TCTTCTGCAATTTCCATAG |
| 24 | SPE2-F1(TDH3 P)  | 55 | GTTTCGAATAAAACACACATAAAAACAAATGAC TGTCACCATAAAGAATTGAC |</p>
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The embodiments described above are to be understood as a few illustrative examples of the present invention. It will be understood by those skilled in the art that various modifications, combinations and changes may be made to the embodiments without departing from the scope of the present invention. In particular, different part solutions in the different embodiments can be combined in other configurations, where technically possible. The scope of the present invention is, however, defined by the appended claims.

REFERENCES

1. WO 2016/144247
2. Kim SK, Jin YS, Choi IG, Park YC, Seo JH. Enhanced tolerance of Saccharomyces cerevisiae to multiple lignocellulose-derived inhibitors through modulation of
spermidine contents. Metab Eng. 2015 May;29:46-55. doi: 10.1016/j.ymben.2015.02.004


CLAIMS

1. A microbial cell capable of producing spermidine, wherein
   said microbial cell is genetically modified for overexpression of at least one enzyme
   selected from the group consisting of S-adenosylmethionine decarboxylase (EC 4.1.1.50) and
   spermidine synthase (EC 2.5.1.16); and
   said microbial cell is genetically modified for enhanced S-adenosylmethionine (SAM)
   biosynthesis.

2. A microbial cell according to claim 1, wherein said microbial cell is genetically modified
   for overexpression of at least one enzyme selected from a group consisting of ornithine
decarboxylase (ODC) (EC 4.1.1.17), N-acetylglutamate synthase (NAGS) (EC 2.3.1.1),
acetylglutamate kinase (EC 2.7.2.8), N-acetyl-gamma-glutamyl-phosphate reductase (EC
1.2.1.38), acetylornithine aminotransferase (EC 2.6.1.11), acetylornithine deacetylase (EC
3.5.1.16) and ornithine acetyltransferase (EC 2.3.1.35).

3. A microbial according any of the claims 1-2, wherein said microbial cell is genetically
   modified for attenuated activity of ornithine decarboxylase antizyme (OAZ), L-ornithine
transaminase (EC 2.6.1.13) and/or ornithine carbamoyltransferase (OTC) (EC 2.1.3.3) or
deletion or disruption of at least one gene selected from a group consisting of a gene encoding
OAZ, a gene encoding L-ornithine transaminase and a gene encoding OTC.

4. A microbial cell according to any of the claims 1-3, wherein said microbial cell is
   genetically modified for enhanced S-adenosylmethionine (SAM) biosynthesis by
overexpression of S-adenosylmethionine synthetase (MAT) (EC 2.5.1.6).

5. A microbial cell according to any of the claims 1-4, wherein said microbial cell is
   genetically modified for enhanced S-adenosylmethionine (SAM) biosynthesis by
overexpression of methylenetetrahydrofolate reductase (MTHFR) (EC 1.5.1.20).

6. A microbial cell according to any of the claims 1-5, wherein said microbial cell is
   genetically modified for enhanced S-adenosylmethionine (SAM) biosynthesis by
overexpression of phosphoribosyldiphosphate (PRPP) 5-amidotransferase (EC 2.4.2.14).
7. A microbial cell according to any of the claims 1-6, wherein said microbial cell is genetically modified for enhanced S-adenosylmethionine (SAM) biosynthesis by overexpression of at least one enzyme in the methionine salvage pathway selected from a group consisting of methylthioadenosine phosphorylase (MTAP) (EC 2.4.2.28), 5-methylthioribose-1-phosphate isomerase (MRI) (EC 5.3.1.23), methylthioribulose-1-phosphate dehydratase (MDE) (EC 4.2.1.109), 2,3-dioxomethiopentane-1-phosphate enolase/phosphatase (EC 3.1.3.77), acireductone dioxygenase (EC 1.13.1.54) and branched-chain amino acid transaminase (BAT) (EC 2.6.1.5, EC 2.6.1.57, EC 2.6.1.42), preferably MTAP and BAT.

8. A microbial cell according to any of the claims 1-7, wherein said microbial cell is genetically modified for enhanced conversion of L-aspartate-4-semialdehyde to methionine.

9. A microbial cell according to claim 8, wherein said microbial cell is genetically modified for enhanced conversion of L-aspartate-4-semialdehyde to methionine by overexpression of at least one enzyme selected from a group consisting of homoserine dehydrogenase [EC 1.1.1.3]; homoserine O-acetyltransferase [EC 2.3.1.31]; O-acetylhomoserine (thiol)-lyase [EC 2.5.1.49], also referred to as O-acetylhomoserine aminocarboxypropyltransferase; and 5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase (METE) [EC 2.1.1.14].

10. A microbial cell according to any of the claims 1-9, wherein said microbial cell is genetically modified for enhanced conversion of pyruvate to L-aspartate-4-semialdehyde.

11. A microbial cell according to claim 10, wherein said microbial cell is genetically modified for enhanced conversion of pyruvate to L-aspartate-4-semialdehyde by overexpression of at least one enzyme selected from a group consisting of pyruvate carboxylase (EC 6.4.1.1), aspartate aminotransferase (EC 2.6.1.1), aspartate kinase (AK) (EC 2.7.2.4) and aspartic beta-semialdehyde dehydrogenase (EC 1.2.1.1).

12. A microbial cell according to any of the claims 1-11, wherein said microbial cell is genetically modified for overexpression of subunits of the RNA Polymerase II mediator complex, preferably overexpression of Saccharomyces cerevisiae GAL11 (SEQ ID NO: 31).
13. A microbial cell according to any one of the claims 1-12, wherein said microbial cell is genetically modified for enhanced spermine biosynthesis by overexpression of spermine synthase (EC 2.5.1.22).

14. A microbial cell according to any of the claims 1-13, wherein said microbial cell is genetically modified for overexpression of *Saccharomyces cerevisiae* *PDR1* (SEQ ID NO: 30) and/or *PDR2* (SEQ ID NO: 129).

15. A microbial cell according to any of the claims 1-14, wherein said microbial cell is genetically modified for overexpression of S-adenosylmethionine decarboxylase (EC 4.1.1.50) and spermidine synthase (EC 2.5.1.16).

16. A method for producing spermidine comprising:
   - culturing a microbial cell according to any of the claims 1-15 in a culture medium and in culture conditions suitable for production of spermidine from said microbial cell; and
   - collecting spermidine from said culture medium and/or said microbial cell.

17. Use of a microbial cell according to any of the claims 1-15 as a food additive.
Figure 1:

Mitochondrion

- Glucose → pyruvate → acetetyl-CoA → TCA cycle → α-ketoglutarate → N-acetyl-glutamate → glutamate

Cytosol

- N-acetyl-glutamate semialdehyde → N-acetylglutamyl-P → N-acetylglutamate
- N-acetyl-ornithine → ornithine
- glutamate γ-semialdehyde
- Ornithine → citrulline → putrescine
- S-Adenosylmethionine → 5'-Methylthioadenosine → spermidine

Chemical structures:

- S-Adenosylmethionine
- 5'-Methylthioadenosine
- Spermidine
Figure 2:
Figure 3:

![Graph showing spermidine titer (mg/L) for different samples: SPDC3, SPD11, SPD12.]

YP1 (CjCASDH + CjCASDC) - + -
YP2 (VcCASDH + VcCASDC) - - +
Figure 4:

Spermidine titer (mg/L)

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<th>SPD06</th>
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<td>Cglycm + Cgasd + CgaspB</td>
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<td>Kex2p-HOM6</td>
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Figure 5:

![Graph showing Spermidine titer (mg/L) for SPDC4, SPD07, and SPD08, with increased SPD and MEU1 and BAT2 markers.

- Increased SPD: SPDC4 (+), SPD07 (+), SPD08 (+)
- MEU1: SPDC4 (-), SPD07 (+), SPD08 (-)
- BAT2: SPDC4 (-), SPD07 (-), SPD08 (+)
Figure 6:
Figure 7:
Figure 8:

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<th>Relative spermidine levels (Arbitrary units/OD)</th>
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<td>SiMAT</td>
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Figure 9:

![Graph showing relative spermidine levels between SPD13 and SPD16.]

- **Increased SPD**: + for SPD13, + for SPD16
- **ScAtMTHFR**: - for SPD13, + for SPD16
Figure 10:

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<td>PDR2</td>
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Increased SPD: + + +
pTEF1-GAL11: - + -
pCYC1-GAL11: - - +
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N9/88 C12N9/10

ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N

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

Electronic database consulted during the international search (name of database and, where practicable, search terms used)

EPO-Internal, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Date of the actual completion of the international search

4 October 2018

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Authorized officer

Sphins, Matthew

Date of mailing of the international search report

16/10/2018

Form PCT/ISA/210 (second sheet) (April 2005)
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