



## L-ornithine production in eukaryotic cells

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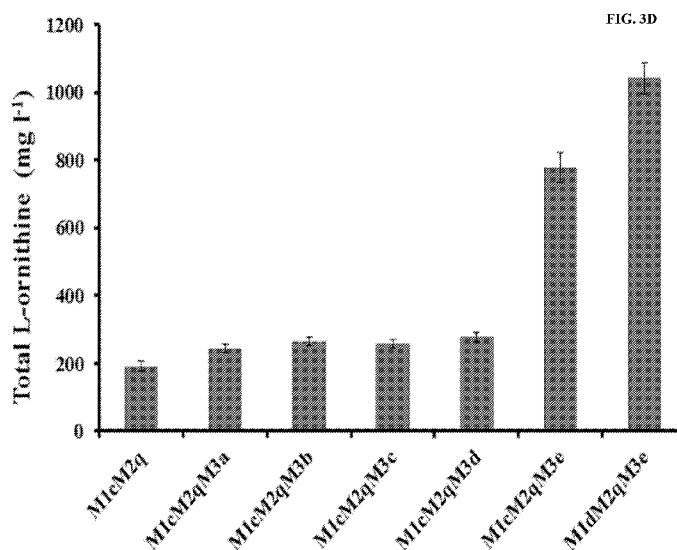
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(54) Title: L-ORNITHINE PRODUCTION IN EUKARYOTIC CELLS



(57) Abstract: The present invention relates to the provision of genetically modified microbial cells, such as yeast cells with an improved ability for producing L-ornithine and its derivatives. Overproduction of L-ornithine is obtained in the first place by the down-regulation or attenuation of specially selected genes, wherein said genes encode enzymes involved in the L-ornithine consumption and/or degradation pathways. Further L-ornithine production ability is improved by down-regulation, attenuation, deletion or over-expression of specially selected genes, wherein said genes encode enzymes and/or proteins involved in the L-ornithine 'acetylated derivatives cycle', L-glutamate synthesis pathways, subcellular trafficking, TCA cycle, pyruvate carboxylation pathway, respiratory electron-transport chain, and the carbon substrates' assimilation machinery. The invention additionally provides a method to produce L-ornithine with said modified eukaryotic cells.



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## L-ORNITHINE PRODUCTION IN EUKARYOTIC CELLS

### TECHNICAL FIELD OF THE INVENTION

The present invention relates to the development of genetically engineered microorganisms that can produce L-ornithine and its derivatives. More specifically the invention relates to the production of L-ornithine, which can, for instance, be used as food additive using genetically engineered eukaryotic cells. Such L-ornithine producing eukaryotic cells, for instance yeast cells, can also be directly used for baking, brewing, or making other food products. In addition, other new chemicals using L-ornithine as precursors in the cell can also be produced when said L-ornithine producing eukaryotic cells are subject to further genetic modifications. These chemicals include but not limited to L-arginine, L- citrulline, putrescine, spermidine, spermine, agmatine and tropane alkaloids, etc.

### BACKGROUND OF THE INVENTION

L-ornithine, the intermediate of L-arginine biosynthesis, has already been widely used as a dietary supplement, as it is known to be beneficial for the treatment of wound healing and liver disease. Furthermore, it is also the precursor of bulk chemicals such as putrescine, an important diamine used as a nylon monomer, and natural products such as tropane alkaloids, which are used as parasympholytics for competitively antagonizing acetylcholine.

L-ornithine is nowadays prepared by various processes, encompassing chemical synthesis and enzymatic catalysis. For instance, EP0464325A2 discloses an enzymic conversion process for the preparation of salts of L-ornithine from L-arginine in the presence of the enzyme L-arginase (EC 3.5.3.1.) extracted from animal liver. To reduce the cost of the enzyme, a whole-cell biotransformation system for the conversion of L-arginine to L-ornithine was also developed by constructing a recombinant *Escherichia coli* with overexpressed arginase (EC 3.5.3.1) encoding gene *ARG* from the bovine liver (Zhan et al. 2013). However, said whole-cell biotransformation system always has the problem of cell permeability, and addition of permeability reagent may lead to subsequent product separation problems. One idea has been to screen for a thermophilic enzyme, higher operation temperature could be used to improve the permeability of the recombinant cells. Arginase (*ARG*) from *Bacillus caldovelox* was found to be a potential thermophilic candidate (Patchett et al. 1991). Recently, the recombinant *E. coli* with *B. caldovelox ARG* gene was constructed, leading to an efficient and simple enzymatic process for the environment-friendly synthesis of L-ornithine from L-arginine (Song et al. 2014).

However, these methods either suffer from issues of expensive substrates, poor enantiopure purity or are environmental unfriendly.

Some L-citrulline or L-arginine auxotroph bacteria belong to the genus *Brevibacterium*, the genus *Corynebacterium*, the genus *Bacillus* and the genus *Arthrobacter* are known to produce L-ornithine. In addition, variants of said L-ornithine producing bacteria having resistance to arginine analogues and/or 2-thiazolealanine

and/or sulfaguanidine and/or 2-fluoropyruvic acid and/or microphenolic acid and/or ornithinol are said to have better L-ornithine producing performance. Metabolic engineering frameworks, which offer the ability to leverage the advantages of biocatalysts (e.g. precision, specificity) and tailor the carbon flow of microbes, have enabled construction of platform cell factories for producing amino acids. Recent advantages in the said metabolic engineering also lead to the strain construction for L-ornithine production (Hwang and Cho 2012; Hwang and Cho 2014; Hwang et al. 2008; Jiang et al. 2013a; Jiang et al. 2013b). For instance, WO 2012008809 A2 discloses a strategy to give the L-ornithine production at a high yield rate and with high efficiency by the fine-tuning of gluconate kinase (GntK) in *Corynebacterium*. In another disclose, US 8741608 B2 suggests that overexpressing of L-ornithine exporter is an efficient strategy to further improve L-ornithine titers. US 20140051132 A1 also discloses an invention where NCgl\_2067-NCgl\_2065 operon in *Corynebacterium* is/are attenuated lead to the improved production of L-amino acids belonging to L-glutamate family which includes L-ornithine. NCgl\_2067-NCgl\_2065 operon was suggested to encode negative regulators which directly controls expression of the related genes in the L-amino acids synthesis belong to L-glutamate.

However, one of the main drawbacks of L-ornithine production in bacterial strains is the phage contamination issues which often result in substantial economic losses. Thus, there is a need for better microbial strategies for the production of L-ornithine, putrescine, spermidine, spermine and other chemicals using these compounds as a precursor

#### SUMMARY OF THE INVENTION

Thus it is an object of the present disclose to provide an improved eukaryotic cell factory, such yeast cell factory that can be used for fermentation based production of L-ornithine. The L-ornithine synthesis pathway, and even the central metabolism in the said cell factory are harnessed to manage the said challenges.

An aspect of the embodiments relates to a eukaryotic cell capable of producing L-ornithine. The eukaryotic cell is genetically modified for attenuated activity of an ornithine carbamoyltransferase.

Another aspect of the embodiments relates to a eukaryotic cell capable of producing L-ornithine. The eukaryotic cell is genetically modified for enhanced L-ornithine biosynthesis from  $\alpha$ -ketoglutarate.

A further aspect of the embodiments relates to a eukaryotic cell capable of producing L-ornithine. The eukaryotic cell is genetically modified for enhanced  $\alpha$ -ketoglutarate biosynthesis.

Yet another aspect of the embodiments relates to a process for production of L-ornithine. The process comprising cultivating a eukaryotic cell according to the embodiments in the presence of a carbon source.

The eukaryotic cells of the embodiments can be further genetically modified for the production of polyamines. A further aspect of the embodiments relates to a process for production of a polyamine selected from the group consisting of putrescine, spermidine and spermine. The process comprising cultivating a eukaryotic cell according

to the embodiments in the presence of a carbon source.

The successful proof of concept production of L-ornithine with *S. cerevisiae* in the present disclose represents the first systematic case implementation to produce amino acids in eukaryotic microbes, which demonstrated the potential to use eukaryotic microbes as the cell factory to produce amino acid or even other amino acid derived chemicals.

According to the present invention microbes can be further engineered to produce L-ornithine-derived products, such as the polyamines putrescine, spermidine and spermine, as well as the arginine-derivative agmatine, which have a variety of industrial applications. This includes overexpression of native and heterologous steps in the biosynthesis of these compounds, as well as elimination of competitive steps. Furthermore, the cellular transport systems are manipulated to allow export of these compounds into the media. We also present several gene targets that can be manipulated in order to allow high tolerance to these compounds when synthesized at large quantities.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. L-arginine leaky auxotroph enables L-ornithine overproduction. (a) The attenuation of *ARG3* was implemented by replacing the original promoter of *ARG3* with the *HXT1* or *KEX2* promoter (*P<sub>HXT1</sub>-ARG3* and *P<sub>KEX2</sub>-ARG3*, respectively). Dashed arrows represent multiple reaction steps. (b) The transcriptional attenuation of Arg3p and knockout of L-ornithine potential consumption step (Car2p) led L-ornithine overproduction. Black filled circle indicates the molecular implementation is included in the strain under test. (c) The transcriptional attenuation of Arg3p decreased the intracellular L-arginine pool. All the strains were cultivated for 72 h in Delft medium. All data are presented as the mean  $\pm$  s.d. (n>3).

FIG.2: Improved L-ornithine production by optimizing the flux from  $\alpha$ -ketoglutarate to L-ornithine 'acetylated derivatives cycle'. Three strategies were proposed to eliminate the obstacles in the Module 2 as follows: (a) Strategy (i), intrinsic pathways including intracellular trafficking steps of Module 2 was strengthened; (b) Strategy (ii), L-glutamate dehydrogenase reaction was proposed to be re-localized to the mitochondria to bypass the potential intracellular trafficking of  $\alpha$ -ketoglutarate and (c). Strategy (iii), L-glutamate and chimeric L-ornithine 'acetylated derivatives cycle' from bacteria was re-localized to cytosol to bypass the potential obstacles of intracellular metabolites' trafficking. (d) Pathway variants of Strategy (i) and Strategy (iii) in Module 2 enable substantial increase of L-ornithine titers. All the strains were cultivated for 72 h in definite Delft medium. All data are presented as the mean  $\pm$  s.d. (n>3).

FIG.3: L-ornithine production improved through strategies to reduce the 'Crabtree effect'. Different strategies to reduce the 'Crabtree effect' on the purpose of improve the carbon channeling from carbon substrate L-glucose to product L-ornithine were proposed. Abbreviation: K, citric acid cycle; O, L-ornithine 'acetylated derivatives cycle'; EMP, glycolysis pathway; PYC, pyruvate; EOH, ethanol; ACT, aldehyde; GLC, glucose; a-KG,  $\alpha$ -

ketoglutarate; MPC, mitochondrial pyruvate carrier; OdC1p, proposed mitochondria  $\alpha$ -ketoglutarate transporter protein; Ort1 p, mitochondria L-ornithine transporter; AgC1p, mitochondria L-glutamate transporter; *MTH1-AT*, truncated version of *MTH1*, AOX, NADH alternative oxidase; Q, ubiquinone; Ndil p, NADH:ubiquinone oxidoreductase. Respiratory chain (complexes III-IV) is shown as a rectangle, and ATP synthase (complex V) as a square. (a) Strategy (i), directly overexpresses the TCA cycle enzymes including the mutated version of Pda1 p which was suggested to improve the TCA cycle flux. The circled + and - symbols denote the reactions/pathways that increased oxidative TCA cycle activity is expected to accelerate or slow down. (b) Strategy (ii), overexpress the NADH alternative oxidase from *H. anomala* (*HaAOXI*) to increase the capacity of the respiration chain which was suggested could reduce the 'Crabtree effect'. The circled + and - symbols denote the reactions/pathways that overexpression of AOX and Ndil p is expected to accelerate or slow down. (c) Strategy (iii), control the glucose uptake rates by overexpression the truncated Mthl p from which the dephosphorylating site was removed. The circled + and - symbols denote the reactions/pathways that overexpression of *MTH1-AT* is expected to accelerate or slow down. (d) Strains with variants pathways in Module 3 led to increase production of L-ornithine. All the strains were cultivated for 72 h in definite Delft medium. All data are presented as the mean  $\pm$  s.d. (n>3) (e) Physiological characterization of strain harboring truncated *MTH1-AT* and the control.  $\mu_{max}$  ( $h^{-1}$ ),  $\gamma_{EOH}$  [g ethanol (g DCW) $^{-1}h^{-1}$ ] and  $r_{glu}$  [g glucose (g DCW) $^{-1}h^{-1}$ ] are shown. All values were calculated in batch culture on glucose during exponential growth phase, identified by the linear relationship between the natural logarithm of culture time and biomass. All data are presented as the mean  $\pm$  s.d. (n>3). (f) Physiological characterization of strain harboring truncated *MTH1-AT* and the control.  $Y_{X/S}$  [(g DCW) (g glucose) $^{-1}$ ],  $Y_{ORN/S}$  [mg L-ornithine (g glucose) $^{-1}$ ] and  $Y_{EOH/S}$  [g ethanol (g glucose) $^{-1}$ ] are shown. All values were calculated in batch culture on glucose during exponential growth phase, identified by the linear relationship between the natural logarithm of culture time and biomass. All data are presented as the mean  $\pm$  s.d. (n>3).

FIG.4: Fed-batch fermentation of engineered strain in 3-l bioreactor. Time course profile of DCW (grey line) and L-ornithine (black line) of strain M1dM2qM3a were shown.

FIG.5: Qualitative verification of L-ornithine produced by proof of concept strain with GC-MS. (a) Gas chromatogram of L-ornithine (retention time 29.75 min). (b) High resolution mass spectrometry of L-ornithine.

FIG.6: RT-PCR indicated significant changes were in strains producing L-ornithine and the control strain. The comparisons are M1c vs. control, M1cM2r vs. control and M1cM2g vs. control. The grey-scale key represents  $\log_2$  [fold change], untextured grey represents up-regulation and dotted-grey represents down-regulation. The intensity of the grey colour correlates with the fold-change.

FIG.7: Proposed underline mechanisms by which the L-ornithine 'acetylated derivatives cycle' flux was boosted when Arg3p was attenuated.

FIG.8: The effect of truncated Gcn4p overexpression on L-ornithine titers. All the strains were cultivated for 72 h in definite Delft medium. All data are presented as the mean  $\pm$  s.d. (n>3).

FIG.9: The single overexpression of related genes in the L-ornithine 'acetylated derivatives cycle' showed no substantial effect to increase the L-ornithine titers. Black filled circle indicates the molecular implementation is included in the strain under test. All the strains were cultivated for 72 h in definite Delft medium. All data are presented as the mean  $\pm$  s.d. (n>3).

5 FIG.10: The effect of *ODC1* overexpression of L-ornithine titers in both GDH1 and GDH3 overexpression strain. All the strains were cultivated for 72 h in definite Delft medium. All data are presented as the mean  $\pm$  s.d. (n>3).

FIG.11: The mitochondria targeting of GDH1 and GDH2 gave substantial decrease to L-ornithine titers. All the strains were cultivated for 72 h in definite Delft medium. All data are presented as the mean  $\pm$  s.d. (n>3).

10 FIG.12: The cytosolic L-ornithine synthesis pathway was functional verified by complementary test. While the control strain could not grow in the medium without L-arginine, the strain harboring the cytosolic pathway restored the ability to grow on L-arginine negative medium. Control represents Strain B0166A (p423GPD) and G04 represents strain B0166A (0RT1). The overnight cultured cells were diluted to OD600 of 1, and 5 $\mu$ l aliquots of dilutions from 10<sup>-1</sup> to 10<sup>-6</sup> were spotted on the corresponding plates.

15 FIG.13: The *KGD2* knock-down gave L-ornithine titers decrease. All the strains were cultivated for 72 h in definite Delft medium. All data are presented as the mean  $\pm$  s.d. (n>3).

FIG.14: Overview of pathway leading to ornithine derivatives.

FIG. 15: Putrescine production in engineered yeast strains with or without an ornithine module. The ornithine module includes pKEX2-ARG3, *Acar2*, GDH1, 0RT1, AGC1 and the cytosolic ornithine synthetic pathway in the plasmid G04. Further engineering for putrescine production includes deletion of 0AZ1 and overexpression of SPE1. Both intra- and extra-cellular levels of putrescine were quantified.

FIG. 16: Spermidine production in engineered yeast strains. Modification to the ornithine metabolism include pKEX2-ARG3, *Acar2*, GDH1, 0RT1, AGC1 and the cytosolic ornithine synthetic pathway in the plasmid G04. Further engineering for spermidine production includes deletion of 0AZ1 and overexpression of SPE1, SPE2 and SPE3.

#### DETAILED DESCRIPTION

Several unique features of Baker's yeast *S. cerevisiae*, including its robustness, GRAS (generally recognized as safe) status, excellent availability of molecular biology tools, wide use in industry, including large scale bioethanol production, and the more efficient ability to express complex enzymes such as cytochrome P450-containing enzymes, make it an attractive chassis host for production various chemicals. Furthermore, as *S. cerevisiae* is also widely used in brewing and baking industries, the L-ornithine producing yeast could represent a plug and play solution for the production of L-ornithine containing drinks and/or baking products or even other



functional food products.

L-arginine biosynthesis is known for its compartmentalization in metabolism where L-ornithine, the precursor of L-arginine is synthesized in the mitochondria using L-glutamate as substrate which is produced in the cytoplasm. After transport to the cytoplasm, L-ornithine is converted to L-arginine after three consecutive steps. In addition, a-ketoglutarate, the precursor of L-glutamate is one of the intermediates of TCA cycle, the flux of which is limited by the so called 'Crabtree effect' leading to the formation of ethanol as main byproduct. It is obvious that engineering *S. cerevisiae* to accumulate amino acids including L-ornithine remains a daunting task.

According to the above mentioned characteristics of L-ornithine metabolism, there are at least four challenges in constructing an L-ornithine over-producing yeast strain: (i) how to tune more metabolic flux stop at L-ornithine rather than toward L-arginine biosynthesis without incapacitating the ability of L-arginine synthesis, as sufficient L-arginine is necessary for cell growth; (ii) how to balance and coordinate the corresponding pathways and enzymes in different subcellular organelles, as pathway perturbation could create a substantial bottleneck due to membranes transportation of intermediates and these intermediates will be redirect to competing pathways; (iii) how to increase the supply of the precursor a-ketoglutarate, as 'Crabtree effect' will limit the TCA cycle efficiency for  $\alpha$ -ketoglutarate biosynthesis during normal batch fermentation with sufficient glucose; (iv) the total pathway optimization calls for intensive perturbations to the related pathways. How to implement the desired engineering strategies efficiently is yet to be demonstrated.

The invention herein relies, unless otherwise indicated, on the use of conventional techniques of metabolic engineering, fermentation engineering, synthetic biology, biochemistry, molecular biology, cell biology, microbiology and recombinant DNA technology.

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

As used herein, the term "modified" refers to a host organism that has been modified to increase production of L-ornithine, putrescine, spermidine, spermine or agmatine, 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 these compounds production 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 into a host cell, and particularly into a host cell which is reduced or negative for ornithine carbamoyltransferase activity. 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).

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 "recombinant" means that a particular nucleic acid (RNA or DNA) is the product of various combinations of restriction, cloning, and/or ligation steps resulting in constructs with structural non-coding or -coding sequences different from endogenous nucleic acids found in the systems of natural.

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 wild-type 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 wild-type prokaryotic cells are defined as the parental cells of the recombinant prokaryotic cells, as used herein.

As used herein, the term "endogenous" when used with respect to a nucleic acid (RNA or DNA) or protein refers to a protein or a nucleic acid which occurs naturally as part of the cell, organism, genome or RNA or DNA sequence where it is present.

As used herein, the term "heterologous" when used with respect to a nucleic acid (RNA or DNA) or protein refers to a protein or a nucleic acid which occurs non-naturally as part of the cell, organism, genome or RNA or DNA sequence where it is present. Heterologous proteins or nucleic acids are not endogenous to the cell where it is introduced, but have been obtained from synthetically produced or one of another cells.

As used herein, the term "gene" indicates to a nucleic acid sequence which contains a template for a nucleic

acid polymerase (in eukaryotes, RNA polymerase II). Genes are transcribed into mRNAs which are then translated into proteins.

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, i.e. present in plasmids.

As used herein, the term "nucleic acid" refers to a ribonucleotide polymer or unless otherwise indicated, the term includes reference to the complementary sequence and the specified sequence thereof.

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, 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 eukaryotic cell to confer to the cell the ability to produce L-ornithine.

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 in eukaryotic host cells may be *PDC*, *GPD1*, *TEF1*, *PGK1* and *TDH*. Other suitable promoters include *HIS3*, *CYC1*, *ADH1*, *PGL*, *GAPDH*, *ADC1*, *URA3*, *TRP1*, *LEU2*, *TPI*, *AOXI* and *EN01*.

As used herein, the term "terminator" is functional in the eukaryotic cell used in the present invention. Natural genes of the host cell are the preferred terminators source.

As used herein, the term "overexpression" refers to increasing the number of copies of a desired nucleic acid sequence. Normally, overexpression leads to an increased level of activity of an enzyme and/or proteins, and/or to an increased level of activity in a desirable location (e.g., in the cytosol or mitochondria). There are a lot of strategies available in the art for overexpression of special nucleotide sequences encoding enzymes and/or proteins in a eukaryotic cell. Particularly, a nucleotide sequence encoding an enzyme/protein may be overexpressed by increasing the copy number of the gene coding for the enzyme/protein in the cell, e.g. by integrating additional copies of the gene in the cell's genome, from an episomal multicopy expression vector, by expressing the gene from a centromeric vector, or by introducing an (episomal) expression vector that comprises multiple copies of the gene. Preferably, overexpression of the enzyme/protein according to the disclosure is obtained with a constitutive strong promoter.

The term "overproducing" is used herein in reference to the production of L-ornithine in a host cell and

indicates that the host cell is producing more of L-ornithine, putrescine, spermidine, spermine or other compounds as disclosed herein 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.

5 As used herein, the term "Crabtree effect" refers to the phenomenon whereby the yeast, *S. cerevisiae*, produces ethanol in high external glucose concentrations and aerobic conditions rather than producing biomass via the tricarboxylic acid (TCA) cycle, which is the common process occurring aerobically in most yeasts e.g. *Kluyveromyces spp.*

10 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.

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.

15 In certain embodiments, the present disclosure relates to a modified eukaryotic cell, preferably, the eukaryotic cell is yeast cell, wherein the yeast cell can overproduce L-ornithine. Said overproduction of L-ornithine is obtained in the first place by the down-regulation or attenuation of specially selected genes, wherein said genes encode enzymes involved in the L-ornithine and/or polyamine consumption and/or degradation pathways. Further L-ornithine production ability is improved by down-regulation, attenuation, deletion or overexpression of specially  
20 selected genes, wherein said genes encode enzymes and/or proteins involved in the L-ornithine 'acetylated derivatives cycle', L-glutamate synthesis pathways, subcellular trafficking, TCA cycle, pyruvate carboxylation pathway, respiratory electron-transport chain, and the carbon subtracts assimilation machinery.

Preferably, the yeast to be modified can be selected from any known genus and species of yeast. In one embodiment, the yeast genus can be *Saccharomyces*, *Kluyveromyces*, *Zygosaccharomyces*, *Candida*,  
25 *Hansenula*, *Torulopsis*, *Kloeckera*, *Pichia*, *Schizosaccharomyces*, *Trigonopsis*, *Brettanomyces*, *Debaromyces*, *Nadsonia*, *Lipomyces*, *Cryptococcus*, *Aureobasidium*, *Trichosporon*, *Lipomyces*, *Rhodotorula*, *Yarrowia*, *Phaffia*, or *Schwanniomyces*, among others. In a further embodiment, the yeast can be *Saccharomyces*, *Yarrowia*, *Zygosaccharomyces*, *Kluyveromyces* or *Pichia spp.* In yet a further embodiment, the yeast can be *Saccharomyces cerevisiae*, *Saccharomyces boulardii*, *Zygosaccharomyces bailii*, *Kluyveromyces*  
30 *lactis*, and *Yarrowia lipolytica*. *Saccharomyces cerevisiae* is commonly used yeast in industrial processes, but the disclosure is not limited thereto. Other yeast species useful in the present disclosure include but are not limited to *Schizosaccharomyces pombe*, *Hansenula anomala*, *Candida sphaerica*, and *Schizosaccharomyces malidevorans*.

In one embodiment the prokaryotic cell can be a bacterial cell or archaeal cell. The recombinant bacterial

cell could be gram positive or gram negative bacteria. The bacteria may also be photosynthetic bacteria (e.g. cyanobacteria). The bacteria can either be Gram-negative or Gram-positive. The genera of Gram negative bacteria include, for example, *Neisseria*, *Spirillum*, *Pasieureiia*, *Brucella*, *Yersinia*, *Franciseiia*, *Haemophilus*, *Bordeteiia*, *Escherichia*, *Salmonella*, *Shigella*, *Klebsiella*, *Proteus*, *Vibrio*, *Pseudomonas*, *Bacteroides*,  
5 *Acetobacter*, *Aerobacter*, *Agrobacierium*, *Azotobacter*, *Spirilla*, *Serratia*, *Vibrio*, *Rhizobium*, *Chlamydia*, *Rickettsia*, *Treponema*, and *Fusobacterium*. The genera of Gram positive bacteria include, for example, *Actinomyces*, *Bacillus*, *Clostridium*, *Corynebactenum*, *Erysipeiothrix*, *Lactobacillus*, *Listeria*, *Mycobacterium*, *Myxococcus*, *Nocardia*, *Staphylococcus*, *Streptococcus*, and *Streptomyces*.

In some embodiments, the present disclosure relates to a modified yeast cell, wherein the activity of the L-ornithine consumption and/or degradation pathway of the yeast cell was reduced. In one preferred embodiment, the yeast cell is *S. cerevisiae*. In one preferred embodiment, the activity of ornithine carbamoyltransferase in *S. cerevisiae* is reduced. In one preferred embodiment, the activity of ornithine carbamoyltransferase in *S. cerevisiae* is reduced by down-regulation, attenuation or deletion the ornithine carbamoyltransferase encoding gene, wherein the said gene is *ARG3*. The attenuation of objective gene can be achieved by known methods in the art. In one preferred embodiment, the attenuation of *ARG3* is achieved by replaced the promoter region of *ARG3* with weak constitutive promoter, for instance the promoter of *KEX2*. It was found that the attenuation of the *ARG3* by the promoter replacement strategy led to the production of L-ornithine which would not achieve with the wild-type strain which has native *ARG3* promoter.  
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In one preferred embodiment, the reduced L-ornithine degradation activity is achieved by reducing the activity of L-ornithine transaminase (OTase) which catalyzes the second step of arginine degradation in eukaryotic cell. In one preferred embodiment, the reduced L-ornithine degradation activity is achieved by reducing the activity of L-ornithine transaminase (OTase) which catalyzes the second step of arginine degradation in *S. cerevisiae*. In one preferred embodiment, the reduction of OTase activity is achieved by deleting the OTase encoding gene *CAR2* in *S. cerevisiae*. The gene deletion can be achieved by known methods in the art. The *CAR2* deletion improves the L-ornithine production.  
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In a preferred embodiment, the activity of arginase which catabolizes L-arginine to L-ornithine and urea is increased in the eukaryotic cell. In a preferred embodiment, the activity of arginase which catabolizes L-arginine to L-ornithine and urea is increased in the modified *S. cerevisiae*. The increasing activity of arginase could be achieved by known methods in the art. For instance, the increasing activity of arginase could be achieved by overexpressing the encoding gene of arginase. The arginase encoding gene can be from any known species, for instance *S. cerevisiae*. In one preferred embodiment, the overexpressing of the *CAR1* gene which encodes the arginase is achieved in the modified *S. cerevisiae*. Surprisingly, the overexpression of *CAR1* gene in the modified yeast cell led to increased production of L-ornithine. Preferably, the yeast cell overexpresses a nucleotide sequence encoding arginase comprising an amino acid sequence that has at least 40%, preferably at least 45,  
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50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 97, 98, 99% sequence identity with the amino sequence of SEQ ID NO: 1. Preferably, the nucleotide sequence encodes arginase comprising the amino acid sequence of SEQ ID NO: 1.

In one preferred embodiment, the carbon channelling from  $\alpha$ -ketoglutarate to L-ornithine is enhanced in the modified eukaryotic cell. In one preferred embodiment, the carbon channelling from  $\alpha$ -ketoglutarate to L-ornithine is enhanced in the modified yeast cell. In a preferred embodiment, the carbon channelling from  $\alpha$ -ketoglutarate to L-ornithine is enhanced in the modified *S.cerevisiae*. Direct enhancing precursor supply pathway was a very commonplace strategy in bacteria to boost more flux to the desired products, yet, the segmentation of precursor intermediates in different subcellular organelles compromised the metabolic engineering endeavors in *S. cerevisiae*. L-ornithine biosynthetic pathway is one of such complicated pathways which is subject to subcellular compartmentalization: the downstream L-ornithine 'acetylated derivatives cycle' and the TCA cycle which gives the precursors of L-glutamate are confined to the mitochondria, while the middle of L-glutamate supply pathway is confined to cytoplasm. According to the metabolism architecture, three strategies were proposed to improve the pathway efficiency between  $\alpha$ -ketoglutarate node of TCA cycle and L-ornithine 'acetylated derivatives cycle' as follows: (i) directly enhance the endogenous pathway in its quondam subcellular organelle and the subcellular trafficking step; (ii) re-localize the L-glutamate synthesis reaction into mitochondria to avoid the subcellular trafficking of L-glutamate and its precursor  $\alpha$ -ketoglutarate and (iii) 'short-circuit' the total L-ornithine pathway started from TCA cycle to cytosol to bypass the subcellular trafficking of L-glutamate and L-ornithine.

In one preferred embodiment, the carbon channelling from  $\alpha$ -ketoglutarate to L-ornithine is enhanced in the modified eukaryotic cell by directly enhancing the endogenous pathway in its quondam subcellular organelle and the subcellular trafficking steps. In one preferred embodiment, the carbon channelling from  $\alpha$ -ketoglutarate to L-ornithine is enhanced in the modified *S. cerevisiae* by directly enhancing the endogenous pathway in its quondam subcellular organelle and the subcellular trafficking steps. In one preferred embodiment, enhancing the endogenous pathway in its quondam subcellular organelle and the subcellular trafficking steps is achieved by overexpressing of any enzymes/proteins or any combinations of said enzymes/proteins in the pathway from  $\alpha$ -ketoglutarate to L-ornithine in the eukaryotic cell. In one preferred embodiment, enhancing the endogenous pathway in its quondam subcellular organelle and the subcellular trafficking steps is achieved by increasing the activity of any enzymes/proteins or any combinations of said enzymes/proteins in the pathway from  $\alpha$ -ketoglutarate to L-ornithine in *S. cerevisiae*. These enzymes/proteins comprise: acetylglutamate synthase (glutamate N-acetyltransferase), acetylglutamate kinase and N-acetyl-gamma-glutamyl-phosphate reductase, mitochondrial ornithine acetyltransferase, acetylornithine aminotransferase, NAD<sup>+</sup>-dependent glutamate dehydrogenase, glutamine synthetase (GS), NAD<sup>+</sup>-dependent glutamate synthase (GOGAT), ornithine transporter of the mitochondrial inner membrane, glutamate uniporter, mitochondrial inner membrane  $\alpha$ -ketoglutarate transporter. In one preferred embodiment, the increased activity of any of the said enzymes/proteins or any combinations of thereof is achieved by overexpression the encoding genes. Preferably, at least one

endogenous or homologous pyruvate carboxylase is/are overexpressed in the eukaryotic cell according to the invention. In one preferred embodiment, the increased activity of any of the said enzymes is achieved by overexpressing the related genes in cytosol or mitochondria. Any genes either endogenous or heterogenous to the modified eukaryotic cell could be chosen as the overexpressing targets. In one preferred embodiment, the overexpression of any genes or any combinations of genes in the list comprising ARG2 (SEQ ID NO: 2), ARG5, 6 (SEQ ID NO: 3), ARG8 (SEQ ID NO: 4), ARG7 (SEQ ID NO: 5), GDH1 (SEQ ID NO: 6), GDH3 (SEQ ID NO: 7), GLN1 (SEQ ID NO: 8), GLT1 (SEQ ID NO: 9), ORT1 (SEQ ID NO: 10), AGC1 (SEQ ID NO: 11) and ODC1 (SEQ ID NO: 12) which are all from *S. cerevisiae* is achieved.

In one preferred embodiment, L-ornithine 'acetylated derivatives cycle' is re-localized to cytosol in the modified eukaryotic cell. In one preferred embodiment, L-ornithine 'acetylated derivatives cycle' is re-localized to cytosol in the modified *S. cerevisiae*. In one preferred embodiment, L-ornithine 'acetylated derivatives cycle' is re-localized to cytosol in the modified *S. cerevisiae* by increasing the enzyme activity of the related enzymes in the said pathway from bacteria cell. In one preferred embodiment, L-ornithine 'acetylated derivatives cycle' is re-localized to cytosol in the modified *S. cerevisiae* by overexpression all the genes in the said pathway from bacteria cell. In one preferred embodiment, L-ornithine 'acetylated derivatives cycle' is re-localized to cytosol in the modified *S. cerevisiae* by overexpression all the genes in the said pathway from bacteria cell, wherein the genes comprising *E. coli argAEc* (SEQ ID NO: 23) and *argBEc* (SEQ ID NO: 24), and *C. glutamicum argCcg* (SEQ ID NO: 25), *argDcg* (SEQ ID NO: 26) and *argJcg* (SEQ ID NO: 27).

In certain preferred embodiment, the efficiency of carbon channeling from substrate to  $\alpha$ -ketoglutarate is enhanced in the modified eukaryotic cell. In certain preferred embodiment, the efficiency of carbon channeling from substrate to  $\alpha$ -ketoglutarate is enhanced in the modified yeast cell. In certain preferred embodiment, the efficiency of carbon channeling from substrate to  $\alpha$ -ketoglutarate is enhanced in the modified eukaryotic cell. In certain preferred embodiment, the efficiency of carbon channeling from substrate to  $\alpha$ -ketoglutarate is enhanced in the modified *S.cerevisiae*.

In one preferred embodiment, the efficiency of carbon channeling from substrate to  $\alpha$ -ketoglutarate is enhanced by increase in the enzymes/proteins activity of at least one enzyme/protein in the pathway from carbon substrate to the  $\alpha$ -ketoglutarate in the modified eukaryotic cell. In one preferred embodiment, the efficiency of carbon channeling from substrate to  $\alpha$ -ketoglutarate is enhanced by increase in the enzymes/proteins activity of at least one enzyme/protein in the pathway from carbon substrate to the  $\alpha$ -ketoglutarate in the modified yeast cell. In one preferred embodiment, the efficiency of carbon channeling from substrate to  $\alpha$ -ketoglutarate is enhanced by increase in the enzymes/proteins activity of at least one enzyme/protein in the pathway from carbon substrate to the  $\alpha$ -ketoglutarate in the modified *S. cerevisiae* cell. In one preferred embodiment, the efficiency of carbon channeling from substrate to  $\alpha$ -ketoglutarate is enhanced by increasing the enzyme/protein activity of at least one enzyme/protein in the pathway from carbon substrate to the  $\alpha$ -ketoglutarate in the modified *S. cerevisiae* cell,

wherein the increasing of the activity of enzymes/proteins is achieved by overexpressing the encoding genes of said enzymes/proteins. Preferably, the eukaryotic cell according to the present disclose, overexpress a nucleotide sequence encoding a pyruvate carboxylase (PYC), preferably a pyruvate carboxylase that is active either at cytosol or mitochondria upon expression of a nucleotide sequence encoding a PYC, for instance a PYC comprising an amino acid sequence according to SEQ ID NO: 13. Preferably, an endogenous or homologous pyruvate carboxylase is overexpressed in the eukaryotic cell according to the invention. It was found that overexpressing an endogenous pyruvate carboxylase (PYC) resulted in increased L-ornithine production levels by the eukaryotic cell according to the present disclosure.

Preferably, the eukaryotic cell according to the present disclose, overexpresss a nucleotide sequence encoding a citrate synthase (CS) which catalyzes the condensation of acetyl coenzyme A and oxaloacetate to form citrate, preferably a citrate synthase that is active either at cytosol or mitochondria upon expression of a nucleotide sequence encoding a CS, for instance a CS comprising an amino acid sequence according to SEQ ID NO: 14. Preferably, an endogenous or homologous pyruvate carboxylase is overexpressed in the eukaryotic cell according to the invention. Surprisingly, it was found that overexpressing an endogenous citrate synthase (CS) resulted in increased L-ornithine production levels by the eukaryotic cell according to the present disclosure.

Preferably, the eukaryotic cell according to the present disclose, the activity of pyruvate dehydrogenase (PDH) is increased. Preferably, the increase activity of PDH is achieved by overexpressing at least one subunit of PDH. Preferably, the nucleotide sequence encoding E1 alpha subunit of the pyruvate dehydrogenase (PDH) complex, preferably E1 alpha subunit of the pyruvate dehydrogenase (PDH) that is active either at cytosol or mitochondria is upon expression of a nucleotide sequence encoding said protein, for instance a E1 alpha subunit of the pyruvate dehydrogenase (PDH) comprising an amino acid sequence according to SEQ ID NO: 15. Preferably, an endogenous or homologous pyruvate carboxylase is overexpressed in the eukaryotic cell according to the invention. Surprisingly, it was found that overexpressing an endogenous pyruvate dehydrogenase (PDH) subunit resulted in increased L-ornithine production levels by the eukaryotic cell according to the present disclosure.

Preferably, the eukaryotic cell according to the present disclose, overexpresses a nucleotide sequence encoding a aconitase, preferably an aconitase is active either at cytosol or mitochondria upon expression of a nucleotide sequence encoding a aconitase, for instance an aconitase comprising an amino acid sequence according to SEQ ID NO: 16. Preferably, an endogenous or homologous aconitase is overexpressed in the eukaryotic cell according to the invention. Surprisingly, it was found that overexpressing an aconitase resulted in increased L-ornithine production levels by the eukaryotic cell according to the present disclosure.

Preferably, the eukaryotic cell according to the present disclose, overexpresses a nucleotide sequence encoding an isocitrate dehydrogenase (IDP) which catalyzes the oxidation of isocitrate to a-ketoglutarate, preferably an IDP is active either at cytosol or mitochondria upon expression of a nucleotide sequence encoding an IDP, for instance an IDP comprising an amino acid sequence according to SEQ ID NO: 17. Preferably, an



endogenous or homologous IDP is overexpressed in the eukaryotic cell according to the invention. It was found that overexpressing an IDP resulted in increased L-ornithine production levels by the eukaryotic cell according to the present disclosure.

In one preferred embodiment, the activity of protein which uptake pyruvate from cytosol to mitochondria is increased in the eukaryotic cell according to the present disclosure. In one preferred embodiment, at least one subunit of the mitochondrial pyruvate carrier from *S. cerevisiae* is subject to overexpression. In one preferred embodiment, *MPC1* (SEQ ID NO: 18) or *MPC2* (SEQ ID NO: 19) or *MPC3* (SEQ ID NO: 20) or any combinations of *MPC1*, *MPC2*, *MPC3* is subject to overexpression. Surprisingly, it was found that overexpressing pyruvate carrier proteins resulted in increased L-ornithine production levels by the eukaryotic cell according to the present disclosure.

In one preferred embodiment, at least one enzyme/protein or any combinations of thereof is/are increased, the enzymes/proteins are as follows: isocitrate dehydrogenase, mitochondrial pyruvate carrier, pyruvate carboxylase, citrate synthase (CS), pyruvate dehydrogenase (PDH).

In one preferred embodiment, the carbon substrate uptake activity is decreased in the eukaryotic cell according to the present disclosure. Many technologies/strategies known in the art can be used to decrease the activity of carbon substrate uptake. In one preferred embodiment, the carbon substrate uptake activity is decreased in the eukaryotic cell according to the present disclosure, wherein the decrease activity of carbon uptake is achieved by decreasing the activity of the transport proteins which transport the carbon substrate from extracellular environment to the cytosol. Preferably, the decreasing of transport proteins which transport the carbon uptake is achieved by increase in the activity of special proteins which regulate the carbon uptake. In one preferred embodiment, stability of one of the proteins in the glucose regulation systems is achieved by overexpression the mutated *MTH1* (SEQ ID NO: 22), encoding the internal deleted version of glucose transporter regulation protein. Surprisingly, more carbon flux from carbon substrate was boosted into L-ornithine and less or no ethanol production was seen with the modified *S. cerevisiae*.

In one preferred embodiment, the activity of the NADH alternative oxidase is increased to increase the efficiency of carbon channelling from carbon substrate to  $\alpha$ -ketoglutarate in the modified eukaryotic cell. In one preferred embodiment, the activity of the NADH alternative oxidase is increased to increase the efficiency of carbon channelling from the carbon substrate to  $\alpha$ -ketoglutarate in the modified yeast cell. In one preferred embodiment, the activity of the NADH alternative oxidase (AOX) is increased to increase the efficiency of carbon channeling from carbon substrate to  $\alpha$ -ketoglutarate in the modified *S. cerevisiae*. In one preferred embodiment, the activity of the NADH alternative oxidase is increased to increase the efficiency of carbon channelling from carbon substrate to  $\alpha$ -ketoglutarate in the modified *S. cerevisiae*, wherein the increased activity of AOX is achieved by overexpression of AOX from *Hansenula anomala* {*HaAOX*} (SEQ ID NO: 28). Surprisingly, more carbon flux from carbon substrate was boosted into L-ornithine and less or no ethanol production was seen with

the modified *S. cerevisiae*.

In one preferred embodiment, the eukaryotic cell according to the present invention may be able to grow on any suitable carbon source known in the art and convert it to L-ornithine. The eukaryotic cell may be able to convert directly plant biomass, hemicelluloses, celluloses, pectines, rhamnase, fucose, maltose, galactose, maltodextrines, ribose, ribulose, or starch, starch derivatives, sucrose, glycerol and lactose. Hence, a preferred host organism expresses enzymes such as hemicellulases (e.g. endo- and exo-xylanases, arabinases) and cellulases (endocellulases and exocellulases) necessary for the conversion of cellulose into glucose monomers and hemicellulose into arabinose and xylose monomers, pectinases able to convert pectines into galacturonic acid and glucuronic acid or amylases to convert starch into glucose monomers. In one preferred embodiment, the modified eukaryotic cell is able to convert a carbon source selected from the group consisting of glucose, xylose, arabinose, fructose, galactose, sucrose, glycerol raffinose and lactose. In another aspect, the present invention relates to a process for the preparation of L-ornithine, comprising fermenting or cultivating the eukaryotic cell according to the present invention, wherein L-ornithine is prepared.

In one preferred embodiment, the L-ornithine produced by the modified eukaryotic cell described herein can be incorporated into one or more food, and/or chemical products.

In one preferred embodiment, the genetically modified eukaryotic cells according to the present disclose described herein can be incorporated into food and/or drink production. In one preferred embodiment, the genetically modified eukaryotic cells according to the present disclose described herein can be incorporated into beer brewing, wherein the beer comprises moderate L-ornithine. In one preferred embodiment, the genetically modified eukaryotic cells according to the present disclose described herein can be incorporated into bread baking, wherein the baking products comprise moderate L-ornithine.

In one preferred embodiment, the genetically modified eukaryotic cells according to the present disclose can be further modified to produce chemical compound which can be produced in the cell using L-ornithine as the precursor.

In certain embodiments, the present disclosure relates to a modified cell, such as yeast or bacterial cell, wherein the cell can overproduce polyamines, such as putrescine, spermidine or spermine. Said overproduction is obtained by combining some or all of the modifications for ornithine production mentioned above with overexpression of the enzymatic steps responsible for production of these compounds, down-regulation of competing and/or inhibitory reactions, overexpression of various export proteins, down-regulation of various uptake proteins and modification of expression of various proteins associated with polyamine toxicity, giving a very effective total process.

In a preferred embodiment, the activity of ornithine decarboxylase (ODC) which catalyzes the conversion of ornithine to putrescine is increased to increase flux towards putrescine in yeast. The increase in activity of ODC could be achieved by known methods in the art. For instance, the increased activity of ODC could be achieved

by overexpressing the encoding gene of ODC. The ODC encoding gene can be from any known species, for instance *S. cerevisiae*. In one preferred embodiment the overexpression of the native *SPE1* gene which encodes the ODC is achieved in the modified *S. cerevisiae*. In addition, heterologous ODC from other eukaryotic or prokaryotic sources can be expressed (e.g. from *Escherichia coli*, *Triticum aestivum*, *Oryza sativa*, *Glycine max*,  
5 *Citrus sinensis*, *Homo sapiens*). Furthermore, a mutated, highly-active ODC from *E. coli* containing modifications in the 1163 and E165 residues can be expressed in yeast (Choi et al., 2015). To further increase the activity of ODC in yeast, the activity of the ODC antizyme, which binds to ODC, inactivates it, and targets it for degradation, could be decreased by deletion or promoter exchange. These modifications can be combined with the modifications for ornithine over-production described above. It has also been previously shown that deletion of  
10 Methylthioadenosine phosphorylase (encoded by *MEU1*) in yeast results in an increase in ODC activity and large elevation in polyamine pools (Subhi et al., 2003). Therefore, deletion or down-regulation of this gene can be combined with the above modifications. Furthermore, down-regulation of spermidine synthase (SPDS), which catalyzes conversion of putrescine to spermidine, can also be combined with the modifications above to increase flux towards putrescine.

15 In another embodiment, flux towards spermidine is increased in yeast by increasing the activities of S-adenosylmethionine decarboxylase (SAMDC) and spermidine synthase (SPDS), which catalyze conversion of putrescine into spermidine. This can be achieved by, for example, overexpression of the native *SPE2* and *SPE3* genes which encode SAMDC and SPDS in the modified *S. cerevisiae*. In addition, heterologous SAMDC and SPDS from other eukaryotic or prokaryotic sources can be expressed (e.g. from *Escherichia coli*, *Triticum*  
20 *aestivum*, *Oryza sativa*, *Glycine max*, *Citrus sinensis*, *Homo sapiens*). To further increase flux towards spermidine production, the *SPE4* gene, which encodes spermine synthase (SPS), can be down-regulated. Flux towards spermidine can also be increased by overexpressing yeast polyamine oxidase (*FMS1*), which catalyzes conversion of spermine to spermidine, or spermine oxidase (SMOX; EC 1.5.3.16) from other sources (e.g. mammalian) for the same reaction. These modifications can be combined with the modifications for ornithine  
25 and/or putrescine over-production described above.

In another embodiment, flux towards spermine production is increased by increasing the activity of spermine synthase (SPS), which catalyzes the conversion of spermidine to spermine. This can be achieved by, for example, overexpression of the native *SPE4* gene, which encodes SPS in the modified *S. cerevisiae*. In addition, heterologous SPS from other eukaryotic or prokaryotic sources can be expressed (e.g. from *Triticum aestivum*,  
30 *Oryza sativa*, *Glycine max*, *Citrus sinensis*, *Homo sapiens*). These modifications can also be combined with increase in the activities of ODC, SAMDC and/or SPDS, as well as down-regulation of the ODC antizyme. In addition, to reduce conversion of spermine to spermidine, yeast *FMS1* can also be down-regulated.

In another embodiment, flux towards polyamines can be increased by down-regulation of competing reactions. For example, polyamine acetyltransferase (encoded by *PAA1* in yeast) can acetylate various

polyamines such as putrescine, spermidine and spermine. To reduce this, this enzyme can be down-regulated or deleted.

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 *TP01*, *TP02*, *TP03*, *TP04* and *TP05*; *Escherichia coli* *MdtJl*, 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 *TP01*, *TP02*, *TP03*, *TP04* or *TP05*.

In another embodiment, the resistance of the above strains to polyamine toxicity is increased. Down-regulation 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 over-expressed and/or down-regulated in various combinations to allow for optimal resistance to polyamine toxicity in yeast.

In certain embodiments, the present disclosure relates to a modified eukaryotic cell capable of over-producing agmatine. Said production can be accomplished either independently, or by combining some of the modifications described for ornithine production above with the overexpression of some of the enzymatic steps responsible for agmatine production and down-regulation of competing reactions.

In one embodiment, flux can be directed from ornithine to arginine and/or agmatine production by increasing the activities of ornithine carbamoyltransferase [EC:2.1.3.3], argininosuccinate synthase (EC:6.3.4.5) and argininosuccinate lyase [EC:4.3.2.1]. This can be done, for example, by overexpressing the native yeast *ARG3*, *ARG1*, *ARG10* and/or *ARG4* genes. In addition, heterologous genes encoding these activities can also be expressed. To decrease competing reactions, the native arginase [*CAR1*] genes can be deleted or down-regulated. Furthermore, conversion of arginine to agmatine can be accomplished by introduction of a heterologous gene encoding arginine decarboxylase [EC:4.1.1.19] (e.g. *E. coli* *SpeA* or *Adi*, *Desulfovibrio magneticus* *pdaA*, *Homo sapiens* *AZIN2*, etc. ).

In certain embodiments, putrescine can be produced from agmatine by introduction of genes encoding for enzyme activities of agmatinase (e.g. *E. coli* *SpeB*), which converts agmatine to putrescine and urea (e.g. *E. coli* *SpeB*). In addition, agmatine could also be converted to putrescine by introduction of two heterologous steps: hydrolytic deamination of agmatine to N-carbamoylputrescine by agmatine deiminase (e.g. *AguA* from *Pseudomonas aeruginosa*) and hydrolysis of the carbomoyl group to yield putrescine in a reaction catalyzed by N-carbamoylputrescine amidohydrolyase (e.g. *AguB* from *Pseudomonas aeruginosa*; *PTC* from *Enterococcus faecalis*).

In certain embodiments, spermidine can be produced from agmatine. In some extremophiles, agmatine aminopropyltransferase uses S-adenosylmethionine (dsSAM) as a donor to produce aminopropylagmatine which is subsequently hydrolysed to spermidine and urea by aminopropylagmatine ureohydrolase. Genes encoding enzymes for these activities can also be expressed in yeast to facilitate spermidine production (e.g. *Thermococcus kodakarensis* TK0147, TK0240, TK0474 and TK0882, *Thermus thermophilus* *speE* and *speB*).

In a another embodiment, spermidine can be produced via the condensation of L-aspartate semialdehyde and putrescine via carboxy(nor)spermidine dehydrogenase (CANSDH) yielding carboxyspermidine, followed by decarboxylation by carbox(nor)spermidine decarboxylase (CANSDC). Genes encoding enzyme with these activities can be expressed to facilitate spermidine production (e.g. CANSDH and CANSDC from *Campylobacter jejuni*, *Vibrio cholera*, *Vibrio cholerae* or *Vibrio alginolyticus*). Alternatively, aminopropyl transferase activity which uses dcSAM to synthesize norspermidine from 1,3-diaminopropane from *Clostridium thermohydrosuluricum* can also be expressed.

In certain embodiments, additional reactions that could potentially compete with polyamine synthesis could be deleted or down-regulated in yeast. This can include reactions involved in pathways that lead to GABA ( $\gamma$ -aminobutyric acid), such as those encoded by *UGA1*, *UGA2*, *UGA3* and/or *UGA4*.

In certain embodiments, production of polyamines from bacterial sources can be achieved. For production of L-ornithine and putrescine, organisms like *Escherichia coli* and *Corynebacterium glutamicum* are feasible hosts for production. This makes them good platform strains for the production for spermidine and spermine through overexpression of intrinsic or heterologous spermidine synthase and S-adenosylmethionine decarboxylase. Modifications in *E. coli* WL3110 (orig. K12 W3110 (CGSC, Coli Genetic Stock Center) to increase ornithine and putrescine production respectively can be implemented as described by Quian et al. (2009). For example, such modifications can comprise enhancing the precursor supply (e.g., deletion of *argI* gene), inactivating putrescine degradation and utilization pathways (e.g., deletion of *speG*; *puuPA*, *argR*) and deleting *rpoS*, a stress responsive polymerase sigma factor. In addition to, or combined with the former changes, genes coding for ornithine decarboxylase converting ornithine to putrescine and ornithine biosynthetic genes [*argC-E*] can be overexpressed either through the use of plasmids or chromosomal integration. This former platform strain can additionally be transformed with either plasmids for overexpression of endogenous or heterologous *speE* (E.C.: 2.5.1.16; SEQ ID NO: 37) and *speD* (EC 4.1.1.50; SEQ ID NO: 39) genes. Spermidine exporters derived from, for example, *E. coli*, including the *mdtJI* complex genes (SEQ ID NOs: 40-41) or Bit from, for example, *B. subtilis* (SEQ ID NO: 42) represent possible candidates for over/co-expression with the "platform" stain described herein to increase the production of spermidine/spermine. In other embodiments, in order to increase the production of spermine, heterologous spermine synthases (E.C. 2.5.1.22) from, for example, eukaryotic sources, including *S. cerevisiae*, *Triticum aestivum*, *Oryza sativa*, *Glycine max*, *Citrus sinensis*, *Homo sapiens* can be expressed.

In yet other embodiments, polyamines can be produced in *C. glutamicum*. Modifications to increase ornithine

production in *C. glutamicum* strain ATCC 13032 can be achieved through, for example, deletion of *proB* and *argF*, which block competing pathways, deletion of *argR* gene, which is the repressor of the L-arginine operon, overexpression of *argCJBD* from *C. glutamicum* (ATCC 21831 start codon change of *pgi* and *zwf*), which lead to enrichment of the NADPH pool, and/or replacing native promoter of *tkf* operon with a strong *sod* promoter. In some embodiments, in order to increase putrescine production the platform strain can be engineered to overexpress the ornithine decarboxylase gene, *speC* (EC 4.1.1.19; SEQ ID NO: 44) from *E. coli* and/or by deletion of the carbamoyl-transferase *argF* gene.

In some embodiments, in order to increase spermidine production in the platform strain, the endogenous spermidine synthase gene *speE* (SEQ ID NO: 45) and *E. coli* S-adenosylmethionine decarboxylase *speD* (EC 4.1.1.50; SEQ ID NO: 39) can be overexpressed. The former strains can additionally be engineered to increase the export, as described elsewhere herein. In other embodiments in order to increase spermidine, heterologous spermine synthases (E.C. 2.5.1.22) from eukaryotic sources can be overexpressed. For example, the former spermine synthases can be derived from, for example, *S. cerevisiae* [*Spe4*, GI: 3201942; SEQ ID NO: 43), *Triticum aestivum*, *Oryza sativa*, *Glycine max*, *Citrus sinensis* and/or *Homo sapiens*.

The following examples are included to demonstrate preferred embodiments of the disclosure. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the disclosure, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the disclosure.

In general, any modification may be applied to a cell to impart or increase accumulation and/or production of L-ornithine or a chemical compound which can be produced in the cell using L-ornithine. In many cases, the modification comprises a genetic modification, wherein the genetic modifications may be introduced into cells by any available means including transfer (e.g., via transformation or mating) of nucleic acids and/or chemical mutation.

An aspect of the embodiments relates to a eukaryotic cell capable of producing L-ornithine. The eukaryotic cell is genetically modified for attenuated activity of an ornithine carbamoyltransferase.

In an embodiment, the eukaryotic cell is genetically modified for attenuated expression of a gene encoding the ornithine carbamoyltransferase in the eukaryotic cell.

In an embodiment, the eukaryotic cell has a native promoter of the gene encoding the ornithine carbamoyltransferase replaced by a weak constitutive promoter. In a particular embodiment, the eukaryotic cell has a native promoter of the gene *ARG3* replaced by a promoter of the gene *KEX2*.

In an embodiment, the eukaryotic cell is genetically modified by deletion or disruption of a gene encoding an L-ornithine transaminase. In a particular embodiment, the eukaryotic cell is genetically modified by deletion or disruption of the gene *CAR2*.

The above disclosed embodiments relates to a eukaryotic cell, such as fungal cell and preferably a yeast cell, genetically modified according to module 1 (M1) as described herein.

In an embodiment, the eukaryotic cell is genetically modified for enhanced L-ornithine biosynthesis from  $\alpha$ -ketoglutarate.

In an embodiment, the eukaryotic cell is genetically modified for cytosolic L-ornithine biosynthesis from  $\alpha$ -ketoglutarate.

In an embodiment, the eukaryotic cell comprises at least one of gene selected from a group consisting of a gene encoding a cytosolic glutamate N-acetyltransferase, a gene encoding a cytosolic N-acetylglutamate kinase, a gene encoding a cytosolic N-acetyl-gamma-glutamyl-phosphate reductase, a gene encoding a cytosolic acetylornithine aminotransferase and a gene encoding a cytosolic ornithine acetyltransferase. In a particular embodiment, the eukaryotic cell comprises the genes *argA<sub>Ec</sub>* and *argB<sub>Ec</sub>* from *Escherichia coli* and the genes *argC<sub>Cg</sub>*, *argD<sub>Cg</sub>* and *argJ<sub>Cg</sub>* from *Corynebacterium glutamicum*.

In an embodiment, the yeast is genetically modified for overexpression of at least one gene selected from a group consisting of a gene encoding an N-acetyl-gamma-glutamyl-phosphate reductase, a gene encoding a mitochondrial ornithine acetyltransferase, a gene encoding an acetylornithine aminotransferase and a gene encoding an acetylglutamate synthase. In a particular embodiment, the yeast is genetically modified for overexpression of the genes *ARG5*, *ARG7*, *ARG8* and *ARG2*,

In an embodiment, the eukaryotic cell is genetically modified for overexpression of at least one gene selected from a group consisting of a gene encoding a L-ornithine transporter, a gene encoding a L-glutamate transporter and a gene encoding NADP<sup>+</sup>-dependent glutamate dehydrogenase. In a particular embodiment, the eukaryotic cell is genetically modified for overexpression of the genes *ORT1*, *AGC1*, *GDH1*.

The above disclosed embodiments relates to a eukaryotic cell, such as fungal cell and preferably a yeast cell, genetically modified according to module 2 (M2) as described herein. As mentioned above, the genetical modification according to module 2 is preferably applied to a eukaryotic cell that also is genetically modified according to module 1. However, the embodiments are not limited. Thus, an aspect of the embodiments relates

to a yeast cell genetically modified according to module 2 but not necessarily according to module 1. This aspect of the embodiments thereby relates to a eukaryotic cell capable of producing L-ornithine. The eukaryotic cell is genetically modified for enhanced L-ornithine biosynthesis from  $\alpha$ -ketoglutarate. The above described optional but preferred embodiments relating to the genetical modification according to module 2 also applies to the aspect of a eukaryotic cell genetically modified according to module 2 but not necessarily according to module 1.

In an embodiment, the eukaryotic cell is genetically modified for enhanced  $\alpha$ -ketoglutarate biosynthesis.

In an embodiment, the eukaryotic cell is genetically modified for attenuated glucose uptake.

In an embodiment, the eukaryotic cell is genetically modified for overexpression of a gene encoding a glucose transporter regulator protein. In a particular embodiment, the eukaryotic cell is genetically modified for overexpression of the gene *MTH1* or the gene *MTH1- $\Delta$ T*, wherein the gene *MTH1- $\Delta$ T* is an internal deletion version of the gene *MTH1*.

The above disclosed embodiments relates to a eukaryotic cell, such as fungal cell and preferably a yeast cell, genetically modified according to module 3 (M3) as described herein. As mentioned above, the genetical modification according to module 3 is preferably applied to a eukaryotic cell that also is genetically modified according to module 1 and module 2. However, the embodiments are not limited. Thus, an aspect of the embodiments relates to a yeast cell genetically modified according to module 3 but not necessarily according to module 1 or module 2. This aspect of the embodiments thereby relates to a eukaryotic cell capable of producing L-ornithine. The eukaryotic cell is genetically modified for enhanced  $\alpha$ -ketoglutarate biosynthesis. The above described optional but preferred embodiments relating to the genetic modification according to module 3 also applies to the aspect of an eukaryotic cell genetically modified according to module 3 but not necessarily according to module 1 or module 2.

Thus, the embodiments encompass a eukaryotic cell genetically modified according to module 1, a eukaryotic cell genetically modified according to module 2, a eukaryotic cell genetically modified according to module 3, a eukaryotic cell genetically modified according to module 1 and module 2, a eukaryotic cell genetically modified according to module 1 and module 3, a eukaryotic cell genetically modified according to module 2 and module 3 and a eukaryotic cell genetically modified according to module 1, module 2 and module 3.

In an embodiment, the eukaryotic cell is genetically modified for overexpression of a gene encoding an arginase. In a particular embodiment, the eukaryotic cell is genetically modified for overexpression of the gene



*CARL*

The above described embodiments and particular embodiments can be combined in any suitable manner. Hence, the present invention also encompasses various combinations of different genetical modifications of the above described embodiments to form a eukaryotic cell capable of producing L-ornithine.

- 5 In an embodiment, the eukaryotic cell is genetically modified by:
- having a native promoter of the gene *ARG3* replaced by a promoter of the gene *KEX2*;
  - deletion or disruption of the gene *CAR2*;
  - overexpression of the genes *ORT1*, *AGC1*, *GDH1*;
  - expression of the genes *argAtx* and *argBtx* from *Escherichia coli* and the genes *argCc*, *argDcg* and *argJcg*
- 10 from *Corynebacterium glutamicum*; and
- overexpression of the gene *MTH1-ΔT*.

In an embodiment, the eukaryotic cell is also genetically modified for overexpression of the gene *CARL*

- Further aspects of the embodiments relates to a eukaryotic cell capable of producing a polyamine using L-ornithine as a starting material or intermediate product in the production of the polyamine. In a preferred
- 15 embodiment, the polyamine is selected from a group consisting of putrescine, spermidine and spermine.

Thus, in embodiment the eukaryotic cell is capable of producing a polyamine selected from the group consisting of putrescine, spermidine and spermine. The eukaryotic cell is also genetically modified for overexpression of a gene encoding ornithine decarboxylase (ODC) and/or attenuated activity of ODC antienzyme.

- In a particular embodiment, the eukaryotic cell is genetically modified for overexpression of the gene *SPE1*
- 20 and deletion or disruption of the gene *OAZL*

In an embodiment, the eukaryotic cell is capable of producing a polyamine selected from the group consisting of spermidine and spermine. The eukaryotic cell is also genetically modified for overexpression of a gene encoding an S-adenosylmethionine decarboxylase and/or a gene encoding a spermidine synthase.

- In a particular embodiment, the eukaryotic cell is genetically modified for overexpression of the genes *SPE2*
- 25 and *SPE3*.

In an embodiment, the eukaryotic cell is capable of producing a polyamine selected from the group consisting of spermidine and spermine. The eukaryotic cell is also genetically modified for expression of a gene encoding a carboxynorspermidine dehydrogenase and/or a gene encoding a carboxynorspermidine decarboxylase.

In a particular embodiment, the eukaryotic cell is genetically modified for expression of:

- i) the genes *Cj0172c* and *Cj1515c* from *Capylobacter jejuni*; or
- ii) the genes *VC1624* and *VC1623* from *Vibrio cholera*; or
- iii) the genes *Lys1* and *nspC* from *Bacteriodes uniformis*.

5 In an embodiment, the eukaryotic cell is capable of producing spermine. The eukaryotic cell is also genetically modified for overexpression of a gene encoding a spermine synthase.

In a particular embodiment, the eukaryotic cell is genetically modified for overexpression of the gene *SPE4*.

In an embodiment relating to the eukaryotic cell capable of producing a polyamine, the eukaryotic cell is genetically modified for overexpression of a gene encoding a polyamine transporter. In a particular embodiment,  
10 the eukaryotic cell is genetically modified for overexpression of at least one gene selected from the group consisting of *TP01*, *TP02*, *TP03*, *TP04* and *TP05*.

In an embodiment, the eukaryotic cell capable of producing L-ornithine and optionally the polyamine is a fungal cell, preferably a yeast cell.

In an embodiment, the yeast cell is selected from a genus consisting of the group consisting of  
15 *Saccharomyces*, *Cryptococcus*, *Trichosporon*, *Zygosaccharomyces*, *Debaromyces*, *Pichia*,  
*Schizosaccharomyces*, *Trigonopsis*, *Brettanomyces*, *Aureobasidium*, *Nadsonia*, *Lipomyces*, *Torulopsis*,  
*Kloeckera*, *Phaffia*, *Rhodotorula*, *Candida*, *Hansenula*, *Kluyveromyces*, *Yarrowia*, and *Schwanniomyces*. In a particular embodiment, the yeast cell is selected from a genus consisting of the group consisting of  
20 *Saccharomyces*, *Yarrowia*, *Zygosaccharomyces*, *Kluyveromyces* and *Pichia* spp. In an embodiment, the yeast cell is preferably selected from the group consisting of *Saccharomyces cerevisiae*, *Saccharomyces boulardii*,  
*Zygosaccharomyces bailii*, *Kluyveromyces lactis*, *Yarrowia lipolytica*, *Schizosaccharomyces pombe*, *Hansenula anomala*, *Candida sphaerica*, and *Schizosaccharomyces malidevorans*, such as *Saccharomyces cerevisiae*.

A further aspect of the embodiments relates to a process for production of L-ornithine. The process comprising cultivating a eukaryotic cell according to any of the embodiments disclosed herein and in particular  
25 mentioned in the foregoing in the presence of a carbon source.

Yet another aspect of the embodiments relates to a process for production of a polyamine selected from the group consisting of putrescine, spermidine and spermine. The process comprising cultivating a eukaryotic cell according to the embodiments above relating to a eukaryotic cell capable of producing a polyamine in the

presence of a carbon source.

In an embodiment, the carbon source is selected from a group consisting of hemicelluloses, celluloses, pectines, rhamnose, fucose, maltose, galactose, maltodextrines, ribose, ribulose, starch, sucrose, glycerol, lactose, glucose, xylose, arabinose, fructose, galactose and glycerol raffinose and lactose.

5

## EXAMPLES

All engineered yeast strains were constructed (Table 1 -3) from *S. cerevisiae* strain CEN.PK 113-1 1C (*MATa SUC2 MAL2-8c his3A1 ura3-52*).

10

### Example 1

#### Strain construction of ornithine-overproducing strains

All the plasmids used in this study can be found in Table 4. Plasmids (G01, G02, G03, G04, Y01, Y02, Y03 Y04 and Y03) were constructed according to MOPE strategy and DNA assembler (Shao et al., 2009). The gene expressing modules consisted of a promoter, a structural gene, a terminator, and the promoter of the next module for homologous recombination. The promoter TEF1p, TDH3p, PGK1p and HXT7p, terminator *FBA1t*, *CYC1t*, *TDH2t* and *ADH2t*, were PCR-amplified from the genomic DNA of *S. cerevisiae* CEN.PK.113-5D. The *TPIp* and the terminator *pYX212t* were PCR-amplified from plasmid pYX21 2. Genes *ARG2*, *ARG5*, *6*, *ARG7*, *ARG8*, *CIT1*, *AC02*, *IDP1*, *PYC2*, *GLT1* and *GLN1* were amplified from the genomic DNA of *S. cerevisiae* CEN.PK.113-5D. PDA1 and mutated mPDA1 were PCR-amplified from plasmid pRS416-PDA1 and pRS416-PDA1 [S313A] respectively (Oliviera et al., 2012). *HoAOXI*, *argJcg*, *argCcg*, *argDcg*, and *argBcg* were codon-optimized and purchased from GenScript. *argAEc* and *argBEc* were PCR-amplified from the genomic DNA of *E. coli*. The mutated *MTH1-AT* was PCR-amplified from the genomic DNA of *S. cerevisiae* TAM (Oud et al., 2012). *argBcg*, and *argJcg* were targeted to the mitochondria using the N-terminal mitochondrial localization signal from subunit IV of the yeast cytochrome c oxidase (CoxIV) (Avalos et al., 2013). All modules were constructed with the one-step PCR strategy similar to overlap extension PCR. The expression modules were co-transformed by electroporation with linearized vector pYX212 or p423GPD into *S. cerevisiae* CEN.PK 113-11 C, and the recombinants appeared on the corresponding plates after 2-4 days. Selected colonies formed on the plates were cultured in 5 mL of YPD liquid medium at 30 °C for 72 h. Recovered plasmids were checked by PCR to verify the assembled pathways. Alternatively, positive plasmids were also transformed into *E. coli* DH5a, recovered, digested by the relative restriction endonuclease, and analyzed by gel electrophoresis. Other plasmids used in this study were constructed according to the regular cloning strategy.

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To replace the *ARG3* promoter, the *HXT1* promoter and *KEX2* promoter were amplified from genomic DNA by PCR. The DNA cassette including the new promoter, the *kanMX* cassette and both 5' and 3' parts of the *ARG3*

promoter was constructed following the strategy of MOPE. Following the transformation of these cassettes into *S. cerevisiae*, the correct transformants were selected and verified by colony PCR. Following a similar strategy, *ORT1*, *ODC1*, *AGC1*, *GDH1*, *GDH2*, *GDH3*, *HaAOXI* and *MTH1-ΔT* were integrated into the chromosome of *S. cerevisiae* background strains, yielding strains with modification in the *URA3*, *YPRCT3* or *KGD2* sites of chromosome (Flagfeldt et al., 2009). Similar to *argBcg* and *argJcg*, *GDH1* and *GDH2* were targeted to the mitochondria using the N-terminal mitochondrial localization signal from subunit IV of the yeast cytochrome c oxidase (CoxIV). Variation combinations of plasmids and background strains yielded L-ornithine producing strains.

Yeast strains without plasmids were maintained on YPD plates containing 10 g l<sup>-1</sup> yeast extract, 20 g l<sup>-1</sup> casein peptone, 20 g l<sup>-1</sup> glucose and 20 g l<sup>-1</sup> agar. Plasmid carrying yeast strains were selected on synthetic dextrose (SD) agar containing 6.9 g l<sup>-1</sup> yeast nitrogen base w/o amino acids (Formedium, Hunstanton, UK), 20 g l<sup>-1</sup> glucose, and 20 g l<sup>-1</sup> agar. Strains containing the *kanMX* cassette were selected on YPD plates containing 200 mg l<sup>-1</sup> G418 (Formedium, Hunstanton, UK). Defined minimal medium (Delft medium) as described before was used for both batch cultivations and fed-batch fermentations of L-ornithine producing strains (Scalcinati et al., 2012). Luria Bertani (LB) broth with 80 mg l<sup>-1</sup> ampicillin was used for maintenance of *E. coli* DH5a harboring appropriate plasmids. L-ornithine was quantified using a ninhydrin colorimetric assay as described previously (Chinard, 1952).

## Example 2

### L-arginine leaky auxotroph enables L-ornithine overproduction

As an intermediate of L-arginine biosynthesis in *S. cerevisiae*, L-ornithine can be converted to L-citrulline catalyzed by ornithine carbamoyltransferase (*ARG3*) in cytoplasm after export from the mitochondria, and L-ornithine biosynthesis rate is limited by the presence of L-arginine due to the feedback inhibition and repression of the key enzymes. However, deletion of *ARG3* results in a L-arginine auxotrophy and a need of L-arginine supplementation, which adds additional costs and might take problems in large-scale process control. Thus, as part of this invention, we tested whether fine-tuning the *ARG3* expression rather than full blocking would be a better strategy, as L-arginine can still be synthesized at controllable lower level to support the growth and also can limit the negative regulation of L-arginine on L-ornithine biosynthesis. Since promoter replacement can be a feasible strategy, we first weakened the *ARG3* expression by replacing its native promoter with the promoter of *HXT1* (Low-affinity glucose transporter of the major facilitator superfamily) and *KEX2* (Ca<sup>2+</sup> dependent serine protease involved in protein processing)(Scalcinati et al. 2012) (Fig. 1a). To replace the *ARG3* promoter, the *HXT1* promoter and *KEX2* promoter were amplified from genomic DNA of *S. cerevisiae* strain CEN.PK113-5D by PCR. The DNA cassette including the new promoter, the *kanMX* cassette and both 5' and 3' parts of the *ARG3* promoter was constructed following the strategy of MOPE as described in Example 1 above. Following the transformation of these cassettes into *S. cerevisiae*, the correct transformants were selected and verified by colony PCR. This resulted in strains M1a (*HXT1*) and M1b (*KEX2*).

This strategy resulted in increased production of ornithine (Fig. 1b), with both strains in which the *ARG3* promoter was replaced with a weaker promoter displaying improved overall production. The choice of promoter was also highly relevant to ornithine production, wherein the strain with the weakest promoter (*KEX2*) had a 75% higher titer (42 mg/L) compared to the strain with the *HXT1* promoter (24 mg/L as shown in Figure 1b).

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### Example 3

#### Pathway re-localization and subcellular trafficking engineering elevates L-ornithine synthesis

After the optimization of L-ornithine consumption, we optimized the L-ornithine biosynthesis pathway from  $\alpha$ -ketoglutarate, the intermediate of the TCA cycle. This part of L-ornithine synthesis is notable for its complicated metabolic compartmentation, where key metabolites, such as L-glutamate,  $\alpha$ -ketoglutarate and L-ornithine, are synthesised in different organelle. Thus, the specific biosynthesis pathway as well as the intermediates transportation/shutting should be co-ordinately optimized.

We started at managing the nature of compartmentalization to efficiently improve the L-ornithine titers (Fig. 2a, 2b and 2c). We first set out to enhance the L-ornithine synthesis pathway from L-glutamate in mitochondria.

*CAR2* was initially deleted from strain **M1b** using a bipartite strategy (Erdeniz et al., 1997). Two overlapping fragments of the *kanMX* resistance marker cassette flanked by *loxP* sites were amplified via PCR from plasmid pUG6 (Giildener et al., 1996). Sequences upstream and downstream of *CAR2* were also amplified. Due to overlapping ends (introduced through the primer sequences) the *GAF2*-upstream fragments could be fused to the 5' *kanMX* fragment and the 3' *kanMX* fragment to the individual *GAF2*-downstream fragments by fusion PCR using the outer primers for amplification. The two overlapping PCR fragments thus generated for each gene deletion were transformed into yeast using the lithium acetate method (Gietz and Woods, 2002). This resulted in the strain **ORN-E(KanMX)**. The strain with the *CAR2* deletion displayed a slight increase in ornithine production (Fig.1b). Next, the genes *ARG5*, 6 (EC. 1.2.1.38; 2.7.2.8; SEQ ID NO: 3), *ARG7* (EC 2.3.1.35; SEQ ID NO: 5) and *ARG8* (EC 2.6.1.11; SEQ ID NO: 4) were amplified from the genomic DNA of *S. cerevisiae* CEN.PK.113-5D and constructed into the G01 plasmid according to MOPE strategy and DNA assembler as described in Example 1 above. The strain **ORN-E(KanMX)** was transformed with either two empty plasmids (pYX212 and p423GPD) yielding strain **M1c**, or with an empty URA-based plasmid (pYX212) and G01 to yield strain **M1cM2f**.

Systematic overexpression of *ARG5*, 6, *ARG7* and *ARG8* in strain M1cM2f, increased L-ornithine titers to 59 mg l<sup>-1</sup>, representing a 31 % increase as compared to the control (strain M1c)(Fig. 2d). This part of the pathway is known as the acetylated derivatives cycle because the acetyl group that is added to L-glutamate in the first step of the pathway is recycled via N-acetylglutamate generated in the fifth step. We speculated that if the activity of the first step which is catalysed by N-acetyl-ornithine synthase (NAGS, encoding by *ARG2*; EC 2.3.1.1) was increased, the further L-ornithine titers should improve. To overexpress *ARG2* (SEQ ID NO: 2), this gene was amplified from the genome of *S. cerevisiae* CEN.PK113-5D strain and cloned into a plasmid together with *ARG5*,

6, *ARG7* and *ARG8* using the MOPE strategy, resulting in plasmid G02. This plasmid was co-transformed into strain **ORN-E(KanMX)** along with pYX212, resulting in strain **M1cM2g**. This strain produced 80 mg l<sup>-1</sup> of L-ornithine, leading to an 36% increasing as compared to the strain expressing only *ARG5*, *6*, *ARG7* and *ARG8* (strain M1cM2f)(Fig. 2d). Indeed, while the *ARG2* overexpression could improve the efficiency of initial step in the said L-ornithine pathway, it also fulfils an anaplerotic role to replenish the pathway intermediates that are lost due to degradation or cell division.

We also investigated whether overexpression of the transcription factor Gcn4p could improve L-ornithine production by up-regulation of corresponding genes such as above mentioned *ARG5*, *6*, *ARG7* and *ARG8* in L-ornithine synthetic pathway. Thus, we used different-strength promoters to fine-tune expression of a truncated version of *GCN4* (*tGCN4*), in which residues 99-106 were truncated to circumvent the rapid degradation through the ubiquitin pathway. No obvious difference was observed in the L-ornithine titers of most of these constructs, although overexpression of *GCN4* using the GPD1 promoter did result in a 16% increase in ornithine titers after 24 h of cultivation (Fig. 8).

Distinguished from L-ornithine synthesis in bacteria, L-ornithine is first synthesised in mitochondria and then exported by Ort1 p to the cytosol for L-arginine biosynthesis in yeast and hence we assessed whether increasing the expression level of *ORT1* could further boost the L-ornithine titers. First, the KanMX gene was removed from strain **ORN-E(KanMX)** by introduction of Cre-recombinase-mediated recombination between the two flanking *loxP* sites using plasmid pSH47 as described previously (Giildener et al., 1996), followed by removal of pSH47 by plating of the strain on 5-FOA. The *ORT1* (SEQ ID NO: 10) gene was then amplified from the genome of *S. cerevisiae* CEN.PK113-5D strain and integrated into the genome, resulting in strain **ORN-F**. This strain was co-transformed with pYX212 and G02, resulting in strain **M1cM2h**. This increased the L-ornithine titers to 115 mg l<sup>-1</sup>, representing a 44% increase as compared to the control strain (M1cM2g)(Fig. 2d).

We then engineered L-glutamate biosynthesis and its intracellular trafficking for precursor supply. We speculated that improving the internal L-glutamate trafficking step could increase the glutamate flux to further increase L-ornithine synthesis. Thus, the glutamate uniporter/aspartate-glutamate exchanger encoding gene *AGC1* (SEQ ID NO: 11) was overexpressed in strain M1cM2h as described above, and L-ornithine titers in resulting strain M1cM2k increased to 149 mg l<sup>-1</sup>, which was a 30% increase as compared to strain M1cM2h (115 mg l<sup>-1</sup>) (Fig. 2d). Once again, the internal trafficking steps were proved to be rate limiting in metabolic pathways and overexpressing related transporters could be efficient to boost pathway flux to chemicals of interest. Beside the glutamate transportation,  $\alpha$ -ketoglutarate transport from mitochondria to cytosol for glutamate synthesis was also enhanced by overexpressing the mitochondrial  $\alpha$ -ketoglutarate exporter OdC1p (strain M1cM2o). However, we did not observe any substantial improvement in L-ornithine titers (Fig. 10). This result suggested that either the OdC1p could not efficiently export  $\alpha$ -ketoglutarate or that the synthesis of  $\alpha$ -ketoglutarate could be rate limiting.

After resolving the L-glutamate transportation, the L-glutamate biosynthesis might become limiting for L-ornithine overproduction. There are three pathways that contribute to L-glutamate synthesis in *S. cerevisiae*. Two pathways are mediated by two isoforms of glutamate dehydrogenase, encoded by *GDH1* (EC: 1.4.1.4; SEQ ID NO: 6) and *GDH3* (EC: 1.4.1.4; SEQ ID NO: 7), while the third one catalyzed by combined activities of glutamine synthetase (encoded by *GLN1*; EC:6.3.1.2; SEQ ID NO: 8) and glutamate synthase (encoded by *GLT1*; EC 1.4.1.14; SEQ ID NO: 9). We then overexpressed the three pathways separately in strain M1cM2k respectively, resulting strain in M1cM2l (*GDH1*), M1cM2m (*GDH3*) and M1cM2n (*GLN1* and *GLT1*). Strain M1cM2l (overexpressing *GDH1*) displayed a further 16% increase in ornithine production, when compared to strain M1cM2k, and the final L-ornithine titer reached 173 mg l<sup>-1</sup> (Fig. 2d). However, we could not see any further improvement when either *GDH3* (strain M1cM2m) or *GLN1* and *GLT1* (strain M1cM2n) were expressed. These results indicated that *Gdh1p* is more efficient for synthesis of L-glutamate than *Gdh3p* and even the GS-GOGAT system.

The separated compartmentalization of metabolites should hinder the substrate channeling for consecutive enzyme catalysis. In our case,  $\alpha$ -ketoglutarate (mitochondria), L-glutamate (cytoplasm) and L-ornithine (mitochondria) are synthesized in different organelles. Thus, re-localization of these steps into same organelle might be helpful for L-ornithine production. We first introduced the single cytosolic step of L-glutamate biosynthesis into mitochondria by re-localizing the most efficient glutamate dehydrogenase *Gdh1p* into mitochondria. Considering *Gdh1p* is NADPH dependent and mitochondria is not rich in NADPH, we also set out to target and overexpress *Gdh2p* (EC 1.4.1.2) which is NADH dependent, into the mitochondria. Though the reaction catalyzed by *Gdh2p* favours L-glutamate degradation, but not synthesis in *S. cerevisiae*, the high level of  $\alpha$ -ketoglutarate and NADH in mitochondria might reverse the reaction towards L-glutamate synthesis from  $\alpha$ -ketoglutarate. Contrary to expectations, mitochondrial re-localization of *Gdh1p* (strain M1cM2i) and *Gdh2p* (M1cM2j) decreased the L-ornithine titers significantly (Fig. 11). The lack of increase of L-ornithine production by mitochondrial *Gdh2p* targeting might be attributed to its role in L-glutamate degradation which reduced the availability of the precursor for L-ornithine synthesis, while the decrease of L-ornithine production in mitochondrial *Gdh1p* targeting might be caused by the absence of functional targeting, which even intervene in mitochondrial function.

We then alternatively re-localized L-ornithine synthetic pathway to cytosol, where the precursor L-glutamate is synthesized. We introduced a chimeric heterologous cytosolic L-ornithine synthetic pathway in strain M1cM2q, where the first two enzymes encoding genes *argAEc* (SEQ ID NO: 23) and *argBEc* (SEQ ID NO: 24) were from *E. coli* and other three enzymes encoding genes *argJcg* (SEQ ID NO: 27), *argCcg* (SEQ ID NO: 25) and *argDcg* (SEQ ID NO: 26) were cloned from *C. glutamicum*. The successful complementation of L-arginine auxotrophic *S. cerevisiae* strain with *ORT1* disruption (Fig. 12), demonstrated that the cytosolic pathway was functional to provide the precursor L-ornithine for L-arginine biosynthesis. We also observed that M1cM2q gave an 11% increase of L-

ornithine, as compared to strain M1cM2l to 192 mg l<sup>-1</sup> in shake flask fermentation (Fig. 2d). These results demonstrated re-localization of the complete pathway into the cytosol was helpful for L-ornithine production.

#### Example 4

5 'Crabtree negative' *S. cerevisiae* construction enables efficient carbon channeling to L-ornithine

After efficiently channelling  $\alpha$ -ketoglutarate toward L-ornithine, we set out to enhance  $\alpha$ -ketoglutarate supply pathway by optimizing Module 3. However, the optimization of this part will be more difficult in *S. cerevisiae* due to the notable 'Crabtree effect', i.e. the major carbon flux is channeled to ethanol via aerobic-fermentation when *S. cerevisiae* is growing exponentially on glucose aerobically. This 'Crabtree effect' actually compromised the carbon flux to TCA cycle providing  $\alpha$ -ketoglutarate during L-ornithine biosynthesis (Fig. 3a).

Previous studies showed that the TCA cycle flux was to some extent controlled by phosphorylation of pyruvate dehydrogenase and that one mutation in the pyruvate dehydrogenase complex E1  $\alpha$  subunit Pdalp (EC1.2.4.1) can bypass the regulation (Oliveira et al. 2012). In this invention, we overexpressed both wild-type and mutated *PDA1* to drive more flux to L-ornithine synthesis. In addition, we overexpressed the potential corresponding genes that catalyze steps from pyruvate to  $\alpha$ -ketoglutarate, including one of the pyruvate carboxylase isomers encoding gene *PYC2* (EC:6.4.1.1), citrate synthase encoding gene *CIT1* (EC:2.3.3.16), aconitase encoding gene *ACO1* (EC 4.2.1.3) and isocitrate dehydrogenase encoding gene *IDP1* (EC 1.1.1.42). The strain M1cM2qM3a, carrying the overexpression of *PDA1*, *PYC2*, *CIT1*, *ACO1* and *IDP1*, had L-ornithine titers of 245 mg l<sup>-1</sup>, representing a 28% increase as compared to parent strain M1cM2q (Fig. 3d), and overexpression of mutated *mPDA1* based on *PYC2*, *CIT1*, *ACO1*, *IDP1* (strain M1cM2qM3b) just had a slight further L-ornithine increase to 264 mg l<sup>-1</sup> (Fig. 3d) as compared to strain M1cM2qM3a. The detailed physiological investigation showed that M1cM2qM3b and M1cM2qM3a exhibited approximately 2-fold increased L-ornithine/glucose yield as compared to the parent, whereas there was no substantial difference when compared with the control in terms of biomass yield and maximum specific growth rate. The ethanol yield on glucose made no difference. These results demonstrated that the 'Crabtree effect' could not be substantially alleviated by directly overexpressing the TCA cycle enzymes, but the metabolic flux of the TCA cycle could be improved at a smaller extent.

If the 'Crabtree effect' was due to the limited capacity of the respiration chain and the overexpression of NADH alternative oxidase (AOX) substantially alleviated the overflow to ethanol and if AOX overexpression up-regulated almost every step of the TCA cycle, this may be helpful for increasing production of TCA cycle derived chemicals.. We overexpressed *AOX* from *Hansenula anomala* (*HaAOXI*) and also *NDI1* (SEQ ID NO: 21) encoding the internal NADH dehydrogenase, which mediated the delivery of the equivalents to the respiratory chain. The overexpression of *HaAOXI* (strain M1cM2qM3c) enabled the L-ornithine titers to reach 258 mg l<sup>-1</sup>, representing a 35% increase as compared the parent strain M1cM2q (Fig. 3d). Furthermore, combined



overexpression of *NDI1* and *HaAOXI* further increased L-ornithine production to 278 mg l<sup>-1</sup> (Fig. 3d). These results indicated that NADH dehydrogenation was a rate limiting step in NADH oxidation, and overexpression of alternative NADH oxidase was an efficient strategy to boost the TCA flux for the production of TCA derived amino acids. It should be emphasized that the overexpression of *HaAOXI* and *NDI1* (strain M1cM2qM3d) increased the L-ornithine production to the same level as boosting the TCA cycle's enzyme activity by direct overexpression of related genes (strain M1cM2qM3b) (Fig. 3d).

Although aforementioned strategy had to some extent positive effect on L-ornithine production, the 'Crabtree effect' needed further alleviation. Overexpression of *MTH1-ΔT* (strain M1cM2qM3e) increased L-ornithine titers up to 778 mg l<sup>-1</sup> compared to the parent strain M1cM2q (Fig. 3d). It should also be emphasized that while the *MTH1-ΔT* overexpression (M1dM2qM3e) strain produced no ethanol and had low glucose uptake of 0.2 g glucose (g DCW)<sup>-1</sup>h<sup>-1</sup>, the parent strain had ethanol production of 1.3 g ethanol (g DCW)<sup>-1</sup>h<sup>-1</sup> and glucose uptake rate of 2.2 g glucose (g DCW)<sup>-1</sup>h<sup>-1</sup> (Fig. 3e). These results demonstrated that alleviating the 'Crabtree effect' of *S. cerevisiae* was an efficient strategy to boost L-ornithine production by enhancing TCA cycle flux.

We also assessed whether ODC attenuation might redirect more TCA flux to synthesis of L-glutamate, the precursor of L-ornithine. However, attenuation of *KGD2*, encoding one of the components of the mitochondrial α-ketoglutarate dehydrogenase complex, decreased L-ornithine titers 10-folds (Fig. 13). *KGD2* down-regulation (Strain M1dM2qM3f) restored the glucose uptake of 2.2 g glucose (g DCW)<sup>-1</sup>h<sup>-1</sup> and ethanol production of g ethanol (g DCW)<sup>-1</sup>h<sup>-1</sup> compared to the parent strain of *MTH1-ΔT* overexpression.

Besides the molecular manipulation, we explored the possibility of alleviating the 'Crabtree effect' by using an aerobic glucose-limited fed-batch strategy with strain M1cM2qM3a, whose upstream TCA cycle was enhanced as described above. Our engineered strain gave a final L-ornithine titer of 5.1 g l<sup>-1</sup>, whereas the DCW was 56 g l<sup>-1</sup> (Fig. 4).

#### Example 5

##### 'Urea cycle' engineering enables further L-ornithine titer improvement

*S. cerevisiae* has the potential to operate a urea cycle: arginase (encoded by *CAR1*; EC:3.5.3.1; SEQ ID NO: 1) can degrade L-arginine into urea and L-ornithine. Early study showed that in the presence of L-arginine and L-ornithine, ornithine carbamoyltransferase (OTC, Arg3p) and arginase (Carlp), both trimeric proteins, can form the so-called 'epi-arginase' mode of regulation, i.e. a one-to-one complex in which arginase remains active but OTC activity is inhibited. This regulation prevents the formation of an L-ornithine futile cycle when the biosynthesis of L-arginine is repressed and the catabolism of L-arginine activated. Our previous result showed that the strain with attenuated Arg3p still maintained 2/3 of the L-arginine intracellular concentration. It is speculated that overexpression of Carlp could enable even lower intracellular L-arginine. *CAR1* overexpression (strain M1dM2qM3e) gave the final L-ornithine titers to 1041 mg l<sup>-1</sup>, representing a further 34% increase as compared to

the parent strain M1cM2qM3e and 23-fold when compared with strain M1c (Fig. 3d). Our result indicated that *CAR1* overexpression pushed more flux into the L-ornithine synthesis cycle.

#### Example 6

5 Overexpression of the putrescine biosynthetic pathway in *S. cerevisiae*

To increase putrescine production, the gene *OAZ1*, coding for the ornithine decarboxylase antizyme was deleted from strain **ORN-L(KanMX)** and strain **CEN.PK113-11C**. First, the KanMX marker from **ORN-L(KanMX)** was looped out by introduction of Cre-recombinase-mediated recombination between the two flanking *loxP* sites using plasmid pSH47 as described previously (Giildener et al., 1996), followed by removal of pSH47 by plating  
10 of the strain on 5-FOA, resulting in strain **ORN-L**. Next, *OAZ1* was deleted from **ORN-L** and **CEN.PK113-11C** using a bipartite strategy (Erdeniz et al., 1997). Two overlapping fragments of the *kanMX* resistance marker cassette flanked by *loxP* sites were amplified via PCR from plasmid pUG6 (Giildener et al., 1996). Sequences upstream and downstream of *OAZ1* were also amplified. Due to overlapping ends (introduced through the primer sequences) the O-Azi-upstream fragments could be fused to the 5' *kanMX* fragment and the 3' *kanMX* fragment  
15 to the individual O-Azi-downstream fragments by fusion PCR using the outer primers for amplification. The two overlapping PCR fragments thus generated for each gene deletion were transformed into yeast using the lithium acetate method (Gietz and Woods, 2002). This resulted in strains **PUT-A(KanMX)** (expressing the ornithine pathway) and **WT-PUT-A(KanMX)** (not-expressing the ornithine platform). Subsequent transformation of **WT-PUT-A(KanMX)** with p426GPD and p423GPD resulted in strain **WT-PUT1**. **PUT-A(KanMX)** was transformed with  
20 plasmids p426GPD and G04, resulting in strain PUT-1.

In parallel, following the same procedure, we also constructed the integration cassette for *SPE1* overexpression which codes for ornithine decarboxylase (EC 4.1.1.17). *SPE1* was amplified from *S. cerevisiae* (CEN.PK113-11C) genome (SEQ ID NO: 29) and placed under the control of *TEF1* promoter and PRM9t terminator (Chen et al., 2012a) using fusion PCR procedures. The cassette was integrated to *OAZ1* site of ORN-  
25 L and CEN.PK113-11C, resulting in strains **PUT-B(KanMX)** and **WT-PUT-B(KanMX)**, respectively. **WT-PUT-B(KanMX)** was then transformed with plasmids p426GPD and p423GPD, resulting in strain **WT-PUT2**. **PUT-B(KanMX)** was transformed with plasmids p426GPD and G04, resulting in strain PUT-2.

To further increase production of putrescine, the export of these polyamines can be increased by overexpressing genes associated with polyamine export or deleting genes associated with polyamine uptake. To  
30 increase putrescine production by *S. cerevisiae*, the gene *TP01* (SEQ ID NO: 32) was amplified from the genome of *S. cerevisiae* (CEN.PK113-11C) using PCR. This gene was then cloned into **p412GPD** under the control of the *TDH3* promoter using standard cloning procedures, resulting in plasmid **p412GPD-TP01**. This plasmid was transformed into strain **WT-PUT-B(KanMX)** together with plasmid p423GPD, resulting in strains **WT-PUT3**. **p412GPD-TP01** was also transformed into strain **PUT-B(KanMX)** together with the vector G04, resulting in

strain **PUT3**.

The resulting strains were cultivated in minimal media. In order to quantify polyamine production, samples were treated as follows: cultivation sample was prepared by taking 1 ml of liquid culture. Cells were spun down by centrifugation. The supernatant was directly used for derivatization. Cells were subject to Hot Water (HW) Extraction. This procedure was adapted from (Canelas et al. 2009). Tubes containing 1 ml of demineralized water were preheated in a water bath at 95-100 °C for 10 min. Then, the hot water was quickly poured over the cell pellet; 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 the accurate measurement of polyamine concentration, samples were diluted, if necessary, to give polyamine concentration within the standard curve range. For derivatization, the procedure was adapted from (Kim et al. 2015). In brief, 0.25 ml of saturated NaHCO<sub>3</sub> 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. Samples filtered through a 25 mm syringe filter (0.45 µm Nylon) can be used for HPLC detection. The following chromatographic condition are used: C18 (100 mm × 4.6 mm i. d., 2.6 µm, Phenomenex Kinetex), excitation wavelength 340nm, emission wavelength 515nm, Sample injecting 10µl, column temperature 40°C, Detector sensitivity 7, acquisition starts at 3.4 min. The mobile phase is water and methanol with the speed of 1mL /min. The elution program is as follows: 0-23 min 50% to 95% MeOH, 23-27 min 95% MeOH, 27-32 min 95% to 100% MeOH, 32-37 min 100% to 50% MeOH.

Both intracellular and extracellular putrescine levels were quantified (Fig. 15). Levels of putrescine significantly increased upon deletion of OAZ1, and increased further upon introduction of SPE1 (Fig.15). Furthermore, strains engineered for increased ornithine production (strains PUT1, PUT2 and PUT3) produced significantly more putrescine compared to strains without any ornithine pathway modifications (Fig. 15), displaying the benefits of the ornithine platform for polyamine production. In addition, a slight increase in putrescine levels was observed for strains overexpressing the transporter TP01 (Fig. 15).

### Example 7

#### Overexpression of the spermidine biosynthetic pathway in *S. cerevisiae*

To increase spermidine production by *S. cerevisiae*, the genes *SPE2* (SEQ ID NO: 30) and *SPE3* (SEQ ID NO: 31), encoding S-adenosylmethionine decarboxylase (EC 4.1.1.50) and spermidine synthase (EC 2.5.1.16) (respectively), were amplified from the genome of *S. cerevisiae* (CEN.PK113-11C) using PCR.

Following the same procedure, we also constructed the integration cassette for *SPE3* overexpression which codes for Spermidine synthase (EC 2.5.1.16). *SPE3* was amplified from *S. cerevisiae* (CEN.PK113-11C) genome (SEQ ID NO: 31) and placed under the control of *PGK1* promoter and pYX212t terminator using fusion PCR

procedures. *SPE1* which codes for ornithine decarboxylase (EC 4.1.1.17) together with TEF1 promoter and PRM9t terminator was also included in the same cassette. The cassette was integrated into *OAZ1* site of ORN-L, resulting in strain **SPD-A(KanMX)**. Subsequent transformation of plasmid p426GPD and G04 resulted in strain **SPD1**.

5 In parallel, following the same procedure, we also constructed the integration cassette for *SPE2* (SEQ ID NO: 30) encoding S-adenosylmethionine decarboxylase (EC 4.1.1.50). *SPE2* was amplified from *S. cerevisiae* (CEN.PK113-11C) genome (SEQ ID NO: 30) and placed under the control of *TDH3* promoter and DIT1t terminator using fusion PCR procedures. *SPE1* which codes for ornithine decarboxylase (EC 4.1.1.17) together with TEF1 promoter and PRM9t terminator was also included in the same cassette. Also, *SPE3* which was amplified from *S.*  
 10 *cerevisiae* (CEN.PK113-11C) genome (SEQ ID NO: 31) under the control of PGK1 promoter and pYX212t terminator using fusion PCR procedures was as part of the integration cassette. The full cassette was integrated into the *OAZ1* site of ORN-L, resulting in strain **SPD-B(KanMX)**. Subsequent transformation of this strain with plasmids p426GPD and G04 resulted in strain **SPD2**.

The resulting strains were cultivated in minimal media and processed for spermidine quantification as  
 15 described in Example 6 above. Fig. 16 shows the modified strains to produce significantly more spermidine than what is found in the control (CEN.PK113-11C) strain, with strain SPD2 producing the highest levels.

### Example 8

#### Overexpression of an alternative pathway for spermidine production

20 In some bacterial species, spermidine can be produced via the condensation of L-aspartate semialdehyde and putrescine via carboxynorspermidine dehydrogenase (CANSDH) yielding carboxyspermidine, followed by decarboxylation by carboxynorspermidine decarboxylase (CANSDC). Genes encoding enzymes with these activities can be expressed to facilitate spermidine production. CANSDH and CANSDC from several organisms can be used. *CjCANSDH* (*Cj0172c*, SEQ ID NO: 46) and *CjCANDC* (*Cj1515c*, SEQ ID NO: 47) can be codon-  
 25 optimized based on sequences from *Campylobacter jejuni*. *VcCANSDH* (*VC1624*, SEQ ID NO: 48) and *VcCANDC* (*VC1623*, SEQ ID NO: 49) can be codon-optimized based on sequences from *Vibrio cholerae*. *BuCANSDH* (*Lys1*, SEQ ID NO: 50) and *BuCANDC* (*nspC*, SEQ ID NO: 51) can be codon-optimized based on sequences from *Bacteroides uniformis*. Each pair of CANSDH and CANSDC can be cloned into the plasmid pYX212 under the promoter of TPIp and PGK1p respectively by the standard Gibson Assembly protocols with a commercial Kit from  
 30 NEB. The resulting plasmids, YP1, YP2 and YP3 each encode a CANSDH and CANSDC from *C. jejuni*, *V. cholerae* and *B. uniformis*, respectively. These plasmids can be separately transformed into strain **PUT-B(KanMX)** together with the vector G04 resulting in strains **SPD-B1**, **SPD-B2** and **SPD-B3**, capable of producing high levels of spermidine.

## Example 9

Overexpression of the spermine biosynthetic pathway in *S. cerevisiae*

To increase spermine production in *S. cerevisiae*, the gene *SPE1*, which codes for ornithine decarboxylase (EC 4.1.1.17), can be amplified from *S. cerevisiae* (CEN.PK113-11C) genome (SEQ ID NO: 29) and cloned into  
 5 vector pSP-GM1 under the control of a *TEF1* promoter (Chen et al., 2012a) using standard cloning procedures. The former resulting in **pSP-GM1-SPE1**. The genes *SPE2* (SEQ ID NO: 30) and *SPE3* (SEQ ID NO: 31), encoding S-adenosylmethionine decarboxylase (EC 4.1.1.50) and spermidine synthase (EC 2.5.1.16) (respectively), can be amplified from the genome of *S. cerevisiae* (CEN.PK113-11C) using PCR. *SPE2* can be cloned into **pSP-GM1-SPE1** under the control of the *PGK1* promoter using standard cloning procedures, resulting in plasmid **pSP-GM1-SPE12**. *SPE3* can be cloned into pSP-GM1 (Chen et al., 2012a) under the control of a *TEF1* promoter, resulting  
 10 in plasmid **pSP-GM1-SPE3**. The *TEFp-SPE3-tADH1* cassette can amplified from pSP-GM1-SPE3 and cloned into **pSP-GM1-SPE12** in reverse orientation, resulting in plasmid **pSP-GM1-SPD** (expressing the genes *SPE1*, *SPE2* and *SPE3*). Next, the gene *SPE4* (SEQ ID NO: 43), encoding spermine synthase (EC 2.5.1.22) can be amplified from the genome of *S. cerevisiae* (CEN.PK113-11C) using PCR and cloned into **pSP-GM1-SPE3** under  
 15 the control of the *PGK1* promoter, resulting in plasmid **pSP-GM1-SPE34**. The cassette encoding *tCYC1-SPE4-PGK1p-pTEF1-SPE3-tADH1* can be then amplified from pSP-GM1-SPE34 and cloned into pSP-GM1-SPE12 in reverse orientation, resulting in plasmid **pSP-GM1-SPM** (expressing the genes *SPE1*, *SPE2*, *SPE3* and *SPE4*). pSP-GM1-SPM can be transformed into strain **PUT-A(KanMX)** together with the vector G04 resulting in strain **AKYSPM1**, capable of producing high levels of spermine.

20

## Example 10

Increased export of polyamines in *S. cerevisiae*

To further increase production of putrescine, spermidine or spermine, the export of these polyamines can be increased by deleting genes associated with polyamine uptake. The *AGP2*, *SAM3* and *DUR3* genes, which are  
 25 involved in polyamine uptake, can be deleted consecutively from strain **PUT-B(KanMX)** as described in Example 6 above, resulting in strain **AKYDOAZ-Trans2**. Next, **AKYDOAZ-Trans2** can be co-transformed with plasmids pSP-GM1-PUT and G04, resulting in strain **AKYPUT2**, capable of producing high levels of putrescine. The strain **AKYDOAZ-Trans2** can also be transformed with the plasmids pSP-GM1-SPD and G04, resulting in strain **AKYSPD2**, capable of producing high levels of spermidine. The strain **AKYDOAZ-Trans3** can also transformed  
 30 with the plasmids pSP-GM1-SPM and G04, resulting in strain **AKYSPM2**, capable of producing high levels of spermine.

## Example 11

Production of agmatine in *S. cerevisiae*

To create a strain capable of producing agmatine, the gene encoding arginase (*CAR1*) can be deleted from strain CEN.PK1 13-1 1C using KanMX-bipartite deletion strategy as described in Example 6, resulting in strain **AKYDCAR1**. Next, the genes encoding for ornithine carbamoyltransferase (EC 2.1.33), argininosuccinate synthase (EC 6.3.4.5) and argininosuccinate lyase (EC 4.3.2.1), encoded by *ARG3*, *ARG1* and *ARG4* (SEQ ID NO: 34-36) can be amplified from the genome of *S. cerevisiae* (CEN.PK1 13-1 1C). *ARG3* and *ARG1* can be cloned into pSP-GM1 under the control of the *TEF1* and *PGK1* promoters, resulting in plasmid **pSP-GM1-ARGA**. *ARG4* can be cloned into pSP-GM1, under the control of the *TEF1* promoter, resulting in plasmid **pSP-GM1-ARGB**. The *TEF1p-ARG4-tADH1* cassette can be amplified from pSP-GM1-ARGB, and cloned in reverse orientation into plasmid pSP-GM1-ARGA, resulting in plasmid **pSP-GM1-ARGC** (expressing *ARG3*, *ARG1* and *ARG4*). The gene encoding arginine decarboxylase (EC 4.1.1.19) (*speA*) (SEQ ID NO: 37) can be amplified from *E. coli* (DH5a) and cloned into plasmid pLYC04 (Chen et al., 2012b) under the control of the *TEF1* promoter, resulting in plasmid **pLYC04-speA**. The plasmids pSP-GM1-ARGC and pLYC04-speA can be co-transformed into strain **AKYDCAR1**, resulting in strain **AKYAGM1**, capable of producing agmatine.

To create a yeast strain capable of converting agmatine to spermidine, a bidirectional *PGK1p-TEF1p* promoter cassette can be amplified from plasmid pSP-GM1 via PCR. Next, the genes encoding S-adenosylmethionine decarboxylase (*SP£2*) (SEQ ID NO: 30) and spermidine synthase (*SPE3*) (SEQ ID NO: 31) can be amplified from the *S. cerevisiae* (CEN.PK1 13-1 1C) genome using overhang-containing primers that allow fusion of these genes to the *PGK1p-TEF1p* cassette (*SPE2* under the control of *TEF1p* and *SPE3* under the control of *PGK1p*). The *SPE3-PGK1p-TEF1p-SPE2* cassette can then be cloned into vector **pXI-5** (Mikkelsen et al., 2012), resulting in vector **pXI-5-SPE32**, allowing the targeting of these genes into the Chr XI: 11779..118967 region of the yeast genome. This vector can be linearized and transformed into strain **AKYDCAR1**, followed by looping-out of the URA3 marker, resulting in strain **AKYAGM2** (containing the two integrated genes).

The gene encoding agmatinase (EC 3.5.3.11) (*speE*) (SEQ ID NO: 38) can be amplified from *E. coli* and cloned into **pLYC04-speA** under the control of the *PGK* promoter, resulting in plasmid **pLYC04-speAB**. The plasmids **pLYC04-speASB** and **pSP-GM1-ARGC** can be co-transformed into strain **AKYAGM2**, resulting in strain **AKYAGM3**, capable of converting agmatine into putrescine and spermidine.

To create a strain capable of producing spermine via the agmatine route, the *PGK1p-SPE4* cassette can be amplified from plasmid **pSP-GM1-SPE34** and cloned into vector **pX-4** (Mikkelsen et al., 2012), resulting in vector **pX-4-SPE4**, capable of targeting *SPE4* to the Chr X: 236336..237310 region of the yeast genome. This vector was linearized and transformed into strain **AKYAGM2**, followed by looping out of the URA3 marker, resulting in strain **AKYAGM4**.

The plasmids **pLYC04-speAB** and **pSP-GM1-ARGC** can be co-transformed into strain **AKYAGM4**, resulting in strain **AKYAGM5**, capable of converting agmatine into putrescine, spermidine and spermine.

## Example 12

Production of spermidine/spermine in *Escherichia coli*

Modifications in *E. coli* WL3110 (orig. K12 W3110 (CGSC, Coli Genetic Stock Center); (Park et al. 2007)) to increase ornithine and putrescine production respectively can be implemented as described in Quian et al. (2009).

5 For example these comprise enhancing precursor supply (deletion of *argI* gene), inactivating putrescine degradation and utilization pathways (deletion of *speG*; *puuPA*, *argR*) and deletion of *rpoS*, a stress responsive polymerase sigma factor. Also genes coding for ornithine decarboxylase converting ornithine to putrescine and ornithine biosynthetic genes [*argC-E*] can be overexpressed either through plasmids or chromosomal integration. This platform strain can be transformed with either plasmids for overexpression of endogenous or heterologous

10 *speE* (E.C.: 2.5.1.16; SEQ ID NO: 37) and *speD* (EC 4.1.1.50; SEQ ID NO: 39) 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 (Gibson et al. 2009). Alternatively chromosomal replacement of promoters of these genes with

15 a constitutive active strong hybrid-promoter like  $P_{trc}$  can be performed. To facilitate increased spermidine export, genes coding for exporters e.g. the *mdtJI* complex genes (SEQ ID NO: 40-41) can be overexpressed through plasmid based expression or promoter exchange as described above. The gene coding for the spermidine exporter *bit* from *B. subtilis* (SEQ ID NO: 42) can be amplified from *B. subtilis* genomic DNA and also heterologously expressed under *E. coli* promoter control (e.g.  $P_{trc}$ ). For the tailored production of spermine

20 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 sinensis*, *Homo sapiens*. For example spermine synthase gene *SPE4* (GI: 3201942; SE ID NO: 43) can be amplified from *S. cerevisiae* genomic DNA (CEN.PK113-11C) using PCR with 30 bp overhang primers and cloned via Gibson cloning (Gibson et al. 2009) into expression vector pTRC.

25

## Example 13

Production of spermidine/spermine in *Corynebacterium glutamicum*

Modifications to increase ornithine production in *C. glutamicum* strain ATCC 13032 can be performed as described in Kim et al. (2015): deletion of *proB* and *argF* (block competing pathways); deletion of *argR* gene

(repressor of the L-arginine operon); overexpression of *argCJBD* from *C. glutanicum* ATCC 21831; start codon change of *pgi* and *zwf* (enrichment of NADPH pool), replacing native promoter of *tkl* operon with a strong *sod* promoter. For increased putrescine production the strain can be further engineered as described in Schneider et al. (2010): The strain was modified with overexpression of ornithine decarboxylase *speC* (EC 4.1.1.19; SEQ ID NO: 44) from *E. coli* and chromosomal deletion of carbamoyl-transferase *argF*. For increased spermidine production endogenous spermidine synthase gene *speE* (SEQ ID NO: 45) can be either overexpressed through plasmid based expression or integrating of multiple copies into the genome. For example, *E. coli*-*C. glutanicum* shuttle vector pMS2 (ATCC®67189TM) with kanamycin as selective marker is used for plasmid based overexpression of heterologous *speD*. For plasmid construction two fragments can be amplified: fragment containing the *P<sub>tac</sub>* promoter and *lacI* gene derived from pTAC-MAT-Tag®-1 (Sigma) and the *speD* gene from *E. coli* genomic DNA (SEQ ID NO: 39). 30 bp overhanging primers can be used to amplify fragments and subsequently cloned via Gibson cloning (Gibson et al. 2009) into the shuttle vector pMS2 with *speD* under *P<sub>tac</sub>* control creating the expression vector pFDAMS2. After transformation into modified *C. glutanicum* strain ATCC 13032 strain (see above) it can be cultivated as described in Schneider et al. 2010. To facilitate increased spermidine export, genes coding for exporters e.g. *mdtJl* from *E. coli* (SEQ ID NO: 40-41) or *bit* from *B. subtilis* (SEQ ID NO: 42) can be amplified from *E. coli* genomic DNA respective *B. subtilis* genomic DNA and also heterologously expressed in *C. glutanicum* as described above. For the tailored production of spermine heterologous spermine synthases (E.C. 2.5.1.22) from eukaryotic sources can be expressed via plasmid-based expression (see above). For example spermine synthases can be derived from *S. cerevisiae* [*Spe4*, G<sub>1</sub>: 3201942; SEQ ID NO: 43), *Triticum aestivum*, *Oryza sativa*, *Glycine max*, *Citrus sinensis*, *Homo sapiens*.

Table 1: Background strains constructed for ornithine production.



No	Strain name	Genotype
1	ORN-A	<i>MATa SUC2 MAL2-8c ura3-52 his3-M PARG3::PHXT1</i>
2	ORN-B	<i>MATa SUC2 MAL2-8c ura3-52 his3-M PARG3::PKEX2</i>
3	ORN-E(KanMX)	<i>MATa SUC2 MAL2-8c ura3-52 his3-AI PARG3::PKEX2 car2 ::LoxP-KanMX-LoxP</i>
4	ORN-F(KanMX)	<i>MATa SUC2 MAL2-8c ura3-52 his3-AI PARG3::PKEX2 car2 ::LoxP-KanMX-LoxP-PTPI-ORT 1-T<sub>p</sub>YX212</i>
5	ORN-G(KanMX)	<i>MATa SUC2 MAL2-8c ura3-52 his3-AI PARG3::PKEX2 car2 ::LoxP-KanMX-LoxP-T<sub>c</sub>T<sub>ci</sub> -AGC1-PtHXTi-PTPi-ORT1-T<sub>p</sub>YX2i2</i>
6	ORN-F	<i>MATa SUC2 MAL2-8c ura3-52 his3-AI PARG3::PKEX2 car2 ::LoxP-P<sub>7</sub>Pi-ORT1-TpYxm</i>
7	ORN-G	<i>MATa SUC2 MAL2-8c ura3-52 his3-AI PARG3::PKEX2 car2 ::LoxP-T<sub>c</sub>rci-AGC1-PtHXTi-Prpi -ORT1-TpYX2i2</i>
8	ORN-H(KanMX)	<i>MATa SUC2 MAL2-8c ura3-52 his3-AI PARG3::PKEX2 car2 ::LoxP-P<sub>7</sub>Pi-ORT1-TpYxm ura3:: LoxP-KanMX-LoxP-PTEFi-GDH1-T<sub>D</sub>iTi</i>
9	ORN-I(KanMX)	<i>MATa SUC2 MAL2-8c ura3-52 his3-AI PARG3::PKEX2 car2 ::LoxP-P<sub>7</sub>Pi-ORT1-TpYxm ura3:: LoxP-KanMX-LoxP-TTDH2-ODC1-PpGKi-PTEFi-GDH1-TDiTi</i>
10	ORN-J(KanMX)	<i>MATa SUC2 MAL2-8c ura3-52 his3-AI PARG3::PKEX2 car2::LoxP-PTPI-ORT1-TpYX212 ura3:: LoxP-KanMX-LoxP-PTEF1-MLS-GDH1-TDIT1</i>
11	ORN-K(KanMX)	<i>MATa SUC2 MAL2-8c ura3-52 his3-AI PARG3::PKEX2 car2::LoxP-PTPI-ORT1-TpYX212 ura3:: LoxP-KanMX-LoxP-PTEF1-MLS-GDH2-TDIT1</i>
12	ORN-J	<i>MATa SUC2 MAL2-8c ura3-52 his3-AI PARG3::PKEX2 car2::LoxP-PTPI-ORT1-TpYX212 ura3:: LoxP-PTEF1-MLS-GDH1-TDIT1</i>
13	ORN-L(KanMX)	<i>MATa SUC2 MAL2-8c ura3-52 his3-AI PARG3::PKEX2 car2::LoxP-T<sub>c</sub>rci-AGC1-PtHXTi-Prpi -ORT1-TpYX2i2 ura3:: LoxP-KanMX-LoxP-PTEF1-GDH1-TDIT1</i>

14	ORN-M(KanMX)	<i>MATa SUC2 MAL2-8c ura3-52 his3-M PARG3::PKEX2 car2::LoxP-TcrCi-AGC1-P<sub>HXTi</sub>-PTPi -ORT1-T<sub>p</sub>YX2i2 ura3:: LoxP-KanMX-LoxP-PTEF1-GDH3-TDIT1</i>
15	ORN-N(KanMX)	<i>MATa SUC2 MAL2-8c ura3-52 his3-M PARG3::PKEX2 car2::LoxP-TcTci-AGC1-P<sub>HXTi</sub>-PTPi -ORT1-T<sub>p</sub>YX2i2 ura3:: LoxP-KanMX-LoxP-TTDH2-ODC1-PPGK1-PTEF1-GDH3-TDIT1</i>
16	ORN-O(KanMX)	<i>MATa SUC2 MAL2-8c ura3-52 his3-M PARG3::PKEX2 car2::LoxP-TcrCi-AGC1-P<sub>HXTi</sub>-PTPi -ORT1-T<sub>p</sub>YX2i2 ura3:: LoxP-KanMX-LoxP-TTDH2-ODC1-PPGK1-PTEF1-GDH1-TDIT1</i>
17	ORN-P(KanMX)	<i>MATa SUC2 MAL2-8c ura3-52 his3-M PARG3::PKEX2 car2::LoxP-TcrCi-AGC1-P<sub>HXTi</sub>-PTPi -ORT1-T<sub>p</sub>YX2i2 YPRT3:: LoxP-KanMX-LoxP-PTEF1-AOX1-TPRM9</i>
18	ORN-Q(KanMX)	<i>MATa SUC2 MAL2-8c ura3-52 his3-AI PARG3::PKEX2 car2::LoxP-TcTci-AGC1-P<sub>HXTi</sub>-PTPi -ORT1-T<sub>p</sub>YX2i2 YPRT3:: LoxP-KanMX-LoxP-TpYX2i2-NDI1-PPGK1-PTEF1-AOX1-TPRM9</i>
19	ORN-R(KanMX)	<i>MATa SUC2 MAL2-8c ura3-52 his3-AI PARG3::PKEX2 car2::LoxP-TcTci-AGC1-P<sub>HXTi</sub>-PTPi -ORT1-T<sub>p</sub>YX2i2 ura3:: LoxP-KanMX-LoxP-PTEF1-MLS-GDH1-TDIT1</i>
20	ORN-L	<i>MATa SUC2 MAL2-8c ura3-52 his3-AI PARG3::PKEX2 car2::LoxP-TcrCi-AGC1-P<sub>HXTi</sub>-Prpi -ORT1-T<sub>p</sub>YX2i2 ura3:: LoxP-PTEF1-GDH1-TDIT1</i>
21	ORN-0	<i>MATa SUC2 MAL2-8c ura3-52 his3-AI PARG3::PKEX2 car2::LoxP-TcTci-AGC1-P<sub>HXTi</sub>-PTPi -ORT1-T<sub>p</sub>YX2i2 ura3:: LoxP-TTDH2-ODC1-PPGK1-PTEF1-GDH1-TDIT1</i>
22	ORN-S(KanMX)	<i>MATa SUC2 MAL2-8c ura3-52 his3-AI PARG3::PKEX2 car2::LoxP-TcrCi-AGC1-P<sub>HXTi</sub>-PTPi -ORT1-T<sub>p</sub>YX2i2 ura3:: LoxP-PTEF1-GDH1-TDIT1 kgd2:: LoxP-KanMX-LoxP</i>
23	ORN-T(KanMX)	<i>MATa SUC2 MAL2-8c ura3-52 his3-AI PARG3::PKEX2 car2::LoxP-TcrCi-AGC1-P<sub>HXTi</sub>-PTPi -ORT1-T<sub>p</sub>YX2i2 ura3:: LoxP-PTEF1-GDH1-TDIT1 kgd2:: LoxP-KanMX-LoxP-TPRM9t-MTH1-PTEF1</i>
24	ORN-U(KanMX)	<i>MATa SUC2 MAL2-8c ura3-52 his3-AI PARG3::PKEX2 car2::LoxP-TcrCi-AGC1-P<sub>HXTi</sub>-PTPi -ORT1-T<sub>p</sub>YX2i2 ura3:: LoxP-PTEF1-GDH1-TDIT1 kgd2:: LoxP-KanMX-LoxP-TPRM9t-AOX1-PTEF1</i>

25	ORN-V(KanMX)	<i>MATa SUC2 MAL2-8c ura3-52 his3-M PARG3::PKEX2 car2::LoxP-TcrCi-AGC1-P<sub>HXTI</sub>-PTP<sub>i</sub> -ORT1-T<sub>p</sub>YX2i2 ura3:: LoxP-PTEF1-GDH1-TDIT1 YPRT3:: LoxP-KanMX-LoxP-PTEF1-MTH1-TPRM9</i>
26	ORN-WN(KanMX)	<i>MATa SUC2 MAL2-8c ura3-52 his3-M PARG3::PKEX2 car2::LoxP-TcrCi-AGC1-P<sub>HXTI</sub>-PTP<sub>i</sub> -ORT1-T<sub>p</sub>YX2i2 ura3:: LoxP-PTEF1-GDH1-TDIT1 YPRT3:: LoxP-KanMX-LoxP-PTEF1-AOX1-TPRM9</i>
27	ORN-W(KanMX)	<i>MATa SUC2 MAL2-8c ura3-52 his3-M PARG3::PKEX2 car2::LoxP-TcrCi-AGC1-P<sub>HXTI</sub>-PTP<sub>i</sub> -ORT1-T<sub>p</sub>YX2i2 ura3:: LoxP-PTEF1-GDH1-TDIT1 YPRT3:: LoxP-KanMX-LoxP-PTEF1-AOX1-TPRM9</i>
28	ORN-X(KanMX)	<i>MATa SUC2 MAL2-8c ura3-52 his3-M PARG3::PKEX2 car2::LoxP-TcrCi-AGC1-P<sub>HXTI</sub>-PTP<sub>i</sub> -ORT1-T<sub>p</sub>YX2i2 ura3:: LoxP-TTDH2-ODC1-PPGK1-PTEF1-GDH1-TDIT1 YPRr3:: LoxP-KanMX-LoxP-PTEF1-AOX1-TPRM9</i>
29	ORN-Y(KanMX)	<i>MATa SUC2 MAL2-8c ura3-52 his3-AI PARG3::PKEX2 car2::LoxP-TCTC1-AGC1-F1HXT1-PTPI-ORT1-T<sub>p</sub>YX2i2 ura3:: LoxP-TTDH2-ODC1-PPGK1-PTEF1-GDH1-TDIT1 kgd2:: LoxP-KanMX-LoxP-TPRM9t-AOX1-PTEF1</i>
30	ORN-Z(KanMX)	<i>MATa SUC2 MAL2-8c ura3-52 his3-AI PARG3::PKEX2 car2::LoxP-TcTci-AGC1-P<sub>HXTI</sub>-PTP<sub>i</sub> -ORT1-T<sub>p</sub>YX2i2 ura3:: LoxP-TTDH2-ODC1-PPGK1-PTEF1-GDH1-TDIT1 kgd2:: LoxP-KanMX-LoxP</i>
31	B0166A CEN.PK	<i>MATa ORT1A</i>
32	CEN.PK113-11C	<i>MATa SUC2 MAL2-8<sup>c</sup> ura3-52 his3-AI</i>

Table 2: Strains used for module and full ornithine pathway optimization

No	Strain name	Module 1		Module 2		Module 3		Reference		
		Module	Desc	Module	Desc	Module	Desc	Background strain	Plasmid 1(URA)	Plasmid 2(HIS)

1	M1a	M1a	<b>P</b> HXT1- ARG3	Null	Null	Null	Null	ORN-A	pYX212	p423GPD
2	M1b	M1b	<b>P</b> KEX2- ARG3	Null	Null	Null	Null	ORN-B	pYX212	p423GPD
3	M1c	M1c	<b>P</b> KEX2- ARG3; CAR2A	Null	Null	Null	Null	ORN- <b>E</b> (KanMX)	pYX212	p423GPD
4	M1cM2a	M1c	<b>P</b> KEX2- ARG3; CAR2A	M2a	ARG2	Null	Null	ORN- <b>E</b> (KanMX)	pSPGM 1-ARG2	p423GPD
5	M1cM2b	M1c	<b>P</b> KEX2- ARG3; CAR2A	M2b	ARG5.6	Null	Null	ORN- <b>E</b> (KanMX)	pSPGM 1- ARG5.6	p423GPD
6	M1cM2c	M1c	<b>P</b> KEX2- ARG3; CAR2A	M2c	ARG7	Null	Null	ORN- <b>E</b> (KanMX)	pSPGM 1-ARG7	p423GPD
7	M1cM2d	M1c	<b>P</b> KEX2- ARG3; CAR2A	M2d	MLS- argBcg	Null	Null	ORN- <b>E</b> (KanMX)	pYX212 -argBEc	p423GPD
8	M1cM2e	M1c	<b>P</b> KEX2- ARG3; CAR2A	M2e	MLS- argJcg	Null	Null	ORN- <b>E</b> (KanMX)	pYX212 -argJcg	p423GPD
9	M1cM2f	M1c	<b>P</b> KEX2- ARG3; CAR2A	M2f	ARG5.6; ARG7; ARG8	Null	Null	ORN- <b>E</b> (KanMX)	pYX212	G01
10	M1cM2g	M1c	<b>P</b> KEX2- ARG3; CAR2A	M2g	ARG5.6; ARG7; ARG8;	Null	Null	ORN- <b>E</b> (KanMX)	pYX212	G02

					ARG2					
11	M1cM2h	M1c	P <sub>KEX2</sub> - ARG3; CAR2Δ	M2h	ARG5,6; ARG7; ARG8; ARG2; ORT1	Null	Null	ORN-F	pYX212	GO2
12	M1cM2i	M1c	P <sub>KEX2</sub> - ARG3; CAR2Δ	M2i	ARG5,6; ARG7; ARG8; ARG2; ORT1; MLS- GDH1	Null	Null	ORN- J(KanMX)	pYX212	GO2
13	M1cM2j	M1c	P <sub>KEX2</sub> - ARG3; CAR2Δ	M2j	ARG5,6; ARG7; ARG8; ARG2; ORT1; MLS- GDH2	Null	Null	ORN- K(KanMX)	pYX212	GO2
14	M1cM2k	M1c	P <sub>KEX2</sub> - ARG3; CAR2Δ	M2k	ARG5,6; ARG7; ARG8; ARG2; ORT1; AGC1	Null	Null	ORN-G	pYX212	GO2
15	M1cM2l	M1c	P <sub>KEX2</sub> - ARG3;	M2l	ARG5,6; ARG7;	Null	Null	ORN- L(KanMX)	pYX212	GO2

			CAR2Δ		ARG8; ARG2; ORT1; AGC1; GDH1					
16	M1cM2m	M1c	P <sub>KEX2</sub> - ARG3; CAR2Δ	M2m	ARG5,6; ARG7; ARG8; ARG2; ORT1; AGC1; GDH3	Null	Null	ORN- M(KanMX)	pYX212	GO2
17	M1cM2n	M1c	P <sub>KEX2</sub> - ARG3; CAR2Δ	M2n	ARG5,6; ARG7; ARG8; ARG2; ORT1; AGC1; GLN1; GLT1	Null	Null	ORN-G	YO1	GO2
18	M1cM2o	M1c	P <sub>KEX2</sub> - ARG3; CAR2Δ	M2o	ARG5,6; ARG7; ARG8; ARG2; ORT1; AGC1; GDH3; ODC1	Null	Null	ORN- N(KanMX)	pYX212	GO2

19	M1cM2p	M1c	P <sub>KEX2</sub> - ARG3; CAR2A	M2p	ARG5.6; ARG7; ARG8; ARG2; ORT1 ; AGC1 ; GDH1 ; ODC1	Null	Null	ORN- O(KanMX)	pYX212	G04
20	M1cM2q	M1c	P <sub>KEX2</sub> - ARG3; CAR2A	M2q	<i>argAEc</i> ; <i>argBEc</i> ; <i>argCcg</i> ; <i>argDcg</i> ; <i>argJcg</i> ; <i>ORT1</i> ; <i>AGC1</i> ; <i>GDH1</i>	Null	Null	ORN- O(KanMX)	pYX212	G04
21	M1cM2r	M1c	P <sub>KEX2</sub> - ARG3; CAR2A	M2r	PADH1- tGCN4	Null	Null	ORN- E(KanMX)	pYX212	PADH1- tGCN4
22	M1cM2s	M1c	P <sub>KEX2</sub> - ARG3; CAR2A	M2s	PTEF1- tGCN4	Null	Null	ORN- E(KanMX)	pYX212	PTEF1- tGCN4
23	M1cM2t	M1c	P <sub>KEX2</sub> - ARG3; CAR2A	M2t	PGPD1- tGCN4	Null	Null	ORN- E(KanMX)	pYX212	PGPD1- tGCN4
24	M1cM2qM3 a	M1c	P <sub>KEX2</sub> - ARG3; CAR2A	M2q	<i>argAEc</i> ; <i>argBEc</i> ; <i>argCcg</i> ;	M3a	PDA1 ; CIT1 ; AC02	ORN- L(KanMX)	Y04	G04

					<i>argDcg;</i> <i>argJcg;</i> <i>ORT1;</i> <i>AGC1;</i> <i>GDH1</i>		;IDP1 ;  PYC2			
25	M1cM2qM3 b	M1c	P <sub>KEX2</sub> - ARG3; CAR2A	M2q	<i>argAtc;</i> <i>argBEc;</i> <i>argCcg;</i> <i>argDcg;</i> <i>argJcg;</i> <i>ORT1;</i> <i>AGC1;</i> <i>GDH1</i>	M3b	mPDA  1;CIT  1;AC  02;ID  P1;PY  C2	ORN-  L(KanMX)	Y05	G04
26	M1cM2qM3 c	M1c	P <sub>KEX2</sub> - ARG3; CAR2A	M2q	<i>argAtc;</i> <i>argBtc;</i> <i>argCcg;</i> <i>argDcg;</i> <i>argJcg;</i> <i>ORT1;</i> <i>AGC1;</i> <i>GDH1</i>	M3c	HcAO  X 1	ORN-  W(KanMX)	pYX212	G04
27	M1cM2qM3 d	M1c	P <sub>KEX2</sub> - ARG3; CAR2A	M2q	<i>argAEc;</i> <i>argBEc;</i> <i>argCcg;</i> <i>argDcg;</i> <i>argJcg;</i> <i>ORT1;</i> <i>AGC1;</i>	M3d	HcAO  X 1;  NDI 1	ORN-  WN(KanMX  )	pYX212	G04



					<i>GDH1</i>					
28	M1cM2qM3 e	M1c	<i>P<sub>KEX2-</sub></i> ARG3; CAR2A	M2q	<i>argAEc;</i> <i>argBEc;</i> <i>argCcg;</i> <i>argDcg;</i> <i>argJcg;</i> <i>ORT1;</i> <i>AGC1;</i> <i>GDH1</i>	M3e	tMTH1	ORN- V(KanMX)	pYX212	G04
29	M1cM2qM3f	M1c	<i>P<sub>KEX2-</sub></i> ARG3; CAR2A	M2q	<i>argAEc;</i> <i>argBEc;</i> <i>argCcg;</i> <i>argDcg;</i> <i>argJcg;</i> <i>ORT1;</i> <i>AGC1;</i> <i>GDH1</i>	M3f	tMTH1 ; AKGD 2	ORN- T(KanMX)	pYX212	G04
30	M1dM2q	M1d	<i>P<sub>KEX2-</sub></i> ARG3; CAR2A; CAR1	M2q	<i>argAEc;</i> <i>argBEc;</i> <i>argCcg;</i> <i>argDcg;</i> <i>argJcg;</i> <i>ORT1;</i> <i>AGC1;</i> <i>GDH1</i>	Null	Null	ORN- L(KanMX)	TPIp- CAR1	G04
31	M1dM2qM3 c	M1d	<i>P<sub>KEX2-</sub></i> ARG3; CAR2A;	M2q	<i>argAEc;</i> <i>argBEc;</i> <i>argCcg;</i>	M3c	<i>HaAO</i>  X 1			

			<i>CAR1</i>		<i>argDcg;</i> <i>argJcg;</i> <i>ORT1;</i> <i>AGC1;</i> <i>GDH1</i>					
32	M1dM2qM3 e	M1d	<i>pKEX2-</i> <i>ARG3;</i> <i>CAR2A;</i> <i>CAR1</i>	M2q	<i>argAtc;</i> <i>argBEc;</i> <i>argCcg;</i> <i>argDcg;</i> <i>argJcg;</i> <i>ORT1;</i> <i>AGC1;</i> <i>GDH1</i>	M3e	<i>tMTH1</i>	ORN- V(KanMX)	TPIp- CAR1	G04
33	M1dM2qM3f	M1d	<i>pKEX2-</i> <i>ARG3;</i> <i>CAR2A;</i> <i>CAR1</i>	M2q	<i>argAtc;</i> <i>argBtc;</i> <i>argCcg;</i> <i>argDcg;</i> <i>argJcg;</i> <i>ORT1;</i> <i>AGC1;</i> <i>GDH1</i>	M3f	<i>tMTH1</i> ; AKGD 2	ORN- T(KanMX)	TPIp- CAR1	G04
34	B0166A(OR T1)	Null	Null	Null	Null	Null	Null	B0166A CEN.PK	pYX212	G04

Table 3: List of strains used for polyamine production

Strain name	Background genotype	Plasmids
PUT-A(KanMX)	ORN-LAoaz1	None
WT-PUT-	CEN.PK113-11C Aoaz1	None

A(KanMX)		
PUT1	ORN-LAoaz1	p426GPD, G04
WT-PUT1	CEN.PK113-11C Aoaz1	p426GPD, p423GPD
PUT-B(KanMX)	ORN-L Aoaz1 SPE1	none
WT-PUT-B(KanMX)	CEN.PK113-11C Aoaz1 SPE1	none
PUT2	ORN-L Aoaz1 SPE1	p426GPD , G04
WT-PUT2	CEN.PK113-11C Aoaz1 SPE1	p426GPD, p423GPD
PUT3	ORN-L Aoaz1 SPE1	P412GPD-TP01. G04
WT-PUT3	CEN.PK113-11C Aoaz1 SPE1	P412GPD-TP01, p423GPD
SPD-A(KanMX)	ORN-L Aoaz1 SPE1 SPE3	none
SPD1	ORN-L Aoaz1 SPE1 SPE3	p426GPD, G04
SPD2	ORN-L Aoaz1 SPE1 SPE3 SPE2	p426GPD, G04
SPD-B(KanMX)	ORN-L Aoaz1 SPE1 SPE3 SPE2	none
SPD-B1	ORN-L Aoaz1 SPE1	YP1. G04
SPD-B2	ORN-L Aoaz1 SPE1	YP2, G04
SPD-B3	ORN-L Aoaz1 SPE1	YP3, G04
AKYSPM1	ORN-V(KanMX) <i>Aoaz1</i>	pSP-GM1-SPM1, G04
AKYDOAZ-Transl	AKYDOAZ <i>TP01 TP05</i>	none
AKYDOAZ-Trans2	PUT-A(KanMX) <i>Asam3 Adur3</i>	none
AKYPUT2	AKYDOAZ <i>TP01 TP05 Asam3 Adur3</i>	pSP-GM1-PUT, G04
AKYSPD2	AKYDOAZ <i>TP01 TP05 Asam3 Adur3</i>	pSP-GM1-SPD, G04
AKYSPM2	AKYDOAZ <i>TP01 TP05 Asam3 Adur3</i>	pSP-GM1-SPM, G04
AKYDCAR1	CEN.PK113-11C $\Delta$ <i>car1</i>	none

AKYAGM1	CEN.PK113-11C $\Delta car1$	pSP-GMI-ARGC, pLYC04-speA
AKYAGM2	CEN.PK113-11C <i>Acar1</i> <i>SPE2</i> <i>SPE3</i>	
AKYAGM3	CEN.PK113-11C <i>Acar1</i> <i>SPE2</i> <i>SPE3</i>	pSP-GMI-ARGC, pLYC04-speAB
AKYAGM4	CEN.PK113-11C <i>Acar1</i> <i>SPE2</i> <i>SPE3</i> <i>SPE4</i>	
AKYAGM5	CEN.PK113-11C <i>Acar1</i> <i>SPE2</i> <i>SPE3</i> <i>SPE4</i>	pSP-GMI-ARGC, pLYC04-speAB
<i>C. glutanicum</i> ATCC 13032	Kalinowski et al . (2003)	Background strain for spermidine/spermine production in <i>C. glutanicum</i>
<i>E. coli</i> WL31 10	orig. K12 W3110 (CGSC, Coli Genetic Stock Center); (Park et al. 2007))	Background strain for spermidine/spermine production in <i>E. coli</i>

Table 4: Plasmids used in this study

No	Plasmid/Alias Name	Genes Insert	Skeleton Vector	Marker	Reference
1	G01	<i>ARG5,6;ARG7;ARG8</i>	p423GPD	HIS3	This Study
2	G02	<i>ARG5,6 ; ARG7; ARG8; ARG2</i>	p423GPD	HIS3	This Study
3	G03	<i>ARG5,6 ARG7; ARG8; ARG2; CAR1</i>	p423GPD	HIS3	This Study
4	G04	<i>argBEc; argAE; c argCc g; argDc g; argJcg</i>	p423GPD	HIS3	This Study
5	Y01	<i>GLN1; GLT1</i>	pYX212	URA3	This Study
6	Y02	<i>PYC2;CIT1; IDP1</i>	pYX212	URA3	This Study
7	Y03	<i>GLN1;GLT1;PYC2; CIT1; IDP1</i>	pYX212	URA3	This Study
8	Y04	<i>PDA1;AC02 ;PYC2; CIT1; IDP1</i>	pYX212	URA3	This Study
9	Y05	<i>mPDA1;AC02; PYC2; CIT1; IDP1</i>	pYX212	URA3	This Study

10	pSPGM1-ARG2	<i>ARG2</i>	pSPGMI	URA3	This Study
11	pSPGMI- ARG5.6	<i>ARG5,6</i>	pSPGMI	URA3	This Study
12	pSPGM1-ARG7	<i>ARG7</i>	pSPGMI	URA3	This Study
13	pYX212- MLSargBcg	<i>MLS-argBcg</i>	pYX212	URA3	This Study
14	pYX212-MLS- argJcg	<i>MLS-argJcg</i>	pYX212	URA3	This Study
15	PADH1- tGCN4	<i>tGCN4</i>	p423ADH	HIS3	This Study
16	PTEF1- tGCN4	<i>tGCN4</i>	p423TEF	HIS3	This Study
17	PGPD1- tGCN4	<i>tGCN4</i>	p423GPD	HIS3	This Study
18	TPIp-CAR1	<i>CAR1</i>	pYX212	URA3	(Johansson et al., 2014)
19	pRS416-PDA1 [S313A]	PDA1 [S313A]	pRS416	URA3	(Oliviera et al., 2012)
20	pRS416-PDA1	PDA1	pRS416	URA3	(Oliviera et al., 2012)
21	pSP-GM1-SPE1	<i>PTEFI-SPE1</i>	pSP-GM1	URA3	This study
22	pSP-GM1-SPE12	<i>PTEFI-SPE1, PPGKI-SPE2</i>	pSP-GM1	URA3	This study
23	pSP-GM1-SPE3	<i>PTEFI-SPE3</i>	pSP-GM1	URA3	This study
24	pSP-GM1-SPD	<i>PTEFI-SPE1, PPGKI-SPE2, PTEFI-SPE3</i>	pSP-GM1	URA3	This study
25	pSP-GM1-SPE34	<i>PTEFI-SPE3, PPGKI-SPE4</i>	pSP-GM1	URA3	This study
26	pSP-GM1-SPM	<i>PTEFI-SPE1, PPGKI-SPE2, PTEFI-SPE3, PPGKI-SPE4</i>	pSP-GM1	URA3	This study
27	pXI-3-TP015	<i>PTEFI-TP01, PPGKI-TP05</i>	pXI-3	URA3	This study
28	pSP-GM1-ARGA	<i>PTEFI-ARG3, PPGKI-ARG1</i>	pSP-GM1	URA3	This study
29	pSP-GM1-ARGB	<i>PTEFI-ARG10, PPGKI-ARG4</i>	pSP-GM1	URA3	This study

30	pSP-GM1-ARGC	<i>PTEFI-ARG3, PPGKI-ARG1</i> <i>PTEFI-ARG10, PPGKI-ARG4</i>	pSP-GM1	URA3	This study
31	pIYC04-speA	<i>PrEFi -speA</i>	pIYC04	HIS3	This study
32	pXI-5-SPE32	<i>PTEFI-SPE2, PPGKI-SPE3</i>	pXI-5	URA3	This study
33	pIYC04-speAB	<i>PrEFi -speA, PpGKi-speB</i>	pIYC04	HIS3	This study
34	pX-4-SPE4	<i>PTEFI-SPE4</i>	pX-4	URA3	This study
35	pSP-GM1	<i>Episomal backbone</i>		URA3	(Chen et al., 2012a)
36	pIYC04	<i>Episomal backbone</i>		HIS3	(Chen et al., 2012b)
37	pXI-3	<i>Integrative backbone</i>		URA3	(Mikkelsen et al., 2012)
38	pXI-5	<i>Integrative backbone</i>		URA3	(Mikkelsen et al., 2012)
39	pX-4	<i>Integrative backbone</i>		URA3	(Mikkelsen et al., 2012)
40	pMS2 ATTC®67189TM	shuttle vector <i>E. coli-C. glutanicum</i>		ampR, kanR	US Patent 4,920,054
41	pTAC-MAT-Tag®- 1 (Sigma)	<i>LaCl, tac</i> promoter amplification		ampR	Rosenberg et al. (1987).
42	pFDAMS2	<i>Episomal expression vector for C. glutanicum</i>	pMS2	ampR	Example 11

		(fac promoter control target gene (e.g. <i>speD</i> ))			
43	pTRC-LIC	<i>Episomal expression vector for E. coli;</i> (tac promoter from pKK233-2)	pTRC99a	ampR	GenBank Accession: EF460847
44	p426GPD	<i>Episomal backbone</i>		URA3	Mumberg et al., 1995
45	pYX212	<i>Episomal backbone</i>		URA3	R&D systems
46	p423GPD	<i>Episomal backbone</i>		HIS3	Mumberg et al., 1995
47	pTPOI	<i>TDH3p -TP01- CYC1t</i>	p426GPD	URA3	This study
48	YP1	(TPIp-C/C/4SDH-FBA1t)+(PGK1 p- <i>CjCASDC-CYCU</i> )	pYX212	URA3	This study
49	YP2	(TPIp-VcC/4SDtf-FBA1t)+(PGK1p- <i>VcCASDC-CYCU</i> )	pYX212	URA3	This study
50	YP3	(TPIp-BivC/4SDtf-FBA1t)+(PGK1p- <i>BuCASDC-CYCU</i> )	pYX212	URA3	This study

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## CLAIMS

1. An eukaryotic cell capable of producing L-ornithine, wherein said eukaryotic cell is genetically modified for attenuated activity of an ornithine carbamoyltransferase.
- 5 2. The eukaryotic cell according to claim 1, wherein said eukaryotic cell is genetically modified for attenuated expression of a gene encoding said ornithine carbamoyltransferase in said eukaryotic cell.
3. The eukaryotic cell according to claim 2, wherein said eukaryotic cell has a native promoter of said gene encoding said ornithine carbamoyltransferase replaced by a weak constitutive promoter.
- 10 4. The eukaryotic cell according to claim 3, wherein said eukaryotic cell has a native promoter of the gene *ARG3* replaced by a promoter of the gene *KEX2*.
5. The eukaryotic cell according to any of the claims 1 to 4, wherein said eukaryotic cell is genetically modified  
15 by deletion or disruption of a gene encoding an L-ornithine transaminase.
6. The eukaryotic cell according to claim 5, wherein said eukaryotic cell is genetically modified by deletion or disruption of the gene *CAR2*.
- 20 7. The eukaryotic cell according to any of the claims 1 to 6, wherein said eukaryotic cell is genetically modified for enhanced L-ornithine biosynthesis from  $\alpha$ -ketoglutarate.
8. An eukaryotic cell capable of producing L-ornithine, wherein said eukaryotic cell is genetically modified for enhanced L-ornithine biosynthesis from  $\alpha$ -ketoglutarate.
- 25 9. The eukaryotic cell according to claim 7 or 8, wherein said eukaryotic cell is genetically modified for cytosolic L-ornithine biosynthesis from  $\alpha$ -ketoglutarate.

10. The eukaryotic cell according to claim 9, wherein said eukaryotic cell comprises at least one of gene selected from a group consisting of a gene encoding a cytosolic glutamate N-acetyltransferase, a gene encoding a cytosolic N-acetylglutamate kinase, a gene encoding a cytosolic N-acetyl-gamma-glutamyl-phosphate reductase, a gene encoding a cytosolic acetylornithine aminotransferase and a gene encoding a cytosolic  
5 ornithine acetyltransferase.
11. The eukaryotic cell according to claim 10, wherein said eukaryotic cell comprises the genes *argA* and *argBfc* from *Escherichia coli* and the genes *argCca*, *argDcg* and *argJcg* from *Corynebacterium glutamicum*.
- 10 12. The eukaryotic cell according to claim 7 or 8, wherein said yeast is genetically modified for overexpression of at least one gene selected from a group consisting of a gene encoding an N-acetyl-gamma-glutamyl-phosphate reductase, a gene encoding a mitochondrial ornithine acetyltransferase, a gene encoding an acetylornithine aminotransferase and a gene encoding an acetylglutamate synthase.
- 15 13. The eukaryotic cell according to claim 12, wherein said yeast is genetically modified for overexpression of the genes *ARG5*, *8ARG7*, *ARG8* and *ARG2*.
14. The eukaryotic cell according to any of the claims 7 to 13, wherein said eukaryotic cell is genetically modified for overexpression of at least one gene selected from a group consisting of a gene encoding a L-ornithine  
20 transporter, a gene encoding a L-glutamate transporter and a gene encoding NADP-dependent glutamate dehydrogenase.
15. The eukaryotic cell according to claim 14, wherein said eukaryotic cell is genetically modified for overexpression of the genes *ORT1*, *AGC1*, *GDH1*.
- 25 16. The eukaryotic cell according to any of the claims 1 to 15, wherein said eukaryotic cell is genetically modified for enhanced  $\alpha$ -ketoglutarate biosynthesis.

17. An eukaryotic cell capable of producing L-ornithine, wherein said eukaryotic cell is genetically modified for enhanced a-ketoglutarate biosynthesis.
18. The eukaryotic cell according to claim 16 or 17, wherein said eukaryotic cell is genetically modified for attenuated glucose uptake.
19. The eukaryotic cell according to any of the claims 16 to 18, wherein said eukaryotic cell is genetically modified for overexpression of a gene encoding a glucose transporter regulator protein.
20. The eukaryotic cell according to claim 19, wherein said eukaryotic cell is genetically modified for overexpression of the gene *MTH1* or the gene *MTH 1-Δ T*, wherein said gene *MTH1-AT* is an internal deletion version of said gene *MTH1*.
21. The eukaryotic cell according to any of the claims 1 to 20, wherein said eukaryotic cell is genetically modified for overexpression of a gene encoding an arginase.
22. The eukaryotic cell according to claim 21, wherein said eukaryotic cell is genetically modified for overexpression of the gene *CARL*.
23. The eukaryotic cell according to any of the claims 1 to 22, wherein said eukaryotic cell is genetically modified by:
- having a native promoter of the gene *ARG3* replaced by a promoter of the gene *KEX2*;
  - deletion or disruption of the gene *CAR2*;
  - overexpression of the genes *ORT1*, *AGC1*, *GDH1*;
  - expression of the genes *argA<sub>Ec</sub>* and *argB<sub>Ec</sub>* from *Escherichia coli* and the genes *argC<sub>Cg</sub>*, *argD<sub>Cg</sub>* and *argJ<sub>Cg</sub>* from *Corynebacterium glutamicum*; and
  - overexpression of the gene *MTH 1-Δ T*.

24. The eukaryotic cell according to claim 23, wherein said eukaryotic cell is genetically modified for overexpression of the gene *CARL*
25. The eukaryotic cell according to any of the claims 1 to 24, wherein  
5 said eukaryotic cell is capable of producing a polyamine selected from the group consisting of putrescine, spermidine and spermine; and  
said eukaryotic cell is genetically modified for overexpression of a gene encoding ornithine decarboxylase (ODC) and/or attenuated activity of ODC antienzyme.
- 10 26. The eukaryotic cell according to claim 25, wherein said eukaryotic cell is genetically modified for overexpression of the gene *SPE1* and deletion or disruption of the gene *OAZ1*.
27. The eukaryotic cell according to any of the claims 1 to 26, wherein  
said eukaryotic cell is capable of producing a polyamine selected from the group consisting of spermidine  
15 and spermine; and  
said eukaryotic cell is genetically modified for overexpression of a gene encoding an S-adenosylmethionine decarboxylase and/or a gene encoding a spermidine synthase.
28. The eukaryotic cell according to claim 27, wherein said eukaryotic cell is genetically modified for  
20 overexpression of the genes *SPE2* and *SPE3*.
29. The eukaryotic cell according to any of the claims 1 to 28, wherein  
said eukaryotic cell is capable of producing a polyamine selected from the group consisting of spermidine  
and spermine; and  
25 said eukaryotic cell is genetically modified for expression of a gene encoding a carboxynorspermidine dehydrogenase and/or a gene encoding a carboxynorspermidine decarboxylase.
30. The eukaryotic cell according to claim 29, wherein said eukaryotic cell is genetically modified for expression

of:

- iv) the genes *Cj0172c* and *Cj1515c* from *Campylobacter jejuni*;
- v) the genes *VC1624* and *VC1623* from *Vibrio cholera*; or
- vi) the genes *Lys1* and *nspC* from *Bacteriodes uniformis*.

5

31. The eukaryotic cell according to any of the claims 1 to 30, wherein said eukaryotic cell is capable of producing spermine; and said eukaryotic cell is genetically modified for overexpression of a gene encoding a spermine synthase.

10 32. The eukaryotic cell according to claim 31, wherein said eukaryotic cell is genetically modified for overexpression of the gene *SPE4*.

33. The eukaryotic cell according to any of the claims 25 to 32, wherein said eukaryotic cell is genetically modified for overexpression of a gene encoding a polyamine transporter.

15

34. The eukaryotic cell according to claim 33, wherein said eukaryotic cell is genetically modified for overexpression of at least one gene selected from the group consisting of *TP01*, *TP02*, *TP03*, *TP04* and *TP05*.

20 35. The eukaryotic cell according to any of the claims 1 to 34, wherein said eukaryotic cell is a fungal cell, preferably a yeast cell.

36. The eukaryotic cell according to claim 35, wherein said yeast cell is selected from a genus consisting of the group consisting of *Saccharomyces*, *Cryptococcus*, *Trichosporon*, *Zygosaccharomyces*, *Debaromyces*, *Pichia*, *Schizosaccharomyces*, *Trigonopsis*, *Brettanomyces*, *Aureobasidium*, *Nadsonia*, *Lipomyces*, *Torulopsis*,  
25 *Kloeckera*, *Phaffia*, *Rhodotomla*, *Candida*, *Hansenula*, *Kluyveromyces*, *Yarrowia*, and *Schwanniomyces*, preferably from the group consisting of *Saccharomyces*, *Yarrowia*, *Zygosaccharomyces*, *Kluyveromyces* and *Pichia* spp, more preferably said yeast cell is selected from the group consisting of *Saccharomyces cerevisiae*, *Saccharomyces boulardii*, *Zygosaccharomyces bailii*, *Kluyveromyces lactis*, *Yarrowia lipolytica*,

*Schizosaccharomyces pombe*, *Hansenula anomala*, *Candida sphaerica*, and *Schizosaccharomyces malidevorans*, such as *Saccharomyces cerevisiae*.

37. A process for production of L-ornithine, said process comprising cultivating a eukaryotic cell according to  
5 any of the claims 1 to 24, 35, 36 in the presence of a carbon source.

38. A process for production of a polyamine selected from the group consisting of putrescine, spermidine and spermine, said process comprising cultivating a eukaryotic cell according to any of the claims 25 to 36 in the presence of a carbon source.

10

39. The process according to claim 37 or 38, wherein said carbon source is selected from a group consisting of hemicelluloses, celluloses, pectines, rhamnose, fucose, maltose, galactose, maltodextrines, ribose, ribulose, starch, sucrose, glycerol, lactose, glucose, xylose, arabinose, fructose, galactose and glycerol raffinose and lactose.

15



FIG. 1A

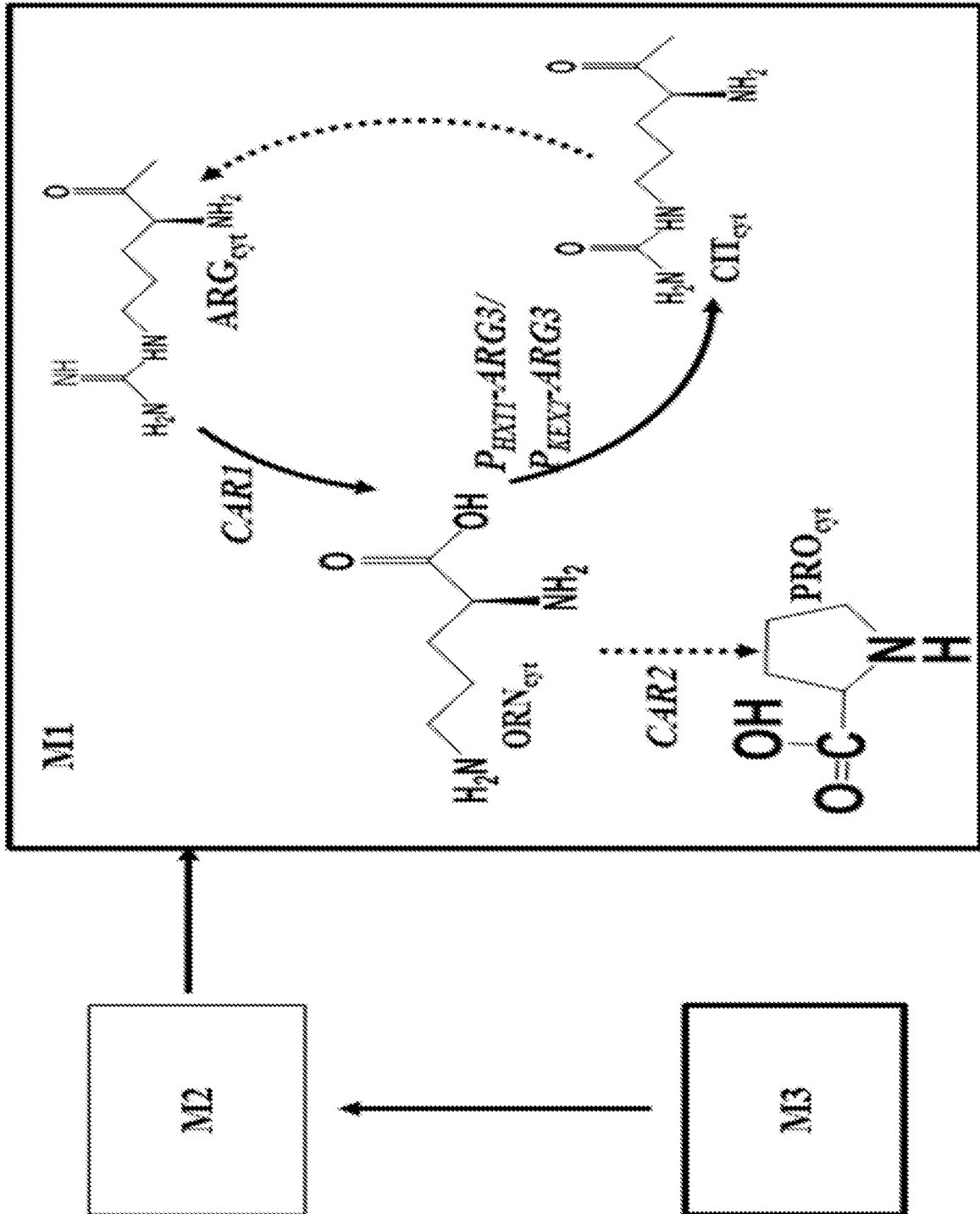
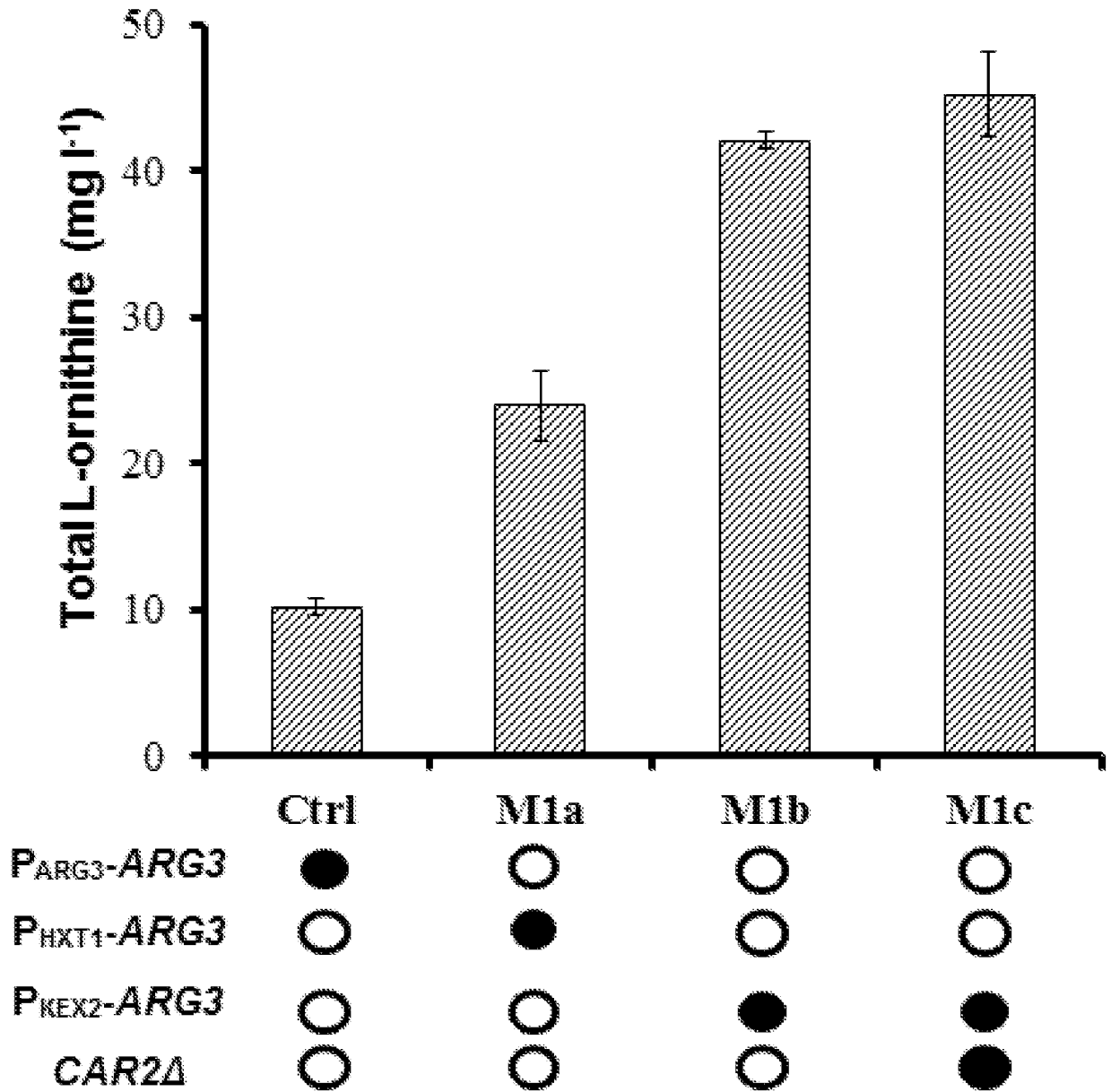


FIG. 1B



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FIG. 1C

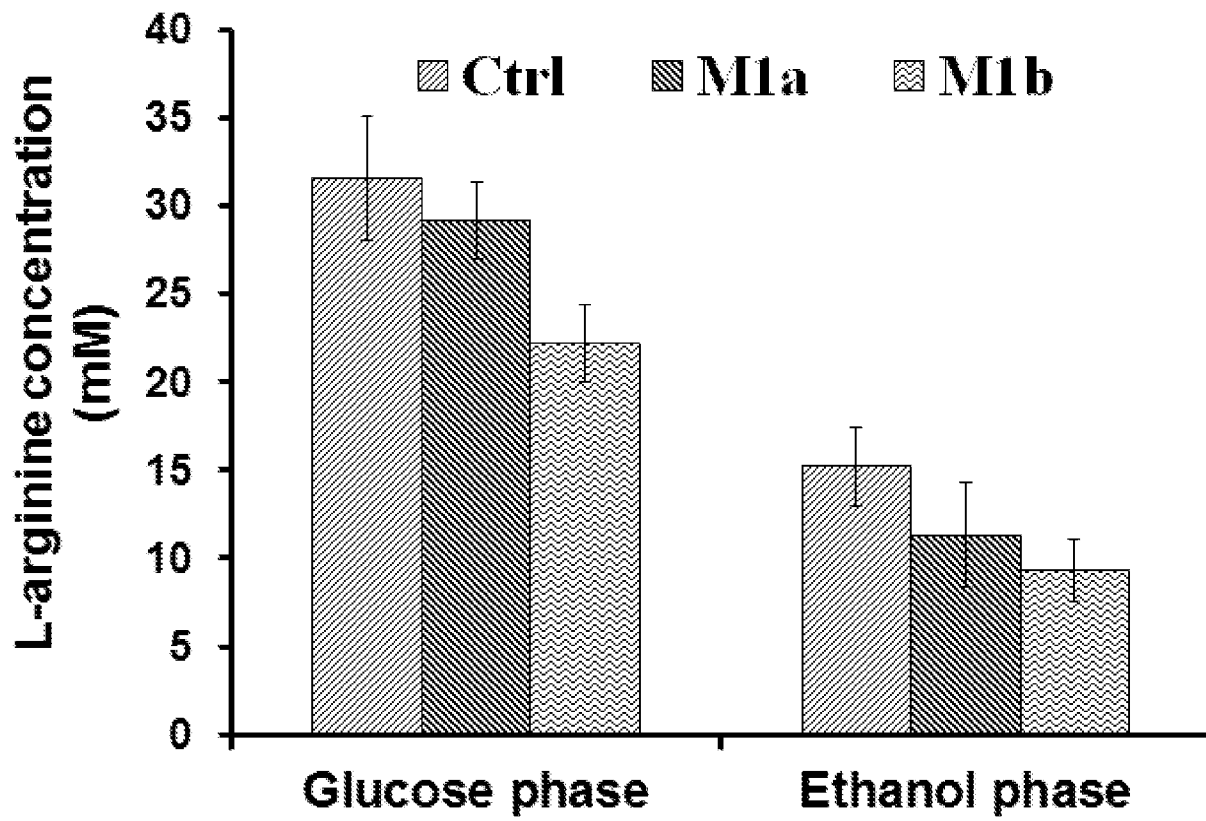


FIG.2A

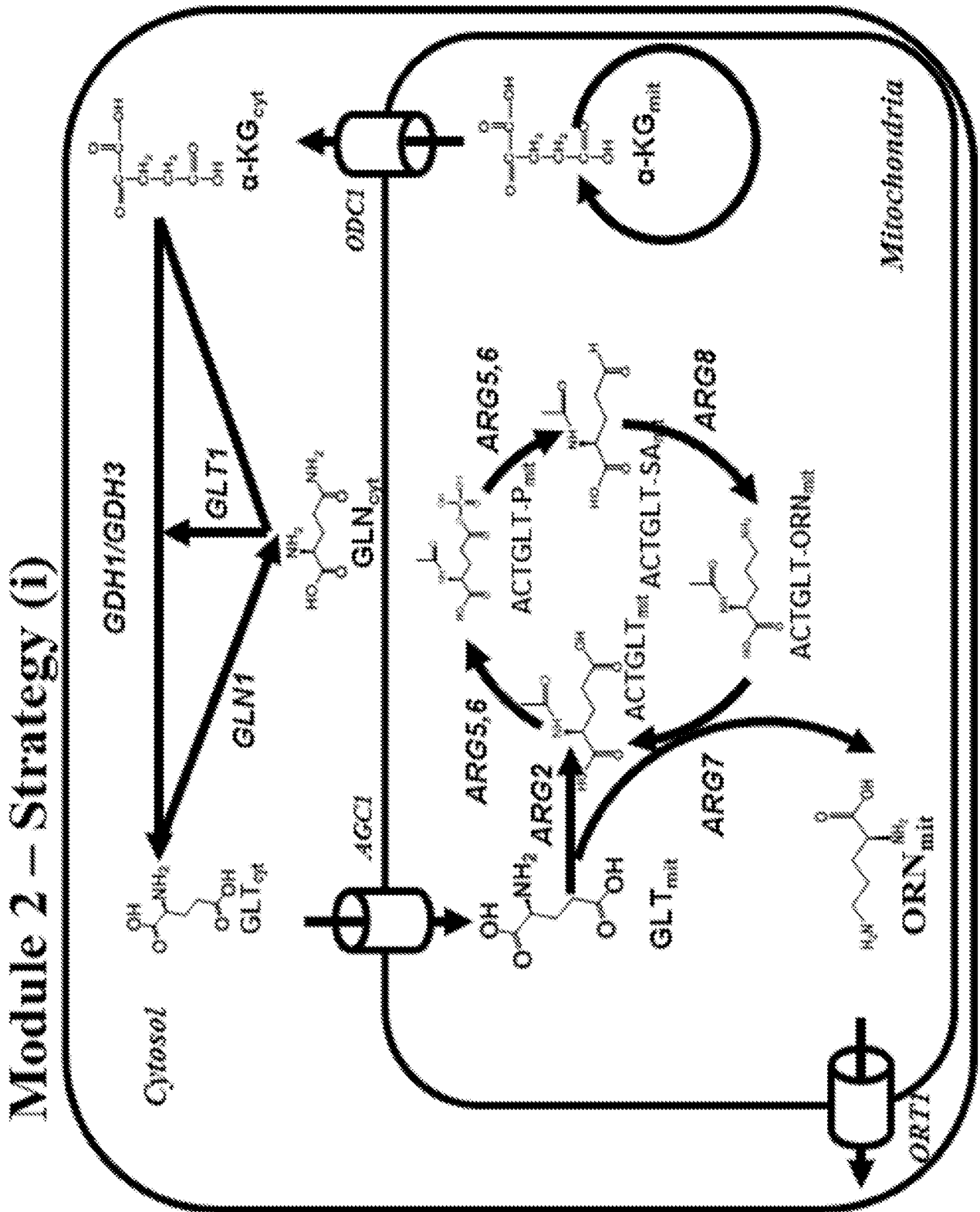


FIG.2B

Module 2 – Strategy (ii)

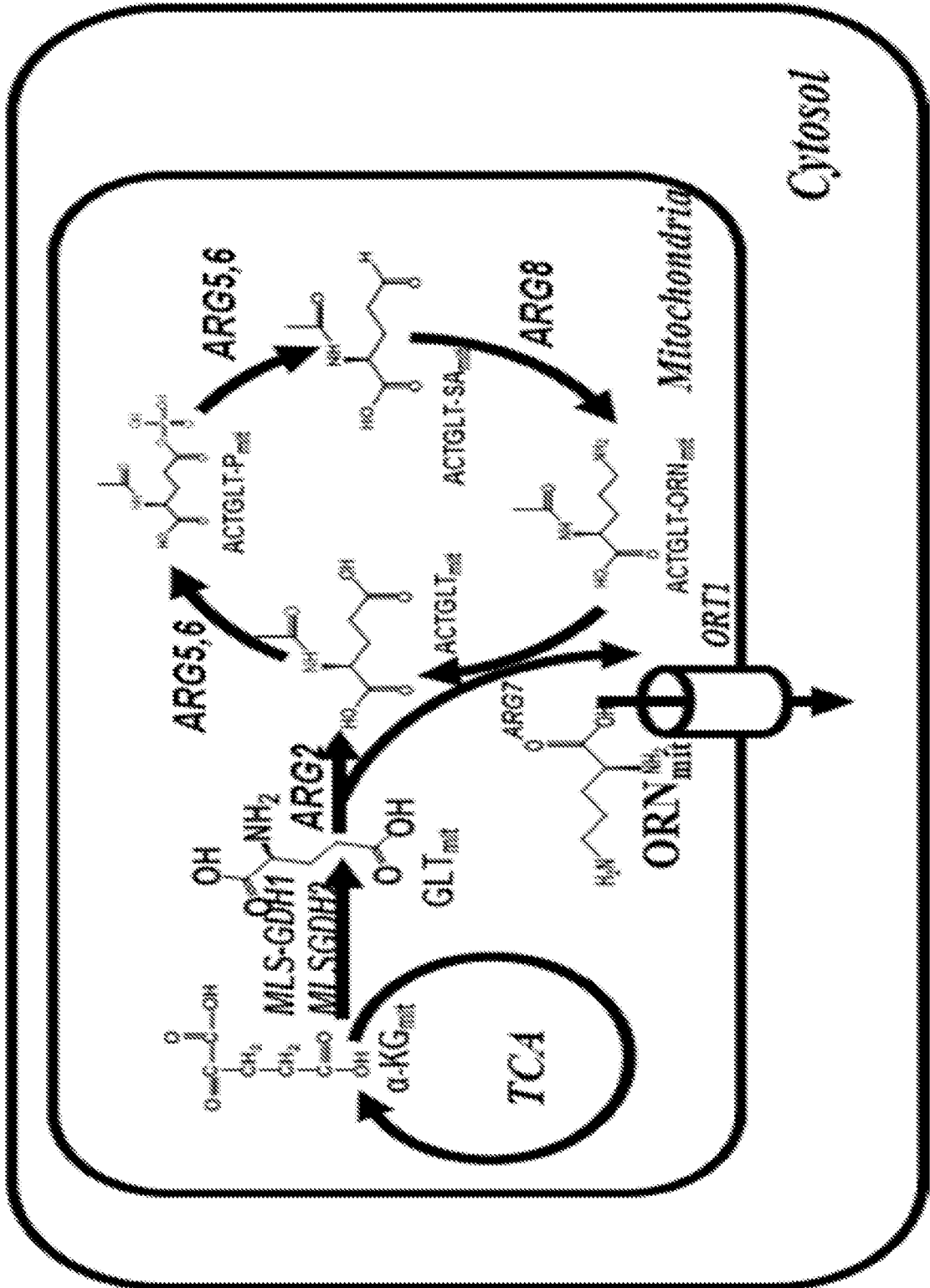




FIG. 2D

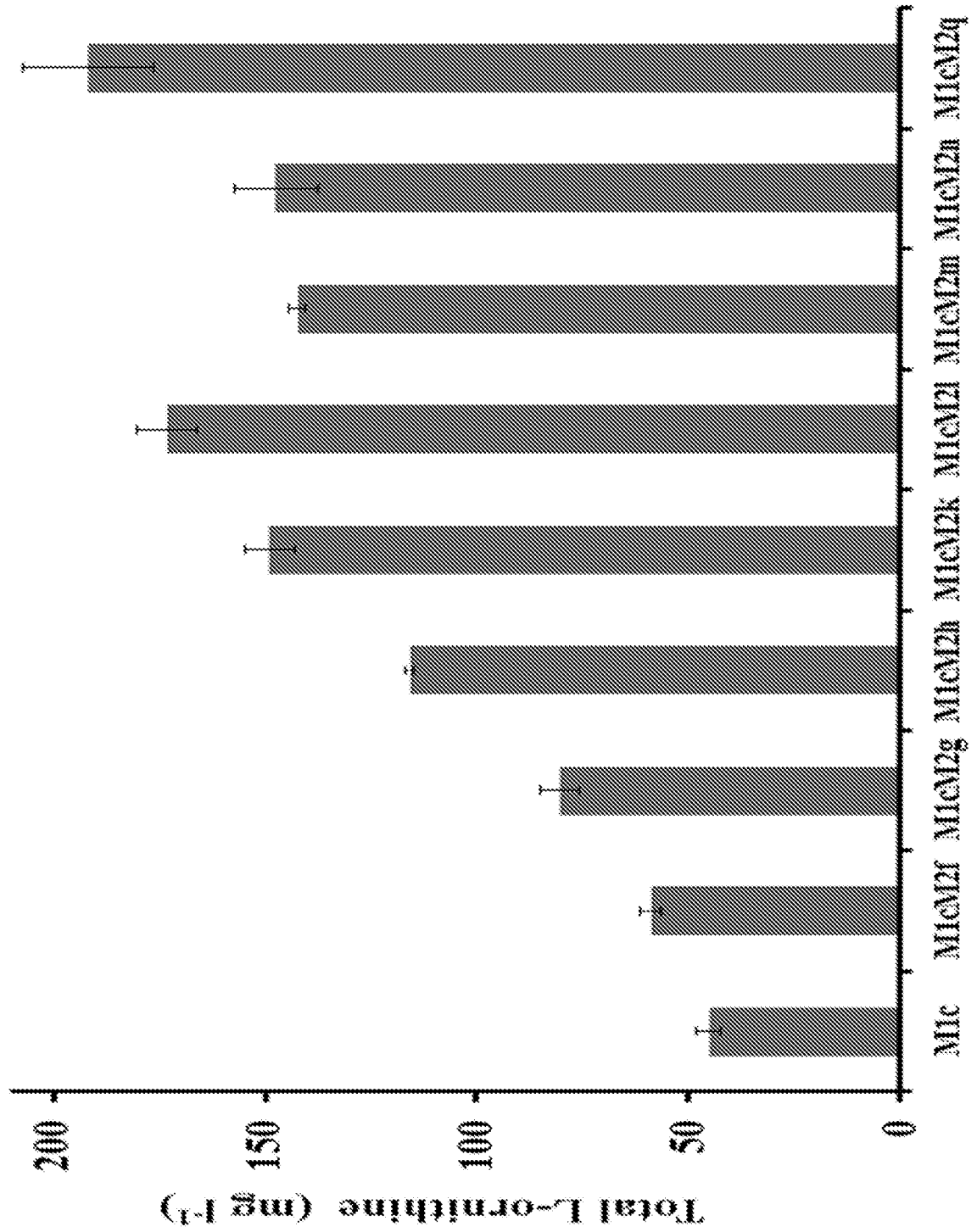


FIG. 3A

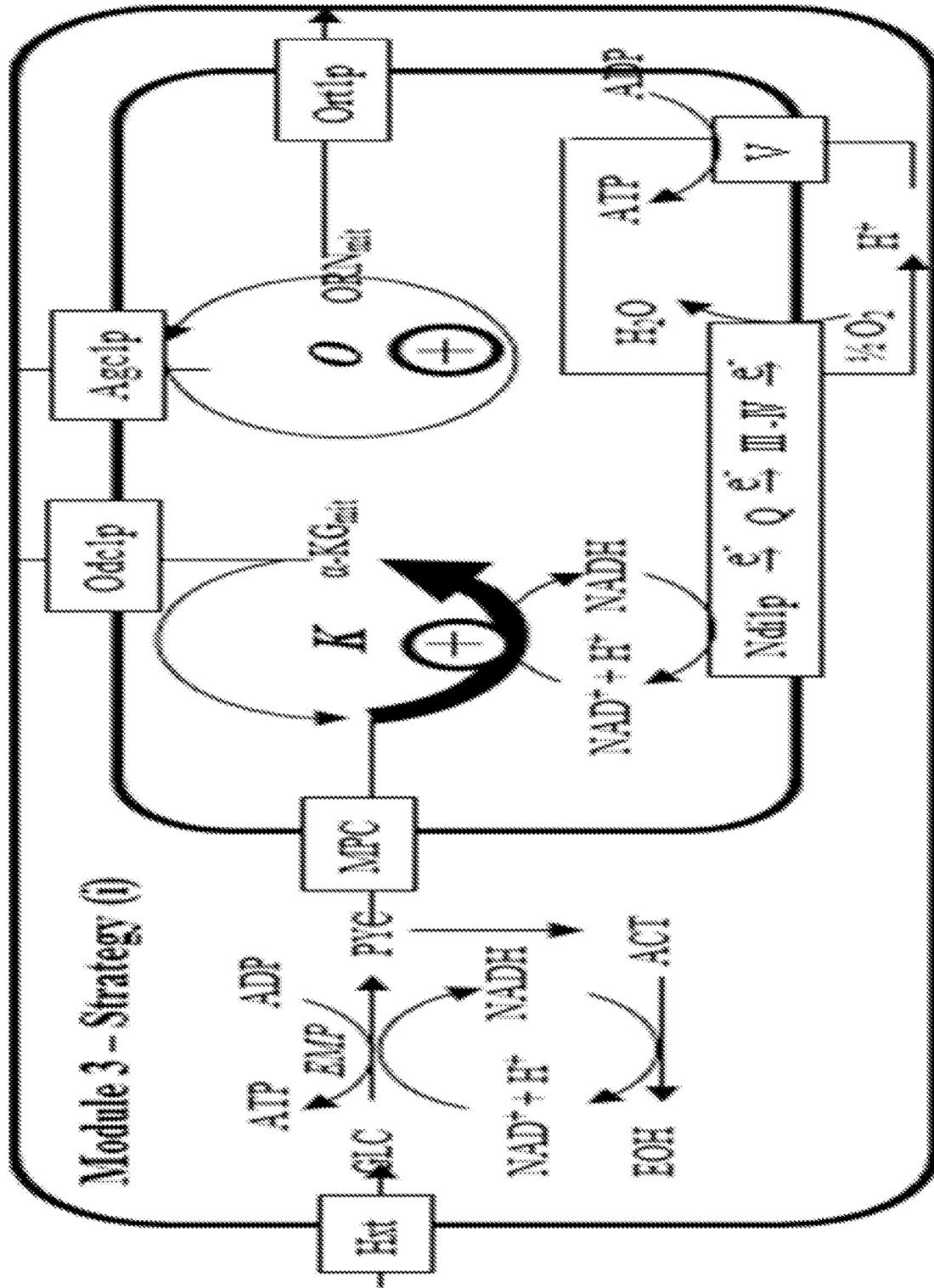




FIG. 3B

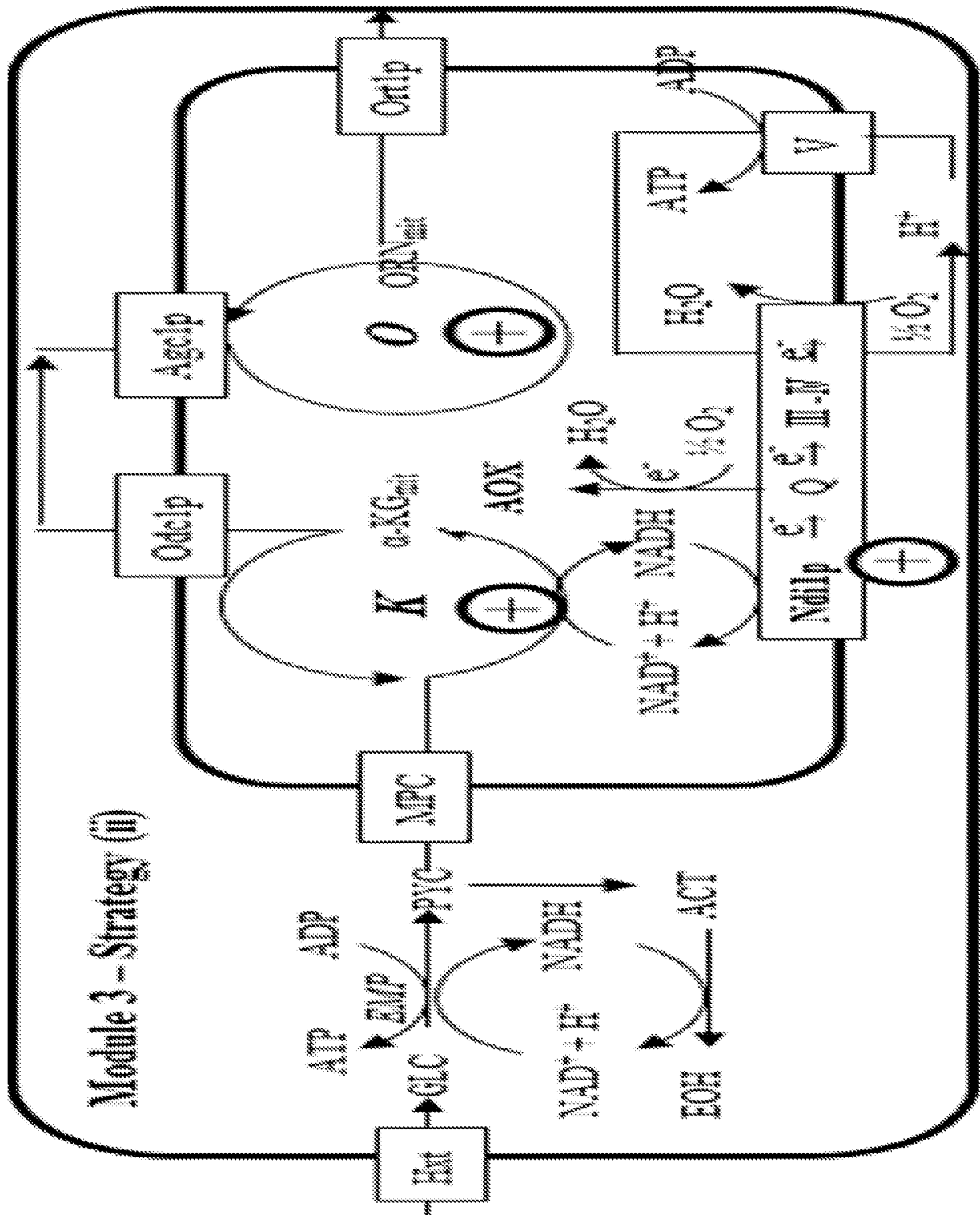
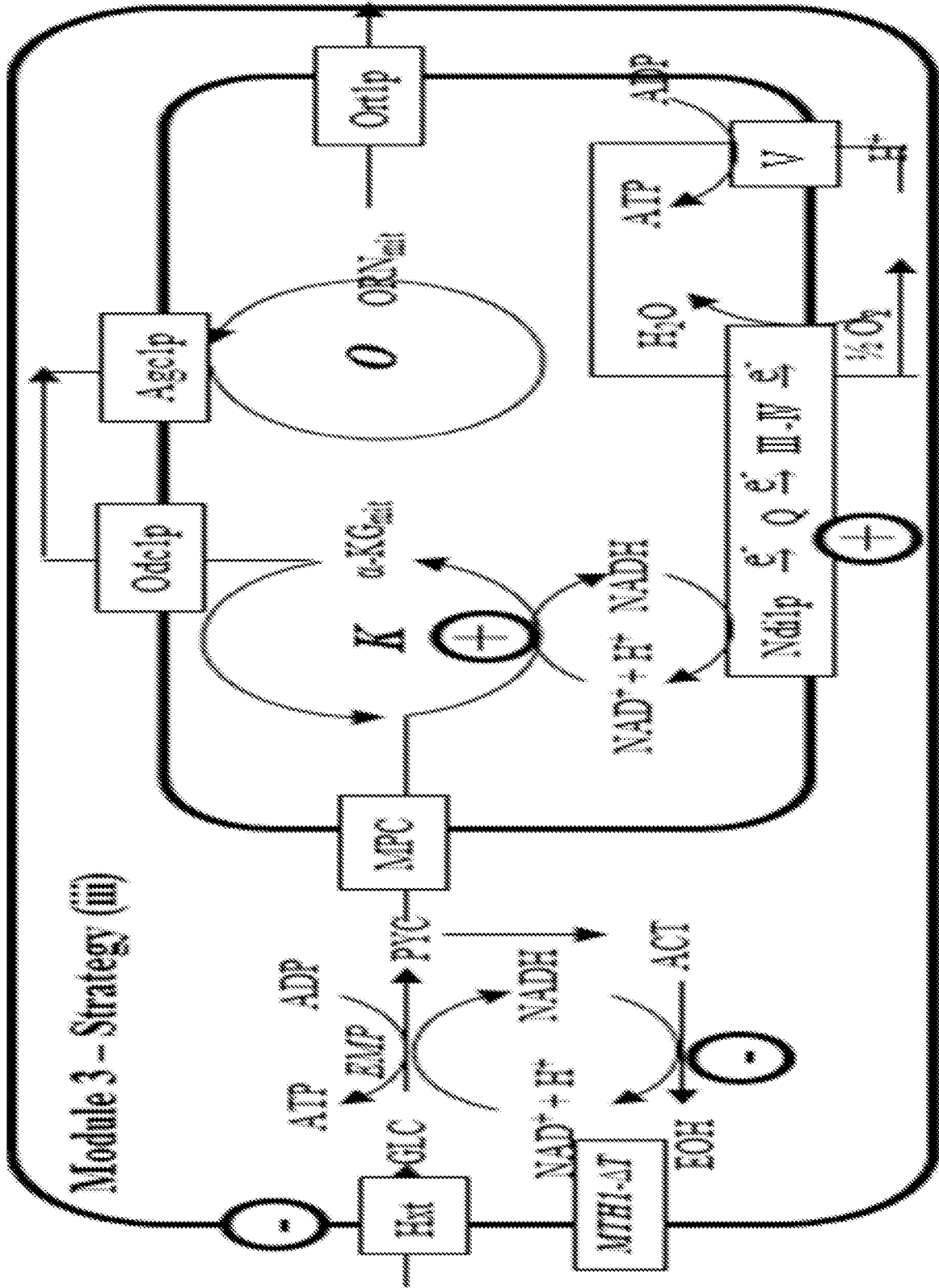
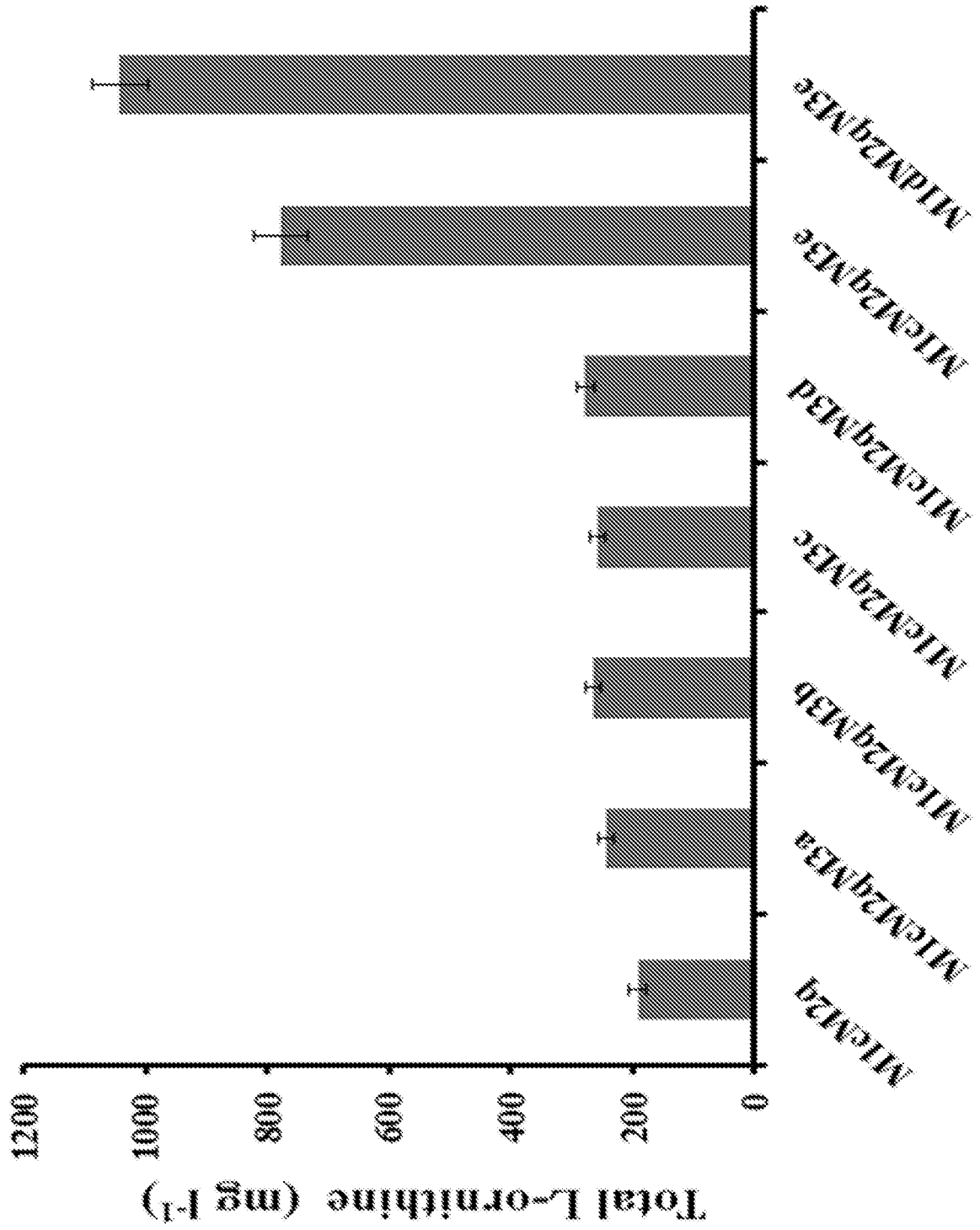


FIG. 3C



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FIG. 3D



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FIG. 3E

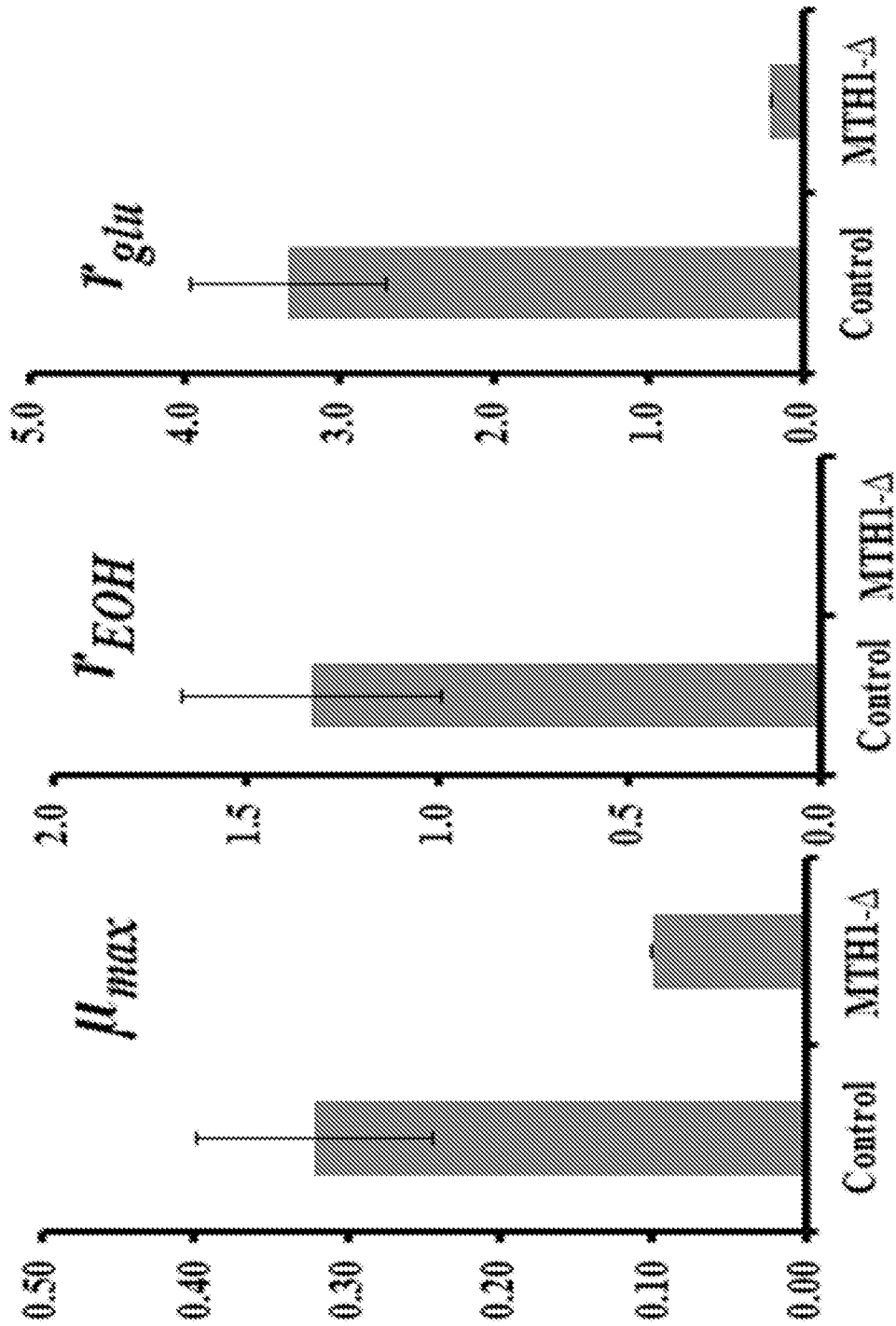


FIG. 3F

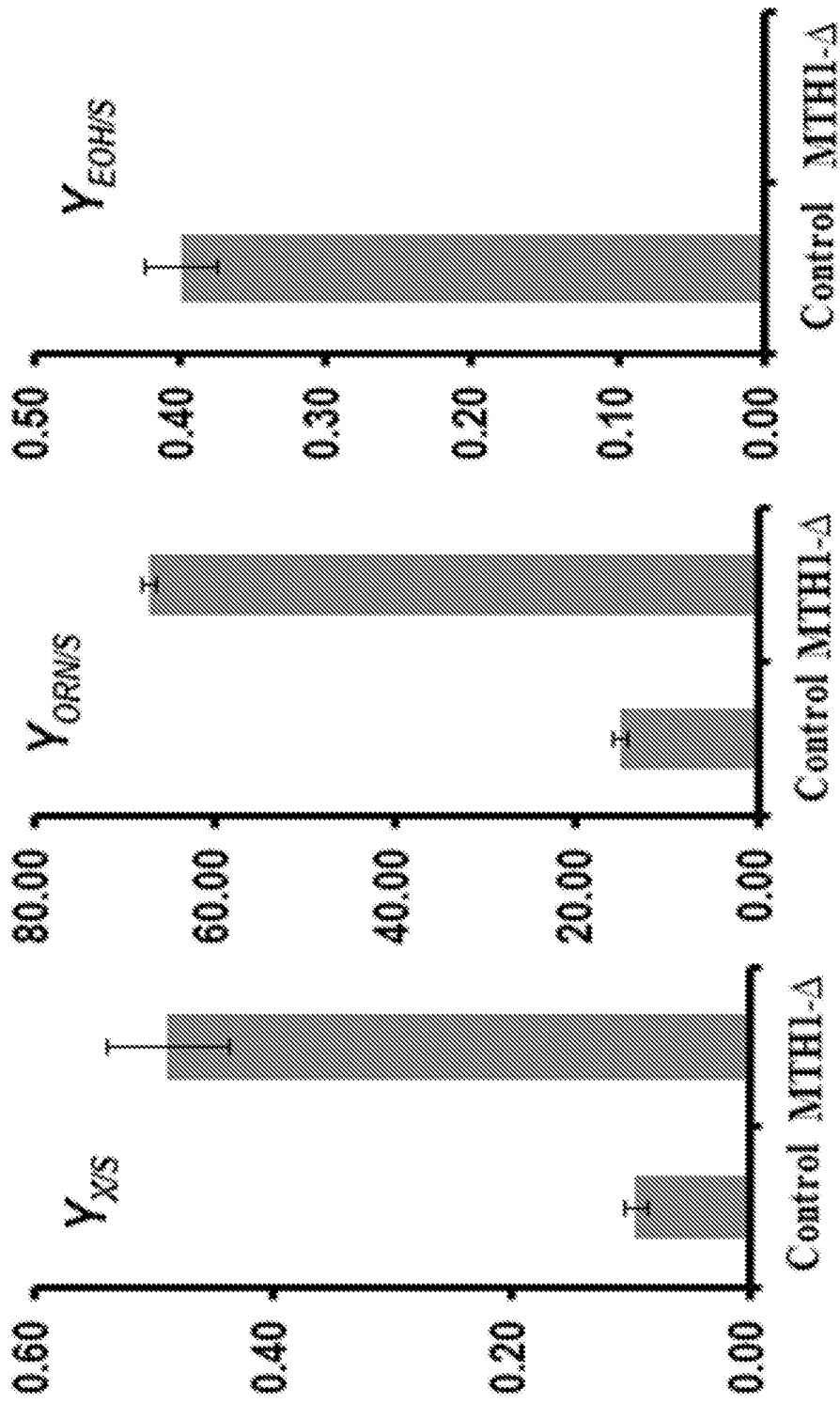


FIG. 4

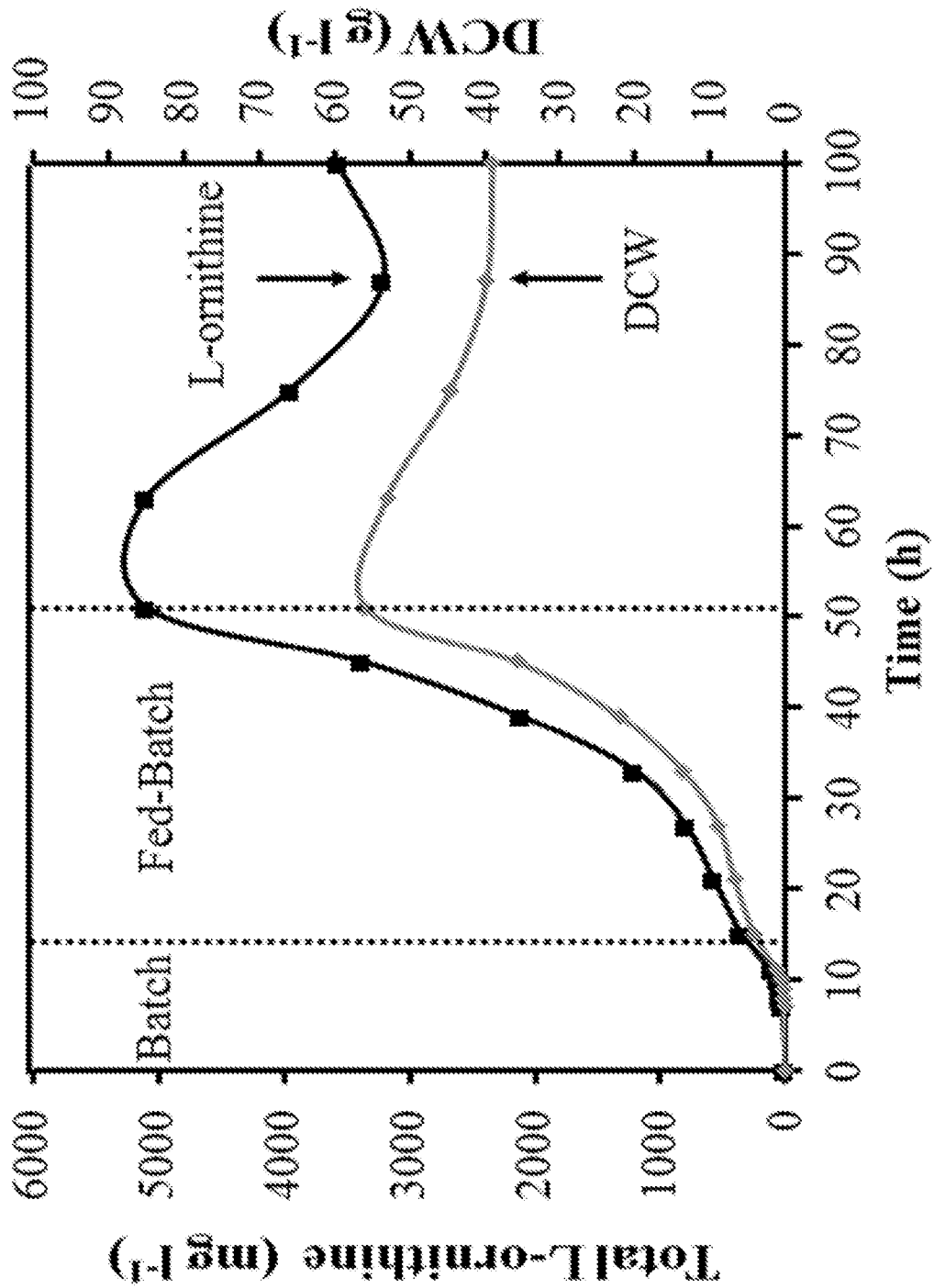
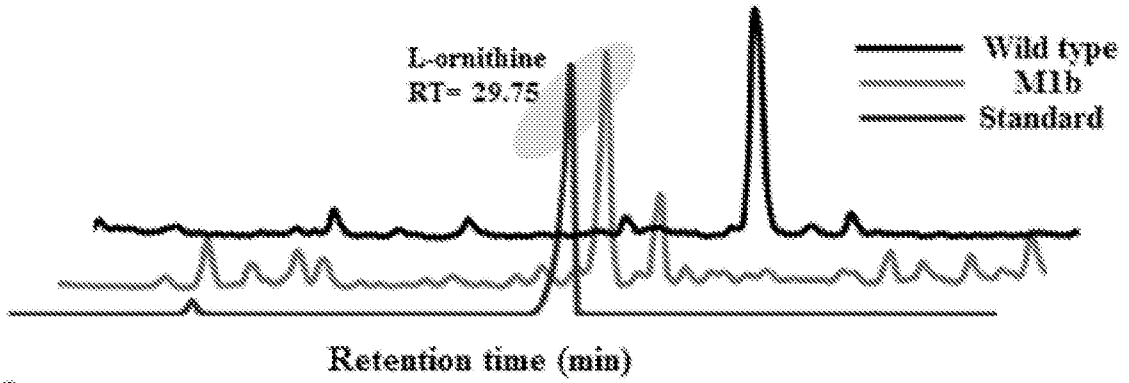


FIG. 5

**a**



**b**

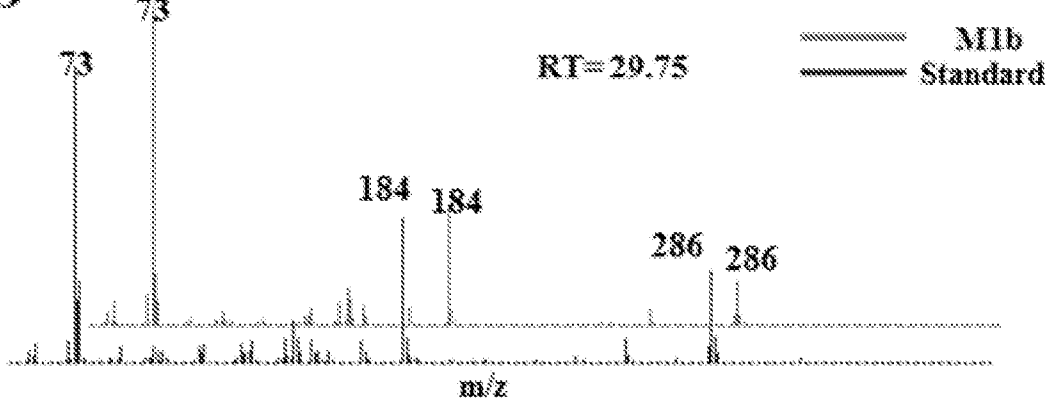


FIG. 6

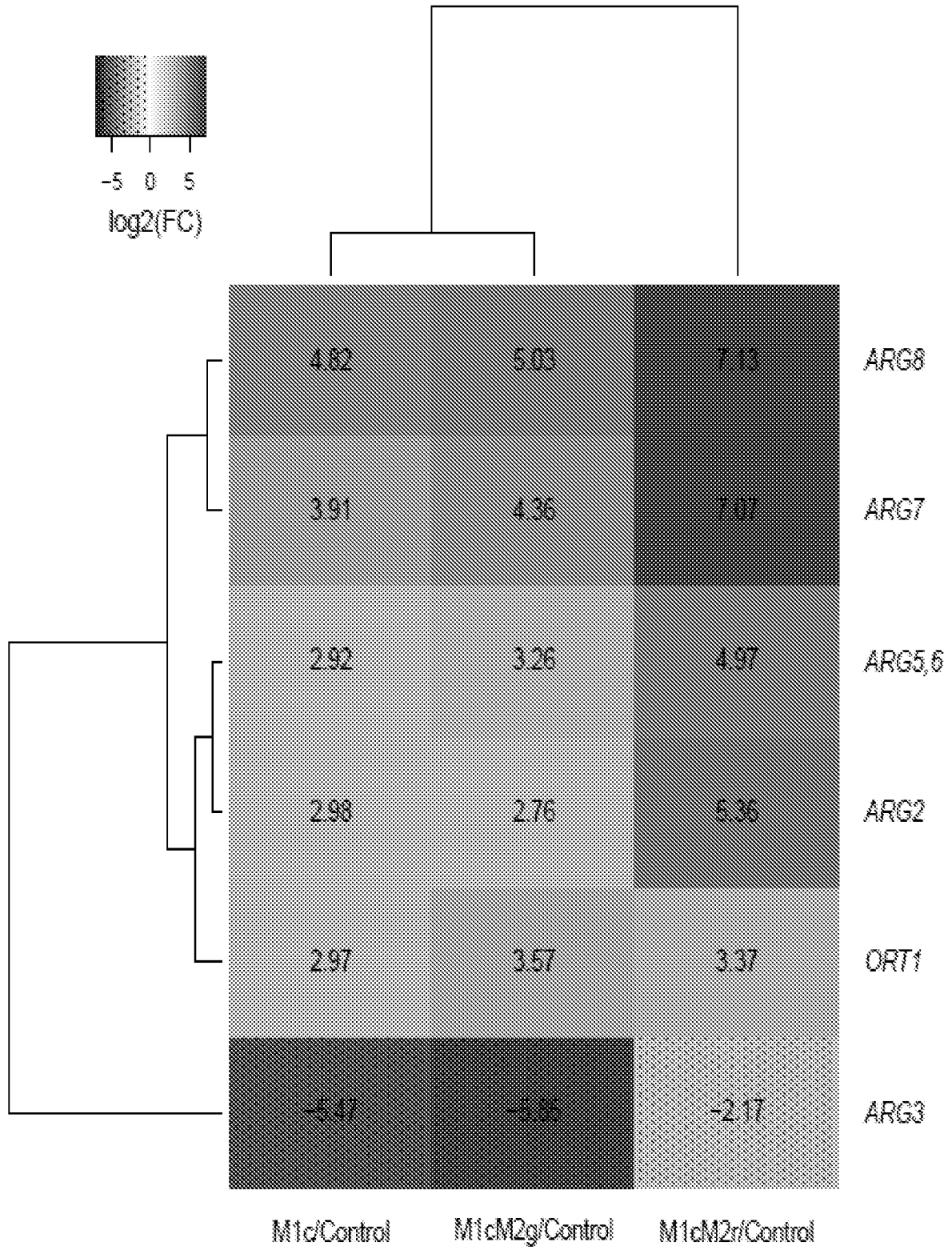




FIG. 7

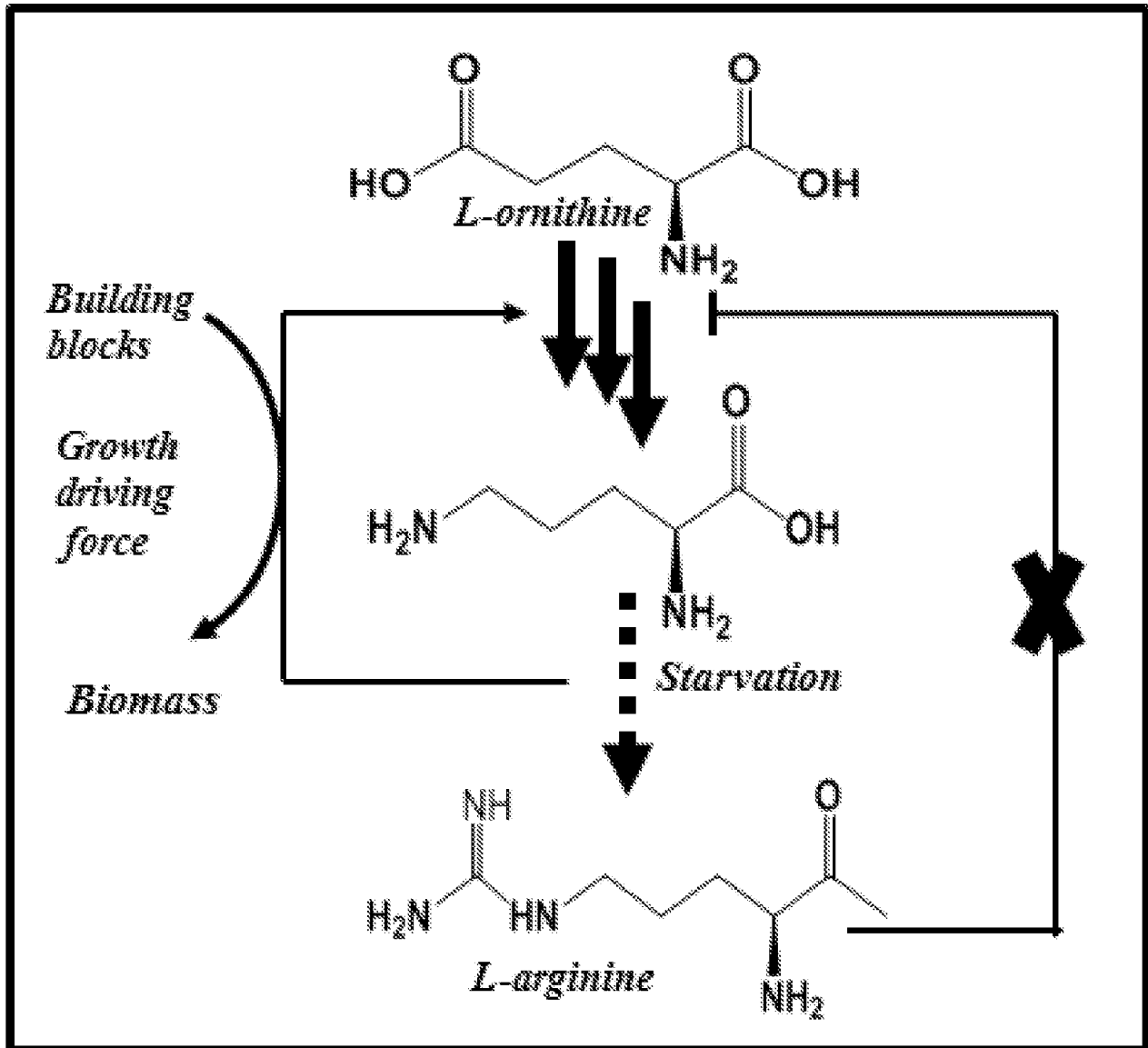


FIG. 8

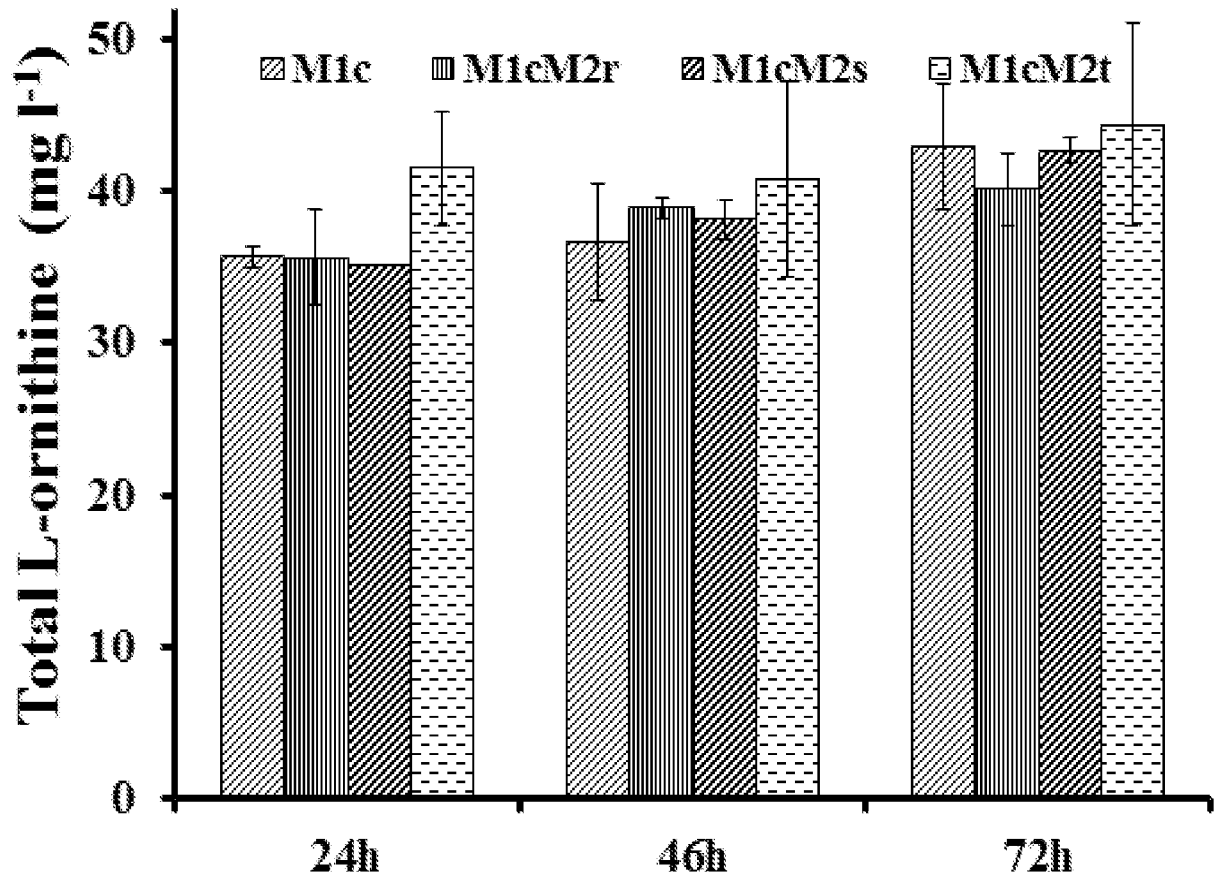


FIG. 9

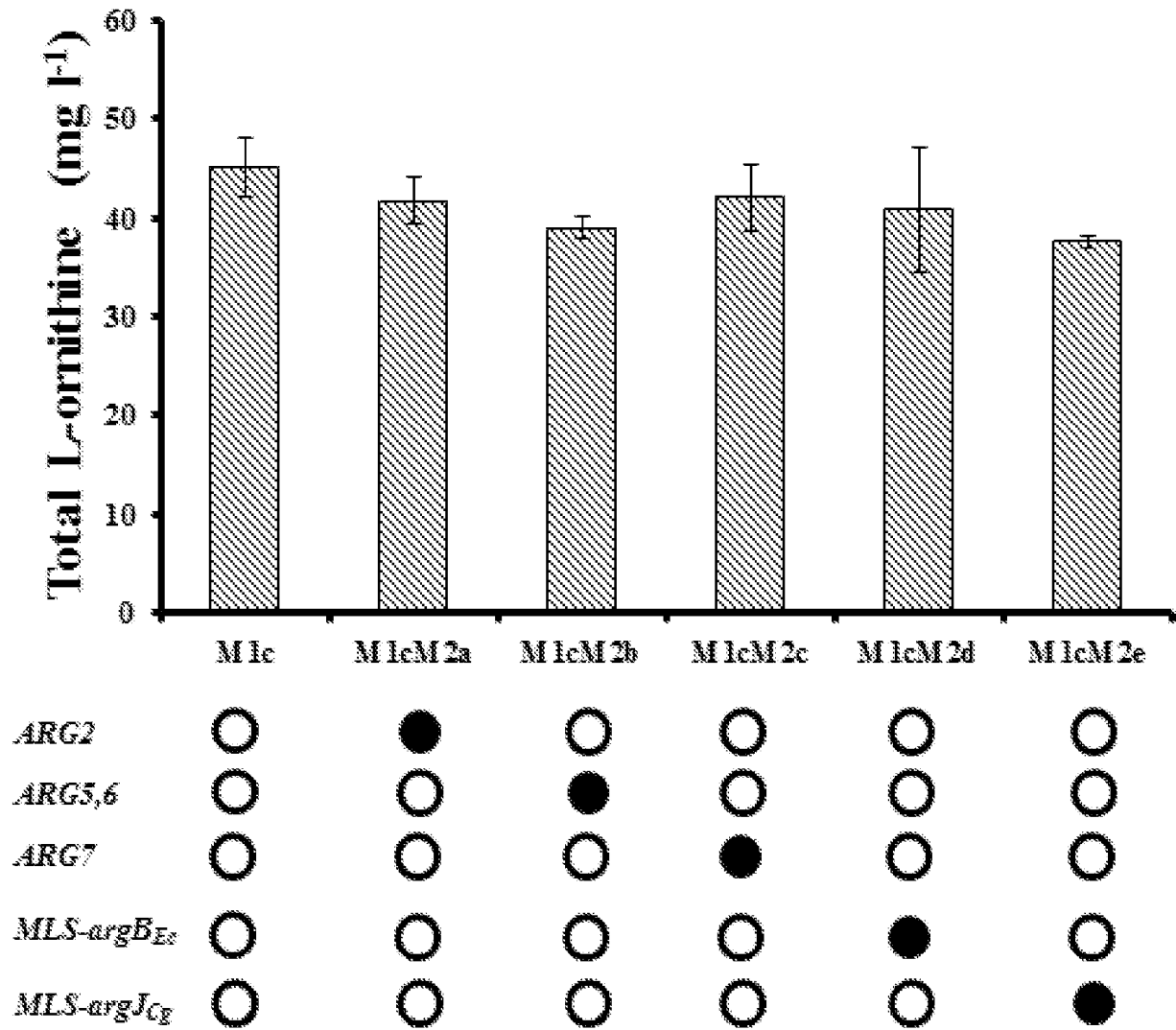


FIG. 10

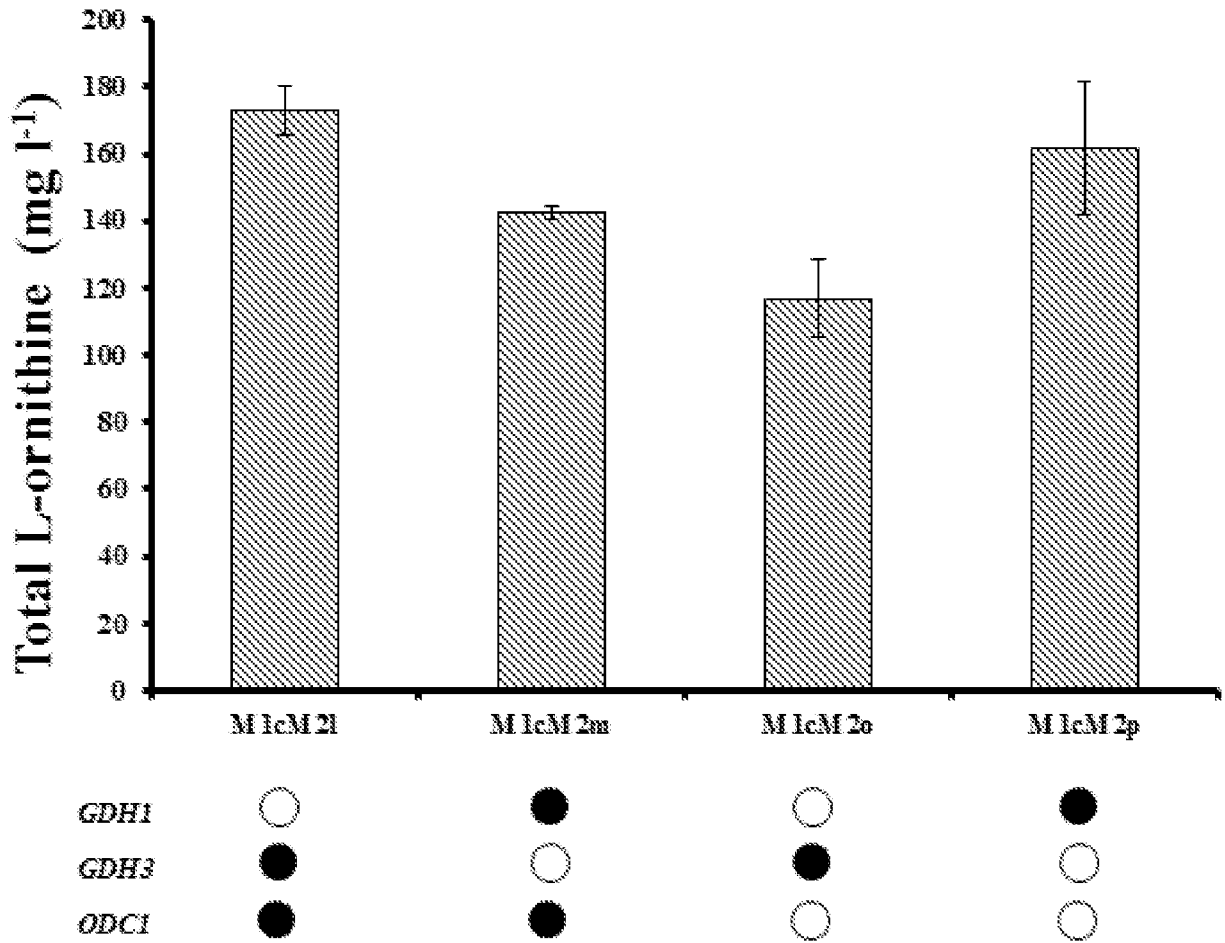


FIG. 11

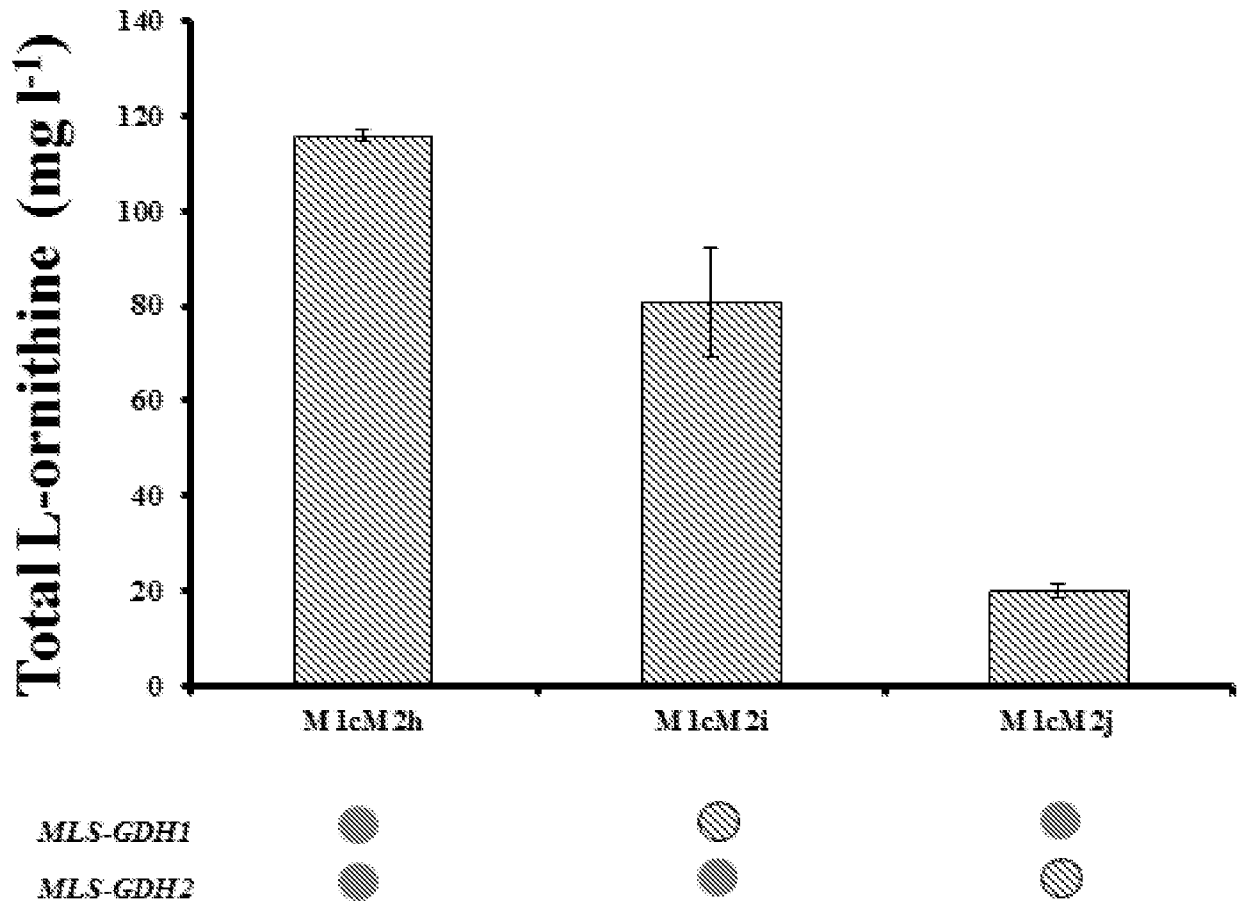


FIG. 12

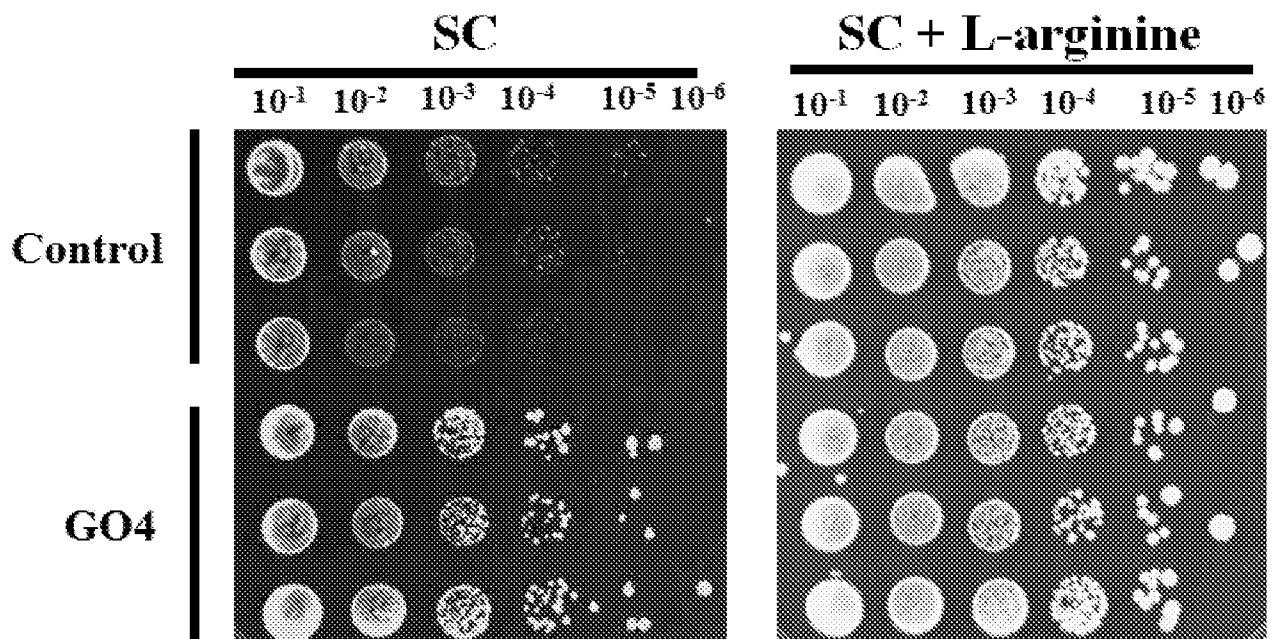


FIG. 13

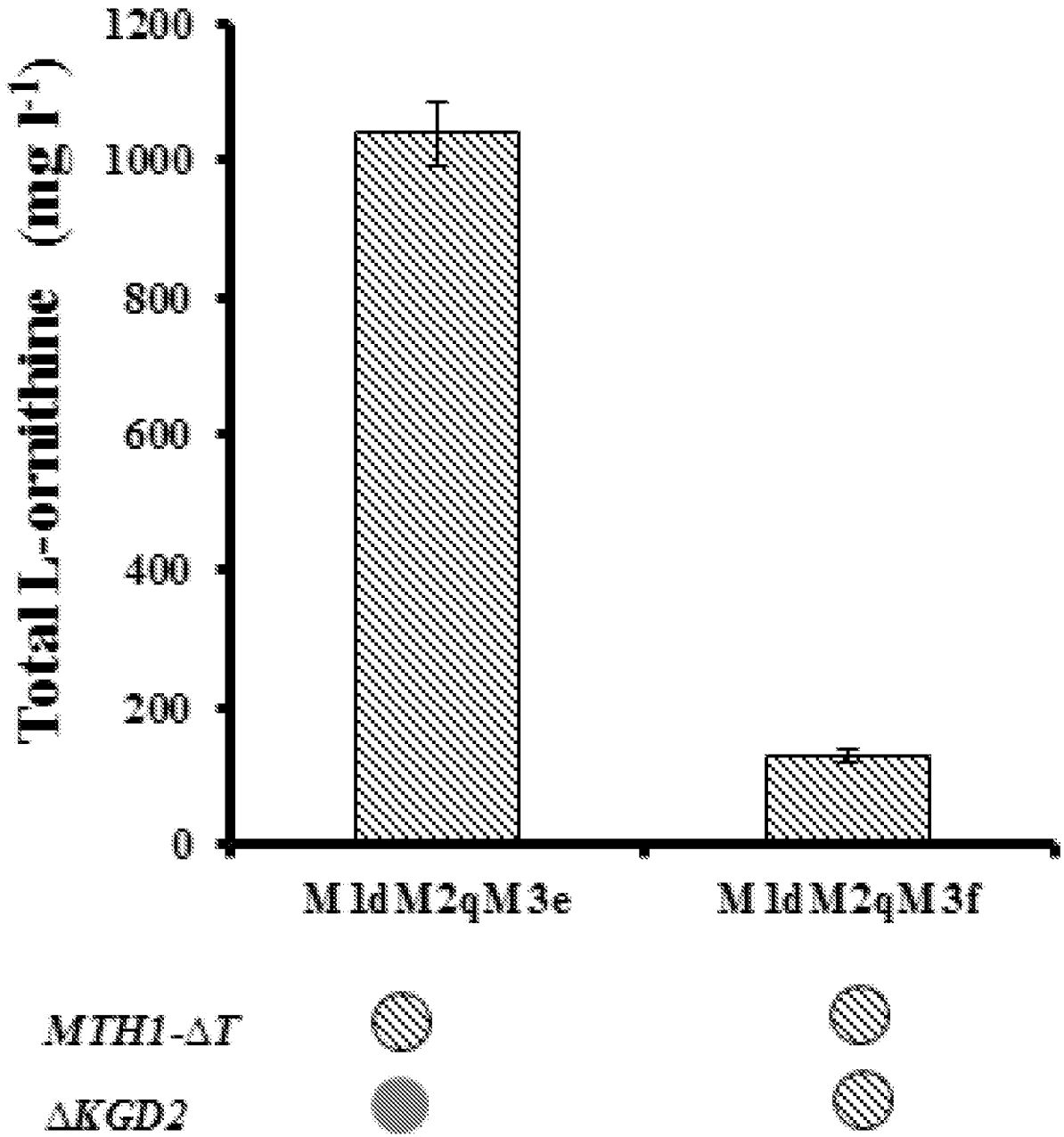


FIG. 14

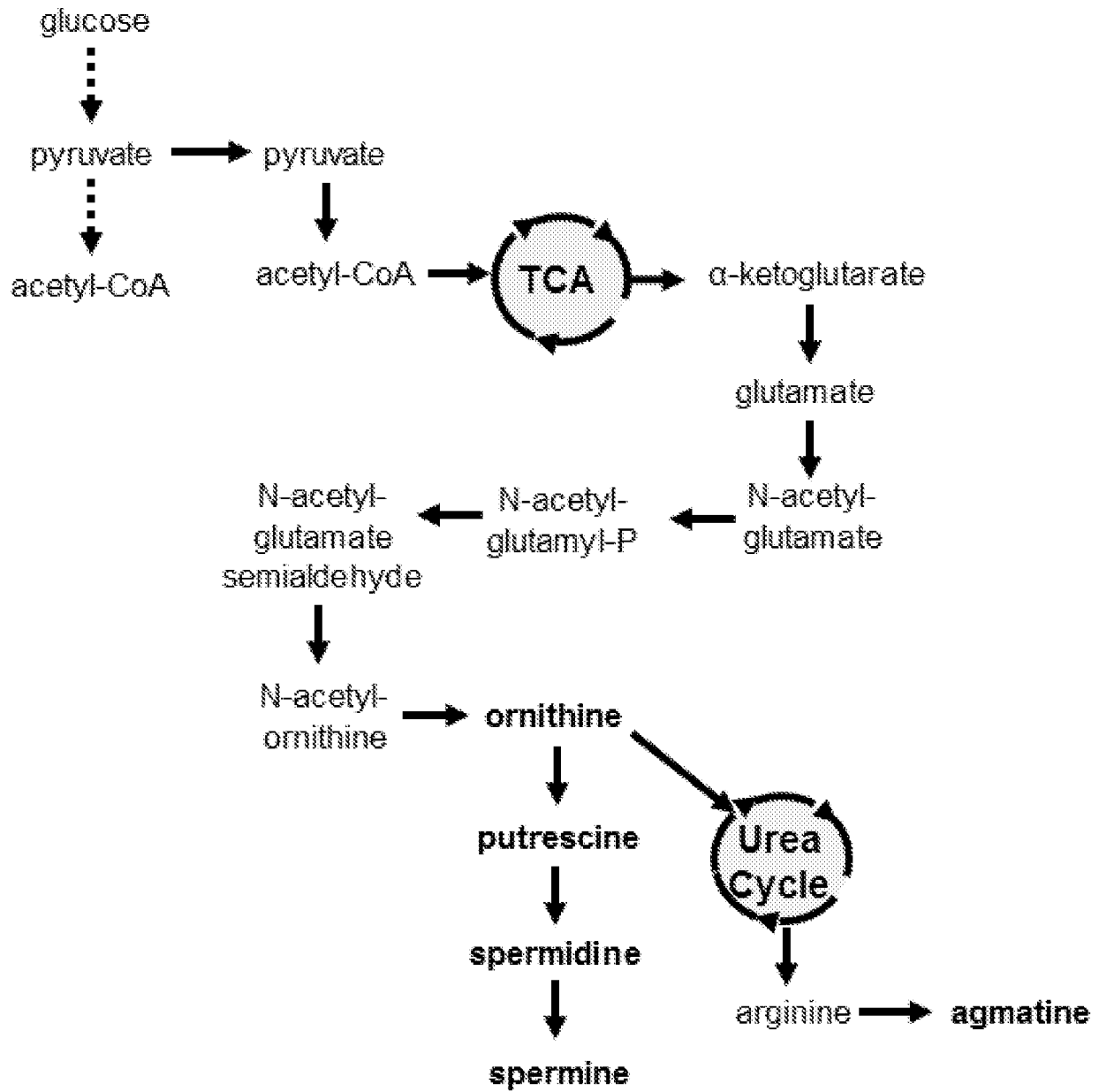




FIG. 15

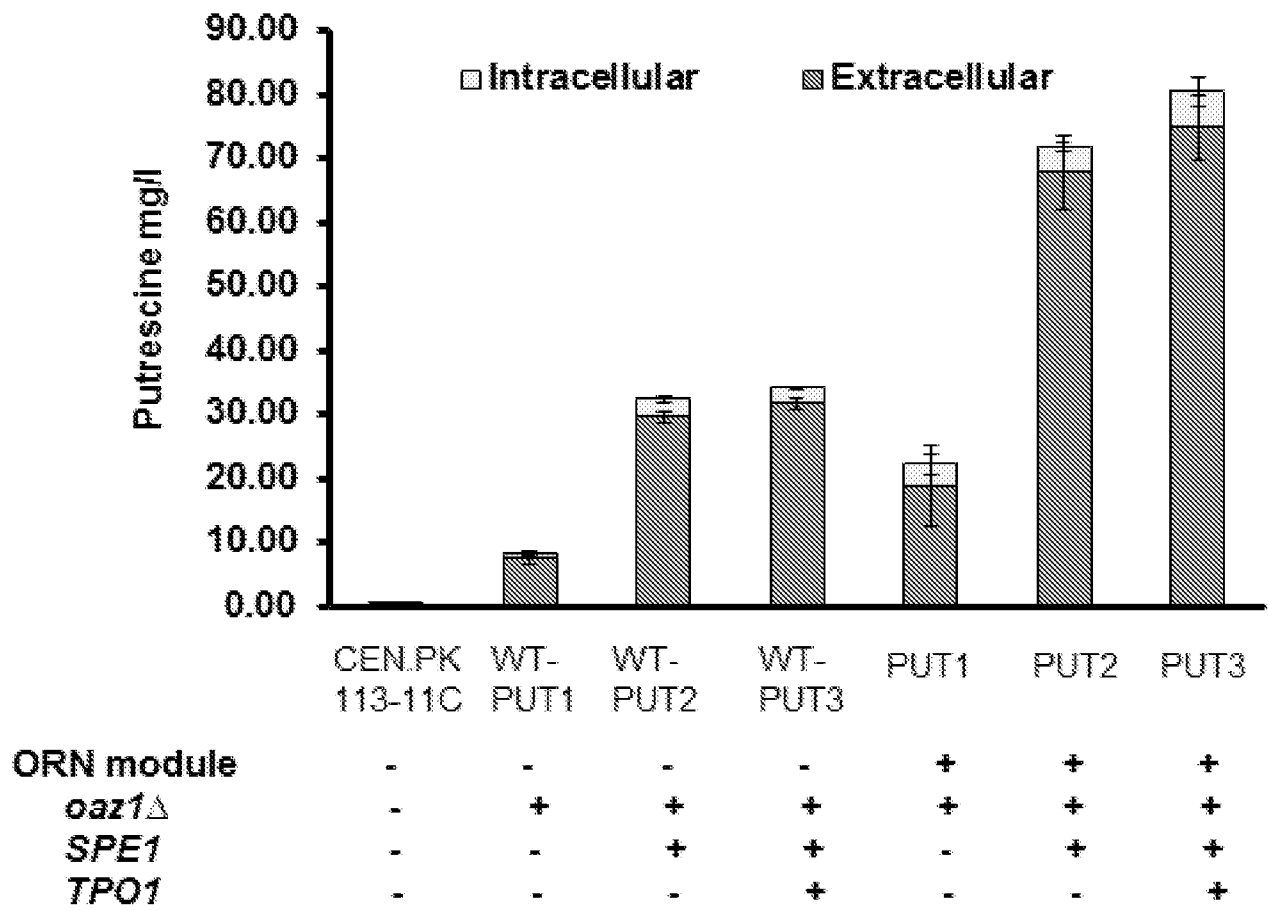
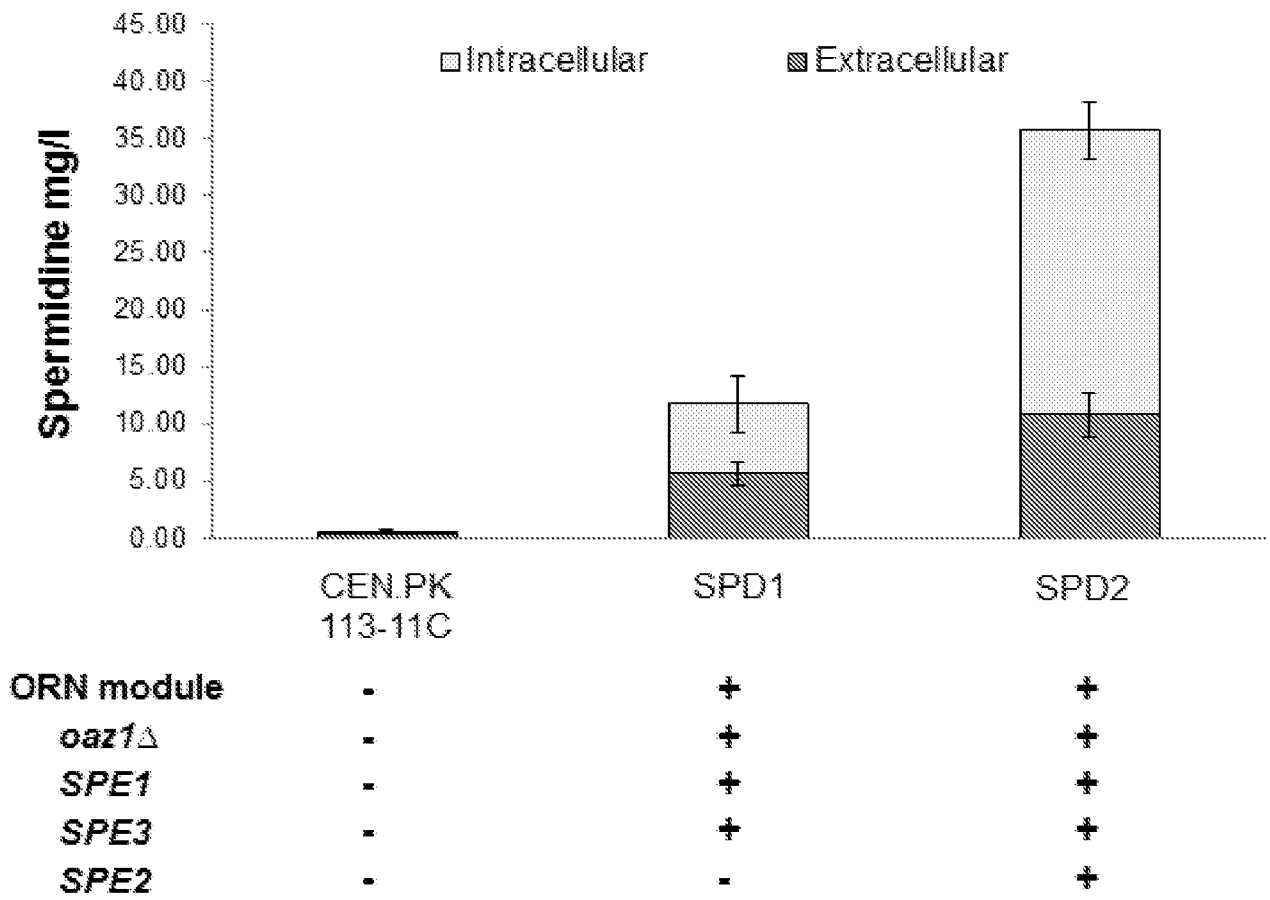


FIG. 16



**INTERNATIONAL SEARCH REPORT**

International application No PCT/SE2016/05Q193
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**A. CLASSIFICATION OF SUBJECT MATTER**  
 INV. C12N9/10 C12N15/52 C12P13/00 C12P13/10  
 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 C12P  
 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Qin J G et al.: "Multiplex amino acid metabolism engineering for increased production of L-ornithine in yeast", Proceedings Metabolic Engineering X, June 15-19, 2014. Westin Bayshore, Vancouver, BC June 2014 (2014-06), XP002758704, Retrieved from the Internet: URL: <a href="http://www3.aische.org/proceedings/Abstract.aspx?PaperID=355976">http://www3.aische.org/proceedings/Abstract.aspx?PaperID=355976</a> [retrieved on 2016-06-10] the whole document ----- - / - -	1-7, 9-16, 18-39

Further documents are listed in the continuation of Box C.       See patent family annex.

\* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent but published on or after the International filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search  13 June 2016	Date of mailing of the international search report  30/08/2016
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Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer  Lejeune, Robert
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INTERNATIONAL SEARCH REPORT

International application No  
PCT/SE2016/05Q193

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	S. J. COOPER ET AL: "High-throughput profiling of amino acids in strains of the Saccharomyces cerevisiae deletion collection" , GENOME RESEARCH, vol . 20, no. 9, 7 July 2010 (2010-07-07) , pages 1288-1296, XP055279428, ISSN: 1088-9051, DOI : 10.1101/gr.105825.110	1,2,35 , 36
A	table 1	3-7, 9-16, 18-34
X	----- A. STEINLE ET AL: "Metabolic Engineering of Saccharomyces cerevisiae for Production of Novel Cyanophycins with an Extended Range of Constituent Amino Acids" , APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol . 75, no. 11, 3 April 2009 (2009-04-03) , pages 3437-3446, XP055279448, US ISSN: 0099-2240, DOI : 10.1128/AEM.00383-09	1,2,35 , 36
A	the whole document	3-7, 9-16, 18-34
A	----- JAE HO SHIN ET AL: "Metabolic engineering of microorganisms for the production of L-arginine and its derivatives", MICROBIAL CELL FACTORIES, BIOMED CENTRAL, vol . 13, no. 1, 3 December 2014 (2014-12-03) , page 166, XP021206106, ISSN: 1475-2859, DOI : 10.1186/S12934-014-0166-4	1-7, 9-16, 18-39
A	----- EP 2 650 358 A2 (CJ CHEILJEDANG CORP [KR]) 16 October 2013 (2013-10-16)	1-7, 9-16, 18-39
T	the whole document	
T	----- JIUFU QIN ET AL: "Modular pathway rewiring of Saccharomyces cerevisiae enables high-level production of L-ornithine" , NATURE COMMUNICATIONS, vol . 6, 8 September 2015 (2015-09-08) , page 8224, XP055279414, DOI : 10.1038/ncomms9224	1-7, 9-16, 18-39
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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/SE2016/05Q193

## Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3.  Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Incompletely) ; 9-16, 18-39(partial ly)

### Remark on Protest

The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

No protest accompanied the payment of additional search fees.

**FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210**

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-7(completely) ; 9-16, 18-39(partially)

An eukaryotic cell capable of producing L-ornithine, wherein said eukaryotic cell is genetically modified for attenuated activity of an ornithine carbamoyltransferase.

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2. claims: 8(completely) ; 9-16, 18-39(partially)

An eukaryotic cell capable of producing L-ornithine, wherein said eukaryotic cell is genetically modified for enhanced L-ornithine biosynthesis from  $\alpha$ -ketoglutarate.

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3. claims: 17(completely) ; 18-39(partially)

An eukaryotic cell capable of producing L-ornithine, wherein said eukaryotic cell is genetically modified for enhanced  $\alpha$ -ketoglutarate biosynthesis.

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# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/SE2016/05Q 193

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 2650358	A2	16-10-2013	
		AU 2011339171 AI	18-07-2013
		CN 103282486 A	04-09-2013
		EP 2650358 A2	16-10-2013
		JP 5860476 B2	16-02-2016
		JP 2013544532 A	19-12-2013
		KR 20120064045 A	18-06-2012
		RU 2013131039 A	20-01-2015
		US 2013344545 AI	26-12-2013
		W0 2012077994 A2	14-06-2012
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