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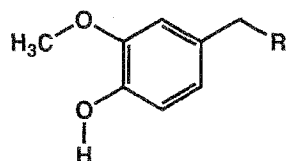
(57) Abstract: The present invention concerns a recombinant yeast comprising a set of exogenous nucleic acids, with each nucleic acid encoding a polypeptide involved in the biosynthesis pathway of a vanilloid, said set of exogenous nucleic acids encoding a set of polypeptides selected in a group comprising: • a set of enzymes allowing conversion of an aromatic amino acid into a vanilloid; and • a set of enzymes allowing conversion of a dehydroshikimic acid into a vanilloid; and wherein said recombinant yeast is devoid of a functional gene adh6 encoding for an alcohol dehydrogenase, and wherein said recombinant yeast has a reduced ability to de-carboxylate phenylacrylic acids, which encompasses that said yeast does not comprise a functional *fdl* gene and/ or a functional *padl* gene.



IMPROVED PRODUCTION OF VANILLOIDS BY FERMENTATION

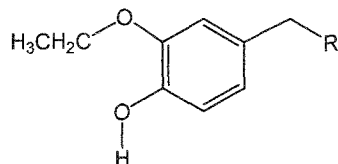
BACKGROUND

Vanilloids are defined as chemical compounds possessing a vanillyl group
5 whose chemical structure is shown below:



wherein R is selected from the group consisting of an hydrogen H, a methyl -
CH₃, an ethyl -CH₂CH₃, an aldehyde and a carboxylic acid. Optionally, the OH group can
be substituted with an O-glycosyl group. In a non-limitative manner, vanilloids include
10 vanillyl alcohol, vanillin, vanillic acid, vanillin β-D-glucoside, acetovanillon,
vanillylmandelic acid, homovanillic acid and capsaicin.

By extension, vanilloids also preferably includes chemical compounds
possessing a group whose chemical structure is shown below:



15 wherein R is selected from the group consisting of an hydrogen H, a methyl -
CH₃, an ethyl -CH₂CH₃, an aldehyde and a carboxylic acid. Optionally, the OH group can
be substituted with an O-glycosyl group. In a non-limitative manner, these chemical
compunds include ethylvanillyl alcohol, ethylvanillin, ethylvanillic acid, ethylvanillin β-
D-glucoside, acetoethylvanillon, ethylvanillylmandelic acid and homoethylvanillic acid.

20 Among vanilloids, vanillin, of chemical name 4-hydroxy-3-
methoxybenzaldehyde, is one of the most important aromatic flavor compound used in
foods, beverages, fragrances, pharmaceuticals and polymers. Vanillin was historically
extracted from *Vanilla planifolia*, *Vanilla tahitiensis* and *Vanilla pompona* pods. The
demand getting higher today, less than 5% of worldwide vanillin production comes from
25 natural vanilla pods. Currently, chemical synthesis is the most important process for
producing vanillin. There is a growing interest in other sources of vanillin and in particular
in bioconversion processes. The use of microbial cells and their enzymes as biocatalysts

for the synthesis of chemicals and flavor compounds have attracted much attention lately. Advantageously, under certain criteria, the products of such bioconversion may be considered 'natural' by legislations, such as the EU one.

Bioconversion processes are based on the following substrates: lignin, phenolic stilbenes, isoeugenol, eugenol, ferulic acid, sugars, phenolic stilbenes, waste residues and aromatic amino acids. The recent review from Kaur and Chakraborty (Kaur B, Chakraborty D. "Biotechnological and molecular approaches for vanillin production: a review." Appl Biochem Biotechnol. 2013 Feb; 169(4):1353-72) list several biosynthetic pathways and appropriate cells used for bioconversion of vanilloids.

De novo synthesis from glucose and using metabolically engineered yeast strains was recently described (Hansen EH, Møller BL, Kock GR, Büchner CM, Kristensen C, Jensen OR, Okkels FT, Olsen CE, Motawia MS, Hansen J. "De novo biosynthesis of vanillin in fission yeast (*Schizosaccharomyces pombe*) and baker's yeast (*Saccharomyces cerevisiae*)". Appl Environ Microbiol. 2009 May;75(9):2765-74). The engineered pathway involves a 3-dehydroshikimate dehydratase (3DSD), an aromatic carboxylic acid reductase (ACAR) and an O-methyltransferase, as shown in figure 2, pathways 1 and 2. In *S. cerevisiae*, because the ACAR enzyme requires an activation mediated by a phosphopantetheinyl transferase, the latter enzyme was also introduced in the yeast. Prevention of reduction of vanillin to vanillyl alcohol was achieved by deleting the host alcohol dehydrogenase encoding gene *adh6*. Later, the same authors improved their engineered pathway by using mutants of human Catechol O-methyltransferase having better substrate specificity, thereby limiting the production of isovanillin (WO 2013/022881).

Another engineered pathway for producing a vanilloid in yeast is inspired from the natural biosynthesis pathway observed in the *Vanilla planifolia* orchid, such as described in patent application US 2003/0070188. This engineered pathway comprises the following enzymes: phenylalanine/tyrosine ammonia lyase (PAL/TAL), cinnamate-4-hydrolase (C4H), 4-coumarate coenzyme A ligase (4CL), enoyl-CoA hydratase/aldolase (ECH), a hydroxybenzoic acid hydroxylase (HBH) and a caffeic acid O-methyltransferase (COMT), as shown in figures 1 and 2, pathway 3. This pathway uses aromatic amino acids such as phenylalanine and tyrosine as primary substrates. Enzymes of this pathway have

been successfully introduced into yeast, able to bio-synthesize vanilloids (patent application EP13156021.1, not published, herein incorporated by reference).

However, even if these engineered pathways are effective for producing vanilloids, they do not allow production of these compounds in amounts which are compatible with the economical and industrial requirements. Different propositions have been made to improve the production of vanilloids by fermentation in cells.

Among them, alcohol dehydrogenases ADH6 & ADH7 are known to convert vanillin into its corresponding vanillyl alcohol. Studies have suggested the deletion of the *adh6* gene in vanillin-producing yeasts (Hansen *et al.*, 2009, previously cited).

The article from Brochado *et al.* (Brochado AR, Matos C, Møller BL, Hansen J, Mortensen UH, Patil KR. "Improved vanillin production in baker's yeast through in silico design". Microb Cell Fact. 2010 Nov 8;9:84) suggested the deletion of genes encoding pyruvate decarboxylase (PDC1) and glutamate dehydrogenase (GDH1), since the deletion of these genes increases the respiratory capacity of the cell and the availability of co-factors (ATP, NADPH...) for the biosynthesis pathway of vanilloids.

Most of phenolic compounds such as vanillin show some toxicity for many living organisms with increased concentration of vanilloids. In case of *Saccharomyces cerevisiae*, growth defect is significant with concentrations as low as 0.5 g/l. To avoid the impaired growth of producing microorganisms, it has been proposed to isolate the produced vanilloids from the culture medium, in particular with resins. Another suggested solution is promoting conversion of vanillin into vanillin β -D-glucoside, this glycosylation inhibiting its toxic effect (Hansen *et al.*, 2009; Brochado *et al.*, 2010, both previously cited).

However, the highest amount of vanillin produced in bioconversion process with these genetically modified microorganisms is still limited to about 1 μ M; indeed several problems subsist and have to be solved to improve vanillin production. Specific disadvantages linked to bioconversion processes are in particular the following:

- In living cells, intermediates of the biosynthesis pathway are subject to degradation by endogenous enzymes. In particular, phenylacrylic acids such as cinnamic acid and coumaric acid can be decarboxylated by endogenous yeasts enzymes and converted into styrene and 4-vinylphenol. Also, aldehydes compounds in the pathway are subject to reduction in their corresponding primary alcohol by various alcohol

dehydrogenases. Finally, the aromatic amino acids, first intermediates of the pathway, are also subject to consumption in concurrent metabolic pathways.

- Efficiency of bioconversion is linked to the growth ability of the producing cells; therefore, it is essential to determine the best conditions of growth for said producing cells. Different parameters can be modified, in order to optimize the growth of cells and therefore the metabolic pathways, for example: volume of the culture, temperature, growth medium, source of carbon, management of the toxic effect of vanillin, etc.

Inventors present here genetically modified yeast cells and specific culture conditions, both allowing a significant improvement of vanilloid production from a source of carbon, by fermentation.

SUMMARY OF THE INVENTION

The present invention is related to a genetically modified yeast, optimized for the production of a vanilloid or a precursor thereof from a source of carbon, comprising enzymes required for production of a vanilloid or a precursor thereof, and wherein the said genetically modified yeast has a reduced ability to decarboxylate phenylacrylic acids. Advantageously, the genetically modified yeast of the invention presents also a decreased ability for degrading aldehydes, and ensures an improved availability of aromatic amino acids.

The present invention is also related to an improved method for producing a vanilloid or a precursor thereof by fermentation. Culture conditions such as carbon sources, biphasic media bioconversion and volume of the culture were explored to optimize vanillin production.

The present invention is also related to a method for producing a vanilloid or a precursor thereof from a specific source of carbon, by fermentation of genetically modified yeasts.

The present invention is also related to a method for producing a vanilloid or a precursor thereof, and separating it from the culture medium by biphasic culture media to avoid the toxic effect of vanillin. In particular the step of the yeast culture is performed in an appropriate medium comprising two phases, an aqueous phase and a water immiscible solvent.

Thus, the present invention is also related to a method for producing a vanilloid or precursor thereof in a unicellular microorganism able to produce said vanilloid or precursor thereof, comprising a step of cultivating said microorganism in an appropriate medium comprising an aqueous phase and a water immiscible phase, said water
5 immiscible phase preferably consisting of polymer of propylene glycol, fatty alcohol, fatty acid, fatty ester or an oil.

In addition, the present invention is related to a composition comprising a vanilloid or a precursor thereof obtainable by the method as disclosed above, and to the use of said composition as a flavoring in the human and animal nutrition field, in
10 pharmacy, and as a fragrance in the cosmetics, perfumery and detergency industries.

FIGURES

FIGURE 1A, 1B: Biosynthetic pathways of vanillin from aromatic amino acids. The figure shows the schematic diagram wherewith carbon source is converted into
15 vanillin. Phenylalanine undergoes several reactions: deamination, hydroxylation of 4 position of the phenyl ring, reduction chain reaction and hydroxylation of 3 position of the phenyl ring, and O-methylation of the 3 position of the phenyl ring.

FIGURE 2A, 2B: Global reaction scheme of vanillin biosynthesis from glucose. The shikimic acid pathway (pathways 1 & 2 – Figure 2A) and the aromatic amino acid pathway (pathway 3 – Figure 2B) are represented. PAL relates to a phenylalanine
20 ammonia lyase. C4H relates to a cinnamic acid hydroxylase. 4CL relates to a 4-coumarate-CoA ligase. HBH relates to a hydroxybenzoic acid hydroxylase. ECH relates to an enoyl-CoA hydratase / crotonase.

FIGURE 3: 3A: conversion of 4-hydroxybenzaldehyde by deletion strains
25 $\Delta ald2$, $\Delta gre3$, $\Delta ald6$ and $\Delta adh6$ and a wild-type (WT) strain; 3B: conversion of 3,4-dihydroxybenzaldehyde by the same strains than figure 3A; 3C: conversion of vanillin by the same strains than figure 3A; 3D: conversion of vanillin by the strain Y00, Y00 $\Delta adh6$ and the multi-deleted strains Y00 $\Delta adh6 \Delta gre3$ and Y00 $\Delta adh6 \Delta ald2$.

FIGURE 4: Assembly of vanillin pathway by fragments containing
30 homologous gene sequences. This figure shows the co-transformation of 9 fragments comprising the 6 genes for vanillin production starting from phenylalanine and 1 gene

coding for the carboxylic acid reductase. HIS3 and LEU2 are the flanking markers enabling the double selection of the recombinant pathway.

FIGURE 5: Grey bars: Metabolites total concentration from vanillin pathway according to then host strain (cinnamic acid, coumaric acid, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 3,4-dihydroxybenzaldehyde, vanillin, vanillic acid, vanillyl alcohol). Striped bars: Total concentration of final products from vanillin pathway according to then host strain (vanillin, vanillic acid and vanillyl alcohol). Black bars: vanillin concentration.

FIGURE 6: Metabolite profile of supernatant of culture from strains comprising a recombinant pathway according to figure 4, and the following deletions: $\Delta adh6\Delta aro10\Delta fdc1$ and $\Delta adh6\Delta aro10\Delta fdc1\Delta pdc1$ in 2mL culture volume. 34AD = 3,4-dihydroxybenzaldehyde; 34AC = 3,4-dihydroxybenzoic acid; VANAL = vanillyl alcohol; VAN = vanillin; VANAC = vanillic acid.

FIGURE 7: Vanillin production of a strain comprising a recombinant pathway according to figure 4, and the following deletions: $\Delta adh6\Delta fdc1\Delta pad1$ in a 50 mL Erlenmeyer flask.

FIGURE 8: Cytotoxicity assays with vanillin toward yeast in the presence or absence of PPG1200 done with a strain comprising a recombinant pathway according to figure 4, and the following deletions: $\Delta adh6\Delta pdc1\Delta pad1$.

FIGURE 9: Extractive bioconversion of vanillin using 30% polypropylene glycol 1200 (PPG). Diagram presents concentration of vanillin (VAN), vanillic acid (VANAC), vanillyl alcohol (VANAL), 3,4-dihydroxybenzoic acid (34hbzAC) and 3,4-dihydroxybenzaldehyde (34hbzAD) in control culture (YPGal) and in biphasic culture (YPAGal/PPG1200) from culture supernatant of a strain comprising a recombinant pathway according to figure 4, and the following deletions: $\Delta adh6\Delta pad1\Delta fdc1$.

FIGURE 10: Vanillin producing cells (strains comprising a recombinant pathway according to figure 4, and the following deletions: $\Delta adh6\Delta pdc1\Delta fdc1$) were precultured in YPD (10 g/l bacto-yeast extract, 20 g/l bacto-peptone and 2% dextrose) or YPGal (10 g/l bacto-yeast extract, 20 g/l bacto-peptone and 3% galactose) medium in a 10 mL for 24h, and used to inoculate 50 mL appropriate medium in a 250 mL Erlenmeyer flask. Vanillin concentration (μM) was monitored after 3 days of culture depending on carbon source used for yeast growth. YPAD = YPGal; YPF (10 g/l bacto-yeast extract,

20 g/l bacto-peptone and 2% fructose); YPAGal (10 g/l bacto-yeast extract, 20 g/l bacto-peptone, 30g/l galactose); YPGE (10 g/l bacto-yeast extract, 20 g/l bacto-peptone, 2% glycerol and 2% ethanol); YPG (10 g/l bacto-yeast extract, 20 g/l bacto-peptone, 2% glycerol); YPE (10 g/l bacto-yeast extract, 20 g/l bacto-peptone and 2% ethanol); YPDE
5 (10 g/l bacto-yeast extract, 20 g/l bacto-peptone, 2% dextrose and 2% ethanol).

DETAILED SPECIFICATION

10 The present invention is related to a recombinant yeast, optimized for the production of a vanilloid or a precursor thereof, comprising a set of exogenous nucleic acids, with each nucleic acid encoding a polypeptide involved in the biosynthesis pathway of a vanilloid, said set of exogenous nucleic acids encoding a set of polypeptides selected in a group comprising:

- 15 - a set of enzymes allowing conversion of an aromatic amino acid into a vanilloid; and
- a set of enzymes allowing conversion of an dehydroshikimic acid into a vanilloid;

and wherein said recombinant yeast is devoid of a functional gene *adh6*
20 encoding for an alcohol dehydrogenase,

and wherein said recombinant yeast has a reduced ability to decarboxylate phenylacrylic acids, which encompasses preferentially that said yeast does not comprise a functional *fdc1* gene and/ or a functional *pad1* gene.

This invention also pertains to a recombinant yeast, optimized for the
25 production of a vanilloid or a precursor thereof, expressing a set of polypeptides involved in the biosynthesis pathway of a vanilloid, said set of polypeptides being encoded by one or more exogenous nucleic acids, and said set of polypeptides being selected in a group comprising:

- 30 - a set of enzymes allowing conversion of an aromatic amino acid into a vanilloid; and
- a set of enzymes allowing conversion of an dehydroshikimic acid into a

vanilloid;

and wherein said recombinant yeast is devoid of a functional gene *adh6* encoding for an alcohol dehydrogenase,

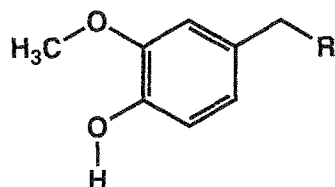
and wherein said recombinant yeast has a reduced ability to decarboxylate phenylacrylic acids, which encompasses that said yeast does not comprise a functional *fdcl* gene and/ or a functional *padl* gene.

The following terms are used in the present application and are defined hereunder.

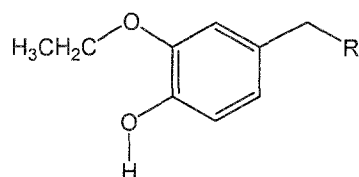
Yeasts are eukaryotic unicellular microorganisms. Yeasts are chemoorganotrophs, as they use organic compounds as a source of energy and do not require sunlight to grow. Over 1,500 species are currently known. An extensively studied genus of yeast is *Saccharomyces*.

By "recombinant yeast" or "genetically modified yeast" is meant a yeast organism whose genetic material has been modified by genetic engineering methods, either by suppression or inactivation of genes, and/or by addition of exogenous genetic material.

By "vanilloid" is meant a chemical compound which possesses a vanillyl group whose chemical structure is shown below:

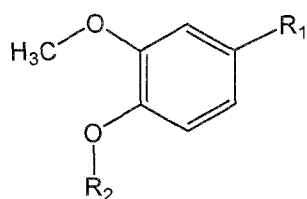


wherein R is selected from the group consisting of H, -CH₃, -CH₂CH₃, an aldehyde and a carboxylic acid. Optionally, the OH group can be substituted with an O-glycosyl group. By extension, vanilloids also preferably include chemical compounds possessing a group whose chemical structure is shown below:

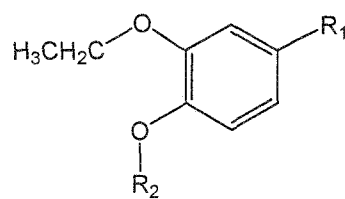


wherein R has the same meaning as defined above.

The present invention relates to a method for producing a vanilloid of formula (I) or of formula (II), preferably of formula (I):



(I)



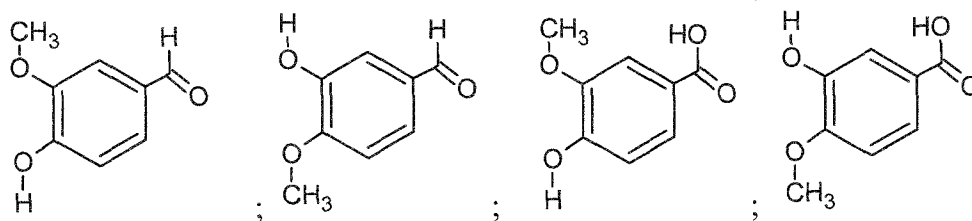
(II)

R^1 being selected from the group consisting of $-\text{CHO}$; $-\text{COOH}$; $-\text{COOR}^3$; $-\text{CH}_2\text{OH}$; $-\text{CH}_2\text{COOH}$; $-\text{C}(=\text{O})\text{CH}_3$; $-\text{CR}^3(\text{OH})\text{COOH}$ and CHR^3COOH , wherein R^3 is a lower alkyl,

5 R^2 being different from a methyl ($-\text{CH}_3$), in particular R^2 being selected from the group consisting of H, sulfate, phosphate and a glycoside. According to a preferred embodiment, R^2 can be H.

Vanilloids include, in a non limitative manner, vanillyl alcohol, vanillin, vanillic acid, vanillin-glycoside, acetovanillon, vanillylmandelic acid, homovanillic acid,
10 capsaicin and ethylvanillin.

Chemical structures of vanillin, isovanillin, vanillic acid and isovanillic acid are respectively given here below:



15 As used herein, an “exogenous nucleic acid encoding a polypeptide” means a nucleic acid comprising a one or more expression regulatory sequences and an Open Reading Frame (which may also be termed “ORF” or “coding sequence”), that has been initially introduced into a yeast cell by a genetic engineering method, thus allowing the said yeast cell to express a polypeptide encoded by the said ORF. Most preferably, an
20 exogenous nucleic acid also comprises at least a transcription terminator. Nucleic acids are exogenous to the host cell, i.e. are introduced in said host cell; they can be naturally absent or present in said host cell, but in all cases they are introduced by human manipulation. Most preferably, an “exogenous nucleic acid encoding a polypeptide” is not naturally present in the genome of the said yeast cell. An example of an exogenous nucleic acid is a
25 nucleic acid which originates from a yeast species different from the host yeast cell

species. Another example of an exogenous nucleic acid is a construct comprising an endogenous ORF which is placed under the control of a promoter which is not the naturally-occurring promoter of the said endogenous ORF.

As used herein, a “set” of exogenous nucleic acids means a plurality of exogenous nucleic acids as defined above, the said plurality of exogenous nucleic acids allowing when expressed in yeast cells wherein they have been introduced, the production of the corresponding set of polypeptides. The examples herein illustrate embodiments of yeast cells in which a set of exogenous nucleic acids has been introduced, the said set of exogenous nucleic acids encoding a set of polypeptides selected in a group comprising:

- a set of enzymes allowing conversion of an aromatic amino acid into a vanilloid; and
- a set of enzymes allowing conversion of an dehydroshikimic acid into a vanilloid.

In some embodiments, a set of exogenous nucleic acids is comprised in a unique polynucleotide, which unique polynucleotide has been introduced in the yeast organism, e.g. within a specific location in a yeast chromosome.

In other embodiments, a set of exogenous nucleic acids is initially comprised in a plurality of polynucleotides which are then fused before introduction in the yeast organism, e.g. within a specific location in a yeast chromosome.

In still other embodiments, a set of exogenous nucleic acids is comprised in a plurality of polynucleotides, each polynucleotide being introduced in the yeast organism, e.g. within a plurality of locations in a yeast chromosome.

As used herein, a recombinant yeast organism “comprises” exogenous nucleic acids when the said exogenous nucleic acids are part of the genetic material thereof, either (i) because the said exogenous nucleic acids are integrated into the chromosomal genetic material of the said yeast organism, (ii) or because the said exogenous nucleic acids are included within the genetic material of the said yeast organism outside chromosomes, which encompasses inclusion of the exogenous nucleic acids into nucleic acid vectors, e.g. plasmids. The term “biosynthesis pathway” as used in the present application refers to the different enzymes involved in specific biochemical steps and intermediates enabling the biosynthesis of products from a substrate *in vivo* by recombinant host cells. A biosynthesis pathway may be termed “artificial” when one or more of the enzymes comprised therein

are encoded by exogenous nucleic acids, i.e. nucleic acids that have been introduced 'artificially' into a host cell, most preferably by using genetic engineering methods.

The term "aromatic amino acid" designates an amino acid that includes an aromatic ring. Among the twenty standard amino acids, four of them consist of aromatic amino acids: phenylalanine, tryptophan, histidine and tyrosine.

The term "dehydroshikimic acid" also called 3-DHS designates the compound of the systemic name (4S,5R)-4,5-D-dihydroxy-3-oxocyclohexene-1-carboxylic acid, whose complete formula is shown in figure 2.

Recombinant yeasts with genetic modifications optimizing the synthesis of vanilloids

Different genetic modifications are proposed for optimizing the biosynthesis of vanilloids in recombinant yeasts comprising exogenous nucleic acids coding for polypeptides involved in the biosynthesis of vanilloids.

As previously reported, prevention of the reduction of vanillin into vanillyl alcohol can be achieved by the inactivation or the deletion of at least one gene coding for host alcohol dehydrogenases, in particular by the inactivation or the deletion of the gene *adh6*.

In the present invention, the recombinant yeast is devoid of a functional gene *adh6*.

The expression "a functional gene is absent" or "said yeast does not comprise a functional gene" or "said yeast is devoid of a functional gene" means that the said gene does not allow the production of the expected functional polypeptide, e.g. does not allow the production of the expected active enzyme. In some embodiments of a yeast cell that is devoid of a functional given gene, the said gene is not expressed in the yeast cell, e.g. the said gene is not transcribed or the corresponding transcription product is not translated into a protein, e.g. into an enzyme, having its usual activity. In some embodiment, the said gene may be 'inactivated'. The man skilled in the art knows various means to obtain such absence of functionality/inactivation of a gene, such as:

- introduction of a mutation into the gene, in particular generation of a stop codon inducing the expression of a non-functional, truncated protein;

- introduction of an 'insert' into the gene, inactivating its correct transcription; e.g. interruption of the gene sequence by introduction of one or more exogenous nucleic acids, which encompasses introduction of a set of exogenous nucleic acids,

5 - replacement of the natural promoter of the gene by a non-functional promoter, or complete suppression of the promoter,

- deletion, complete or partial of the coding sequence of the gene.

In some embodiments, the introduction of a mutation into the gene may be achieved by mutagenesis, which includes random and directed mutagenesis.

10 In some preferred embodiments, "absence of a functional gene" refers to an endogenous gene having been inactivated by introduction of a DNA insert, or refers to a gene whose coding sequence has been partially or completely deleted.

Phenylacrylic acids include cinnamic acid, coumaric acid and ferulic acid, all of them present in numerous plants. These carboxylic acids are intermediates in the pathway of catabolism of aromatic amino acids. Mukai *et al.* have shown that genes encoding PAD1 and FDC1 are essential for the decarboxylation of phenylacrylic acids in *S. cerevisiae* (Mukai N, Masaki K, Fujii T, Kawamukai M, Iefuji H. "PAD1 and FDC1 are essential for the decarboxylation of phenylacrylic acids in *Saccharomyces cerevisiae*." J Biosci Bioeng. 2010 Jun;109(6):564-9).

20 The expression "reduced ability to decarboxylate phenylacrylic acids" means, for the purpose of the present description, that the intermediates in the vanilloid biosynthesis pathway, such as cinnamic acid, coumaric acid, and ferulic acid, are less converted by decarboxylases than in a wild-type corresponding yeast, and therefore that the availability of these compounds for the biosynthesis pathway of vanilloid is increased.

25 In particular, the intermediates in the vanilloid pathway may comprise or consist of cinnamic acid, and/or p-coumaric acid, and/or ferulic acid.

According to some embodiments, the yeast cell is genetically modified so as to possess a low capacity of decarboxylating phenylacrylic acids, because of:

30 - the absence of at least one functional gene coding for a Phenylacrylic acid decarboxylase (PAD) and/or a Ferulic acid Decarboxylase (FDC); and/or

- the decreased or null enzymatic activity of the encoded proteins PAD and/or FDC; and/or

- the decreased expression of at least one nucleic acid coding for said PAD and/or said PDC.

According to one preferred embodiment of the invention, the yeast cell is genetically modified so as to possess a low capacity of decarboxylating phenylacrylic acids, because of the absence of at least one functional gene coding for a Phenylacrylic acid decarboxylase (PAD) and/or a Ferulic acid Decarboxylase (FDC), and most preferably, coding for the decarboxylases Pad1p (Phenylacrylic acid decarboxylase) and/or Fdc1p (Ferulic acid Decarboxylase). Respective contribution of the Pad1p and Fdc1p proteins to the decarboxylation of phenylacrylic acids is disclosed in the article of Mukai et al. (2010), previously cited.

According to one embodiment, a yeast having reduced ability to decarboxylate phenylacrylic acids encompasses a recombinant yeast that does not comprise a functional gene coding for the decarboxylase Pad1p.

According to one embodiment, a yeast having reduced ability to decarboxylate phenylacrylic acids encompasses a recombinant yeast that does not comprise a functional gene coding for the decarboxylase Fdc1p.

According to one embodiment, a yeast having reduced ability to decarboxylate phenylacrylic acids encompasses a recombinant yeast that does not comprise a functional gene coding for the decarboxylase Pad1p and a functional gene coding for the decarboxylase Fdc1p.

In a specific embodiment of the invention, the genetically modified yeast is further modified to decrease the consumption of aromatic amino acids in other biosynthesis pathways than the vanilloid biosynthesis pathway. In particular, the expression and/or activity of enzymes using aromatic amino acids as substrates could be decreased, except for the enzymes phenylalanine ammonia lyase and tyrosine ammonia lyase.

Advantageously, the genetically modified yeast does not comprise a functional gene *aro10*. *Aro10* gene encodes a phenylpyruvate decarboxylase that is involved in the consumption of phenylalanine and tyrosine (Vuralhan Z, Morais MA, Tai SL, Piper MD, Pronk JT. « *Identification and characterization of phenylpyruvate decarboxylase genes in Saccharomyces cerevisiae* » Appl Environ Microbiol. 2003 Aug; 69(8):4534-41). The

disruption of *aro10* causes an improved availability of phenylalanine for the biosynthesis pathway towards vanilloids.

In vanillin pathway, many intermediate precursors are aldehydes, which are known to be substrates of many endogenous enzymes leading to their corresponding alcohol or acid derivative. In recombinant yeasts according to the invention, aldehydes tend to be oxidized immediately after the reduction chain reaction, and as a consequence no vanillin is detected. In order to lower the conversion of aldehydes into their corresponding acid derivatives, a carboxylic acid reductase protein may be added, that is able to convert aromatic carboxylic acid into corresponding aldehyde. The CAR enzyme requires activation by phosphopantetheinylation (this post-translation modification is done by PPTase).

In a preferred embodiment of the invention, the genetically modified yeast does not comprise a functional gene *ald6*, coding for an aldehyde dehydrogenase. Its disruption causes an improved availability of aldehyde intermediates in the biosynthesis pathway towards vanilloids.

Advantageously, the genetically modified yeast is further devoid of a functional gene *pdcl*. *Pdcl* gene encodes a pyruvate decarboxylase, converting pyruvate into acetaldehyde. Pyruvate is a key metabolite in *S. cerevisiae* metabolism and the branch point between respiratory and fermentation metabolism. It has been shown that deletion of *pdcl* results in a reduction of 30% of total pyruvate decarboxylase activity; leading to an increased production of vanillin (Brochado et al., 2010, previously cited).

In a specific embodiment, a recombinant yeast comprising a set of exogenous nucleic acids encoding a set of enzymes allowing conversion of an aromatic amino acid into a vanilloid, presents one of the following phenotypes:

25 $\Delta\text{ahd6}\Delta\text{fdc1}$, $\Delta\text{ahd6}\Delta\text{fdc1}\Delta\text{aro10}$, $\Delta\text{ahd6}\Delta\text{fdc1}\Delta\text{ald6}$, $\Delta\text{ahd6}\Delta\text{fdc1}\Delta\text{pdcl}$,
 $\Delta\text{ahd6}\Delta\text{fdc1}\Delta\text{aro10}\Delta\text{ald6}$, $\Delta\text{ahd6}\Delta\text{fdc1}\Delta\text{aro10}\Delta\text{pdcl}$, $\Delta\text{ahd6}\Delta\text{fdc1}\Delta\text{ald6}\Delta\text{pdcl}$,
 $\Delta\text{ahd6}\Delta\text{fdc1}\Delta\text{aro10}\Delta\text{ald6}\Delta\text{pdcl}$, $\Delta\text{ahd6}\Delta\text{pad1}$, $\Delta\text{ahd6}\Delta\text{pad1}\Delta\text{aro10}$, $\Delta\text{ahd6}\Delta\text{pad1}\Delta\text{ald6}$,
 $\Delta\text{ahd6}\Delta\text{pad1}\Delta\text{pdcl}$, $\Delta\text{ahd6}\Delta\text{pad1}\Delta\text{aro10}\Delta\text{ald6}$, $\Delta\text{ahd6}\Delta\text{pad1}\Delta\text{aro10}\Delta\text{pdcl}$,
 $\Delta\text{ahd6}\Delta\text{pad1}\Delta\text{ald6}\Delta\text{pdcl}$, $\Delta\text{ahd6}\Delta\text{pad1}\Delta\text{aro10}\Delta\text{ald6}\Delta\text{pdcl}$, $\Delta\text{ahd6}\Delta\text{fdc1}\Delta\text{pad1}$,
30 $\Delta\text{ahd6}\Delta\text{fdc1}\Delta\text{pad1}\Delta\text{aro10}$, $\Delta\text{ahd6}\Delta\text{fdc1}\Delta\text{pad1}\Delta\text{ald6}$, $\Delta\text{ahd6}\Delta\text{fdc1}\Delta\text{pad1}\Delta\text{pdcl}$,
 $\Delta\text{ahd6}\Delta\text{fdc1}\Delta\text{pad1}\Delta\text{aro10}\Delta\text{ald6}$, $\Delta\text{ahd6}\Delta\text{fdc1}\Delta\text{pad1}\Delta\text{aro10}\Delta\text{pdcl}$, $\Delta\text{ahd6}\Delta\text{fdc1}\Delta\text{pad1}\Delta\text{al}$

d6Apdc1, Δ ahd6 Δ fdc1 Δ pad1 Δ aro10 Δ ald6Apdc1 or any other combination of these deletions.

In another specific embodiment, a recombinant yeast comprising a set of exogenous nucleic acids encoding a set of enzymes allowing conversion of an dehydroshikimic acid into a vanilloid, presents one of the following phenotypes:

Δ ahd6 Δ fdc1, Δ ahd6 Δ fdc1 Δ aro10, Δ ahd6 Δ fdc1 Δ ald6, Δ ahd6 Δ fdc1 Δ pdc1, Δ ahd6 Δ fdc1 Δ aro10 Δ ald6, Δ ahd6 Δ fdc1 Δ aro10 Δ pdc1, Δ ahd6 Δ fdc1 Δ ald6Apdc1, Δ ahd6 Δ fdc1 Δ aro10 Δ ald6Apdc1, Δ ahd6 Δ pad1, Δ ahd6 Δ pad1 Δ aro10, Δ ahd6 Δ pad1 Δ ald6, Δ ahd6 Δ pad1 Δ pdc1, Δ ahd6 Δ pad1 Δ aro10 Δ ald6, Δ ahd6 Δ pad1 Δ aro10 Δ pdc1, Δ ahd6 Δ pad1 Δ ald6Apdc1, Δ ahd6 Δ pad1 Δ aro10 Δ ald6Apdc1, Δ ahd6 Δ fdc1 Δ pad1, Δ ahd6 Δ fdc1 Δ pad1 Δ aro10, Δ ahd6 Δ fdc1 Δ pad1 Δ ald6, Δ ahd6 Δ fdc1 Δ pad1 Δ pdc1, Δ ahd6 Δ fdc1 Δ pad1 Δ aro10 Δ ald6, Δ ahd6 Δ fdc1 Δ pad1 Δ aro10 Δ pdc1, Δ ahd6 Δ fdc1 Δ pad1 Δ ald6Apdc1, Δ ahd6 Δ fdc1 Δ pad1 Δ aro10 Δ ald6Apdc1 or any other combination of these deletions.

Enzymes involved in the biosynthesis pathway of vanilloids from aromatic amino acids

In the present description, enzymes are identified by their specific activities. This definition thus includes all polypeptides that have the defined specific activity also present in other organisms. Enzymes with similar activities can be identified by homology. Using the references given in GenBank for known genes, those skilled in the art are able to determine the equivalent genes in other organisms, bacterial strains, yeasts, plants, etc. This routine work is advantageously done using consensus sequences that can be determined by carrying out sequence alignments with genes derived from other microorganisms, and designing degenerate probes to clone the corresponding gene in another organism. These methods of molecular biology are well known to those skilled in the art, and are described, for example, in Green & Sambrook (Molecular Cloning: a Laboratory Manual. 4th ed. 2012 Cold Spring Harbor Lab., Cold Spring Harbor, New York).

According to a first embodiment of the invention, the artificial vanillin pathway uses endogenous aromatic amino acids (AAA) as primary substrates. Endogenously produced phenylalanine or tyrosine is diverted to the synthesis of vanillin.

Exogenous phenylalanine and/or tyrosine can also be added into the culture medium of the yeast. Six enzymes are required for the conversion of L-phenylalanine/tyrosine to vanillin (see figures 1A and 1B).

In particular, phenylalanine is converted to coumarate by the successive actions
5 of the enzymes phenylalanine ammonia lyase (PAL) and cinnamate-4-hydroxylase (C4H). The following step is the reduction chain reaction of the coumaric acid leading to the 4-hydroxybenzaldehyde. The reaction is initiated by the activation of coumaric acid to coumaroyl-CoA provided by 4CL enzyme (4-coumarate coenzyme A ligase), followed by a β -oxidation performed by ECH enzyme (enoyl-CoA hydratase/aldolase, an enzyme of a
10 crotonase family). The next step is the hydroxylation on 3-position of the phenyl ring carried out by HBH enzyme (hydroxybenzoic acid hydroxylase, 3-monooxygenase enzyme family). The final step is the 3-O-methylation leading to vanillin final product. This step is catalyzed by the COMT enzyme (caffeic acid O-methyltransferase, O-methyltransferase enzyme family). In order to lower endogenous conversion of aldehyde intermediate
15 product, carboxylic acid reductase (CAR) protein is preferentially added. The CAR enzyme requires activation by phosphopantetheinylation, and this is achieved by co-expression of a phosphopantetheinyl transferase (PPTase).

According to a specific embodiment of the invention, the aromatic acid is phenylalanine, and the recombinant yeast comprises a set of exogenous nucleic acids
20 coding for the following enzymes: phenylalanine ammonia lyase (PAL), cinnamate-4-hydroxylase (C4H), 4-coumarate coenzyme A ligase (4CL), enoyl-coA hydratase/aldolase (ECH), a hydroxybenzoic acid hydroxylase (HBH) and a caffeic acid O-methyltransferase (COMT).

According to another specific embodiment of the invention, the aromatic
25 amino acid is tyrosine, and the genetically modified yeast comprises a set of exogenous nucleic acids coding for the following enzymes: tyrosine ammonia lyase (TAL), cinnamate-4-hydroxylase (C4H), 4-coumarate coenzyme A ligase (4CL), enoyl-CoA hydratase/aldolase (ECH), a hydroxybenzoic acid hydroxylase (HBH), and a caffeic acid O-methyltransferase (COMT).

30 The phenylalanine ammonia lyase (PAL) is an enzyme catalyzing the tyrosine deamination reaction classified EC 4.3.1.23. The tyrosine ammonia lyase (TAL) is an enzyme catalyzing the phenylalanine deamination reaction classified EC 4.3.1.24. Another

enzyme is known for catalyzing the phenylalanine or tyrosine deamination reaction, classified EC 4.3.1.25, and called PAL/TAL. In a specific embodiment, the PAL enzyme is issued from *Petunia species* or *Populus species*. In another specific embodiment, a PAL/TAL enzyme from maize (Rösler, Krekel, Amrhein, Schmid. “Maize phenylalanine ammonia-lyase has tyrosine ammonia-lyase activity” Plant Physiol. 1997 January; 113(1): 175–179) is introduced into the recombinant yeast. This enzyme is able to convert phenylalanine into cinnamic acid and tyrosine into coumaric acid.

The cinnamic acid hydroxylase (C4H) is an enzyme catalyzing the cinnamate 4-hydroxylation, classified EC 1.14.13.11. It is a P450-dependent enzyme. In a specific embodiment, the C4H enzyme is issued from *Petunia species* or *Glycin species*.

The 4-coumarate coenzyme A ligase (4CL) is an enzyme catalyzing the CoA esterification of coumaric acid, classified EC 6.2.1.12. This enzyme belongs to the family of ligases, specifically those forming carbon-sulfur bounds as acid-thiol ligases. In a specific embodiment, the 4CL enzyme is issued from *Populus species*.

The enoyl-CoA hydratase/aldolase (ECH) is an enzyme catalyzing the hydration of 2-trans-enoyl-CoA into 3-hydroxyacyl-CoA, belonging to the family of crotonases, and is classified EC 4.2.1.17. In a specific embodiment, the ECH enzyme is issued from *Pseudomonas fluorescens* or *Azotobacter vinelandii*.

The hydroxybenzoic acid hydrolase (HBH) is an enzyme catalyzing the hydroxylation of 4-hydroxybenzaldehyde. The HBH enzyme is also known as 4-hydroxybenzoate-3-monooxygenase (EC 1.14.13.2). In a specific embodiment, the HBH enzyme is issued from *Pseudomonas fluorescens* or *Azotobacter vinelandii*.

The caffeic acid O-methyltransferase (COMT) is an enzyme catalyzing the chemical conversion of 3,4-dihydroxy-trans-cinnamate (caffeate) to 3-methoxy-4-hydroxy-trans-cinnamate (ferulate). This enzyme is also capable of converting protocatechuic aldehyde to vanillin. This enzyme is classified EC 2.1.1.68. Eventually, the same reaction can be catalyzed by the caffeoyl-CoA O-methyl transferase belonging to EC 2.1.1.104.

Preferentially, the genetically modified yeast further comprises exogenous nucleic acid coding for a carboxyreductase (CAR, EC1.2.99.6) and a phosphopantetheinyl transferase (PPTase). In a specific embodiment, the CAR enzyme is issued from the organism *Nocardia iowensis* or *Micobacterium smegmatis* and the PPTase is issued from *Nocardia iowensis* or *Micobacterium smegmatis*.

According to a specific embodiment of the invention, and as described in example 1, the artificial vanillin pathway is introduced into the locus bud31 of modified yeast cells. Nine synthetic DNA fragments are needed to introduce artificial vanillin pathway in yeast genome (Figure 4). All genes possess one promoter and one terminator.

5 Gene promoters allow constitutive expression of proteins from the pathway. Fragment hybridizes and recombines together in the region of the entire ORF of each couple of gene. By that way, the whole pathway is assembled in the yeast cell, and then integrated into the chromosome. Tg 5' and Tg 3' correspond to the target sequences on the yeast genome that corresponds to the insertion site in the BUD 31 locus of the yeast chromosome triggering

10 the homologous integration into the desired chromosome site. HIS3 and LEU2 are the flanking markers enabling the double selection of the recombinant pathway. After assembly of the fragments by homologous recombination in yeast, a functional complete pathway of 30Kbp is reconstituted and the double selection permits the isolation of recombinants.

15 In a particular embodiment of the invention, the genetically modified yeast further comprises exogenous nucleic acids coding for a vanillyl alcohol oxidase (EC 1.1.3.38) that catalyzes the chemical conversion of vanillyl alcohol and O₂ into vanillin and H₂O₂. Presence of this enzyme allows a better yield of vanillin since all vanillyl alcohol produced via the artificial biosynthetic pathway can be converted into vanillin. A

20 specifically preferred vanillyl alcohol oxidase is the enzyme described in patent US 5,721,125.

Enzymes involved in the biosynthesis pathway of vanilloids from dehydroshikimic acid

25 According to a second embodiment of the invention, the artificial vanillin pathway derives from the acid dehydroshikimic pathway, such as disclosed in Hansen *et al.*, 2009 (cited above). In particular, the genetically modified yeast comprises a set of exogenous nucleic acids coding for the following enzymes able to convert an acid dehydroshikimic into a vanilloid: a 3-dehydroshikimate dehydratase (3DSD), an aromatic

30 carboxylic acid reductase (ACAR) and a catechol O-methyltransferase or a caffeic acid O-methyltransferase (both designed as COMT). Preferentially, the genetically modified yeast further comprises a phosphopantetheinyl transferase (PPTase).

In a specific aspect of the invention, and for all disclosed modified yeast, the exogenous nucleic acid encoding the caffeic acid O-methyltransferase (COMT) is preferentially issued from the plants *Medicago sativa*, *Vanilla planifolia* or *Rosa chinensis*. Interestingly, these enzymes have a high selectivity for methylation, and their
5 final product is pure vanillin (100% of selectivity) instead of a mixture of vanillin and isovanillin.

Recombinant yeasts

In the sense of the present invention, it is understood that exogenous nucleic
10 acids encoding polypeptides that are introduced into the yeast are "codon-optimized" to be expressed efficiently into the yeast. The man skilled in the art knows some 'Codon Optimization Tools' that allows the preparation of synthetic genes from one host organism for an optimized expression in another, in particular in the yeast according to the invention.

15 Exogenous nucleic acids encoding the heterologous polypeptides are introduced into the yeast by transformation and this manipulation results into incorporation and expression of exogenous genetic material. Transformation of yeast can be performed by means well known by the man skilled in the art : yeast cells may be treated with enzymes to degrade their cell walls, may be exposed to alkali cations such as
20 lithium acetate, or to polyethylene glycol. Electroporation using electric shock is also a technique that allows exogenous DNA to enter into yeasts. Some of useful techniques are reviewed in the review (Kawai S, Hashimoto W, Murata K. "Transformation of *Saccharomyces cerevisiae* and other fungi: methods and possible underlying mechanism." Bioeng Bugs. 2010 Nov-Dec;1(6):395-403).

25 In a preferred embodiment of the invention, transformations of competent yeast cells are performed as described by Gietz and Woods (Gietz and Woods. "Transformation of yeast by the LiAc/ss Carrier DNA/PEG method." Meth. Enzymol., 350, 87-96).

An equimolar mix of fragments is used to transform yeast.

30 According to a specific aspect of the invention, the polypeptides are encoded by exogenous genes stably integrated into the yeast genome.

Preferentially, the nucleic acids encoding heterologous polypeptides are integrated into the yeast genome by the technique of homologous recombination. An example of integration into genome of the yeast is shown in figure 4. Preferably, in a set of exogenous nucleic acids as described herein, the exogenous nucleic acids are assembled
5 into a cluster and said cluster is integrated into the yeast genome. In particular, polynucleotides encoding a series of enzymes are provided as full-length polynucleotides of different origin, wherein :

- the 5'-terminal sequence is of the polynucleotide encoding the first enzyme in the series, and

- 10 - the 3'-terminal sequence is of the polynucleotide encoding the last enzyme in the series.

Most preferably, each nucleic acid encoding a polypeptide included in an exogenous nucleic acid as described herein comprises, from its 5'-end to its 3'-end, (1) a regulatory sequence comprising one or more promoter sequences, the said regulatory
15 sequence being functional in the yeast organism wherein it has been introduced, (ii) an ORF encoding a polypeptide of interest and (iii) one or more transcription terminator sequences.

The polynucleotides encoding the enzymes may be in the order of the consecutive enzymatic reactions required for the biosynthesis pathway which is under
20 consideration, or in any other order, provided that the required enzymes are actually produced.

The invention also relates to a recombinant yeast having a reduced alcohol dehydrogenase activity, in the genome of which has been inserted a DNA construct comprising :

- 25 - one or more nucleic acids encoding a phenylalanine ammonia lyase (PAL) or a tyrosine ammonia lyase (TAL),

- one or more nucleic acids encoding a cinnamate-4-hydrolase (C4H),

- one or more nucleic acids encoding a 4-coumarate coenzyme A ligase (4CL),

- one or more nucleic acids encoding a enoyl-CoA hydratase/aldolase (ECH),

- 30 - one or more nucleic acids encoding a hydroxybenzoic acid hydroxylase (HBH) and

- one or more nucleic acids encoding a caffeic acid O-methyltransferase (COMT),

and wherein in said recombinant yeast the decarboxylation of phenylacrylic acids is attenuated, in particular said yeast does not comprise a functional gene *fdc1* and/or *pad1*.

The invention also relates to a recombinant yeast such as described above, in the genome of which has been inserted a DNA construct comprising:

- one or more exogenous nucleic acids encoding a carboxyreductase (CAR)

and

- one or more exogenous nucleic acids encoding a heterologous phosphopantetheinyl transferase (PPTase).

The invention also relates to a recombinant yeast having a reduced alcohol dehydrogenase activity, in the genome of which has been inserted a DNA construct comprising:

- one or more nucleic acids encoding a 3-dehydroshikimate dehydratase (3DSD),

- one or more nucleic acids encoding an aromatic carboxylic acid reductase (ACAR)

- one or more nucleic acids encoding a phosphopantetheinyl transferase (PPTase), and

- one or more nucleic acids encoding a caffeic acid O-methyltransferase (COMT),

and wherein said recombinant yeast does not comprise a functional gene *ald6*.

According to the invention, the yeast is from the genera *Saccharomyces* or *Schizosaccharomyces*. In particular the modified yeast belongs to the following species: *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Pichia pastoris*, *Yarrowia lipolytica*.

Culture process for producing a vanilloid or a precursor thereof

The present invention is also related to a method for producing a vanilloid or a precursor thereof, comprising the following successive steps:

a) providing a recombinant yeast comprising enzymes necessary for vanilloid biosynthesis,

b) cultivating said yeast in an appropriate medium comprising a source of carbon chosen from glucose, fructose, galactose, arabinose, lactose, ethanol, glycerol, and mixtures thereof; and

c) isolating said vanilloid or precursor thereof from the medium.

The phrase “recombinant yeast comprising enzymes necessary for vanilloid biosynthesis” designates a genetically modified yeast, expressing enzymes allowing the biosynthesis of a vanilloid in said yeast by any biosynthesis pathway.

In particular, exogenous nucleic acids are inserted into said yeast, said nucleic acids coding for enzymes involved in the biosynthesis pathway of a vanilloid, being chosen among the following groups of enzymes:

- enzymes able to convert an aromatic amino acid into a vanilloid; or
- enzymes able to convert an acid dehydroshikimic into a vanilloid.

In particular, said recombinant yeast does not comprise a functional gene *adh6*. More particularly, said yeast does not comprise a functional gene *adh6* neither a functional gene *fdc1*. More particularly, said yeast does not comprise a functional gene *adh6* neither a functional gene *pad1*.

In a preferred aspect of the invention, said recombinant yeast does not comprise functional genes *adh6*, *pad1*, *fdc1*.

In another preferred aspect of the invention, said recombinant yeast does not comprise functional genes *adh6*, *pad1*, *fdc1*.

In another preferred aspect of the invention, said recombinant yeast does not comprise functional genes *adh6*, *pad1*, *fdc1*.

In another preferred aspect of the invention, said recombinant yeast does not comprise functional genes *adh6*, *fdc1*, *pad1* and *aro10*.

In a specific aspect of the invention, at least one of the genetically modified yeasts described in the present application is cultivated in this method.

Terms ‘biosynthesis’, ‘bioconversion’ ‘fermentative production’ and ‘production’ have the same meaning in the sense of the invention, and designates the production of at least one vanilloid by a recombinant yeast cultivated in appropriate conditions.

The term "precursor" as used herein refers to a substrate molecule that is subject to enzymatic reactions and can be converted into a product by this specific reaction. By "a precursor of vanilloid" is meant a precursor or an intermediate in the biosynthesis pathway of a vanilloid, which is in particular a hydroxybenzaldehyde or a
5 respective acid or a respective alcohol, an aromatic amino acid, or an acid dehydroshikimic. This term includes in particular: protocatechuic aldehyde, 4-hydroxyaldehyde, protocatechuic alcohol, 4-hydroxybenzoic acid, 4-hydroxybenzyl alcohol, protocatechuic acid, protocatechuic alcohol, 4-hydroxyaldehyde, 4-hydroxyl alcohol, cinnamic acid, coumaric acid, caffeic acid and ferulic acid.

10 According to the invention the term 'cultivating' is used to denote the growth of yeast. The term "appropriate medium" designates a medium (e.g., a sterile, liquid media) comprising nutrients essential or beneficial to the maintenance and/or growth of the cell such as carbon sources or carbon substrates, nitrogen sources, urea, ammonium sulfate, ammonium chloride, ammonium nitrate and ammonium phosphate; phosphorus
15 sources; metal salts, for example magnesium salts, cobalt salts and/or manganese salts; as well as growth factors such as amino acids and vitamins. Typical medium is composed of 10 g/l of yeast extract, 20 g/l of peptone and 20 g/l of carbon source.

The term "source of carbon" or "carbon substrate" according to the present invention denotes any source of carbon that can be used by those skilled in the art to
20 support the normal growth of a yeast. Yeasts use sugars as their main carbon and energy source, but non-conventional carbon sources can be accepted too. The major source for energy production in the yeast is glucose and glycolysis is the general pathway for conversion of glucose to pyruvate, whereby production of energy in form of ATP is coupled to the generation of intermediates and reducing power in form of NADH for
25 biosynthetic pathways. Fructose is also a hexose that can be uptake and metabolized by yeast. Galactose is a 'non-conventional' nutrient for yeast, which however can be used as a sole carbon source when glucose is absent from the medium. In yeast cells supplied with glucose, the GAL genes are repressed. They are activated a thousand fold in cells that are starved for glucose, and this one of the few pathways in yeast which is regulated in a nearly
30 'all-or-nothing' mode. Enzymes implied in conversion of galactose to glucose 1-phosphate are: galactose kinase, galactose-1-phosphate-uridylyltransferase, and UDP-glucose-4-epimerase. Glycerol functions as a compatible solute in osmoregulation in osmotolerant

yeasts that are capable of growing in high sugar or salt environments. Many types of yeast can grow on glycerol as a sole carbon source under aerobic conditions, but glycerol is a non-fermentable carbon source for many yeast species, including *S. cerevisiae*. To serve as a carbon source, glycerol after internalization has to convert by glycerol kinase to glycerol-3-phosphate, which is then transformed into dihydroxyacetone phosphate by glycerol-3-phosphate dehydrogenase that is a substrate in gluconeogenesis.

Many types of yeast have the capability of metabolizing ethanol or methanol. In presence of ethanol ADH2, the enzyme that converts ethanol back into acetaldehyde, is expressed. Acetaldehyde is subsequently converted into acetyl-CoA, the substrate for the citric acid cycle.

The source of carbon is selected among the group consisting of glucose, fructose, mannose, xylose, arabinose, galactose, lactose, ethanol, cellobiose, glycerol and polysaccharides such as cellulose, and mixtures thereof. An especially preferred substrate is ethanol.

According to another preferred embodiment of the invention, the volume of the culture is at least one liter. Indeed, when the culture volume is low (about 1 ml), vanillin is produced but is quickly converted to vanillic acid. This conversion is not observed in stable continuous culture in larger volume, of at least one liter.

The cultivation process comprises a fermentation process (batch, fed-batch or continuous mode) under controlled conditions of pH, temperature, pO₂ and aeration, conditions well known to a person skilled in the art.

In a specific embodiment, the vanilloid isolated from the medium is selected from the group consisting of: vanillin, vanillic acid, vanillyl alcohol and vanillin-glycoside.

In another specific embodiment, the vanilloid precursor isolated from the medium is selected from the group consisting of protocatechuic aldehyde, protocacheuic acid, protocacheuic alcohol, 4-hydroxyaldehyde, 4-hydrobenzoic acid, 4-hydroxyl alcohol, cinnamic acid, coumaric acid, caffeic acid and ferulic acid.

According to a specific embodiment, the present invention is related to a method for producing a vanilloid comprising the following successive steps:

a) providing a recombinant yeast comprising enzymes necessary for vanilloid biosynthesis,

b) cultivating said yeast in an appropriate medium comprising a source of carbon chosen from glucose, fructose, galactose, arabinose, lactose, ethanol, glycerol, and mixtures thereof;

c) eventually, isolating said vanilloid from the medium;

and further comprising a step d:

d) converting precursors of vanilloid remaining in the medium into vanilloid.

Culture process using bi-phase culture

Vanillin cytotoxicity is a bottleneck in the microbial bioconversion including unicellular microorganisms such as yeasts. We proceeded to an extractive bioconversion of vanillin using polypropylene glycol. In the two-phase process, the bioconversion is performed in a medium comprising an aqueous phase containing the biocatalyst (cells) and hydrophobic organic solvent (water-immiscible phase) containing organic compounds as substrates and / or products. The organic solvent acts as a reservoir for hydrophobic compounds. The biphasic system enables continuous supply of substrate depending on the metabolic demand and without inhibiting the enzymatic reaction. Moreover, the product of the reaction is progressively extracted into the organic phase. A major advantage is to reduce the cytotoxicity of substrates and/or products of the bioconversion. This system allows raising yields of bioconversion.

PPG is a polymer of propylene glycol also called propane-1,2-diol or 1,2-propanediol. When aqueous solutions (culture medium) and PPG are mixed, the mixture becomes turbid and when it is left for a while, phase separation occurs. Aqueous two-phase system therefore provides mild conditions for cells and proteins. The interfacial tension is very low compared with that of organic solvent /water system. The phase system forms a finely dispersed emulsion with large surface area and short diffusion distances from one phase to the other. This means that conditions are very favorable for mass transfer.

This method is particularly convenient for improving the production of vanilloids or precursors thereof in such unicellular microorganisms.

The phrases “two-phase system”, or “bi-phase medium”, or “bi-phase culture”, or “bi-phasic system” are considered herein to have the same meaning.

Thus, the present invention is also related to a method for producing a

vanilloid or precursor thereof in a unicellular microorganism able to produce said vanilloid or precursor thereof, comprising a step of cultivating said microorganism in an appropriate medium comprising an aqueous phase and a water immiscible phase, said water immiscible phase preferably comprising or consisting of a polymer of propylene glycol, a fatty alcohol, a fatty acid, a fatty ester or an oil, and mixtures thereof.

For example, a fatty acid may be selected from a group comprising or consisting of octanoic acid and oleic acid.

For example, a fatty alcohol may be selected from a group comprising or consisting of n-octanol, lauryl alcohol, and oleyl alcohol.

The phrase “unicellular microorganism” designates any unicellular microorganism, including bacteria, archaea, protozoa, unicellular algae and unicellular fungi, that is either prokaryotic or eukaryotic; which also includes wild-type, naturally-occurring, non-naturally-occurring, genetically modified or recombinant microorganisms such as recombinant yeasts.

A “genetically modified microorganism” includes microorganisms obtained or obtainable by random or directed mutagenesis.

Examples of unicellular microorganisms include strains belonging to the order of *Actinomycetales*, which includes a suborder selected from the group consisting of *Actinomycineae*, *Actinopolysporineae*, *Catenulisporineae*, *Corynebacterineae*, *Frankineae*, *Glycomycineae*, *Kineosporiineae*, *Micrococcineae*, *Micromonosporineae*, *Propionibacterineae*, *Pseudonocardineae*, *Streptomycineae* and *Streptosporanginea*; which includes recombinant strains from the genus *Amycolatopsis* or *Streptomyces*; which includes strains *Amycolatopsis* sp. ATCC 39116, HR167 and DSMZ 9992.

Still, and in a non-limitative manner, other examples of unicellular microorganisms include yeasts, lactic acid bacteria and *Escherichia Coli* strains.

Some of the above-mentioned unicellular microorganisms are naturally able to produce a vanilloid or precursor thereof, while others require to be genetically modified and/or cultivated in a manner that is suitable for expressing enzymes allowing the biosynthesis of said vanilloid or precursor.

The phrase “unicellular microorganism able to produce a vanilloid or precursor thereof” designates, in particular, unicellular microorganisms expressing

enzymes allowing the biosynthesis of a vanilloid or precursor thereof by any biosynthesis pathway.

According to one preferred embodiment, the unicellular microorganism is a recombinant yeast comprising enzymes necessary for vanilloid biosynthesis.

According to another preferred embodiment, the unicellular microorganism is a strain belonging to the order of *Actinomycetales*.

Strains belonging to the order of *Actinomycetales* which are specifically considered are strains wherein at least one gene encoding an enzyme having vanillin reductase activity is non-functional.

The phrase "recombinant yeast comprising enzymes necessary for vanilloid biosynthesis" designates a genetically modified yeast, expressing enzymes allowing the biosynthesis of a vanilloid in said yeast by any biosynthesis pathway.

In particular, exogenous nucleic acids are inserted into said microorganism, including said yeast, said nucleic acids coding for enzymes involved in the biosynthesis pathway of a vanilloid, being chosen among the following groups of enzymes:

- enzymes able to convert an aromatic amino acid into a vanilloid; or
- enzymes able to convert an acid dehydroshikimic into a vanilloid.

In particular, said recombinant yeast does not comprise a functional gene *adh6*. More particularly, said yeast does not comprise a functional gene *adh6* neither a functional gene *fdc1*. More particularly, said yeast does not comprise a functional gene *adh6* neither a functional gene *fdc1*. More particularly, said yeast does not comprise a functional gene *adh6* neither functional genes *fdc1* nor *pad1*.

In another preferred aspect of the invention, said recombinant yeast does not comprise functional genes *adh6*, *pad1*, *pdcl*.

In another preferred aspect of the invention, said recombinant yeast does not comprise functional genes *adh6*, *fdc1*, *pad1* and *aro10*.

In a specific aspect of the invention, the genetically modified yeasts described in the present application are cultivated according to this method, in a bi-phase medium, wherein said medium comprises two phases, an aqueous phase and a water immiscible phase, said water immiscible phase preferably consisting of: polymer of propylene glycol, fatty alcohol, fatty acid, fatty ester or an oil.

Thus, the present invention is also related to a method for producing a
5 vanilloid or a precursor thereof, and/or as defined above, comprising the following steps:

a) providing a recombinant yeast comprising enzymes necessary for vanilloid
biosynthesis,

b) cultivating said yeast in an appropriate medium comprising a precursor
chosen from glucose, fructose, galactose, arabinose, lactose, ethanol, glycerol, and
10 mixtures thereof; and

c) isolating said vanilloid or precursor thereof from the medium,

wherein the step b) is performed in an appropriate medium comprising two
phases, an aqueous phase and a water immiscible phase, said water immiscible phase
preferably comprising or consisting of polymer of propylene glycol, fatty alcohol, fatty
15 acid, fatty ester or an oil.

According to some embodiments, the vanilloid or precursor thereof in step c)
is isolated from the aqueous phase only, from the water immiscible phase only, or from a
mixture thereof.

The appropriate medium comprises precursors of the step b) or mixtures of
20 thereof, either in pure grade or technical grade.

The cultivation process comprises a fermentation process (batch, fed-batch or
continuous mode) under controlled conditions of pH, temperature, pO_2 and aeration,
conditions well known to a person skilled in the art.

According to one exemplary embodiment, said water immiscible phase
25 comprises or consists of polymers of polypropylene glycol (PPG). A polymer of
polypropylene glycol in the sense of the invention may include polymers of PPG having
an average molecular weight ranging for instance from about 200 to about 4000 g/mol,
and mixtures thereof; which includes 250; 425; 725; 1000; 1200; 2000; and 2700 g/mol.

In a non-limitative manner, such polymers of PPGs are commercially available
30 under the DOW-P reference (The Dow Chemical Company©), and/or commercialized by
Sigma Aldrich©.

One example of such polymer of PPG is PPG 1200.

Still, in a non-limitative manner, the water-immiscible phase is obtained by mixing a solution comprising a hydrophobic organic solvent, such as a polymer of polypropylene glycol, with an appropriate medium in a manner suitable for phase separation to occur; thereby forming in said appropriate medium an aqueous phase and a water immiscible phase.

In a non-limitative manner, a precursor can be added to the appropriate medium either before the occurrence of phase separation, during phase separation or after separation. In view of the above, and still in a non-limitative manner, the precursor can be added before mixing or after mixing the solution comprising said hydrophobic organic solvent.

According to said embodiment, the precursor is preferably chosen from: glucose, fructose, galactose, arabinose, lactose, ethanol, glycerol, and mixtures thereof.

The hydrophobic organic solvent, such as the polymer of polypropylene glycol, may be present in the solution, prior to mixing, in varying amounts ranging from 0.1% to 90% in volume percent of the total volume of said solution; which includes 0.1%; 1%; 5%; 10%; 20%; 30%; 40%; 50%; 60%; 70%; 80% and 90% in volume percent of the total volume of said solution.

According to one preferred embodiment, the present invention is related to a method for producing a vanilloid or a precursor thereof in said unicellular microorganism, including said recombinant yeast, comprising a step of cultivating said microorganism and/or yeast in an appropriate medium comprising at least two phases:

- an aqueous phase comprising a precursor chosen from glucose, fructose, galactose, arabinose, lactose, ethanol, glycerol, and mixtures thereof; and

- a water immiscible phase, comprising or consisting of a hydrophobic organic solvent such as a solvent selected from: a polymer of propylene glycol, a fatty alcohol, a fatty acid, a fatty ester or an oil, and mixtures thereof.

According to one exemplary embodiment, the present invention is related to a method for producing a vanilloid or a precursor thereof in said microorganism and/or yeast, comprising a step of cultivating said microorganism and/or yeast in an appropriate medium comprising at least two phases:

- an aqueous phase comprising a precursor chosen from glucose, fructose, galactose, arabinose, lactose, ethanol, glycerol, and mixtures thereof; and
- a water immiscible phase, comprising or consisting of a polymer of propylene glycol, such as PPG 1200;

5 wherein said appropriate medium is optionally obtained by mixing a solution comprising or consisting of said polymer of polypropylene glycol, with an appropriate medium in a manner suitable for phase separation to occur; thereby forming said appropriate medium said aqueous phase and said water immiscible phase.

10 **Composition comprising vanilloid or a precursor thereof**

The present invention is also related to a composition comprising a vanilloid or a precursor thereof obtainable by the method as disclosed above, and to the use of said composition as a flavoring in the human and animal nutrition field, in pharmacy, and as a
15 fragrance in the cosmetics, perfumery and detergency industries.

Vanillin is a compound well-known for its aromatic properties. However, the flavor profile of an aromatic composition is dependent on for instance byproducts and impurities, and consequently on the preparation process. The composition comprising a vanilloid or a precursor thereof obtainable by the method of the invention may thus show
20 aromatic notes different from other aromatic composition.

Thus, the present invention also relates to a composition comprising a vanilloid obtainable or obtained by one of the methods as disclosed above, from said microorganism and/or yeast, or from the culture supernatant thereof, which includes clarified culture supernatant.

25 A composition comprising said vanilloid, in the sense of the invention, may include pharmaceutical compositions, cosmetic compositions and/or compositions which are suitable for human and animal nutrition, including oral and topical administration.

According to some embodiments the composition is a culture supernatant or a clarified culture supernatant.

30 According to some embodiments, the composition comprising said vanilloid is obtainable or obtained by cultivating said microorganism and/or yeast in an appropriate medium comprising an aqueous phase and a water immiscible phase as previously

described, wherein said composition is collected from the aqueous phase and/or the water immiscible phase.

EXAMPLES

5

Example 1. Introduction of a vanillin biosynthesis pathway into $\Delta adh6$ yeast strain

All of the *Saccharomyces cerevisiae* strains used in this work were isogenic haploids from BY4741 and were obtained from EUROSCARF (haploid a-mater BY00 or α -mater BY10). Yeast strain BY47 derived from a strain collection that contains knock outs of auxotrophic (-ura3, -leu2, -his3) marker genes. Enrichment and propagation of clones were made in YPD liquid cultures (10 g/l bacto-yeast extract, 20 g/l bacto-peptone and 2% dextrose) at 30°C. Recombinants were selected on dropout agar plates (YNB + CSM) in the absence of uracil, leucine or histidine. The gene defects in uracil, histidine and leucine biosynthetic pathway result in auxotrophy. All ORF used for the pathway were synthesized by GeneArt (Germany) and then amplified by PCR. Amplification was performed using high fidelity PhusionTaq (New England Biolabs). Amplicons were cleaned up by using the Wizard PCR Clean-up System (Promega) and used for transformation assays.

15
20

The following genes were used:

Table 1: Reference identities of the genes/proteins used in this example

Protein	Species	Reference NCBI	Length in amino acids	SEQ ID
PAL	<i>Petroselinum crispum</i>	X81158.1 GI:534892	718 AA	NO. 2
	<i>Populus trichocarpa x Populus deltoides</i>	L11747.1 GI:169453	715 AA	NO. 1
C4H	<i>Glycine max</i>	FJ770468.1 GI:225194700	506 AA	NO. 3
	<i>Petroselinum crispum</i>	Q43033.1 GI:3915088	506 AA	NO. 4
4CL	<i>Populus tremuloides</i>	AF041049.1 GI:3258634	570 AA	NO. 5
ECH	<i>Pseudomonas fluorescens</i>	AJ536325.1	276 AA	NO. 6
	<i>Azotobacter vinelandii</i>	YP 002798614.1 GI:226943541	276 AA	NO. 7
HBH	<i>Pseudomonas aeruginosa</i>	ZP_07797957.1 GI:313112178	394 AA	NO. 8
	<i>Azotobacter vinelandii</i>	NC 012560.1 GI:226943557	394 AA	NO. 9
COMT	<i>Medicago sativa</i>	ACYO6328.1 GI:261889456	365 AA	NO. 10
	<i>Vanilla planifolia</i>	AAS64572.1 GI:45444737	365 AA	NO. 11
CAR	<i>Nocardia iowensis</i>	AAR91681.1 GI:40796035	1174 AA	NO. 12
PPTase	<i>Nocardia iowensis</i>	AB183656.1 GI:114848891	222 AA	NO. 13

The complete pathway was assembled using a somatic *in vivo* DNA assembly. Eight fragments containing (F1 to F8) the 6 genes of the vanillin pathway were designed by computational analysis. Fragments hybridize and recombine together in the region of the entire ORF of each couple of gene. That way enabled the whole pathway assembly in the yeast cell, and its integration into the chromosome. T 5' and T 3' correspond to the bud31 target sequences on the yeast genome allowing homologous integration onto the chromosome locus. Overlapping sequences correspond to the 5' part and the 3' part of the marker genes. HIS6 and LEU2 are the flanking markers enabling the double selection of the recombinant pathway. Each gene is under the control of one promoter and one terminator sequences allowing its expression in yeast cells. After assembly of the fragments by homologous recombination in yeast, a functional complete pathway is reconstituted and the double selection permits the isolation of recombinants.

Transformations of competent yeast cells were performed as described by Gietz and Woods (Transformation of yeast by the LiAc/ss Carrier DNA/PEG method. Meth. Enzymol., 350, 87-96). Analysis of clones revealed that the genes had assembled, resulting in correct ORFs.

HPLC was used to measure the production of vanillin and pathway intermediates in *S. cerevisiae* cultures. Analysis showed that chromatograms from cells expressing vanillin pathway genes contained additional peaks compared to a yeast control. These peaks were identified as cinnamic acid, coumaric acid, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, vanillic acid and vanillin were identified. When the culture is not supplemented by exogenous phenylalanine, the PAL protein uses endogenous phenylalanine. The pathway is fully functional as 3,4-dihydroxybenzoic acid and vanillic acid were detected but the yield is reduced 4 times when compared to the phenylalanine supplemented medium. Only 1 μ M of vanillin was detected in the non-adapted strain.

A carboxylic acid reductase protein (CAR) was added with its activating coupling protein phosphopantetheinyl transferase (PPTase). The bicistronic construction was integrated to yeast genome by homologous recombination using URA as selection marker into ADH6 locus. Prevention of reduction of vanillin to vanillyl alcohol was achieved by knockout of the host alcohol dehydrogenase ADH6. In order

to take off selection marker, flanking repeated sequences were added to URA3 gene in order to permit URA3 gene excision. Recombinant cells were selected on –URA selective medium and the right integration was verified by PCR.

5 **Example 2-Improvement of aldehyde stability in *S. cerevisiae***

Vanillin biosynthesis pathway used in our production process implies three aldehydes including the final product vanillin. One way to increase vanillin production yield during a bioproduction using *S. cerevisiae* is to reduce aldehyde oxidase or aldehyde reductase endogenous activity in the production strain. A large number of
10 Euroscarf deletion strains have been screened; these strains were firstly selected on the basis of a biochemical study and rational targeting of potentially interesting genes such as ADH (alcohol dehydrogenases), ALD (aldehyde dehydrogenases), GRE (aldehyde reductases), OYE, AAD (aryl alcohol dehydrogenase) & ARI (aldehyde reductase).

15 **Table 2: Euroscarf deletion strains**

Gene Name	Description	Systematic Name	Alias(es)	Euroscarf Acc N°
ADH5	Alcohol dehydrogenase isoenzyme V	YBR145W	alcohol dehydrogenase ADH5	Y03284
ADH7	NADPH-dependent medium chain alcohol dehydrogenase	YCR105W	ADHVII	Y05821
SFA1	Bifunctional alcohol dehydrogenase and formaldehyde dehydrogenase	YDL168W	ADH5, bifunctional alcohol dehydrogenase/S-(hydroxymethyl)glutathione dehydrogenase	Y03866

ADH4	Alcohol dehydrogenase isoenzyme type IV	YGL256W	NRC465, ZRG5, alcohol dehydrogenase ADH4	Y04623
ADH3	Mitochondrial alcohol dehydrogenase isozyme III	YMR083W	alcohol dehydrogenase ADH3	Y06217
ADH2	Glucose-repressible alcohol dehydrogenase II	YMR303C	ADR2, alcohol dehydrogenase ADH2	Y00891
ADH 6	NADPH-dependent medium chain alcohol dehydrogenase	YMR318C	ADHVI	Y06460
ALD5	Mitochondrial aldehyde dehydrogenase	YER073W	aldehyde dehydrogenase (NAD(P)(+)) ALD5	Y00213
ALD3	Cytoplasmic aldehyde dehydrogenase	YMR169C	aldehyde dehydrogenase (NAD(+)) ALD3	Y00752
ALD2	Cytoplasmic aldehyde dehydrogenase	YMR170C	aldehyde dehydrogenase (NAD(+)) ALD2	Y00753
ALD4	Mitochondrial aldehyde dehydrogenase	YOR374W	ALD7, ALDH2, aldehyde dehydrogenase (NADP(+)) ALD4	Y01661
ALD 6	Cytosolic aldehyde dehydrogenase	YPL061W	ALD1, aldehyde dehydrogenase (NADP(+)) ALD6	Y02767

GRE3	Aldose reductase	YHR104W	trifunctional aldehyde reductase/xylose reductase/glucose 1- dehydrogenase (NADP(+))	Y01932
GRE2	3-methylbutanal reductase and NADPH-dependent methylglyoxal reductase	YOL151W	methylglyoxal reductase (NADPH-dependent) GRE2	Y07320
OYE2	Conserved NADPH oxidoreductase containing flavin mononucleotide (FMN)	YHR179W	Old Yellow Enzyme	Y02873
OYE3	Conserved NADPH oxidoreductase containing flavin mononucleotide (FMN)	YPL171C	ZRG6, Old Yellow Enzyme	Y02081
AAD3	Putative aryl-alcohol dehydrogenase	YCR107W		Y07253
AAD4	Putative aryl-alcohol dehydrogenase	YDL243C		Y03940
AAD 6	Putative aryl-alcohol dehydrogenase	YFL056C		Y05677
AAD10	Putative aryl-alcohol dehydrogenase	YJR155W		10327A

AAD14	Putative aryl-alcohol dehydrogenase	YNL331C		10565A
ARI1	NADPH-dependent aldehyde reductase	YGL157W	carbonyl reductase (NADPH-dependent) ARI1	Y04524

A screening has been performed to evaluate stability of the three aldehydes of the vanilloid pathway with these 22 strains and results have been compared with the same approach with a wild-type *S. cerevisiae* strain. Principle of the assay is to grow yeasts for a first 24h in rich-medium in a 96-well microplates. Then cells are two fold diluted using the same quantity of medium and let grown 24h more in presence of 500μM of the aldehyde to be evaluated (4-hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde and vanillin).

Figure 3 shows the titers obtained for three oxidative states (aldehyde, acid, alcohol) related to the feeding.

Looking at these results, the strongest impact is given by *adh6* deletion. ALD6 deletion also has an impact on the quantity of acid formed (more acid than the wild type cells). ALD6 could work on the conversion of aldehyde to alcohol. The third strong effect concerns ADH3. Actually, alcohol concentration is higher in $\Delta adh3$ cells than in the WT. ADH3 probably catalyzes the conversion of aldehyde into its acid form. A very small effect has been detected for $\Delta gre3$ and $\Delta ald2$. These genes were then assayed in combination with the most promising ones. Looking at all these results, the goal was then to combine gene deletions in the same cell. An expression of the van pathway in such a genetic context could favor vanillin synthesis.

Figures 3 show that the major effect is given by ADH6 deletion in WT cells, as expected. However a little effect is also detected when ADH3 deletion is added to that of ADH6. Indeed, alcohol concentration is a little lower in this strain.

$\Delta ald6$ is another deletion that significantly disturbs the equilibrium between the three oxidation states of 4HB and 3,4diHB. Since it doesn't show a strong impact on vanillin, we did not try it during the first assay of multi-deletion. However, the low impact of the other double deletions convinced us to try a deletion of *ald6* in combination with $\Delta adh6$.

**Example 3. Effect of metabolic engineering on vanillin pathway flux -
Multideletion of *pad1*, *fdc1* & *aro10* genes**

The deletion of three genes coding for enzymes involved in the catabolism of phenylpropanoid compounds (*fdc1*, *pad1* & *aro10*) were tested. Each new gene deletion and combinations thereof were evaluated for the production of vanillin and intermediates of the vanillin pathway. Taking into account the already obtained results in the investigation of metabolic engineering, the tested gene deletions were: *aro10*, *fdc1*, *pad1*, *adh6*, *ald6*, *adh3*.

Aro10 deletion increases the intrinsic concentration of phenylalanine, which is the branching point of our pathway. *Fdc1* and *pad1* deletions prevent the decarboxylation of intermediate metabolites, such as cinnamic and coumaric acids.

Gene deletion is performed using a CreLox deletion system. First, a deletion cassette is obtained by PCR on an appropriate plasmid (pUG72 or pUG73 from Euroscarf). The forward Primers used for amplification of the deletion K7 are designed to add upstream of the 5' side of the selection marker a track of 50 bp homologous to the last 50 bp of the promoter of the gene to be deleted. The reverse ones are designed to add downstream of the 3' side of the selection marker a track of 50 bp homologous to the first 50 bp of the terminator of the gene to be deleted. Once transformed by an appropriate deletion K7, the selection marker (*Ura3* or *Leu2*) is integrated in the yeast genome and it strictly replaces the ORF of the targeted gene. The selection marker is framed by two identical sequences (LoxP) that allow marker excision under the action of a Cre enzyme obtained by a new transformation of the new strain using a pSH62 plasmid (Euroscarf). *His3* selection marker of the pSH62 is then removed by a little growth of the transformed cells in a rich medium. This deletion system has been used to successively delete genes and to retrieve selection markers for next transformations. Each new gene deletion is followed by evaluation of cinnamic and coumaric acids production capacities after a transformation of the cells by a ZmPAL expression cassette.

Several modified yeast strains were evaluated for vanillin production; results are shown in figure 5:

Y10

Y10 Δ *adh6* ;

Y10 Δ *adh6* Δ *pad1* Δ *fdc1*;

Y10 Δ *aro10* Δ *adh6* Δ *fdc1*;

Y10 Δ *aro10* Δ *adh6* Δ *fdc1* Δ *adh3*;

Y10 Δ *aro10* Δ *adh6* Δ *fdc1* Δ *ald6*;

Y10 Δ *aro10* Δ *adh6* Δ *fdc1* Δ *adh3* Δ *ald6*;

5 Recombinant modified clones containing vanillin pathway were cultured in rich medium with galactose as carbon source using 100ml Erlenmeyer. After three days, clarified supernatants were analyzed by HPLC and concentration of metabolites from the pathway was evaluated. Total concentration of metabolites from vanillin pathway was evaluated.

10 Figure 5 shows the impact of deletions on the flow of the vanillin pathway. The total metabolites concentration (grey bars) comprises cinnamic acid, coumaric acid, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 3,4-dihydroxybenzaldehyde, vanillin, vanillic acid and vanillyl alcohol concentrations. Amount of final products (vanillin, vanillyl alcohol and vanillic acid) are
15 indicated with striped bars and vanillin concentration is indicated with black bars.

Aro10 deletion, by increasing the concentration of phenylalanine produced by the yeast, clearly increases the overall flow of the vanillin pathway. The total metabolite concentration is multiplied 4-fold. When the deletion of *ald6* is added, flow is also increased. In contrast, the *adh3* deletion decreases the flow of the pathway.
20 Vanillin produced equilibrates into acid and alcohol oxidation states. The results show that *aro10* deletion has little effect on the final concentration of metabolites (vanillin, vanillic acid & vanillyl alcohol). Besides increasing the flow of the pathway, *ald6* deletion significantly increases the concentration of end metabolites.

Figure 6 shows the profile of supernatants of strains presenting the following
25 deletions: Δ *adh6* Δ *aro10* Δ *fdc1* and Δ *adh6* Δ *aro10* Δ *fdc1* Δ *pdc1*. Clearly, the deletion of *pad1* is beneficial for the vanillin biosynthesis pathway in these strains.

Example 4. Optimization of culture conditions to increase vanillin production - Yeast volume culture

30 The synthesis of vanillin was evaluated based on the volume of culture for bioconversion. From a pre-culture, YPD medium was inoculated to an OD₆₀₀ of 0.1. The cells were then distributed into the different kind of media support from 96-well plate to a

1 liter Erlenmeyer flask. The concentration of vanillin was found to be clearly dependent on the volume of the support. Culture in 96-well plate allows good growth of yeast, but very little metabolite pathway vanillin are produced. Using 24-well plates, vanillin is produced, but quickly converted to vanillic acid. In contrast, when the bioconversion takes place in 1 liter Erlenmeyer flask, the synthesis of vanillin is continuous and stable and reaches 250 μ M after 8 days, and then the concentration drops down (Figure 7).

Example 5. Extractive bioconversion of vanillin using polypropylene glycol (PPG)

The toxicity of vanillin for yeast was evaluated (Figure 8). A yeast culture of *$\Delta adh6\Delta pdc1\Delta pad1$* strain expressing vanillin pathway was inoculated in YPDE to an OD₆₀₀ of 0.05 and yeast growth was monitored in the presence of different concentrations of vanillin (0 to 30 mM). The same experiment was carried out by adding 30% PPG 1200. Figure 7 shows that the toxicity threshold is multiplied by a factor 10.

In a second step we evaluated the bioconversion by our pathway in the presence of PPG. To do this, from a preculture in YPGal medium, an optimized cell expressing the pathway of vanillin was inoculated into 50 ml YPAGal in a 250 ml Erlenmeyer containing 50% of PPG. A control was done without PPG. The concentration of each metabolite was monitored in the control YPGal alone. Typically clarified supernatant was removed and analyzed by HPLC. In the biphasic culture, two phases were studied. Phase containing the supernatant was analyzed. PPG phase was taken and diluted to a volume of acetonitrile to be analyzed by HPLC.

In control culture, total metabolite concentration reaches 710 μ M whereas in biphasic culture concentration reaches 2,800 μ M, 75% (2,100 μ M) were located in PPG phase and 25% (700 μ M) were located in aqueous phase. As to compare to control culture, bioconversion yield is clearly improved when PPG is present and vanillin concentration increased from 100 μ M to 735 μ M (Figure 9). All intermediate metabolites of the pathway are also extractable in PPG, but the emulsion is sufficiently high to allow the availability of substrates to the cell while reducing any toxicity. This leads to a significant increase in the flux of the pathway (4 fold increase). Vanillin concentration was significantly increased (8 fold increase).

Example 6. Optimization of carbon source precursor for yeast growth on vanillin factory cell

Different carbon sources have been tested as substrates to produce vanillin in recombinant modified cells: sugars such as hexoses (glucose, fructose, galactose) as well
5 as alcohol compounds with two carbons (ethanol) or three carbons (glycerol).

Enrichment and propagation of clones were classically made in YPD liquid cultures (10 g/l bacto-yeast extract, 20 g/l bacto-peptone and 2% dextrose) at 30°C.

Several media were used: YPGal (10 g/l bacto-yeast extract, 20 g/l bacto-peptone and 3% galactose); YPF (10 g/l bacto-yeast extract, 20 g/l bacto-peptone and 2% fructose); YPDE (10 g/l bacto-yeast extract, 20 g/l bacto-peptone, 2% dextrose and 2% ethanol); YPGE (10 g/l bacto-yeast extract, 20 g/l bacto-peptone, 2% glycerol and 2% ethanol); YPG (10 g/l bacto-yeast extract, 20 g/l bacto-peptone, 2% glycerol); YPE (10 g/l bacto-yeast extract, 20 g/l bacto-peptone and 2% ethanol).
10

Vanillin producing cells were precultured in YPD or YPGal medium in a 10 mL for 24h, and used to inoculate 50mL appropriate medium in a 250 mL Erlenmeyer flask. Metabolites concentrations were monitored.
15

Figure 10 shows that when yeasts grow on glucose carbon source, vanillin concentration reaches 100 μ M over 3 days, then concentration decreases in favor of vanillic acid. Results are similar when yeasts grow on galactose carbon source, but vanillin is more stable. Fructose carbon source gives concentration of vanillin smaller than both other
20 sugars. However, YPDE and YPGE give same results, vanillin concentration grows. According to concentration of vanillin when yeasts grow on glyceraldehyde carbon source as to compare to ethanol alone, ethanol was the key factor to increase vanillin concentration. Ethanol is a normal by-product of glucose fermentation, transiently accumulated in the yeast culture medium. But when it is added to medium in high
25 concentration (2% or more), it influences yeast metabolism. Besides being a source of carbon for yeast growth, ethanol influences integrity of cellular membrane and carbon metabolism of *S. cerevisiae*. Addition of ethanol significantly changes the permeability of the cytoplasmic membrane, and results in a marked decrease in the mitochondrial
30 membrane potential. In the present case, ethanol significantly increases vanillin synthesis.

Conclusion

The synthesis of vanillin was significantly enhanced by the use of the process as described. Gene deletions have increased the flow of pathway by promoting the synthesis process and reducing the degradation process. The control of culture parameters
5 has allowed the increase of the bioconversion. These combined changes have allowed the increase of the concentration of the recovered vanillin from 1 to 250 μM .

SEQUENCES

PAL pop_715AA (SEQ ID 1)

5 METVTKNQYQNGSLESLCVNQRDPLSWGVAAEAMKGSHLDEVKRMVADYRKPVVKLGGET
 LTIAQVASIAGHDTGDKVELSESARPGVKASSDWVMDSMDKGTDSYGVTTFGFGATSHRR
 TKQGGALQKELIRFLNAGIFGNGTETCHTLPHSATRAAMLVRINTLLQGYSGIRFEILEA
 ITRLLNNNITPCLPLRGTITASGDLVPLSYIAGLLTGRPNKATGPTGEVLDAEAFKAA
 GIESGFFELQPK EGLALVNGTAVGSGGLASMLVFETNVLAVLSELLSAIFAEMNGKPEFT
 10 DHLTHKLKHHHPGQIEAAAIMEHILDGSAYMKA AKKLHETDPLQPKQDRYALRTSPQWL
 PQIEVIRFSTKSIEREINSVNDNPLIDVSRNKAIHGGNFQGTPIGVSMNDVRLAIASIGK
 LLFAQFSELVNDFYNNGLPSNLTASRNPSLDYGFKGAEIAMASYCSELQYLANPVTTHVQ
 SAEQHNQDVNSLGLISSRKTA EAVDILKLMSTTFLVALCQAIDLRHLEENLKSAVKNTVS
 QVSKRVLTGTANGELHPSRFCEKELLKVVDREYVFAYVDDPCSATYPLMQKL RQVFDHA
 LENGENEKNFSTSVFQKIEAFEEELKALLPKEVESARAAYDSGNSAIDNKIKECRSYPLY
 15 K FVREELGTVLLTGEKVQSPGEEFDKVFTAMCQGKIIDPMLECLGEWNGSPLPIC

PALpet_718AA (SEQ ID 2)

MAYVNGTTNGHANGNGLDLCMKKEDPLNWGVAAEALTGSHLDEVKRMVAEYRKPVVKLEG
 ETLTISQVA AISARDDSGVKVELSEEARAGVKASSDWVMDSMNKGTD SYGVTTFGFGATSH
 20 RRTKQGGALQKELIRFLNAGIFGSGAEAGNNTLPHSATRAAMLVRINTLLQGYSGIRFEI
 LEAITKFLNHNITPCLPLRGTITASGDLVPLSYIAGLLTGRPNKAVGPTGVTLSPEEAF
 KLAGVEGGFFELQPK EGLALVNGTAVGSGMASMLVF EANILAVLAEVMSAIFAEMQGKP
 EFTDHLTHKLKHHHPGQIEAAAIMEHILDGSAYVKA AQKLHEMDPLQPKQDRYALRTSPQ
 WLG PQIEVIR SSTKMIEREINSVNDNPLIDVSRNKAIHGGNFQGSPIGVSMNTRLAIAA
 25 IGKLMFAQFSELVNDFYNNGLPSNLSGGRNPSLDYGFKGAEIAMASYCSELQFLANPVTN
 HVQSAEQHNQDVNSLGLISSRK TSEAVEILKLMSTTFLVGLCQAIDLRHLEENLKSTVKN
 TVSQVAKRVLTMGVN GELHPSRFCEKDLLRVVDREYIFAYIDDPCSATYPLMQKLRETLV
 EHALNNGDKERNLSTSIFQKIAAFEDELKALLPKEVETARA ALESGNPAIPNRIKECRSY
 PLYKFVREELGTEYLTGEKVRSPGEEFEKVFTAMSKGEIIDPLLECLESWNGAPLPIC

30

C4Hgly_506AA (SEQ ID 3)

MDLLLLLEKTLIGLFLAAVVAIAVSTLRGRKFKLPPGPLPVPIFGNWLQVGDDL NHRNLT
 LAKKFGDIFLLRMGQRNLVVVSSPELAKEVLHTQGVEFGSRTRNVVFDIFTGKGQDMVFT
 VYGEHWRKMRRIMTVPFFTNKV VQQYRHGWESEAAAVVEDVKKNPDAAVSGTVIRRRQL
 35 MMYNNMYRIMFDRRFES EEDPIFQRLRALNGERSRLAQSF EYNYGDFIPILRPFLKGYLK
 ICEVKETRLKLFKDYFVDERKKLGSTKSTNNNNELKCAIDHILDAQRKGEINEDNVLYI
 VENINVA AIETTLWSIEWGIAELVNHPETIQKLRDEIDRVLGAGHQVTEPD IQKLPYLQA
 VVKETLRLRMAIPLLPHMNLHDAKLG YDIPAESKILVNAWWLANNPAHWKKPEEFRPE
 RFFEEESLVEANGNDFRYLPFGVGRRSCPGIILALPILGITLGR LVQNFELLPPPGQSQI
 40 DTSEKGGQFSLHILKHSTIVAKPRSF

C4Hpet_506AA (SEQ ID 4)

MMDFVLLEKALLGLFIATIVAITISKLRGKKLKLPPGPPIVPVFGNWLQVGDDL NQRNLV
 45 DYAKKFGDLFMLRMGQRNLVVVSSPELAKDVLHTQGVEFGSRTRNVVFDIFTGKGQDMVF
 TVYSEHWRKMRRIMTVPFFTNKV VQQYRFGWEDEAARVVEDVKANPEAATNGIVLRNRLQ
 LLMYNNMYRIMFDRRFESVDDPLFLKLKALNGERSRLAQSF EYHFGDFIPILRPFLRGYL
 KLCQEI KDKRLKLFKDYFVDERKKLESIKSVDNNSLKCAIDHIIEAQQKGEINEDNVLYI
 VENINVA AIETTLWSIEWGIAELVNNPETIQKLRHELDTVLGAGVQICEPDVQKLPYLQA

VIKETLRYRMAIPLLVPHMNLHDAKLAGYDIPAESKILVNAWWLANNPAHWNKPDEFRPE
 RFLEEESKVEANGNDFKYIPFGVGRRSCPGIILALPILGIVIGRLVQNFELLPPPGQSKI
 DTAEGKGQFSLQILKHSTIVCKPRSL

5 4CLpop_570AA (SEQ ID 5)

MMSVATVEPPKPELSPPQNQNAPSSHETDHIFRSKLPDITISNDLPLHAYCFENLSDFSD
 RPCLISGSTGKTYSAETHLISRKVAAGLSNLGIKKGDVIMTLLQNCPEFVFSFIGASMI
 GAVITTANPFYTQSEIFKQFSASRAKLIITQSQYVKNLGDSDCHENNQKPGEDFIVITID
 DPPENCLHFNVLVEASESEMP TVSILPDDPVALPFSSGTTGLPKGVLTHKSLITSVAQQ
 10 VDGEIPNLYLKQDDVVLCLVPLFHFIFSLNSVLLCSLRAGSAVLLMQKFEIGSLLELIQKH
 NVSVAAVVPLVLALAKNPLEANFDLSSIRVVLSGAAPLGKELEDALRSRVPQAILGQGY
 GMTEAGPVLMSCLAFSKQPFPTKSGSCGTVVRNAELKVIDPETGRSLGYNQGEICIRGS
 QIMKGYLNDAEATANTIDVEGWLHTGDIGYVDDDDDEIFIVDRVKEIIKFKGFQVPPAELE
 ALLVNHPSIADAADVPPQKDEVAGEVPVAFVVRSDDLDLSEEAVKEYIAKQVVFYKKLHKV
 15 FFVHSIPKSASGKILRKDLRAKLATATTMS

ECHpfl_276AA (SEQ ID 6)

MSNYEGRWTTVKVEIEDGIAWVILNRPEKRNAMSPTLNRE MIDVLETLEQDPAAGVLVLT
 GAGEAWTAGMDLKEYFREVDAGPEILQEKIRREASQWQWKLRLMYAKPTIAMVNGWCFGG
 20 GFSPLVACDLAICADEATFGLSEINWGI PPGNLVSKAMADTVGHRQSLYYIMTGKTFGGQ
 KAAEMGLVNDVPLARLREVTIELARNLLEKNPVVLRAAKHGFKRCRELTWEQNEDYLYA
 KLDQSRLLDTEGGREGQGMKQFLDDKSIKPGLQAYKR

ECHavi_276AA (SEQ ID 7)

MNKYEGRWKT VIVEIEGGIAWVTLNRPDKRNAMSPTLNREMRDVLETLEQDPAARVLVLT
 GAGSAWTAGMDLKEYFREVDAGPEILQEKIRREACEWQWKLRLMYAKPTVAMVNGWCFGG
 25 GFSPLVACDLAICADEATFGLSEINWGI PPGNLVSKAMADTVGHRQALYYIMTGKTFDGR
 QAAEMGLVNQSVPLAQLRET VATLCQDLLDKNPVVLRAAKNGFKRCRELTWEQNEDYLYA
 KLDQSRLLDEEGGREGMRQFLDEKSIKPGLQAYKR

30

HBHpae_394AA (SEQ ID 8)

MKTQVAIIAGPSGGLLLGQLLHKAGIDNVILERQTPDYVLGRIRAGVLEQGMVDLLREAG
 VDRRMARDGLVHEGVEIAFAGQRRRIDLKRLSGGKTVTVYGQTEVTRDLMEAREACGATT
 35 VYQAAEVRLHDLQGERPYVTFERDGERLRLCDYIAGCDGFHGISRQSI PAERLKVFERV
 YPFGWLGLLADTPPVSHELIYANHPRGFALCSQRSATRSRYVQVPLTEKVEDWSDERFW
 TELKARLPAEVAEKLVTGPSLEKSIAPLRSFVVEPMQHGRFLAGDAAHIVPPTGAKGLN
 LAASDVSTLYRLLLKAYREGRGELLERYSAICLRRIWKAERFSWWMTSVLHRFPD TDAFS
 QRIQQTELEYL GSEAGLATIAENYVGLPYEEIE

40

HBHavi_394AA (SEQ ID 9)

MKTQVAIIAGPSGGLLLGQLLHKAGIDNVILERHSPDYVLGRIRAGVLEQGVVDLLREAG
 VAERMDREGLVHEGIELACSGRRIRLDL KALSGGKTMVYGQTEVTRDLMDARRASGAPI
 VYEAQNVRLSGLKDGMPHVTYEKDGQTHRLDCDYIAGCDGFHGVSRQSI PAEALSHYERV
 45 YPFGWLGLLSDTPPVHEELIYAHTDLG FVLCSQRSPTRSRYYLQVPLSDRVEDWSDERFW
 NELKRRLPGDVANRLVTGPSLEKSIAPLRSYVVEPMQYGRFLVLGDAAHIVPPTGAKGLN
 LAGSDVCYLYRILLKVYREGRTELLEKYSELALRRVWKGERFSWFMTNLLHDFEGSDAFD
 RRMQLADRDYYLDSEAGRV TIAENYVGLPYEEIA

COMTmsa_365AA (SEQ ID 10)

MGSTGETQITPTHISDEEANLFAMQLASASVLPMLKSALELDLLEIIAKAGPGAQISPI
EIASQLPTTNPDPAPVMLDRMLRLLACYNILTCSVRTQQDGKVQRLYGLATVAKYLVKNED
GVSISALNLMNQDKVLMESWYHLKDAVLDDGGIPFNKAYGMTAFHEYHGTDPFRNKVFNKGM
5 SDHSTITMKKILETYTGFEGLKSLVDVGGGTGAVINTIVSKYPTIKGINFDLPHVIEDAP
SYPGVEHVGGDMFVSIPKADAVFMKWICHDSDEHCLKFLKNCYEALPDNGKVIVAECIL
PVAPDSSLATKGVVHIDVIMLAHNPGGKERTQKEFEDLAKGAGFQGFKVHCNAFNTYIME
FLKKV

10 COMTvp1_365AA (SEQ ID 11)

MATWVEHQQQNGSKDVDEEACMYAMQLSSMVVLPMTLRVAVELGILEQIQAGGPDSYLT
AEDLAARLGNSNPLAPVMIERILRLLTSYSILNFTDTVDGEGRTVRSYGAHVCKYLTPN
QDGVSMAPLVLMTNDKVLMEWYHMKDAVTNGGIPFNLAYGMTAFHEYHGKDLRFNKVFNE
GMKNNSIIITKKILERYKRFEDVNVLIDVGGGIGGTISMITAKYPHIHGINFDLPHVVSE
15 APPFQGVHVGGMNFESVPIGDAIFIKWILHDWSDEHCLKLLRNCASLPDKGKVIVVEC
ILPDAPLVTPEAGVFHLDMIMLAHNPGGKERTKKEFKELAMLSGFSNFKALFSYANVWV
MEFNK

20 Carboxylic acid reductase_1174AA (SEQ ID 12)

MAVDSPDERLQRRIAQLFAEDEQVKAARPLEAVSAAVSAPGMRLAQIAATVMAGYADRPA
AGQRAFELNTDDATGRTSLRLLPRFETITYRELWQRVGEVAAAWHHDPENPLRAGDFVAL
LGFTSIDYATLDDLADIHLGAVTVPLQASAAVSQLIAILTETSPRLLASTPEHLDAAVECL
LAGTTPPERLVVFDYHPEDDDQRAAFESARRRLADAGSLVIVETLDAVRARGRDLPAAPLF
25 VPDTHDDPLALLIYTSGSTGTPKGAMYTNRLAATMWQGNQNSQRVGINLNYMPMSH
IAGRISLFGVLARGGTAYFAAKSDMSTLFEDIGLVRPTEIFFVPRVCDMVFORQYQSELDR
RSVAGADLDTLDREVKAHLRQNYLGGFRLVAVVGSAPLAAEMKTFMESVLDLPLHDGYGS
TEAGASVLLDNQIQRPVLDYKLVDVPELGYFRTPDRPHPRGELLLKAETTIPGYKRPV
TAEIFDEDDGFYKTGDIVAELEHDLRVYVDRNNVLKLSQGEFVTVAHLEAVFASSPLIRQ
30 IFIYGSSERSYLLAVIVPTDDALRGRDTATLKSALAESIQRIDANLQPYEIPRDFLIE
TEPFTIANGLLSGIAKLLRPNLKERYGAQLEQMYTDLATGQADELLALRREAADLPVLET
VSRAAKAMLGVASADMRPDAHFTDLGGDSLSALSFSNLLHEIFGVEVPVGVVVSPANELR
DLANYIEAERNNGAKRPTFTSVHGGGSEIRAADLTLDKFDARTLAAADSIPHAPVPAQT
VLLTGANGYLGRFLCLEWLERLDTGGTLCVVRGSDAAAARKRLDSAFDSDGDPGLLEHY
35 QQLAARTLEVLAGDIGDPNLGLDDATWQRLAETVDLIVHPAALVNHVLPYTQLFGPNVVG
TAEIVRLAITARRKPVTYLSTVGVADQVDAEYQEDSDVREMSAVRVVRESYANGYGNSK
WAGEVLLREAHDLCGLPVAVFRSDMILAHSTRYAGQLNVQDVFTRLILSLVATGIAPYSFY
RTDADGNRQRAHYDGLPADFTAAAITALGIQATEGFRTYDVLNPDYDGGISLDEFVDWLVE
SGHPIQRITDYSDFHFRFETAIRALPEKQRQASVLPDLLDAYRNPCPAVRGAILPAKEFQA
40 AVQTAKIGPEQDIPHLAPLIDKYVSDLELLQLL

PPTase_222AA (SEQ ID 13)

45 MIETILPAGVESAEELLEYPEDLKAHPAEHLLIAKSVEKRRRDFIGARHLCARLALAELEGEP
PVAIGKGERGAPIWPRGVVGSLSLTHCDGYRAAAVAHKMRFRSIGIDAEPHATLPEGVLDSV
SLPPEREWLKT'TDSALHLDRLLFCAKEATYKAWWPLTARWLGFEAAHITFEIEDGSADSG
NGTFHSELLVPGQTNDGGTPLLSDGRWLIADGFIILTAIAYA

50

CLAIMS

1. A recombinant yeast comprising a set of exogenous nucleic acids, with each nucleic acid encoding a polypeptide involved in the biosynthesis pathway of a vanilloid, said set of exogenous nucleic acids encoding a set of polypeptides selected in a group comprising:

- a set of enzymes allowing conversion of an aromatic amino acid into a vanilloid; and
- a set of enzymes allowing conversion of an dehydroshikimic acid into a vanilloid;

and wherein the said recombinant yeast is devoid of a functional gene *adh6* encoding for an alcohol dehydrogenase,
and wherein said recombinant yeast has a reduced ability to decarboxylate phenylacrylic acids.

2. A recombinant yeast according to claim 1, wherein said yeast does not comprise a functional *fdc1* gene and/ or a functional *pad1* gene.

3. A recombinant yeast according to claim 1 or 2, wherein said yeast does not comprise a functional gene *aro10*.

4. A recombinant yeast according to anyone of claims 1 to 3, wherein said yeast does not comprise a functional gene *ald6*.

5. A recombinant yeast according to anyone of claims 1 to 4, wherein said yeast does not comprise a functional gene *pdc1*.

6. A recombinant yeast according to anyone of claims 1 to 5, wherein the aromatic acid is phenylalanine or tyrosine, and wherein it comprises a set of exogenous nucleic acids coding for the following enzymes: tyrosine/phenylalanine ammonia lyase (TAL/PAL), cinnamate-4-hydrolase (C4H), 4-coumarate coenzyme A ligase (4CL), enoyl-CoA hydratase/aldolase (ECH), a hydroxybenzoic acid hydroxylase (HBH) and a caffeic acid O-methyltransferase (COMT).

7. A recombinant yeast according to claim 6, wherein it further comprises exogenous nucleic acids coding for a carboxy reductase (CAR) and a phosphopantetheinyl transferase (PPTase).

8. A recombinant yeast according to anyone of claims 6 or 7, wherein it further comprises exogenous nucleic acids coding for a vanillyl alcohol oxidase.

9. A recombinant yeast according to anyone of claims 1 to 5, wherein it comprises a set of exogenous nucleic acids coding for the following enzymes able to convert an acid dehydroshikimic into a vanilloid: a 3-dehydroshikimate dehydratase (3DSD), an aromatic carboxylic acid reductase (ACAR), a phosphopantetheinyl transferase (PPTase) and a caffeic acid O-methyltransferase (COMT).

10. A recombinant yeast according to anyone of claims 6 to 9, wherein the exogenous nucleic acid coding for caffeic acid O-methyltransferase (COMT) is issued from *Medicago sativa*, *Vanilla planifolia* or *Rosa chinensis*.

11. A recombinant yeast according to anyone of claims 1 to 10, wherein it is from the genera *Saccharomyces* or *Schizosaccharomyces*.

12. A recombinant yeast according to anyone of claims 1 to 11, wherein the polypeptides are encoded by exogenous genes stably integrated into the yeast genome.

13. A method for producing a vanilloid or a precursor thereof, comprising:

a) providing a recombinant yeast according to anyone of claims 1 to 12,

b) cultivating said yeast in an appropriate medium comprising a substrate chosen from glucose, fructose, galactose, arabinose, lactose, ethanol, glycerol, and mixtures thereof; and

c) isolating said vanilloid or said precursor from the medium.

14. A method according to claim 13 wherein the substrate is ethanol.

15. A method according to anyone of claims 13 or 14 wherein the vanilloid is selected from the group consisting of:

vanillin, vanillic acid, vanillyl alcohol and vanillin-glycoside,

or wherein the precursor is selected from the group consisting of:

protocatechuic aldehyde, protocatechuic acid, protocatechuic alcohol, 4-hydroxyaldehyde, 4-hydrobenzoic acid, 4-hydroxyl alcohol, cinnamic acid, coumaric acid, caffeic acid and ferulic acid.

16. A method according to anyone of claims 13 to 15 wherein the step b) is performed in an appropriate medium comprising two phases, an aqueous phase and a water immiscible phase, said water immiscible phase preferably consisting of polymer of propylene glycol, fatty alcohol, fatty acid, fatty ester or an oil.

17. A composition comprising a vanilloid or a precursor thereof obtainable by the method as defined in anyone of claims 13 to 16.

18. Use of the composition according to claim 17 as a flavoring in the human and animal nutrition field, in pharmacy, and as a fragrance in the cosmetics, perfumery and detergency industries.

19. A method for producing a vanilloid or precursor thereof in a unicellular
5 microorganism able to produce said vanilloid or precursor thereof, comprising a step of cultivating said microorganism in an appropriate medium comprising an aqueous phase and a water immiscible phase, said water immiscible phase preferably comprising or consisting of a polymer of propylene glycol, fatty alcohol, fatty acid, fatty ester or an oil.

20. A method according to the preceding claim, wherein the unicellular
10 microorganism is a recombinant yeast comprising enzymes necessary for vanilloid biosynthesis.

21. A method according to the preceding claim, comprising the following steps:

a) providing a recombinant yeast comprising enzymes necessary for vanilloid
15 biosynthesis,

b) cultivating said yeast in an appropriate medium comprising a precursor chosen from glucose, fructose, galactose, arabinose, lactose, ethanol, glycerol, and mixtures thereof; and

c) isolating said vanilloid or precursor thereof from the medium.

20

Figure 1A

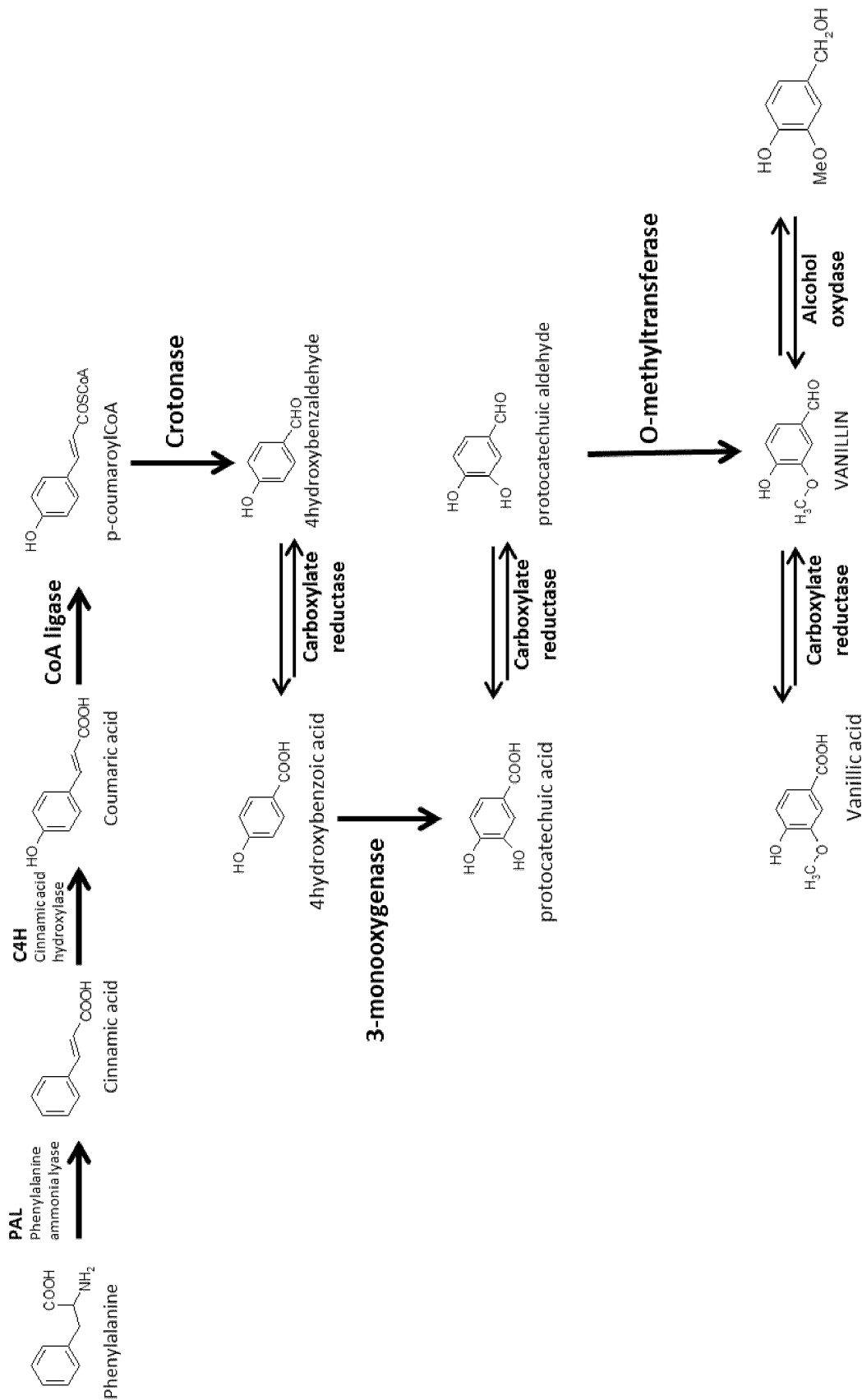


Figure 1B

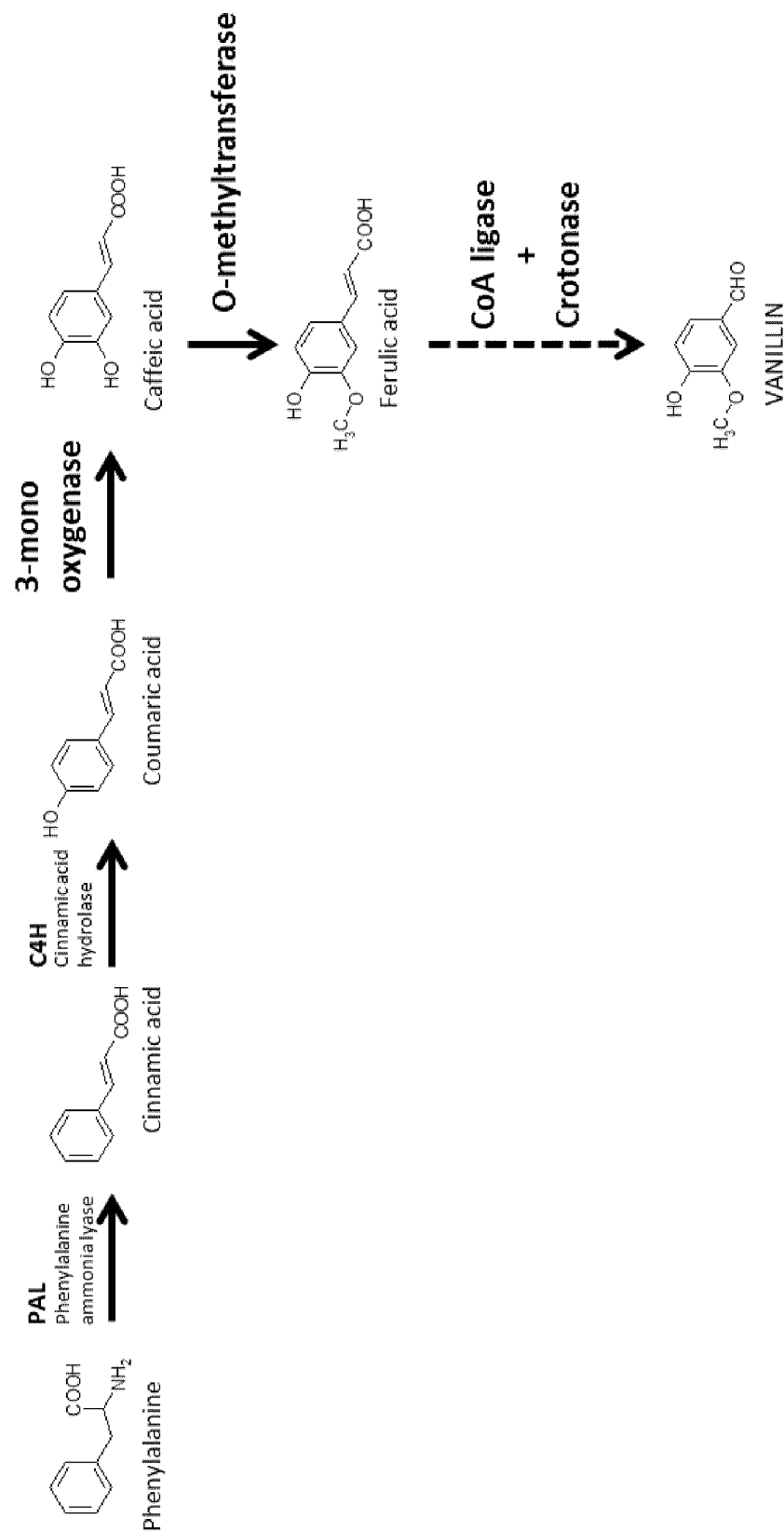


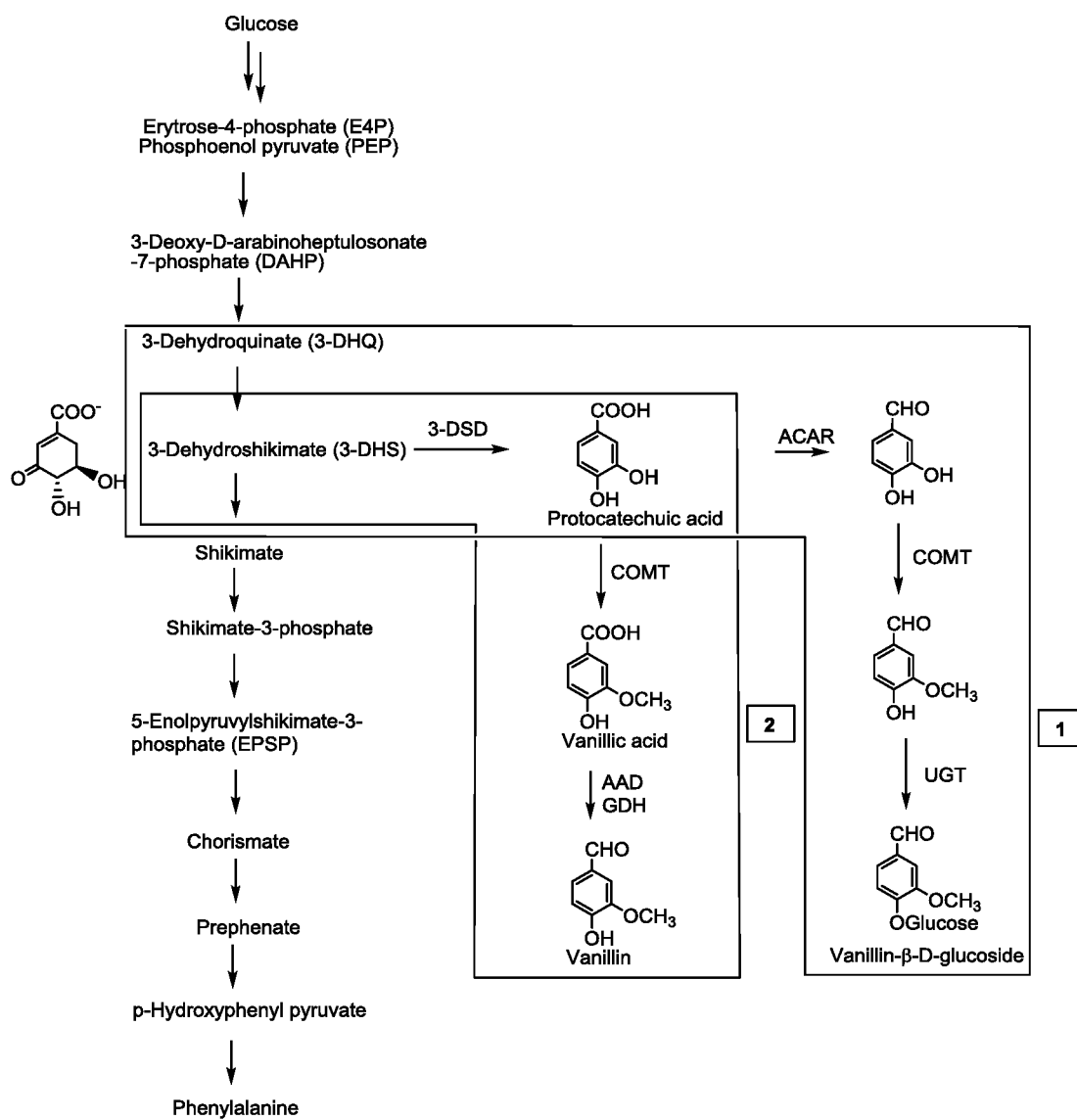
Figure 2A

Figure 2B

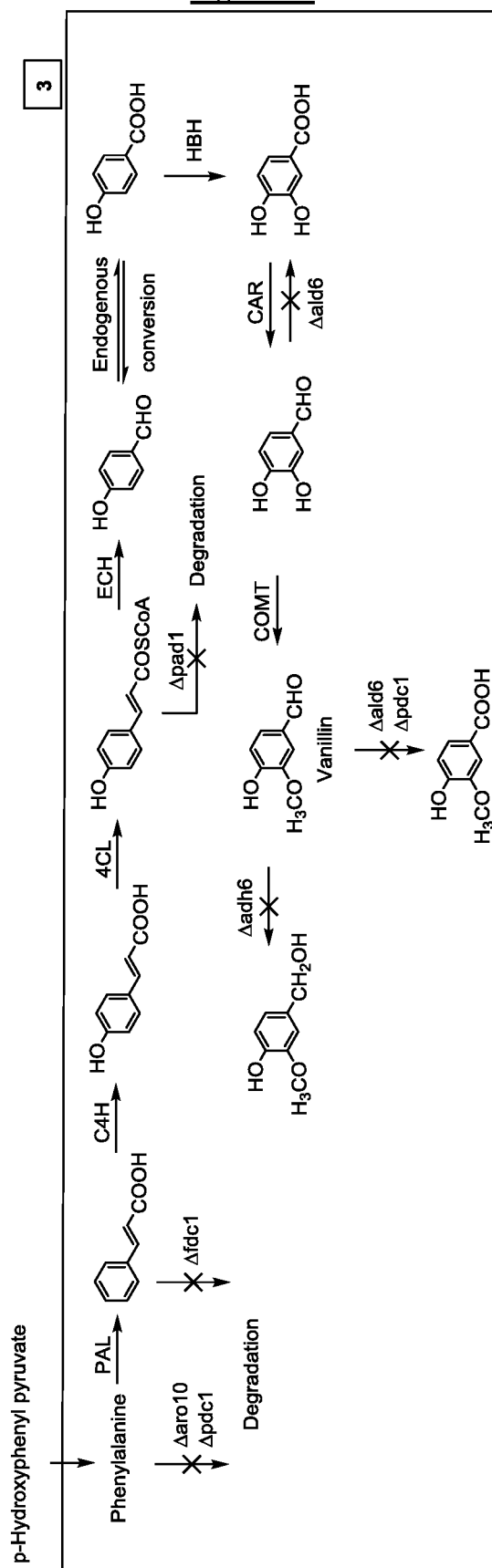


Figure 3A

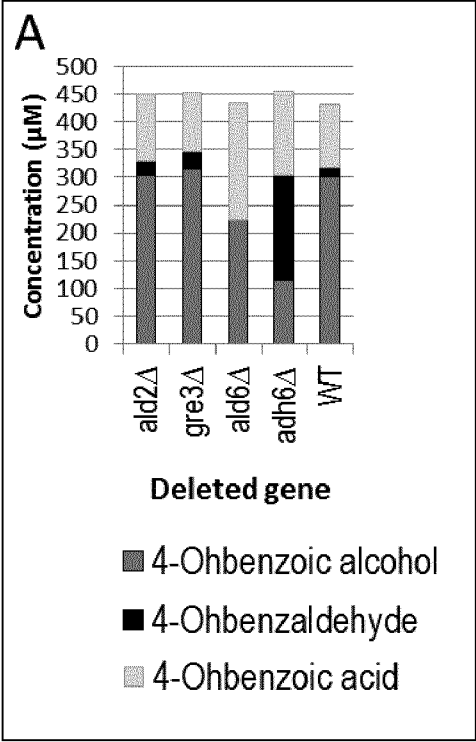


Figure 3 B

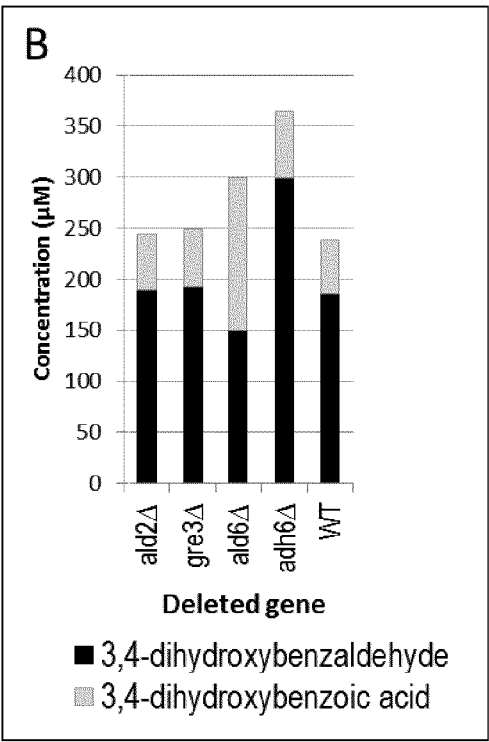


Figure 3 C

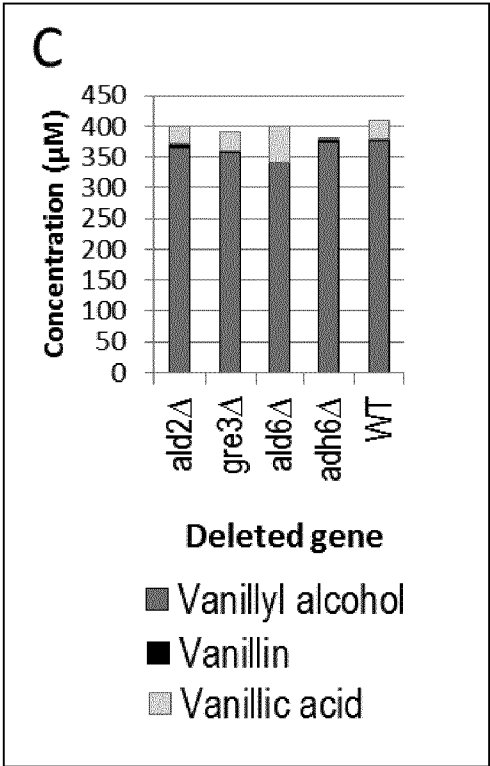


Figure 3 D

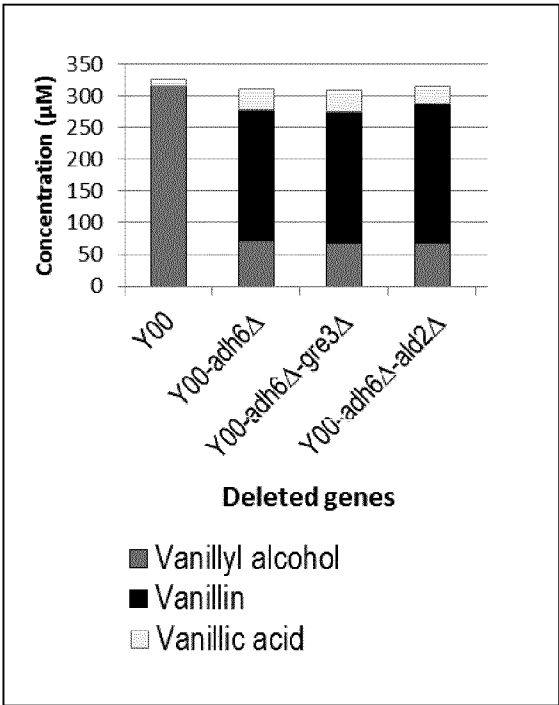


Figure 4

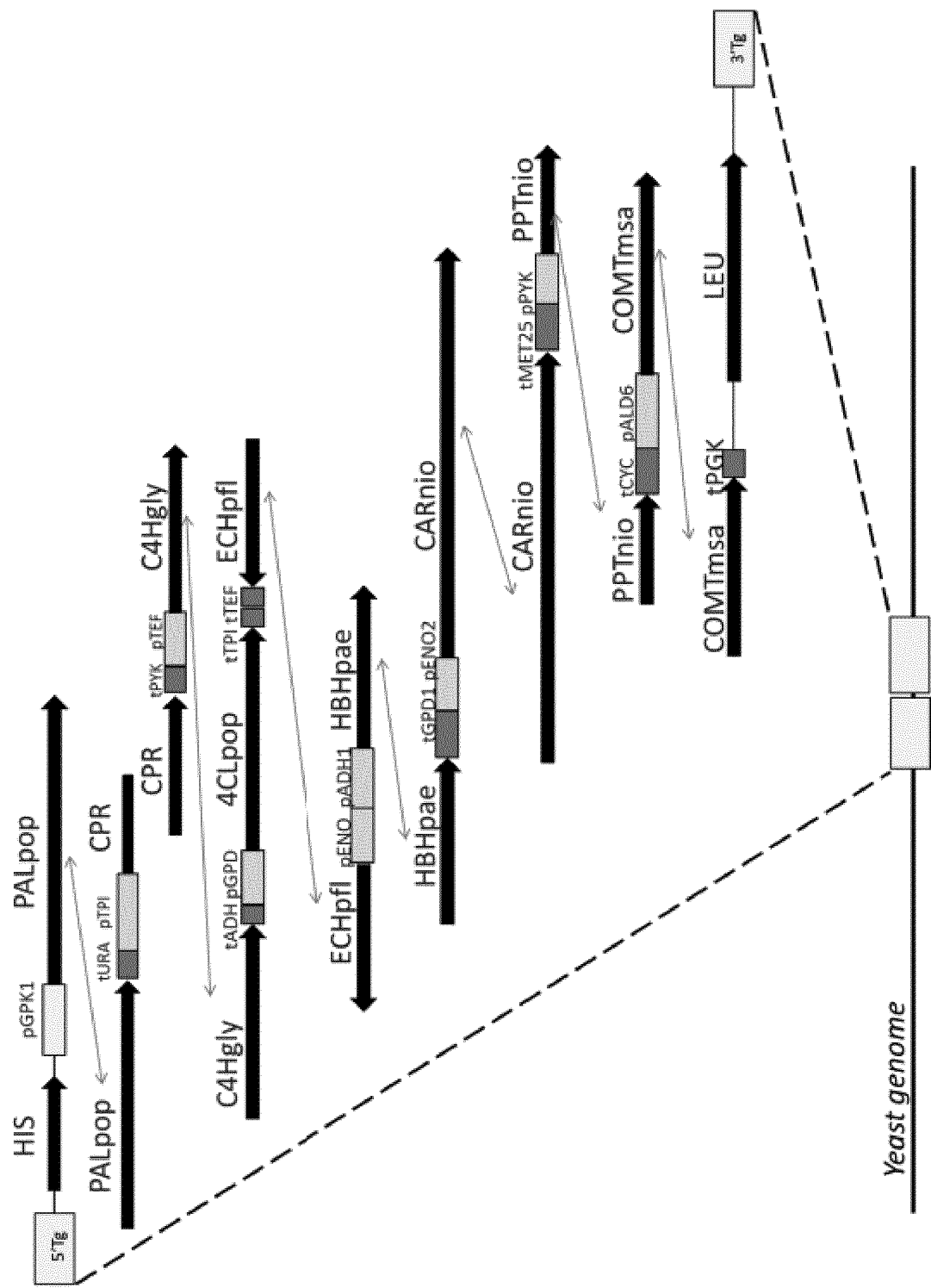


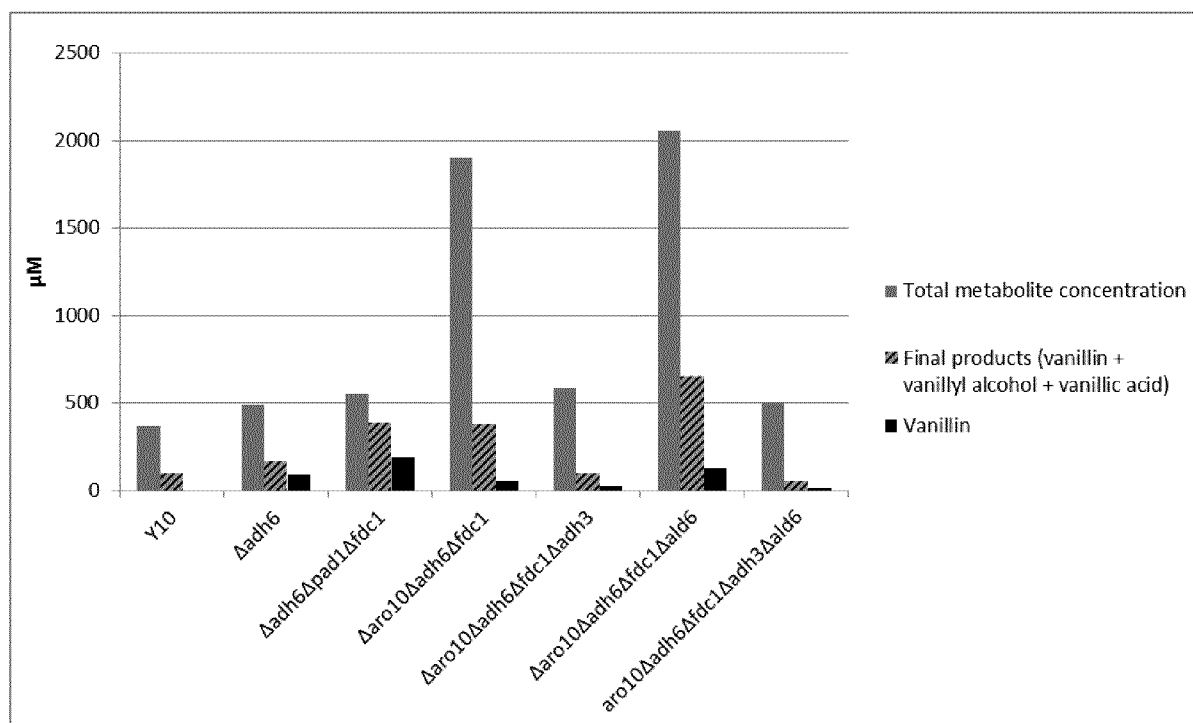
Figure 5

Figure 6

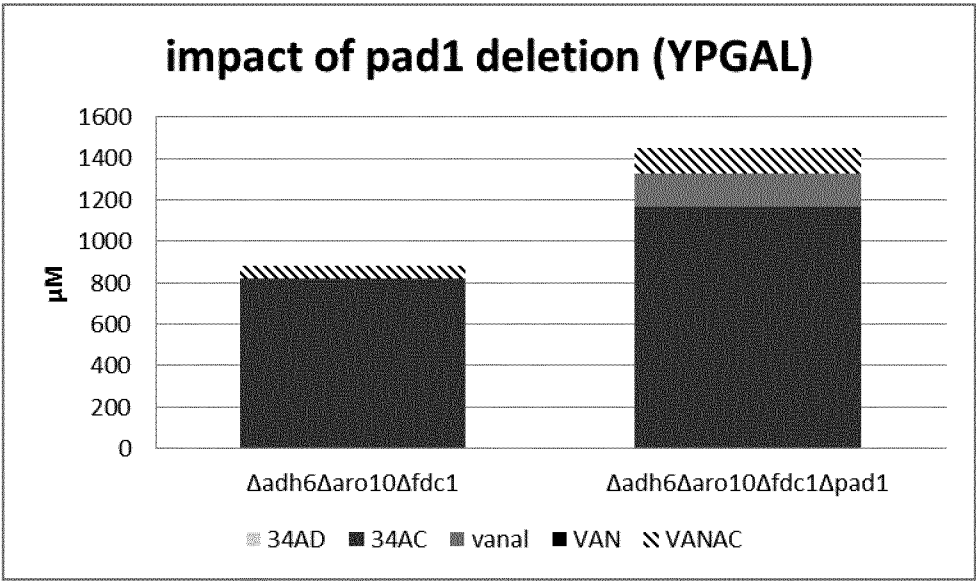


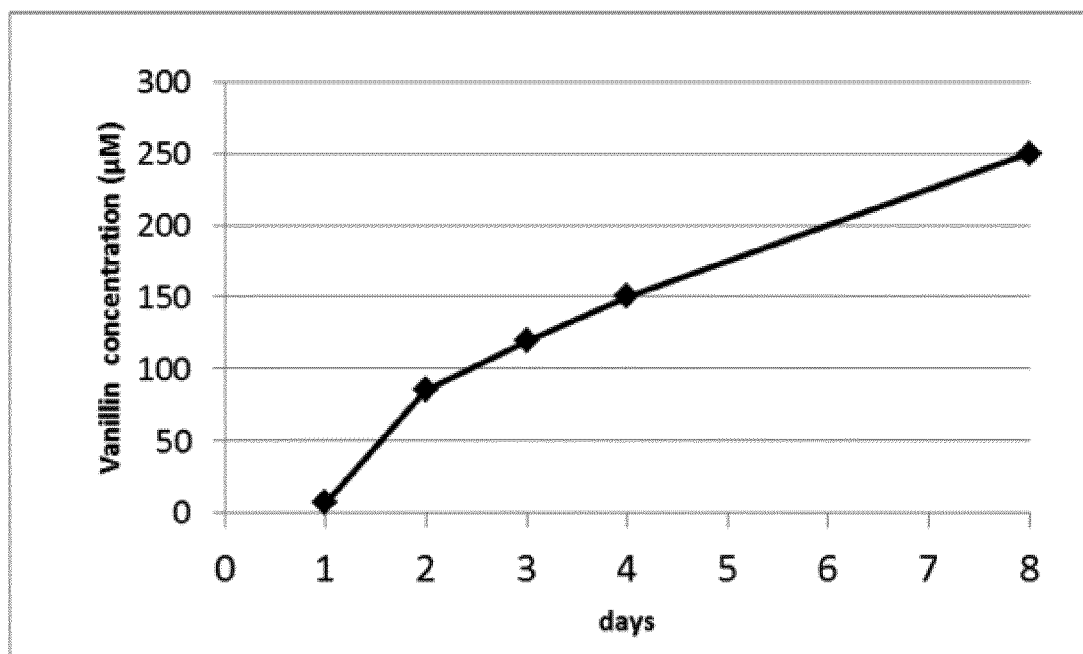
Figure 7

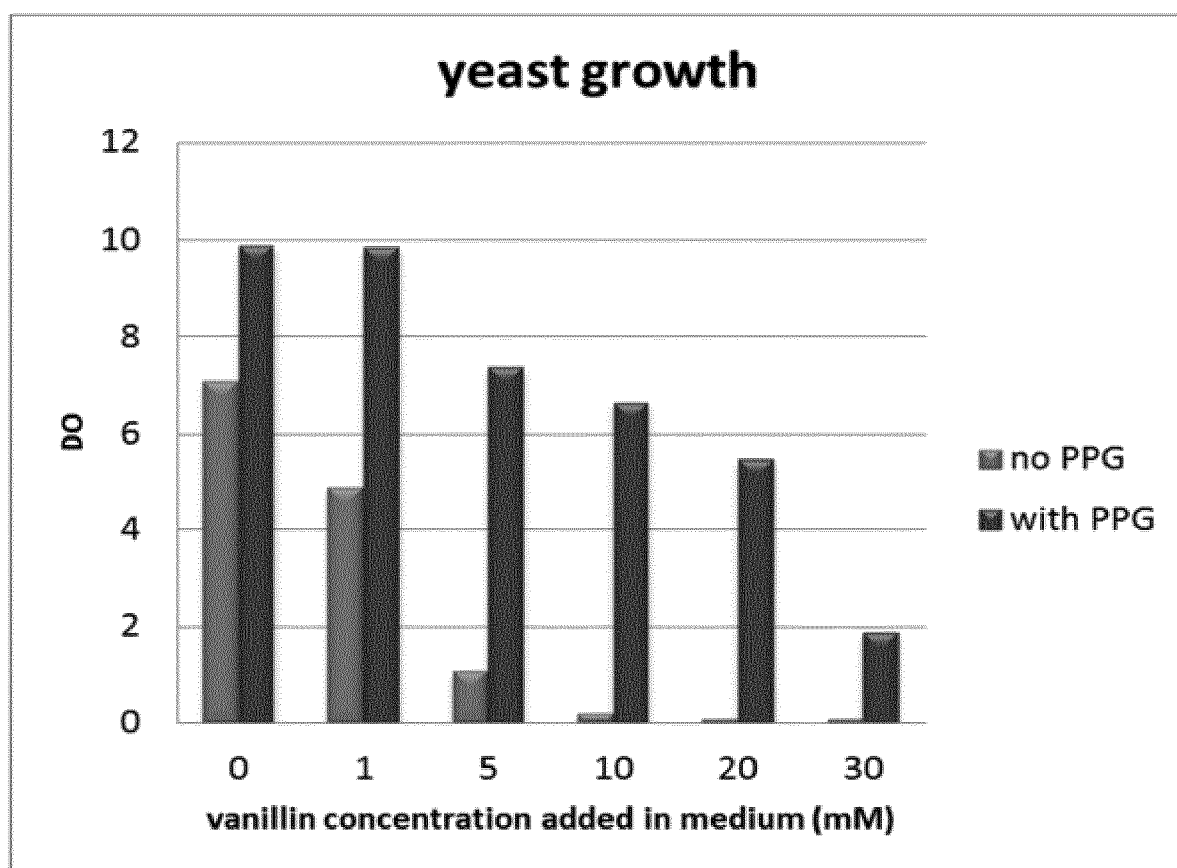
Figure 8

Figure 9

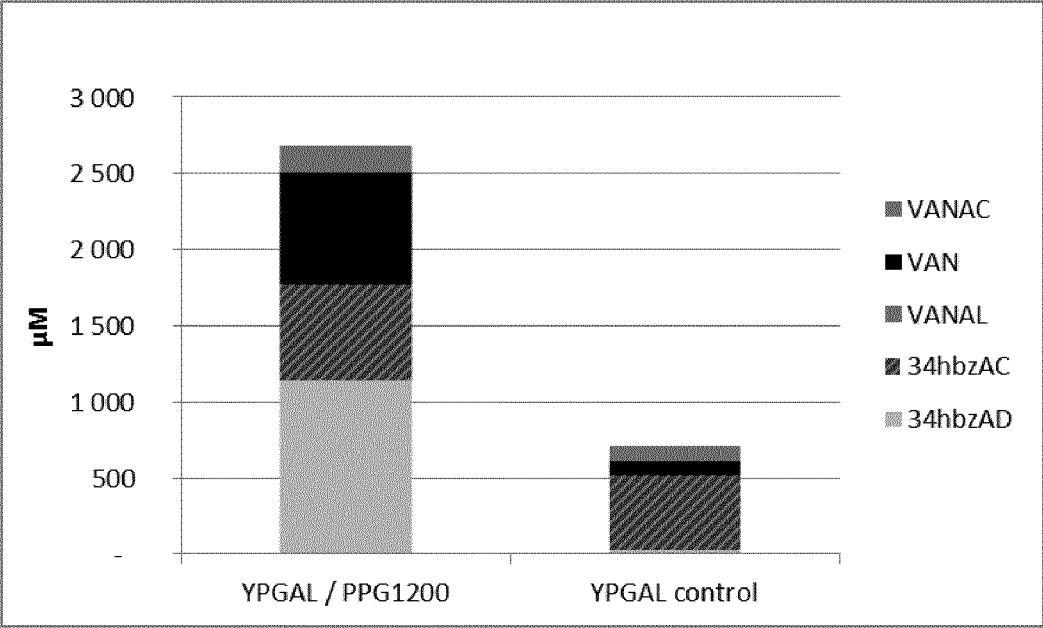
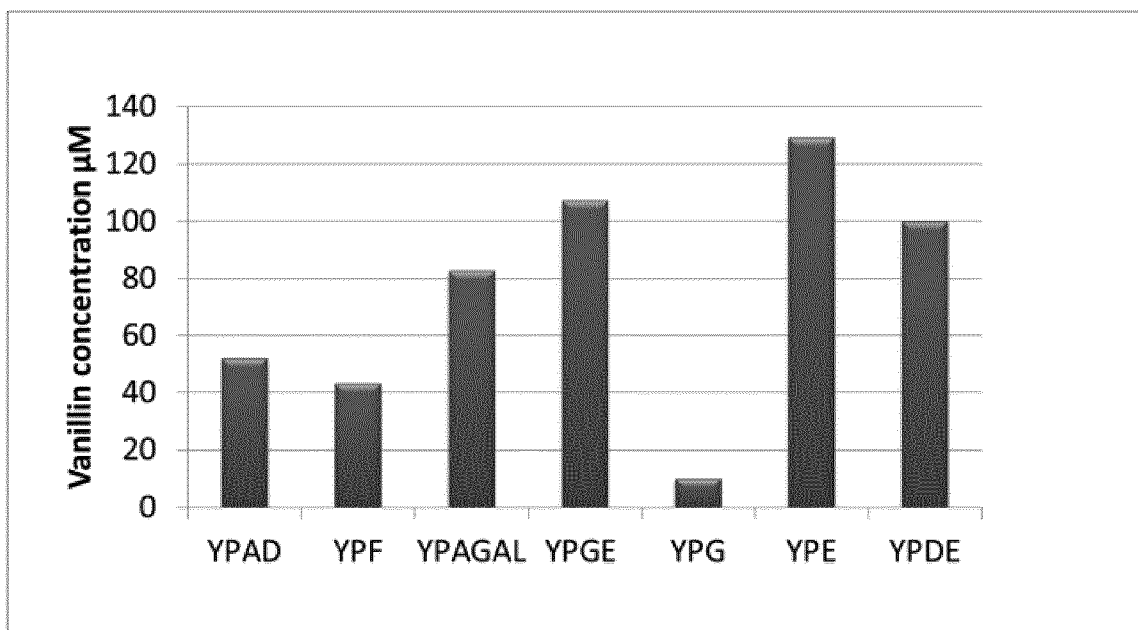


Figure 10

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/063546

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N1/32 C12N15/52 C12P7/24
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, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ESBEN H HANSEN ET AL: "De Novo Biosynthesis of Vanillin in Fission Yeast (Schizosaccharomyces pombe) and Baker's Yeast (Saccharomyces cerevisiae)", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 75, no. 9, 1 May 2009 (2009-05-01), pages 2765-2774, XP002666446, ISSN: 0099-2240, DOI: 10.1128/AEM.02681-08 [retrieved on 2009-03-01]	1-15,17,18
Y	cited in the application the whole document ----- -/--	16



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

"E" earlier application or patent but published on or after the international filing date

"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)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"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

"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

"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

"&" document member of the same patent family

Date of the actual completion of the international search

21 October 2015

Date of mailing of the international search report

30/10/2015

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Authorized officer

R. von Eggelkraut-G.

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2015/063546

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BROCHADO ANA RITA ET AL: "Improved vanillin production in baker's yeast through in silico design", MICROBIAL CELL FACTORIES, BIOMED CENTRAL, LONDON, NL, vol. 9, no. 1, 8 November 2010 (2010-11-08), page 84, XP021077232, ISSN: 1475-2859, DOI: 10.1186/1475-2859-9-84	1-15,17,18
Y	the whole document	16
A	----- JIANG H ET AL: "METABOLIC ENGINEERING OF THE PHENYLPROPANOIC PATHWAY IN SACCHAROMYCES CEREVISIAE", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 71, no. 6, 1 June 2005 (2005-06-01), pages 2962-2969, XP008053136, ISSN: 0099-2240, DOI: 10.1128/AEM.71.6.2962-2969.2005 the whole document	1-18
A	----- SO-YEON SHIN ET AL: "Production of resveratrol from tyrosine in metabolically engineered Saccharomyces cerevisiae", ENZYME AND MICROBIAL TECHNOLOGY, vol. 51, no. 4, 1 September 2012 (2012-09-01), pages 211-216, XP055148585, ISSN: 0141-0229, DOI: 10.1016/j.enzmictec.2012.06.005 the whole document	1-18
X	----- DE WULF O ET AL: "Bioconversion of vanillin to vanillyl alcohol in a two-phase reactor", APPLIED BIOCHEMISTRY AND BIOTECHNOLOGY ; PART A: ENZYME ENGINEERING AND BIOTECHNOLOGY, HUMANA PRESS INC, NEW YORK, vol. 20 - 21, no. 1, 1 January 1989 (1989-01-01), pages 165-180, XP035176080, ISSN: 1559-0291, DOI: 10.1007/BF02936481	19-21
Y	the whole document	16
	----- -/--	

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2015/063546

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LEE I-Y ET AL: "DECARBOXYLATION OF FERULIC ACID TO 4-VINYLGUAIACOL BY BACILLUS PUMILUS IN AQUEOUS-ORGANIC SOLVENT TWO-PHASE SYSTEMS", ENZYME AND MICROBIAL TECHNOLOGY, STONEHAM, MA, US, vol. 23, 1 January 1998 (1998-01-01), pages 261-266, XP000907521, ISSN: 0141-0229, DOI: 10.1016/S0141-0229(98)00044-1 the whole document -----	19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2015/063546

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.:

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: 1-18

A method for producing a vanilloid or precursor thereof, which is based on cultivating the specific recombinant yeast able to produce a vanilloid or precursor thereof as disclosed in claims 1-12 in an appropriate medium comprising a substrate as disclosed in claim 13

2. claims: 19-21

A method for producing a vanilloid or precursor thereof, which is based on cultivating an unicellular microorganism able to produce a vanilloid or precursor thereof in an appropriate biphasic medium as disclosed in claim 19
