Microbial cells and methods for production of hernandulcin

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Publication date:
2023

Document Version
Publisher's PDF, also known as Version of record

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
21 December 2023 (21.12.2023)

WIPO | PCT

(10) International Publication Number
WO 2023/242403 A1

(51) International Patent Classification:
C12N 9/88 (2006.01) *C12N 15/81* (2006.01)
C12N 9/02 (2006.01) *C12P 5/00* (2006.01)

(21) International Application Number:
PCT/EP2023/066249

(22) International Filing Date:
16 June 2023 (16.06.2023)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
22179430.8 16 June 2022 (16.06.2022) EP

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, MG, MK, MN, MU, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SC, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *of inventorship (Rule 4.17(iv))*

(54) Title: MICROBIAL CELLS AND METHODS FOR PRODUCTION OF HERNANDULCIN

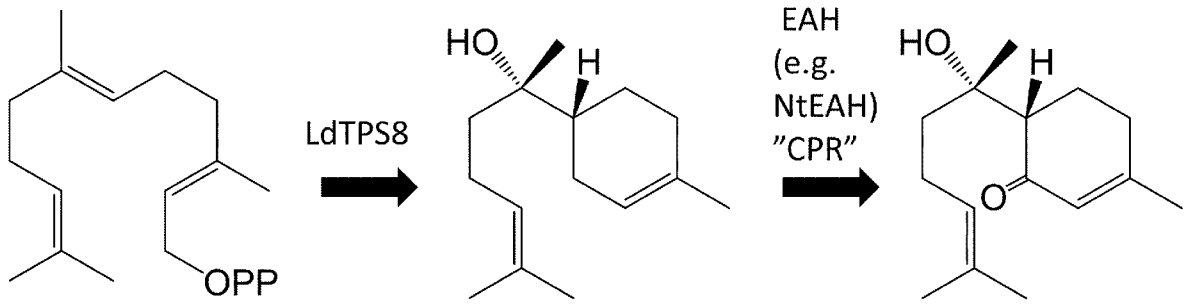

Figure 1

(57) Abstract: The present invention relates to microbial production of the sweet non-calorigenic sesquiterpenoid hernandulcin. Disclosed herein are yeast cells capable of producing hernandulcin and optionally hernandulcin derivatives, said yeast cells expressing at least one (+)-epi-alpha-bisabolol synthase, at least one cytochrome P450 enzyme (CYP) and at least one cytochrome P450 reductase, preferably said CYP the Nicotiana tabacum CYP 5-epi-aristolocene dihydroxylase (NtEAH) or the Datura stramonium cytochrome P450 enzyme DsEAH.

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WO 2023/242403 A1

Published:

— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
— *with sequence listing part of description (Rule 5.2(a))*
— *in black and white; the international application as filed contained color or greyscale and is available for download from PATENTSCOPE*

Microbial cells and methods for production of hernandulcin

Technical field

The present invention relates to microbial cells and in particular yeast cells for production of hernandulcin and optionally derivatives thereof in cells. The present disclosure also provides methods for producing hernandulcin in microbial cells and in particular yeast cells. Herein are also disclosed nucleic acids, expression systems and host cells for performing the present methods.

Background

Hernandulcin is a sweet non-calorigenic sesquiterpenoid found in the Mesoamerican plant Aztec Sweet Herb *Lippia dulcis* (*Phyla scaberrima*). Hernandulcin has been estimated to be approximately 1000 times sweeter than sucrose on a molar basis. While hernandulcin can be extracted from *L. dulcis*, these extracts suffer from low yields and impurities (De Oliveira et al., 2012). However, hernandulcin itself seems to be well-tolerated by animals in animal studies and to be non-mutagenic.

Chemical synthesis of hernandulcin from (−)-isopulegol in six steps with 15% yield has been achieved (Jung et al., 2002). Recently, 182.7 mg/L hernandulcin was produced from cell suspension cultures of *L. dulcis* with addition of the precursor (+)-epi-α-bisabolol (Villa-Ruano et al., 2021). However, the addition of elicitors or precursors and the lack of genetic engineering tools for *L. dulcis* makes this approach unlikely to work for large-scale production.

Therefore, it would be advantageous to produce hernandulcin using engineerable microbes with solid track records for high terpenoid production, which could lead to higher hernandulcin production with less impurities. However, full heterologous hernandulcin production has not been possible yet, since the biosynthetic pathway for hernandulcin is not completely elucidated.

The precursor (+)-epi-α-bisabolol was shown to be produced by a sesquiterpenoid synthase from *L. dulcis* (LdTPS8p) (Attia et al., 2012). Expression of LdTPS8 in a pre-engineered *Saccharomyces cerevisiae* strain resulted in 280 mg/L (+)-epi-α-bisabolol. However, the remaining steps necessary to oxygenate (+)-epi-α-bisabolol into hernandulcin remained unresolved. In one report, co-expression of LdTPS8p and

mammalian cytochromes P450 were attempted to produce hernandulcin in *S. cerevisiae*, but this only lead to the formation of various hydroxylated (+)-epi-α-bisabolol derivatives (Sarrade-Loucheur et al., 2020).

Thus, enzyme(s) catalysing the conversion of (+)-epi-α-bisabolol to hernandulcin need to be identified, in order to fulfil the longstanding desire to establish a microbial cell factory capable of producing hernandulcin.

Summary

The invention is as defined in the claims.

Surprisingly, the inventors have found that the cytochrome P450 enzyme *Nicotiana tabacum* 5-epi-aristolochene dihydroxylase (NtEAH) as set forth in SEQ ID NO 2 is able to catalyse the conversion of (+)-epi-α-bisabolol to hernandulcin. The present disclosure provides microbial cells and in particular yeast cells as well as method and means for producing hernandulcin using *Nicotiana tabacum* 5-epi-aristolochene dihydroxylase (NtEAH) as set forth in SEQ ID NO 2, or a functional variant thereof having at least 65% identity, homology or similarity thereto, capable of producing hernandulcin.

In particular, the present disclosure provides yeast cells capable of producing hernandulcin and/or derivatives thereof from (+)-epi-α-bisabolol, said yeast cells expressing a cytochrome P450 enzyme, such as the *Nicotiana tabacum* 5-epi-aristolochene dihydroxylase (NtEAH), the *Solanum lycopersicum* premnaspirodiene oxygenase-like protein SlEAH (SEQ ID NO 9), the *Datura stramonium* cytochrome P450 enzyme DsEAH (SEQ ID NO 11), the *Capsicum chinense* cytochrome P450 enzyme CcEAH (SEQ ID NO 13) or the *Capsicum annuum* cytochrome P450 71D7-like protein CaEAH (SEQ ID NO 15), or a functional variant thereof having at least 65% identity, homology or similarity to any of the aforementioned. Furthermore, comprised herein are yeast cells, methods and means for production of hernandulcin derivatives.

Thus, provided herein is a yeast cell capable of producing hernandulcin and/or one or more derivatives thereof, said yeast cell expressing:

i. at least one (+)-epi-alpha-bisabolol synthase capable of converting farnesyl diphosphate into (+)-*epi*-α-bisabolol, preferably a heterologous (+)-epi-alpha-bisabolol synthase;

ii. at least one cytochrome P450 enzyme capable of converting (+)-*epi*-α-bisabolol into hernandulcin, preferably a heterologous cytochrome P450 enzyme such as *Nicotiana tabacum* 5-epi-aristolocene dihydroxylase (NtEAH) as set forth in SEQ ID NO 2, *Datura stramonium* DsEAH as set forth in SEQ ID NO 11, or a functional variant thereof having at least 65% identity, homology or similarity thereto; and

iii. optionally at least one cytochrome P450 reductase, preferably a heterologous cytochrome P450 reductase,

whereby said yeast cell is capable of producing hernandulcin and/or one or more derivatives thereof.

Disclosed herein are cytochrome P450 enzymes capable of converting (+)-*epi*-α-bisabolol into hernandulcin, such as plant cytochrome P450 enzyme native to an organism of a genus selected from *Nicotiana, Solanum*, *Datura*, and *Capsicum*, such as *Nicotiana tabacum, Solanum lycopersicum*, *Datura stramonium*, *Capsicum chinense* and *C. annuum*. In particular, disclosed are the plant cytochrome P450 enzymes NtEAH (SEQ ID NO 2), SlEAH (SEQ ID NO 9), DsEAH (SEQ ID NO 11), CcEAH (SEQ ID NO 13) and CaEAH (SEQ ID NO 15), or functional variants thereof having at least 65% identity, homology or similarity to any of the aforementioned.

Further provided herein is an expression system for expression in a yeast cell, said expression system comprising:

i. a nucleic acid encoding at least one (+)-epi-alpha-bisabolol synthase (EC 4.2.3.138), preferably a heterologous (+)-epi-alpha-bisabolol synthase such as *Lippia dulcis* terpene synthase 8 (LdTPS8) as set forth in SEQ ID NO 1 or a functional variant thereof having at least 70% identity, homology or similarity thereto;

ii. a nucleic acid encoding at least one cytochrome P450 enzyme, preferably a heterologous cytochrome P450 enzyme such as *Nicotiana tabacum* 5-epi-aristolocene dihydroxylase (NtEAH) as set forth in SEQ ID NO 2, DsEAH as set forth in SEQ ID NO 11, or a functional variant thereof having at least 65% identity, homology or similarity thereto; and

iii. optionally a nucleic acid encoding at least one cytochrome P450 reductase (EC 1.6.2.4), preferably a heterologous cytochrome P450 reductase, for example a plant cytochrome P450 reductase such as *Arabidopsis thaliana* cytochrome P450 reductase ATR2 (AtATR2) as set forth in SEQ ID NO 3 or a functional variant thereof having at least 70% identity, homology or similarity thereto.

Also provided herein is a method for producing hernandulcin and/or one or more derivatives thereof in a yeast cell, said method comprising the steps of:

i. providing a yeast cell disclosed herein; and
ii. incubating said yeast cell in a medium,

whereby hernandulcin and/or derivatives thereof is produced.

Provided herein is also yeast cells comprising the above expression systems and/or nucleic acids.

Also provided herein is the use of above nucleic acids, expression systems and/or yeast cells for the production of hernandulcin and/or one or more derivatives thereof.

Also provided herein is a composition comprising hernandulcin and/or one or more derivatives thereof obtainable by a method disclosed herein.

Provided herein is also hernandulcin and/or one or more derivatives thereof obtainable by the methods disclosed herein.

Further provided herein is the use of a *Nicotiana, Datura*, *Salanum* or *Capsicum* cytochrome P450 enzyme in a method for producing hernandulcin and/or one or more derivatives thereof, preferably wherein the *Nicotiana* cytochrome P450 enzyme is *Nicotiana tabacum* 5-Epi-aristolochene dihydroxylase (NtEAH, SEQ ID NO 2), the *Datura* cytochrome P450 enzyme is the *Datura stramonium* cytochrome P450 enzyme DsEAH (SEQ ID NO 11), the *Solanum* cytochrome P450 enzyme is *Solanum lycopersicum* premnaspirodiene oxygenase-like protein (SlEAH, SEQ ID NO 9), the *Capsicum* cytochrome P450 enzyme is the *Capsicum chinense* cytochrome P450 enzyme CcEAH (SEQ ID NO 13) and/or the *Capsicum annuum* cytochrome P450 71D7-like protein (CaEAH, SEQ ID NO 15).

Provided herein is also a nucleic acid of an expression system for modifying a yeast cell, said nucleic acid or expression system comprising at least one nucleic acid encoding *Nicotiana tabacum* 5-Epi-aristolochene dihydroxylase (NtEAH, SEQ ID NO 2), SlEAH (SEQ ID NO 9), DsEAH (SEQ ID NO 11), CcEAH (SEQ ID NO 13) or CaEAH (SEQ ID NO 15), and/or a functional variant thereof having at least 65% identity, homology or similarity to any of the aforementioned.

Description of Drawings

Figure 1: Biosynthetic pathway for the production of hernandulcin in yeast. LdTPS8, *Lippia dulcis* (+)-epi-α-bisabolol synthase. NtEAH, *Nicotiana tabacum* 5-Epi-aristolochene dihydroxylase. "CPR", optional cytochrome P450 reductase.

Figure 2: Mass spectra of hernandulcin peaks from authentic standard (RT: 9.33 min) and cell extracts (RT: 9.34 min) from HRN1-producer. A) MS1 spectra. B) MS2 spectra of [M+H-H2O]+.

Figure 3*:* Hernandulcin production in the HRN1-producer. Averages and standard deviations are based on three or two replicates for cell pellet or supernatant, respectively. The HRN1-producer was cultivated in 2.5 mL YPD80 for 72 hours in a 24-deepwell plate.

Figure 4: Relative abundance of hernandulcin detected in the supernatant of a control strain, the HRN1- and HRN2-producer. Data represents the results of single replicates. ST10274 was used as control strain. The strains were cultivated in 2.5 mL YPD80 for 72 hours in a 24-deepwell plate.

Detailed description

Definitions

(+)-epi-alpha-bisabolol synthase is a terpene synthase (TPS), it has an EC number EC 4.2.3.138, and can catalyse the reaction:

(2E,6E)-farnesyl diphosphate + H_2O <=> (+)-epi-alpha-bisabolol + diphosphate

(+)-epi-alpha-bisabolol synthase converts farnesyl diphosphate into (+)-epi-α-bisabolol. A yeast cell expressing (+)-epi-alpha-bisabolol synthase may thus be able to convert farnesyl diphosphate to (+)-epi-α-bisabolol, thus producing (+)-epi-α-bisabolol in the presence of farnesyl diphosphate.

5-epi-aristolocene dihydroxylase (EAH) is an oxidoreductase (EC 1), more specifically a cytochrome P450 enzyme (CYP). EAH is well-known for catalysing the reaction (EC 1.14.14.149):
5-epiaristolochene + 2 [reduced NADPH--hemoprotein reductase] + 2 O_2 <=> capsidiol + 2 [oxidized NADPH--hemoprotein reductase] + 2 H_2O

Thus, EAH is well-known for converting 5-epiaristolochene into capsidiol, and is a recognized cytochrome P450 hydroxylase (Ralston et al., 2001). Surprisingly, the inventors have found that EAH from *Nicotiana tabacum* (NtEAH) can also catalyse the reaction:
(+)-epi-alpha-bisabolol + $2O_2$ + 2 [reduced NADPH--hemoprotein reductase] <=> hernandulcin + $3H_2O$ + 2 [oxidized NADPH--hemoprotein reductase]

Suprisingly, the inventors have also found that DsEAH, SlEAH, CcEAH and CaEAH can catalyse the latter reaction.

Thus, in this disclosure NtEAH, DsEAH, SlEAH, CcEAH and CaEAH can convert (+)-epi-α-bisabolol into hernandulcin. A yeast cell expressing EAH may thus be able to convert (+)-epi-α-bisabolol to hernandulcin, thus producing hernandulcin in the presence of (+)-epi-α-bisabolol. EAH of the present disclosure may have an EC number belonging to EC 1.14.14.- for example EC 1.14.14.1 or another EC number belonging to or falling under EC 1.14.14.-.

Cytochrome P450 reductase (CPR) is an oxidoreductase (EC 1). CPR has an EC number EC 1.6.2.4 and can catalyse the reaction:
NADPH + n oxidized hemoprotein <=> $NADP^+$ + n reduced hemoprotein

CPR converts oxidized hemoprotein into reduced hemoprotein.

Identity, homology or similarity with respect to a polynucleotide or polypeptide, are defined herein as the percentage of nucleotides or amino acids, respectively, in the candidate sequence that are identical, homologous or similar, respectively, to the residues of a corresponding native (may be codon-optimised) nucleotide or amino acid sequence, respectively, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent identity / similarity, and considering any conservative substitutions according to the NCIUB rules ([https://iubmb.qmul.ac.uk/misc/naseq.html; NC-IUB, Eur J Biochem (1985)]) as part of the sequence identity. In particular, the percentage of similarity refers to the percentage of residues conserved with similar physiochemical properties. Neither 5' or 3' extensions nor insertions (for nucleic acids) or N' or C' extensions nor insertions (for polypeptides) result in a reduction of identity, similarity or homology. Methods and computer programs for the alignments are well known in the art. Generally, a given identity between two sequences implies that the similarity between these sequences is at least equal to the identity; for example, if two sequences are 70% identical to one another, they cannot be less than 70% similar to one another – but could be sharing 80% similarity. Thus, throughout the present disclosure, it will be understood that any variant, such as a functional variant, or homologue said to have at least 70% identity, homology, or similarity to a specified sequence (polynucleotide or polypeptide) refers to a sequence having at least 70%, such as at least 71%, such as at least 72%, such as at least 73%, such as at least 74%, such as at least 75%, such as at least 76%, such as at least 77%, such as at least 78%, such as at least 79%, such as at least 80%, such as at least 81%, such as at least 82%, such as at least 83%, such as at least 84%, such as at least 85%, such as at least 86%, such as at least 87%, such as at least 87%, such as at least 88%, such as at least 89%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% identity, similarity, or homology thereto.

Functional variant as term refers herein to functional variants of an enzyme, which retain at least some of the activity of the parent enzyme. Thus, a functional variant of a fluorinase, a phosphorylase, a nucleosidase can catalyse the same conversion as a fluorinase, a phosphorylase, or a nucleosidase, respectively, from which they are derived, although the efficiency of the conversion reaction may be different, e.g. the

efficiency is decreased or increased compared to the parent enzyme or the substrate specificity is modified.

Native as term when referring to a polypeptide, such as a protein or an enzyme, or to a polynucleotide, such as a gene, coding sequence of a gene or genetic element, shall herein be construed to refer to a polypeptide or a polynucleotide which is naturally present in a wild type cell.

Heterologous as term when referring to a polypeptide, such as a protein or an enzyme, or to a polynucleotide, such as a gene, coding sequence of a gene or genetic element, shall herein be construed to refer to a polypeptide or a polynucleotide which is not naturally present in a wild type cell.

Mutation as term when used herein in the context of nucleic acid sequences refers to a change in nucleic acid sequence compared to the parent nucleic acid sequence. The term mutation covers single nucleotide mutations, but also insertions and deletions of multiple nucleotides, i.e. any change that leads to a different nucleic acid sequence than the parent nucleic acid sequence. The term mutation thus encompasses deletions, such as deletions of a whole gene or of a coding sequence of a gene, or a fragment/fraction of a gene or of a coding sequence of a gene.

Reduced activity as term may herein refer to a total or a partial loss of activity of a given polypeptide, such as a protein or an enzyme. In some cases, polypeptides are encoded by essential genes, which cannot be deleted. In these cases, activity of the polypeptide can be reduced by methods known in the art, such as downregulation of transcription or translation, or inhibition of the polypeptide. In other cases, the polypeptide is encoded by a non-essential gene, and the activity may be reduced or it may be completely lost, e.g. as a consequence of a deletion of the gene encoding the polypeptide.

Increased activity as term may herein refer to an improvement of activity of a given polypeptide, such as a protein or an enzyme. Said improvement in activity may be assessed based on the activity of the unmodified polypeptide serving as a reference. The improvement of activity may not be present at all times and/or in all conditions. Thus, the improvement of activity may be dependent on the condition wherein it is

assessed such as a growth condition and therefore be considered condition-dependent. However, the improvement of activity may be present at all time and/or in all conditions. Improvement of activity may be a consequence of a mutation of the gene encoding the polypeptide and/or a mutation in one or more genetic elements influencing the expression of the gene and/or the activity of the polypeptide. Improvement of activity may be achieved by modifying the promoter and/or the terminator of the gene encoding the polypeptide.

Derived from as term when referring to a polypeptide or a polynucleotide derived from an organism means that said polypeptide or polynucleotide is native to said organism, i.e. that it is naturally found in said organism.

Titer as term herein refers to the concentration of a compound or product that accumulates inside a cell and/or in the extracellular media during cultivation of the cell.

Derivative as term herein refers to any molecule, compound or product that has undergone any conversion, either obtained by means of chemicals (chemical synthesis) or catalysed by enzymes (enzymatic conversion) or a combination thereof, whereby another molecule, compound or product is being produced or synthesised. Said produced another molecule, compound or product may be volatile or non-volatile. With respect to a biosynthetic pathway such as a reaction series comprising at least one enzymatically catalysed reaction, a derivative refers to a molecule, compound or product that is further modified compared to any of the substrates, intermediates and/or products of said pathway. By contrast, a precursor of a given molecule, compound or product is a molecule or compound from which the given molecule, compound or product is a derivative. In other words, within a given pathway going from an initial substrate to a final product, a precursor of a molecule is typically upstream of the molecule, while a derivative of a molecule is typically obtained downstream. Thus, according to this definition a precursor of a molecule, compound or product is not a derivative of said molecule, compound or product. With respect to hernandulcin, 4-hydroxy-hernandulcin is an example of a derivative thereof, while hernandulcin is a precursor of 4-hydroxy-hernandulcin.

Production of hernandulcin

The present inventors have discovered that expression of *Nicotiana tabacum* 5-epi-aristolochene dihydroxylase NtEAH (SEQ ID NO 2), *Datura stramonium* cytochrome P450 enzyme DsEAH (SEQ ID NO 11), *Solanum lycopersicum* premnaspirodiene oxygenase-like protein SlEAH (SEQ ID NO 9), *Capsicum chinense* cytochrome P450 enzyme (CcEAH, SEQ ID NO 13) and/or *Capsicum annuum* cytochrome P450 71D7-like protein (CaEAH, SEQ ID NO 15), or functional variants thereof having at least 65% identity, homology or similarity to any of the aforementioned, and optionally at least one cytochrome P450 reductase in yeast cells capable of producing (+)-epi-α-bisabolol results in production of hernandulcin.

Thus, provided herein is a yeast cell capable of producing hernandulcin and/or one or more derivatives thereof, said yeast cell expressing:

i. at least one (+)-epi-alpha-bisabolol synthase capable of converting farnesyl diphosphate into (+)-*epi*-α-bisabolol, preferably a heterologous (+)-epi-alpha-bisabolol synthase such as *Lippia dulcis* terpene synthase 8 (LdTPS8) (EC 4.2.3.138) as set forth in SEQ ID NO 1 or a functional variant thereof having at least 70% identity, homology or similarity thereto; and
ii. at least one cytochrome P450 enzyme capable of converting (+)-*epi*-α-bisabolol into hernandulcin, preferably a heterologous cytochrome P450 enzyme such as *Nicotiana tabacum* 5-epi-aristolocene dihydroxylase (NtEAH) as set forth in SEQ ID NO 2, DsEAH as set forth in SEQ ID NO 11), *Solanum lycopersicum* premnaspirodiene oxygenase-like protein (SlEAH) as set forth in SEQ ID NO 9, CcEAH as set forth in SEQ ID NO 13 and/or *Capsicum annuum* cytochrome P450 71D7-like protein (CaEAH) as set forth in SEQ ID NO 15, or a functional variant thereof having at least 65% identity, homology or similarity thereto,

whereby said yeast cell is capable of producing hernandulcin and/or one or more derivatives thereof.

Also provided herein is a yeast cell capable of producing hernandulcin and/or one or more derivatives thereof, said yeast cell expressing:

i. at least one (+)-epi-alpha-bisabolol synthase capable of converting farnesyl diphosphate into (+)-*epi*-α-bisabolol, preferably a heterologous (+)-epi-alpha-bisabolol synthase such as *Lippia dulcis* terpene synthase 8

(LdTPS8) (EC 4.2.3.138) as set forth in SEQ ID NO 1 or a functional variant thereof having at least 70% identity, homology or similarity thereto;

ii. at least one cytochrome P450 enzyme capable of converting (+)-*epi*-α-bisabolol into hernandulcin, preferably a heterologous cytochrome P450 enzyme such as *Nicotiana tabacum* 5-epi-aristolocene dihydroxylase (NtEAH) as set forth in SEQ ID NO 2, DsEAH as set forth in SEQ ID NO 11), *Solanum lycopersicum* premnaspirodiene oxygenase-like protein (SlEAH) as set forth in SEQ ID NO 9, CcEAH as set forth in SEQ ID NO 13 and/or *Capsicum annuum* cytochrome P450 71D7-like protein (CaEAH) as set forth in SEQ ID NO 15, or a functional variant thereof having at least 65% identity, homology or similarity thereto; and

iii. optionally at least one cytochrome P450 reductase (EC 1.6.2.4), preferably a heterologous cytochrome P450 reductase such as *Arabidopsis thaliana* cytochrome P450 reductase ATR2 (AtATR2) as set forth in SEQ ID NO 3 or a functional variant thereof having at least 70% identity, homology or similarity thereto,

whereby said yeast cell is capable of producing hernandulcin and/or one or more derivatives thereof.

(+)-epi-alpha-bisabolol synthase (TPS)

(+)-epi-alpha-bisabolol synthase is a terpene synthase (TPS) with EC 4.2.3.138 that can convert farnesyl diphosphate (FPP) to (+)-epi-alpha-bisabolol. Thus, in the present disclosure (+)-epi-alpha-bisabolol synthase will sometimes be referred to as (+)-epi-α-bisabolol synthase, TPS8 or TPS8p and the names will be used interchangeably. (+)-epi-alpha-bisabolol synthase may also in the field be referred to as EAS or EASp. In this disclosure, (+)-epi-alpha-bisabolol may also be referred to as (+)-epi-α-bisabolol and the names will be used interchangeably herein.

The (+)-epi-alpha-bisabolol synthase preferably originates from the organism *Lippia dulcis.* The gene encoding the (+)-epi-alpha-bisabolol synthase such as LpTPS8 may be codon-optimized for the yeast cell expressing the (+)-epi-alpha-bisabolol synthase, as is known in the art.

Conversion of FPP to (+)-epi-alpha-bisabolol can be detected using Gas Chromatography (GC) coupled to Mass Spectrometry (MS) or GC coupled to a Flame Ionization Detector (FID) as described for example by Attia et al., 2012.

Cytochrome P450 enzyme (CYP)

In particular the present disclosure relates to cytochrome P450 enzymes (CYPs) capable of converting (+)-epi-alpha-bisabolol into hernandulcin as well as yeast cells and methods for production of hernandulcin by expression and/or use of said CYPs. Said CYPs may also be referred to as "(+)-epi-alpha-bisabolol oxygenases", a "(+)-epi-alpha-bisabolol dihydroxylases" or "at least one cytochrome P450 enzyme".

Thus, provided herein is a yeast cell capable of producing hernandulcin and/or one or more derivatives thereof, said yeast cell expressing:

i. at least one (+)-epi-alpha-bisabolol synthase capable of converting farnesyl diphosphate into (+)-*epi*-α-bisabolol;
ii. at least one cytochrome P450 enzyme capable of converting (+)-*epi*-α-bisabolol into hernandulcin, preferably a heterologous cytochrome P450 enzyme such as a heterologous plant cytochrome P450 enzyme; and
iii. optionally at least one cytochrome P450 reductase (EC 1.6.2.4),

whereby said yeast cell is capable of producing hernandulcin and/or one or more derivatives thereof.

Conversion of (+)-epi-alpha-bisabolol to hernandulcin can be detected using Liquid Chromatography coupled to MS as described for example by De Oliveira et al., 2012, or as in the examples of this disclosure.

<u>5-Epi-aristolochene dihydroxylase (EAH)</u>

5-epi-aristolocene dihydroxylase (EAH) is a cytochrome P450 enzyme (CYP). In the present disclosure, 5-epi-aristolochene dihydroxylase will sometimes also be referred to as EAH, EAHp, and the names will be used interchangeably.

EAH is well-known for converting 5-epiaristolochene (sometimes 5-epi-aristolochene) into capsidiol (Ralston et al., 2001). Interestingly, the inventors have found that EAH from *Nicotiana tabacum* (NtEAH) can catalyse the reaction:

(+)-epi-alpha-bisabolol + $2O_2$ + 2 [reduced NADPH--hemoprotein reductase] <=> hernandulcin + $3H_2O$ + 2 [oxidized NADPH--hemoprotein reductase]

Thus, NtEAH may be considered a cytochrome P450 oxygenase, such as a (+)-epi-alpha-bisabolol oxygenase or a (+)-epi-alpha-bisabolol dihydroxylase. The EAH preferably originates from the organism *Nicotiana tabacum*. *Nicotiana tabacum* 5-epi-aristolochene dihydroxylase may be referred to as NtEAH or NtEAHp herein, and the names will be used interchangeably. NtEAH may be encoded by the nucleic acid set forth in SEQ ID NO 5 (*NtEAH*). The gene encoding EAH such as *NtEAH* may be codon-optimized for the yeast cell expressing the EAH, as is known in the art.

EAH-related CYPs

In addition to NtEAH, further CYPs capable of converting (+)-epi-alpha-bisabolol into hernandulcin are disclosed herein. Said CYPs may be referred to as EAH-related CYPs. In particular CYPs from plants are provided herein. The provided CYPs of the present disclosure can catalyse the reaction

(+)-epi-alpha-bisabolol + $2O_2$ + 2 [reduced NADPH--hemoprotein reductase] <=> hernandulcin + $3H_2O$ + 2 [oxidized NADPH--hemoprotein reductase]

Thus, the cytochrome P450 enzyme disclosed herein, such as the at least one cytochrome P450 enzyme or the at least one plant cytochrome P450 enzyme, may be a CYP native to an organism of a genus selected from *Nicotiana, Solanum*, *Datura*, and *Capsicum*, such as *Nicotiana tabacum, Solanum lycopersicum*, *Datura stramonium*, *Capsicum chinense* and *C. annuum*. Preferably, said cytochrome P450 enzyme is a plant cytochrome P450 enzyme, such as NtEAH (SEQ ID NO 2), SlEAH (SEQ ID NO 9), DsEAH (SEQ ID NO 11), CcEAH (SEQ ID NO 13) or CaEAH (SEQ ID NO 15), or a functional variant thereof having at least 65% identity, homology or similarity to any of the aforementioned.

Thus, in some embodiments the at least one plant cytochrome P450 enzyme is a *Datura* cytochrome P450 enzyme, preferably a *Datura stramonium* cytochrome P450 enzyme, more preferably DsEAH as set forth in SEQ ID NO 11, or a functional variant thereof having at least 65% identity, homology or similarity thereto.

In some further embodiments the at least one plant cytochrome P450 enzyme is a premnaspirodiene oxygenase-like protein, such as a *Solanum* premnaspirodiene oxygenase-like protein, preferably a *Solanum lycopersicum* premnaspirodiene oxygenase-like protein, more preferably the *Solanum lycopersicum* premnaspirodiene oxygenase-like protein (SlEAH) as set forth in SEQ ID NO 9 or a functional variant thereof having at least 65% identity, homology or similarity thereto.

In other embodiments the at least one plant cytochrome P450 enzyme is a cytochrome P450 71D7-like protein, such as a *Capsicum* cytochrome P450 71D7-like protein, preferably a *Capsicum annuum* cytochrome P450 71D7-like protein, more preferably the *Capsicum annuum* cytochrome P450 71D7-like protein (CaEAH) as set forth in SEQ ID NO 15 or a functional variant thereof having at least 65% identity, homology or similarity thereto.

In further other embodiments the at least one plant cytochrome P450 enzyme is a *Capsicum* cytochrome P450 enzyme, preferably a *Capsicum chinense* cytochrome P450 enzyme, more preferably the CcEAH as set forth in SEQ ID NO 13 or a functional variant thereof having at least 65% identity, homology or similarity thereto.

Cytochrome P450 reductase

CYPs may be dependent on reductases such as CPRs that facilitate electron transfer between NAD(P)H and CYP, a so-called redox reaction. CPRs of EC 1.6.2.4 can convert oxidized hemoprotein to reduced hemoprotein. It may thus be necessary for the yeast cell to also express a CPR to assist the CYP. In some embodiments the yeast cell thus expresses, in addition to at least one (+)-epi-alpha-bisabolol synthase and the at least one cytochrome P450 enzyme (CYP), a CPR, preferably a heterologous CPR. In some embodiments, the at least one further CPR is a plant CPR. The reductase assisting the CYP such as NtEAH (SEQ ID NO 2), DsEAH (SEQ ID NO 11), Sl (SEQ ID NO 9), CcEAH (SEQ ID NO 13) or CaEAH (SEQ ID NO 15), or a functional variant thereof having at least 65% identity, homology or similarity thereto may be any reductase capable of assisting the CYP such as a CPR of EC 1.6.2.4, but is not limited hereto.

Many CPRs, such as plant CPRs, are known to be promiscuous and capable of assisting multiple different CYPs. Importantly, the CPR is capable of assiting the CYP catalysing the reaction:

(+)-epi-alpha-bisabolol + $2O_2$ + 2 [reduced NADPH--hemoprotein reductase] <=> hernandulcin + $3H_2O$ + 2 [oxidized NADPH--hemoprotein reductase]

Thus, the person skilled in the art is well capable of identifying a CPR that is capable of assisting the CYP that is capable of converting (+)-epi-α-bisabolol to hernandulcin, such as NtEAH (SEQ ID NO 2), DsEAH (SEQ ID NO 11), Sl (SEQ ID NO 9), CcEAH (SEQ ID NO 13) or CaEAH (SEQ ID NO 15). The CPR preferably originates from a plant such as *Lippia dulcis*, *Arabidopsis thaliana*, *Medicago truncatula* or *Glycyrrhiza uralensis.* Other CPRs that are capable of assisting CYPs in catalysing a redox reaction of interest when expressed in yeast may be identified by the person skilled in the art such as in Istiandari et al. 2021. The gene encoding the CPR may be codon-optimized for the yeast cell expressing the CPR, as is known in the art.

In some embodiments, the at least one CPR and/or the at least one further CPR are heterologous CPRs, optionally plant CPRs. In some embodiments, said CPR and/or plant CPR is selected from a *Lippia dulcis* CPR such as LdCPR1 (SEQ ID NO 7), a *Arabidopsis thaliana* CPR such as AtATR2 (NP_194750.1) or AtATR1 (NP_194183.1), a *Medicago truncatula* CPR such as MtCPR1 (XP_003602898.1) or MtCPR2 (XP_003610109.1), a *Lotus japonicas* CPR such as LjCPR1 or LjCPR2 (BAG68945.1), or *Glycyrrhiza uralensis* CPR such as GuCPR1 (AUG98241.1) or GuCPR2 (QCZ35624.1), or a functional variant thereof having at least 70% identity, homology or similarity thereto, encoding a CPR.

In some embodiments, the at least one further CPR is a *Lippia* CPR. In some embodiments the CPR is a *Lippia dulcis* CPR such as the *Lippia dulcis* CPR LdCPR1 as set forth in SEQ ID NO 7, or a functional variant thereof having at least 70% identity, homology or similarity thereto. LdCPR1 may have an EC number EC 1.6.2.4.

Yeast cell

The present disclosure relates to a yeast cell which has been modified or engineered to be capable of producing (+)-epi-α-bisabolol, hernandulcin and/or derivatives thereof, for

example as outlined in Figure 1. The inventors have found that expression of a (+)-epi-alpha-bisabolol synthase, a cytochrome P450 enzyme such as 5-epiaristolochene 1,3-dihydroxylase and optionally a cytochrome P450 reductase in a yeast cell results in production of hernandulcin. Derivatives of hernandulcin may also be obtained by expressing additional enzymes in the yeast cell. The yeast cell may be engineered as disclosed herein; the nature of the desired product and/or titers will dictate which enzymes should be introduced in the yeast cell and/or how it should be modified.

In one aspect, the present disclosure provides a yeast cell capable of producing hernandulcin and/or one or more derivatives thereof, said yeast cell expressing:

- at least one (+)-epi-alpha-bisabolol synthase, preferably a heterologous (+)-epi-alpha-bisabolol synthase such as *Lippia dulcis* terpene synthase 8 (LdTPS8) as set forth in SEQ ID NO 1 or a functional variant thereof having at least 70% identity, homology or similarity thereto,

wherein the at least one (+)-epi-alpha-bisabolol synthase is capable of converting farnesyl diphosphate (FPP) into (+)-*epi*-α-bisabolol;

- at least one cytochrome P450 enzyme capable of converting (+)-*epi*-α-bisabolol into hernandulcin, preferably a heterologous cytochrome P450 enzyme such as *Nicotiana tabacum* 5-epi-aristolocene dihydroxylase (NtEAH) as set forth in SEQ ID NO 2, DsEAH as set forth in SEQ ID NO 11), *Solanum lycopersicum* premnaspirodiene oxygenase-like protein (SlEAH) as set forth in SEQ ID NO 9, CcEAH as set forth in SEQ ID NO 13 and/or *Capsicum annuum* cytochrome P450 71D7-like protein (CaEAH) as set forth in SEQ ID NO 15, or a functional variant thereof having at least 65% identity, homology or similarity to any of the aforementioned; and
- optionally at least one cytochrome P450 reductase, preferably a heterologous cytochrome P450 reductase such as *Arabidopsis thaliana* cytochrome P450 reductase ATR2 (AtATR2) as set forth in SEQ ID NO 3 or a functional variant thereof having at least 70% identity, homology or similarity thereto,

wherein the at least one cytochrome P450 enzyme can catalyse the conversion of (+)-*epi*-α-bisabolol into hernandulcin,

whereby said yeast cell is capable of producing hernandulcin and/or one or more derivatives thereof.

In some embodiments, the yeast cell expresses at least one further cytochrome P450 reductase (CPR), preferably a heterologous CPR. In preferred embodiments, said at least one further CPR is a CPR with EC number EC 1.6.2.4. In some embodiments, said at least one further CPR is a plant CPR such as a *Lippia* CPR, such as a *Lippia dulcis* CPR. In some embodiments, the *L. dulcis* CPR LdCPR1 as set forth in SEQ ID NO 7, or a functional variant thereof having at least 70% identity, homology or similarity thereto.

In some embodiments, the yeast cell is a *Saccharomyces cerevisiae* cell or a *Yarrowia lipolytica* cell.

Throughout the present disclosure, it will be understood that the yeast cell can produce the compounds of interest listed herein when incubated in a cultivation medium under conditions that enable the yeast cell to grow and produce the desired compound. From the description of the yeast cells provided herein, the skilled person will not have difficulties in identifying suitable cultivation media and conditions to achieve production.

Production of hernandulcin

The yeast cell of the present disclosure can produce hernandulcin. This requires that the yeast cell expresses at least one TPS, such as at least one (+)-epi-alpha-bisabolol synthase, capable of converting farnesyl diphosphate (FPP) to (+)-*epi*-α-bisabolol, and at least one cytochrome P450 enzyme and optionally at least one cytochrome P450 reductase (CPR) for conversion of (+)-*epi*-α-bisabolol to hernandulcin. In some embodiments, the yeast cell expresses at least one further CPR.

In some embodiments, the at least one TPS is a heterologous TPS. In some embodiments the TPS and/or the heterologous TPS is a plant TPS. In some embodiments, the TPS is a (+)-epi-alpha-bisabolol synthase (EC 4.2.3.138).

In preferred embodiments, the at least one TPS is (+)-epi-alpha-bisabolol synthase (EC 4.2.3.138). In some embodiments, the (+)-epi-alpha-bisabolol synthase is a heterologous (+)-epi-alpha-bisabolol synthase. In some embodiments the (+)-epi-alpha-bisabolol synthase is a *Lippia* (+)-epi-alpha-bisabolol synthase, such as a *Lippia dulcis* (+)-epi-alpha-bisabolol synthase. In some embodiments, the *Lippia dulcis* (+)-epi-alpha-bisabolol synthase is *Lippia dulcis* terpene synthase 8 (LpTPS8) as set forth

in SEQ ID NO 1 or a functional variant thereof having at least 70% identity, homology or similarity thereto, wherein LpTPS8 is capable of converting farnesyl diphosphate to (+)-*epi*-α-bisabolol.

In preferred embodiments, the at least one cytochrome P450 enzyme (CYP) capable of converting (+)-*epi*-α-bisabolol into hernandulcin is a heterologous CYP. Said CYP may also be referred to as (+)-epi-alpha-bisabolol oxygenases, or (+)-epi-alpha-bisabolol dihydroxylases. In some embodiments, the CYP is a *Nicotiana* CYP. In other embodiments, the CYP is a CYP native to an organism of a genus selected from *Nicotiana, Solanum*, *Datura*, and *Capsicum*. In some embodiments, the CYP is a cytochrome P450 oxygenase. In some embodiments, the CYP is a heterologous cytochrome P450 oxygenase.

In some embodiments, the CYP is a cytochrome P450 oxygenase from *Nicotiana* such as from *Nicotiana tabacum*. In some embodiments, the CYP is a 5-epi-aristolocene dihydroxylase (EAH). In some embodiments, the CYP is a *Nicotiana* EAH. In some embodiments, the CYP is a *Nicotiana tabacum* 5-epi-aristolocene dihydroxylase (NtEAH). In preferred embodiments the *Nicotiana tabacum* 5-epi-aristolocene dihydroxylase (NtEAH) is the *Nicotiana tabacum* 5-epi-aristolocene dihydroxylase (NtEAH) as set forth in SEQ ID NO 2 or a functional variant thereof having at least 65% identity, homology or similarity thereto, such as at least 66%, such as at least 67%, such as at least 68%, such as at least 69%, such as at least 70%, such as at least 71%, such as at least 72%, such as at least 73%, such as at least 74%, such as at least 75%, such as at least 76%, such as at least 77%, such as at least 78%, such as at least 79%, such as at least 80%, such as at least 81%, such as at least 82%, such as at least 83%, such as at least 84%, such as at least 85%, such as at least 86%, such as at least 87%, such as at least 87%, such as at least 88%, such as at least 89%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% identity, similarity, or homology thereto, wherein NtEAH is capable of converting (+)-*epi*-α-bisabolol into hernandulcin.

In some embodiments the CYP is a cytochrome P450 enzyme from *Capsicum* such as from *Capsicum annuum*. In some embodiments, the CYP is a cytochrome P450 71D7-like protein. In other embodiments, the CYP is a *Capsicum* cytochrome P450 71D7-like

protein. In some embodiments, the CYP is a *Capsicum annuum* cytochrome P450 71D7-like protein capable of converting (+)-*epi*-α-bisabolol into hernandulcin. In preferred embodiments, the *Capsicum annuum* cytochrome P450 71D7-like protein is the *Capsicum annuum* cytochrome P450 71D7-like protein (CaEAH) as set forth in SEQ ID NO 15 or a functional variant thereof having at least 65% identity, homology or similarity thereto, such as at least 66%, such as at least 67%, such as at least 68%, such as at least 69%, such as at least 70%, such as at least 71%, such as at least 72%, such as at least 73%, such as at least 74%, such as at least 75%, such as at least 76%, such as at least 77%, such as at least 78%, such as at least 79%, such as at least 80%, such as at least 81%, such as at least 82%, such as at least 83%, such as at least 84%, such as at least 85%, such as at least 86%, such as at least 87%, such as at least 87%, such as at least 88%, such as at least 89%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% identity, similarity, or homology thereto.

In some embodiments the CYP is a cytochrome P450 enzyme from *Solanum* such as from *Solanum lycopersicum*. In some embodiments, the CYP is a premnaspirodiene oxygenase-like protein. In other embodiments, the CYP is a *Solanum* premnaspirodiene oxygenase-like protein. In some embodiments, the CYP is a *Solanum lycopersicum* premnaspirodiene oxygenase-like protein capable of converting (+)-*epi*-α-bisabolol into hernandulcin. In preferred embodiments, the *Solanum lycopersicum* premnaspirodiene oxygenase-like protein is the *Solanum lycopersicum* premnaspirodiene oxygenase-like protein (SlEAH) as set forth in SEQ ID NO 9 or a functional variant thereof having at least 65% identity, homology or similarity thereto, such as at least 66%, such as at least 67%, such as at least 68%, such as at least 69%, such as at least 70%, such as at least 71%, such as at least 72%, such as at least 73%, such as at least 74%, such as at least 75%, such as at least 76%, such as at least 77%, such as at least 78%, such as at least 79%, such as at least 80%, such as at least 81%, such as at least 82%, such as at least 83%, such as at least 84%, such as at least 85%, such as at least 86%, such as at least 87%, such as at least 87%, such as at least 88%, such as at least 89%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as

at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% identity, similarity, or homology thereto.

In other embodiments, the CYP is a cytochrome P450 enzyme from *Datura* such as *Datura stramonium*. In some embodiments the CYP is a *Datura stramonium* cytochrome P450 enzyme capable of converting (+)-*epi*-α-bisabolol to hernandulcin. In preferred embodiments the *Datura stramonium* cytochrome P450 enzyme is DsEAH as set forth in SEQ ID NO 11, or a functional variant thereof having at least 65% identity, homology or similarity identity, homology or similarity thereto, such as at least 66%, such as at least 67%, such as at least 68%, such as at least 69%, such as at least 70%, such as at least 71%, such as at least 72%, such as at least 73%, such as at least 74%, such as at least 75%, such as at least 76%, such as at least 77%, such as at least 78%, such as at least 79%, such as at least 80%, such as at least 81%, such as at least 82%, such as at least 83%, such as at least 84%, such as at least 85%, such as at least 86%, such as at least 87%, such as at least 87%, such as at least 88%, such as at least 89%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% identity, similarity, or homology thereto.

In other embodiments, the CYP is a cytochrome P450 enzyme from *Capsicum* such as *Capsicum chinense*. In some embodiments the CYP is a *Capsicum chinense* cytochrome P450 enzyme capable of converting (+)-*epi*-α-bisabolol to hernandulcin. In preferred embodiments the *Capsicum chinense* cytochrome P450 enzyme is CcEAH as set forth in SEQ ID NO 13, or a functional variant thereof having at least 65% identity, homology or similarity identity, homology or similarity thereto, such as at least 66%, such as at least 67%, such as at least 68%, such as at least 69%, such as at least 70%, such as at least 71%, such as at least 72%, such as at least 73%, such as at least 74%, such as at least 75%, such as at least 76%, such as at least 77%, such as at least 78%, such as at least 79%, such as at least 80%, such as at least 81%, such as at least 82%, such as at least 83%, such as at least 84%, such as at least 85%, such as at least 86%, such as at least 87%, such as at least 87%, such as at least 88%, such as at least 89%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such

as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% identity, similarity, or homology thereto.

Thus, in another aspect, the present disclosure provides a yeast cell capable of producing hernandulcin and/or one or more derivatives thereof, said yeast cell expressing:

- at least one (+)-epi-alpha-bisabolol synthase, preferably a heterologous (+)-epi-alpha-bisabolol synthase such as *Lippia dulcis* terpene synthase 8 (LdTPS8) as set forth in SEQ ID NO 1 or a functional variant thereof having at least 70% identity, homology or similarity thereto,

wherein the at least one (+)-epi-alpha-bisabolol synthase is capable of converting farnesyl diphosphate (FPP) into (+)-*epi*-α-bisabolol; and

- at least one cytochrome P450 enzyme capable of converting (+)-*epi*-α-bisabolol into hernandulcin, preferably a heterologous cytochrome P450 enzyme such as *Nicotiana tabacum* 5-epi-aristolocene dihydroxylase (NtEAH) as set forth in SEQ ID NO 2, DsEAH as set forth in SEQ ID NO 11), *Solanum lycopersicum* premnaspirodiene oxygenase-like protein (SlEAH) as set forth in SEQ ID NO 9, CcEAH as set forth in SEQ ID NO 13 and/or *Capsicum annuum* cytochrome P450 71D7-like protein (CaEAH) as set forth in SEQ ID NO 15, or a functional variant thereof having at least 65% identity, homology or similarity thereto,

wherein the at least one cytochrome P450 enzyme can catalyse the conversion of (+)-*epi*-α-bisabolol into hernandulcin,

whereby said yeast cell is capable of producing hernandulcin and/or one or more derivatives thereof.

In some embodiments, the yeast cell further expresses at least one cytochrome P450 reductase. In some embodiments, the at least one cytochrome P450 reductase (CPR) is a heterologous CPR, optionally with EC number EC 1.6.2.4. In other embodiments, the at least one CPR is a CPR with EC number EC 1.6.2.4. In some embodiments, the CPR is a plant CPR. In some embodiments, the CPR is an *Arabidopsis* CPR. In some embodiments, the CPR is an *Arabidopsis thaliana* CPR such as the *Arabidopsis thaliana* CPR ATR2 (AtATR2) (EC 1.6.2.4) as set forth in SEQ ID NO 3 or a functional variant thereof having at least 70% identity, homology or similarity thereto. In some embodiments, the at least one CPR is a reductase such as an oxidoreductase or

another reductase capable of assisting the CYP in the conversion of (+)-*epi*-α-bisabolol into hernandulcin by converting oxidized hemoprotein to reduced hemoprotein.

In some embodiments, the at least one CPR and/or the at least one further CPR is the same. In some embodiments, the optionally at least one CPR and/or the at least one further CPR are different.

In one aspect, the present disclosure provides a yeast cell capable of producing hernandulcin and/or one or more derivatives thereof, said yeast cell expressing:

- at least one (+)-epi-alpha-bisabolol synthase, preferably a heterologous (+)-epi-alpha-bisabolol synthase such as *Lippia dulcis* terpene synthase 8 (LdTPS8) as set forth in SEQ ID NO 1 or a functional variant thereof having at least 70% identity, homology or similarity thereto,

wherein the at least one (+)-epi-alpha-bisabolol synthase is capable of converting farnesyl diphosphate (FPP) into (+)-*epi*-α-bisabolol;

- at least one cytochrome P450 enzyme capable of converting (+)-*epi*-α-bisabolol into hernandulcin, preferably a heterologous cytochrome P450 enzyme such as *Nicotiana tabacum* 5-epi-aristolocene dihydroxylase (NtEAH) as set forth in SEQ ID NO 2, DsEAH as set forth in SEQ ID NO 11), *Solanum lycopersicum* premnaspirodiene oxygenase-like protein (SlEAH) as set forth in SEQ ID NO 9, CcEAH as set forth in SEQ ID NO 13 and/or *Capsicum annuum* cytochrome P450 71D7-like protein (CaEAH) as set forth in SEQ ID NO 15, or a functional variant thereof having at least 65% identity, homology or similarity thereto; and
- at least one cytochrome P450 reductase, preferably a heterologous cytochrome P450 reductase such as *Arabidopsis thaliana* cytochrome P450 reductase ATR2 (AtATR2) as set forth in SEQ ID NO 3 or a functional variant thereof having at least 70% identity, homology or similarity thereto,

wherein the at least one cytochrome P450 enzyme can catalyse the conversion of (+)-*epi*-α-bisabolol into hernandulcin,

whereby said yeast cell is capable of producing hernandulcin and/or one or more derivatives thereof.

In some embodiments, the at least one further CPR is a heterologous CPR. In some embodiments, the at least one further CPR is a plant CPR. In preferred embodiments the at least one further CPR has an EC number EC 1.6.2.4. In some embodiments, the

at least one further CPR is a *Lippia* CPR. In some embodiments the CPR is a *Lippia dulcis* CPR such as the *Lippia dulcis* CPR LdCPR1 as set forth in SEQ ID NO 7, or a functional variant thereof having at least 70% identity, homology or similarity thereto. LdCPR1 may have an EC number EC 1.6.2.4.

In some embodiments, the at least one CPR and/or the at least one further CPR are heterologous CPRs, optionally plant CPRs. In some embodiments, said plant CPR is selected from a *Lippia dulcis* CPR such as LdCPR1 (SEQ ID NO 7), a *Arabidopsis thaliana* CPR such as AtATR2 (NP_194750.1) or AtATR1 (NP_194183.1), a *Medicago truncatula* CPR such as MtCPR1 (XP_003602898.1) or MtCPR2 (XP_003610109.1), a *Lotus japonicas* CPR such as LjCPR1 or LjCPR2 (BAG68945.1), or *Glycyrrhiza uralensis* CPR such as GuCPR1 (AUG98241.1) or GuCPR2 (QCZ35624.1), or a functional variant thereof having at least 70% identity, homology or similarity thereto, encoding a CPR.

In further another aspect, the present disclosure provides a yeast cell capable of producing hernandulcin and/or one or more derivatives thereof, said yeast cell expressing:

- at least one (+)-epi-alpha-bisabolol synthase, preferably a heterologous (+)-epi-alpha-bisabolol synthase such as *Lippia dulcis* terpene synthase 8 (LdTPS8) as set forth in SEQ ID NO 1 or a functional variant thereof having at least 70% identity, homology or similarity thereto,

wherein the at least one (+)-epi-alpha-bisabolol synthase is capable of converting farnesyl diphosphate (FPP) into (+)-*epi*-α-bisabolol;

- at least one cytochrome P450 enzyme capable of converting (+)-*epi*-α-bisabolol into hernandulcin, preferably a heterologous cytochrome P450 enzyme such as *Nicotiana tabacum* 5-epi-aristolocene dihydroxylase (NtEAH) as set forth in SEQ ID NO 2, DsEAH as set forth in SEQ ID NO 11), *Solanum lycopersicum* premnaspirodiene oxygenase-like protein (SlEAH) as set forth in SEQ ID NO 9, CcEAH as set forth in SEQ ID NO 13 and/or *Capsicum annuum* cytochrome P450 71D7-like protein (CaEAH) as set forth in SEQ ID NO 15, or a functional variant thereof having at least 65% identity, homology or similarity thereto;
- at least one cytochrome P450 reductase, preferably a heterologous cytochrome P450 reductase such as *Arabidopsis thaliana* cytochrome P450 reductase

ATR2 (AtATR2) as set forth in SEQ ID NO 3 or a functional variant thereof having at least 70% identity, homology or similarity thereto; and

- at least one further CPR, preferably a heterologous CPR, such as the *Lippia dulcis* CPR LdCPR1 as set forth in SEQ ID NO 7, or a functional variant thereof having at least 70% identity, homology or similarity thereto,

wherein the at least one cytochrome P450 enzyme can catalyse the conversion of (+)-*epi*-α-bisabolol into hernandulcin,

whereby said yeast cell is capable of producing hernandulcin and/or one or more derivatives thereof.

In further another aspect, the present disclosure provides a yeast cell capable of producing hernandulcin and/or one or more derivatives thereof, said yeast cell expressing:

- at least one (+)-epi-alpha-bisabolol synthase, preferably a heterologous (+)-epi-alpha-bisabolol synthase such as *Lippia dulcis* terpene synthase 8 (LdTPS8) as set forth in SEQ ID NO 1 or a functional variant thereof having at least 70% identity, homology or similarity thereto,

wherein the at least one (+)-epi-alpha-bisabolol synthase is capable of converting farnesyl diphosphate (FPP) into (+)-*epi*-α-bisabolol;

- at least one cytochrome P450 enzyme capable of converting (+)-*epi*-α-bisabolol into hernandulcin, preferably a heterologous cytochrome P450 enzyme such as *Nicotiana tabacum* 5-epi-aristolocene dihydroxylase (NtEAH) as set forth in SEQ ID NO 2, DsEAH as set forth in SEQ ID NO 11), *Solanum lycopersicum* premnaspirodiene oxygenase-like protein (SlEAH) as set forth in SEQ ID NO 9, CcEAH as set forth in SEQ ID NO 13 and/or *Capsicum annuum* cytochrome P450 71D7-like protein (CaEAH) as set forth in SEQ ID NO 15, or a functional variant thereof having at least 65% identity, homology or similarity thereto; and
- at least one further CPR, preferably a heterologous CPR, such as the *Lippia dulcis* CPR LdCPR1 as set forth in SEQ ID NO 7, or a functional variant thereof having at least 70% identity, homology or similarity thereto,

wherein the at least one cytochrome P450 enzyme can catalyse the conversion of (+)-*epi*-α-bisabolol into hernandulcin,

whereby said yeast cell is capable of producing hernandulcin and/or one or more derivatives thereof.

In some embodiments, the yeast cell expresses at least LpTPS8 and NtEAH as set forth in SEQ ID NO 1 and SEQ ID NO 2, respectively, or functional variants thereof having at least 65% identity, homology or similarity thereto. In some other embodiments, the yeast cell expresses at least LpTPS8 and SlEAH as set forth in SEQ ID NO 1 and SEQ ID NO 9, respectively, or functional variants thereof having at least 65% identity, homology or similarity thereto. In other embodiments, the yeast cell expresses at least LpTPS8 and DsEAH as set forth in SEQ ID NO 1 and SEQ ID NO 11, respectively, or functional variants thereof having at least 65% identity, homology or similarity thereto. In other embodiments, the yeast cell expresses at least LpTPS8 and CcEAH as set forth in SEQ ID NO 1 and SEQ ID NO 13, respectively, or functional variants thereof having at least 65% identity, homology or similarity thereto. In other embodiments, the yeast cell expresses at least LpTPS8 and CaEAH as set forth in SEQ ID NO 1 and SEQ ID NO 15, respectively, or functional variants thereof having at least 65% identity, homology or similarity thereto.

In some embodiments, the yeast cell expresses at least LpTPS8, NtEAH, and AtATR2 as set forth in SEQ ID NO 1, SEQ ID NO 2 and SEQ ID NO 3, respectively, or functional variants thereof having at least 65% identity, homology or similarity thereto. In some embodiments, the yeast cell expresses at least LpTPS8, DsEAH, and AtATR2 as set forth in SEQ ID NO 1, SEQ ID NO 11 and SEQ ID NO 3, respectively, or functional variants thereof having at least 65% identity, homology or similarity thereto. In some embodiments, the yeast cell expresses at least LpTPS8, SlEAH, and AtATR2 as set forth in SEQ ID NO 1, SEQ ID NO 9 and SEQ ID NO 3, respectively, or functional variants thereof having at least 65% identity, homology or similarity thereto. In some embodiments, the yeast cell expresses at least LpTPS8, CcEAH, and AtATR2 as set forth in SEQ ID NO 1, SEQ ID NO 13 and SEQ ID NO 3, respectively, or functional variants thereof having at least 65% identity, homology or similarity thereto. In some embodiments, the yeast cell expresses at least LpTPS8, CaEAH, and AtATR2 as set forth in SEQ ID NO 1, SEQ ID NO 15 and SEQ ID NO 3, respectively, or functional variants thereof having at least 65% identity, homology or similarity thereto. In alternative embodiments, AtATR2 may be replaced by another *Arabidopsis thaliana* CPR such AtATR1 (NP_194183.1), a *Medicago truncatula* CPR such as MtCPR1 (XP_003602898.1) or MtCPR2 (XP_003610109.1), a *Lotus japonicas* CPR such as LjCPR1 or LjCPR2 (BAG68945.1), or a *Glycyrrhiza uralensis* CPR such as GuCPR1 (AUG98241.1) or GuCPR2 (QCZ35624.1), or a functional variant having at least 70%

identity, homology or similarity to SEQ ID NO 7, SEQ ID NO 3, AtATR1, MtCPR1, MtCPR2, LjCPR1, LjCPR2, GuCPR1, or GuCPR2, respectively.

In some embodiments, the yeast cell expresses at least LpTPS8, NtEAH, AtATR2, and LpCPR1 as set forth in SEQ ID NO 1, SEQ ID NO 2, SEQ ID NO 3 and SEQ ID NO 7, respectively, or functional variants thereof having at least 65% identity, homology or similarity thereto. In some embodiments, the yeast cell expresses at least LpTPS8, DsEAH, AtATR2, and LpCPR1 as set forth in SEQ ID NO 1, SEQ ID NO 11, SEQ ID NO 3 and SEQ ID NO 7, respectively, or functional variants thereof having at least 65% identity, homology or similarity thereto.In some embodiments, the yeast cell expresses at least LpTPS8, SlEAH, AtATR2, and LpCPR1 as set forth in SEQ ID NO 1, SEQ ID NO 9, SEQ ID NO 3 and SEQ ID NO 7, respectively, or functional variants thereof having at least 65% identity, homology or similarity thereto. In some embodiments, the yeast cell expresses at least LpTPS8, CcEAH, AtATR2, and LpCPR1 as set forth in SEQ ID NO 1, SEQ ID NO 13, SEQ ID NO 3 and SEQ ID NO 7, respectively, or functional variants thereof having at least 65% identity, homology or similarity thereto. In some embodiments, the yeast cell expresses at least LpTPS8, CaEAH, AtATR2, and LpCPR1 as set forth in SEQ ID NO 1, SEQ ID NO 15, SEQ ID NO 3 and SEQ ID NO 7, respectively, or functional variants thereof having at least 65% identity, homology or similarity thereto. In alternative embodiments, AtATR2 may be replaced by another *Arabidopsis thaliana* CPR such AtATR1 (NP_194183.1), a *Medicago truncatula* CPR such as MtCPR1 (XP_003602898.1) or MtCPR2 (XP_003610109.1), a *Lotus japonicas* CPR such as LjCPR1 or LjCPR2 (BAG68945.1), or a *Glycyrrhiza uralensis* CPR such as GuCPR1 (AUG98241.1) or GuCPR2 (QCZ35624.1), or a functional variant having at least 70% identity, homology or similarity to SEQ ID NO 7, SEQ ID NO 3, AtATR1, MtCPR1, MtCPR2, LjCPR1, LjCPR2, GuCPR1, or GuCPR2, respectively.

In some embodiments, the yeast cell expresses at least LpTPS8 and NtEAH as set forth in SEQ ID NO 1 and SEQ ID NO 2, respectively, or functional variants thereof having at least 65% identity, homology or similarity thereto, and further expresses one or more of a *Lippia dulcis* CPR such as LdCPR1 (SEQ ID NO 7), a *Arabidopsis thaliana* CPR such as AtATR2 (SEQ ID NO 3) or AtATR1 (NP_194183.1), a *Medicago truncatula* CPR such as MtCPR1 (XP_003602898.1) or MtCPR2 (XP_003610109.1), a *Lotus japonicas* CPR such as LjCPR1 or LjCPR2 (BAG68945.1), or a *Glycyrrhiza uralensis* CPR such as GuCPR1 (AUG98241.1) or GuCPR2 (QCZ35624.1), or a

functional variant having at least 70% identity, homology or similarity to SEQ ID NO 7, SEQ ID NO 3, AtATR1, MtCPR1, MtCPR2, LjCPR1, LjCPR2, GuCPR1, or GuCPR2, respectively.

In some embodiments, the yeast cell expresses at least LpTPS8 and DsEAH as set forth in SEQ ID NO 1 and SEQ ID NO 11, respectively, or functional variants thereof having at least 65% identity, homology or similarity thereto, and further expresses one or more of a *Lippia dulcis* CPR such as LdCPR1 (SEQ ID NO 7), a *Arabidopsis thaliana* CPR such as AtATR2 (SEQ ID NO 3) or AtATR1 (NP_194183.1), a *Medicago truncatula* CPR such as MtCPR1 (XP_003602898.1) or MtCPR2 (XP_003610109.1), a *Lotus japonicas* CPR such as LjCPR1 or LjCPR2 (BAG68945.1), or a *Glycyrrhiza uralensis* CPR such as GuCPR1 (AUG98241.1) or GuCPR2 (QCZ35624.1), or a functional variant having at least 70% identity, homology or similarity to SEQ ID NO 7, SEQ ID NO 3, AtATR1, MtCPR1, MtCPR2, LjCPR1, LjCPR2, GuCPR1, or GuCPR2, respectively. In some embodiments, the yeast cell expresses at least LpTPS8 and SlEAH as set forth in SEQ ID NO 1 and SEQ ID NO 9, respectively, or functional variants thereof having at least 65% identity, homology or similarity thereto, and further expresses one or more of a *Lippia dulcis* CPR such as LdCPR1 (SEQ ID NO 7), a *Arabidopsis thaliana* CPR such as AtATR2 (SEQ ID NO 3) or AtATR1 (NP_194183.1), a *Medicago truncatula* CPR such as MtCPR1 (XP_003602898.1) or MtCPR2 (XP_003610109.1), a *Lotus japonicas* CPR such as LjCPR1 or LjCPR2 (BAG68945.1), or a *Glycyrrhiza uralensis* CPR such as GuCPR1 (AUG98241.1) or GuCPR2 (QCZ35624.1), or a functional variant having at least 70% identity, homology or similarity to SEQ ID NO 7, SEQ ID NO 3, AtATR1, MtCPR1, MtCPR2, LjCPR1, LjCPR2, GuCPR1, or GuCPR2, respectively. In some embodiments, the yeast cell expresses at least LpTPS8 and CcEAH as set forth in SEQ ID NO 1 and SEQ ID NO 13, respectively, or functional variants thereof having at least 65% identity, homology or similarity thereto, and further expresses one or more of a *Lippia dulcis* CPR such as LdCPR1 (SEQ ID NO 7), a *Arabidopsis thaliana* CPR such as AtATR2 (SEQ ID NO 3) or AtATR1 (NP_194183.1), a *Medicago truncatula* CPR such as MtCPR1 (XP_003602898.1) or MtCPR2 (XP_003610109.1), a *Lotus japonicas* CPR such as LjCPR1 or LjCPR2 (BAG68945.1), or a *Glycyrrhiza uralensis* CPR such as GuCPR1 (AUG98241.1) or GuCPR2 (QCZ35624.1), or a functional variant having at least 70% identity, homology or similarity to SEQ ID NO 7, SEQ ID NO 3, AtATR1, MtCPR1, MtCPR2, LjCPR1, LjCPR2, GuCPR1, or GuCPR2, respectively. In some

embodiments, the yeast cell expresses at least LpTPS8 and CaEAH as set forth in SEQ ID NO 1 and SEQ ID NO 15, respectively, or functional variants thereof having at least 65% identity, homology or similarity thereto, and further expresses one or more of a *Lippia dulcis* CPR such as LdCPR1 (SEQ ID NO 7), a *Arabidopsis thaliana* CPR such as AtATR2 (SEQ ID NO 3) or AtATR1 (NP_194183.1), a *Medicago truncatula* CPR such as MtCPR1 (XP_003602898.1) or MtCPR2 (XP_003610109.1), a *Lotus japonicas* CPR such as LjCPR1 or LjCPR2 (BAG68945.1), or a *Glycyrrhiza uralensis* CPR such as GuCPR1 (AUG98241.1) or GuCPR2 (QCZ35624.1), or a functional variant having at least 70% identity, homology or similarity to SEQ ID NO 7, SEQ ID NO 3, AtATR1, MtCPR1, MtCPR2, LjCPR1, LjCPR2, GuCPR1, or GuCPR2, respectively.

In some embodiments, the yeast cell may be further engineered to allow production of derivatives of hernandulcin. A yeast cell producing a derivative of hernandulcin may also produce hernandulcin.

Examples of useful yeast cells

The yeast cells disclosed herein producing or being capable of producing the compounds of interest, such as hernandulcin and/or one or more derivatives thereof might be referred to as production organisms, microbial factories, microbial production organisms, hosts, host cells, host organisms, production hosts, cell factories etc.

Various yeast species may be useful as production organisms of hernandulcin and/or one or more derivatives thereof according to the present disclosure. Thus, in some embodiments the yeast cell is a non-pathogenic yeast cell.

In some embodiments, the yeast cell belongs to a genus selected from *Saccharomyces*, *Pichia*, *Yarrowia*, *Kluyveromyces*, *Candida, Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon* and *Lipomyces*.

In some embodiments, the yeast cell belongs to a species selected from *Saccharomyces cerevisiae, Saccharomyces boulardi, Komagetella phaffi* (*Pichia pastoris*), *Kluyveromyces marxianus, Cryptococcus albidus, Lipomyces lipofera, Lipomyces starkeyi, Rhodosporidium toruloides, Rhodotorula glutinis, Trichosporon pullulan* and *Yarrowia lipolytica.* Preferably the yeast cell is a *S. cerevisiae* cell or a *Y. lipolytica* cell. In a preferred embodiment, the yeast cell is a *Y. lipolytica* cell.

The yeast cell may thus be a *Y. lipolytica* cell expressing any of the enzymes described herein. In particular, the *Y. lipolytica* cell may express a TPS such as LdTPS8 (SEQ ID NO 1) and a CYP such as NtEAH (SEQ ID NO 2), SlEAH (SEQ ID NO 9), DsEAH (SEQ ID NO 11), CcEAH (SEQ ID NO 13) or CaEAH (SEQ ID NO 15), or a functional variant thereof having at least 65% identity, homology or similarity to any of the aforementioned. Preferably the CYP is any of NtEAH (SEQ ID NO 2) and/or DsEAH (SEQ ID NO 11).

The yeast cell may be a *S. cerevisiae* cell expressing any of the enzymes described herein. In particular, the *S. cerevisiae* cell may express a TPS such as LdTPS8 (SEQ ID NO 1) and a CYP such as NtEAH (SEQ ID NO 2), SlEAH (SEQ ID NO 9), DsEAH (SEQ ID NO 11), CcEAH (SEQ ID NO 13) or CaEAH (SEQ ID NO 15), or a functional variant thereof having at least 65% identity, homology or similarity to any of the aforementioned. Preferably the CYP is any of NtEAH (SEQ ID NO 2) and/or DsEAH (SEQ ID NO 11).

The yeast cell may be a cell belonging to the species of *Saccharomyces boulardi*, *Komagetella phaffi* (*Pichia pastoris*), *Kluyveromyces marxianus, Cryptococcus albidus, Lipomyces lipofera, Lipomyces starkeyi, Rhodosporidium toruloides, Rhodotorula glutinis* and *Trichosporon pullulan*, expressing any of the enzymes described herein. In particular, the cell of *Saccharomyces boulardi*, *Komagetella phaffi* (*Pichia pastoris*), *Kluyveromyces marxianus, Cryptococcus albidus, Lipomyces lipofera, Lipomyces starkeyi, Rhodosporidium toruloides, Rhodotorula glutinis* and *Trichosporon pullulan* may express a TPS such as LdTPS8 (SEQ ID NO 1) and a CYP such as NtEAH (SEQ ID NO 2), SlEAH (SEQ ID NO 9), DsEAH (SEQ ID NO 11), CcEAH (SEQ ID NO 13) or CaEAH (SEQ ID NO 15), or a functional variant thereof having at least 65% identity, homology or similarity to any of the aforementioned. Preferably the CYP is any of NtEAH (SEQ ID NO 2) and/or DsEAH (SEQ ID NO 11).

Expression systems, nucleic acids and/or polynucleotides useful for obtaining yeast cells capable of producing hernandulcin and/or one or more derivatives thereof are disclosed anywhere herein, in particular in the section “Expression systems and nucleic acids”.

Other modifications

The yeast cells disclosed herein for production of hernandulcin may be further modified. Said modifications may be modifications to enhance the precursor supply, for example supply of native or heterologous metabolites involved in the production of (+)-epi-alpha-bisabolol, hernandulcin and/or derivatives thereof, or to reduce bypass-pathways. Thus, the yeast cell may be any of the yeast cells described herein above, for example a *Y. lipolytica* cell or a *S. cerevisiae* cell, and expressing a TPS, a CYP, and optionally a CPR, and may in addition be further modified as described herein below. Preferably the CYP is NtEAH (SEQ ID NO 2), SlEAH (SEQ ID NO 9), DsEAH (SEQ ID NO 11), CcEAH (SEQ ID NO 13) and/or CaEAH (SEQ ID NO 15).

Production of hernandulcin and/or one or more derivatives thereof requires farnesyl diphosphate (FPP) as a substrate. Thus, and without being bound by theory, it may require that the yeast cell is capable of producing FPP. If the yeast cell is not capable of producing FPP, it may require modification such as engineering of said yeast cell in order to enable it to synthesize FPP. Alternatively or additionally, if the yeast cell is not capable of producing FPP, production of hernandulcin and/or derivatives thereof may require supplementing FPP to the cultivation medium, whereby the yeast cell is capable of converting FPP to hernandulcin and/or one or more derivatives. Further, to this, if the yeast cell can produce FPP, production such as improved production of hernandulcin and/or derivatives thereof, may require that the production of FPP is increased such as improved.

Thus, in some embodiments, if the yeast cell is not capable of producing FPP, it may require modification or engineering of the yeast cell to enable production of FPP by said cell. It may be advantageous to modify the yeast cell in such a manner that FPP metabolism is directed towards increased FPP synthesis, thereby further increasing the titers of hernandulcin and/or one or more derivatives thereof. Thus, in some embodiments, the yeast cell comprises one or more modifications resulting in increased or improved availability of FPP. Such modification may be achieved by increasing or improving the mevalonate (MVA) pathway flux. In other words, it may be achieved by increasing the sesquiterpene pathway flux. Useful modifications is disclosed in Arnesen et al., 2020, in particular useful modifications of a *Y. lipolytica* cell such as one or more of the modifications of *Y. lipolytica* ST9149 disclosed in Arnesen et al., 2020.

In some embodiments, the one or more modifications is selected from a mutation, an insertion and/or a deletion. In some embodiments, the one or more modifications may be overexpression, upregulation, downregulation or deletion of a gene or other genetic element. In some embodiments, the one or more modifications may comprise overexpression of a gene or other genetic element.

In some embodiments, the one or more modifications may comprise modifying the activity of a polypeptide. In some embodiments, the one or more modifications may comprise reducing the activity of a polypeptide. In some embodiments, the one or more modifications may comprise increasing the activity of a polypeptide.

It may be advantageous to improve synthesis of acetyl-CoA and/or upregulate expression of one or more genes of the MVA pathway to improve synthesis of dimethylallyl diphosphate (DMAPP) and/or isopentyl diphosphate (IPP). In some embodiments, improved synthesis of DMAPP and/or IPP may comprising increasing the activity of one or more polypeptides such as proteins of the MVA pathway.

In some embodiments, the yeast cell overexpresses one or more genes involved in the synthesis of acetyl-CoA, such as overexpression of ATP citrate lyase (ACL), for example native ACL, and/or *Salmonella enterica* acetyl-CoA synthetase (SeACS) with a L641P substitution as described in Huang et al. 2018. In some embodiments, the yeast cell is a *Y. lipolytica* cell, and the cell overexpresses native ATP citrate lyase such as *Y. lipolytica* ATP citrate lyase 1 (YlACL1), for example YlACL1 as set forth in accession number: XM_503231, or functional variants thereof having at least 70% identity, homology or similarity thereto. In other embodiments, SeACS is as set forth in accession number: WP_000083882.1, with the lysine residue 641 substituted for proline (L641P) or a functional variant thereof having at least 70% identity, homology or similarity thereto. In some embodiments, the SeACS has a L641P substitution ($SeACS^{L641P}$). Thus, in some embodiments the SeACS is $SeACS^{L641P}$ or functional variants thereof having at least 70% identity, homology or similarity thereto. In some embodiments, the one or more modifications comprise increasing the activity of ACL such as ACL1 or YlACL1, and/or SeACS such as $SeACS^{L641P}$, or functional variants thereof having at least 70% identity, homology or similarity thereto, respectively. For example, the yeast cell may be a *Y. lipolytica* cell expressing $SeACS^{L641P}$, and further

expressing a TPS such as LdTPS8 (SEQ ID NO 1), a CYP such as NtEAH (SEQ ID NO 2), SlEAH (SEQ ID NO 9), DsEAH (SEQ ID NO 11), CcEAH (SEQ ID NO 13) or CaEAH (SEQ ID NO 15), and a CPR and further expressing or a functional variant thereof having at least 65% identity, homology or similarity to any of the aforementioned. In other embodiments, the yeast cell may be a *Y. lipolytica* cell expressing YlACL1, and further expressing a TPS such as LdTPS8 (SEQ ID NO 1), a CYP such as NtEAH (SEQ ID NO 2), SlEAH (SEQ ID NO 9), DsEAH (SEQ ID NO 11), CcEAH (SEQ ID NO 13) or CaEAH (SEQ ID NO 15), and a CPR, or a functional variant thereof having at least 65% identity, homology or similarity to any of the aforementioned.In some embodiments, the yeast cell overexpresses 3-hydroxy-3-methylglutaryl-CoA reductase (HMG), such as native HMG that can catalyse the conversion 3-hydroxy-3-methylglutaryl-CoA to mevalonic acid (mevalonate). In some embodiments, the yeast cell is a *Y. lipolytica* cell, and the cell overexpresses native HMG such as *Y. lipolytica* 3-hydroxy-3-methylglutaryl-CoA reductase 1 (YlHMG1), for example YlHMG1 as set forth in accession number: XP_503558 or a functional variant thereof having at least 70% identity, homology or similarity thereto. In some embodiments, the yeast cell is *S. cerevisiae,* and the cell expresses or overexpress a truncated version of HMG (tHMG). In some embodiments, the one or more modifications comprise increasing the activity of HMG such as tHMG1 or YlHMG1, or functional variants thereof having at least 70% identity, homology or similarity thereto, respectively. Thus, in other embodiments, the yeast cell may be a *Y. lipolytica* cell overexpressing YlHMG1, and further expressing a TPS such as LdTPS8 (SEQ ID NO 1), a CYP such as NtEAH (SEQ ID NO 2), SlEAH (SEQ ID NO 9), DsEAH (SEQ ID NO 11), CcEAH (SEQ ID NO 13) or CaEAH (SEQ ID NO 15), and a CPR and further expressing or a functional variant thereof having at least 65% identity, homology or similarity to any of the aforementioned. In other embodiments, the yeast cell may be a *S. cerevisiae* cell overexpressing tHMG1, and further expressing a TPS such as LdTPS8 (SEQ ID NO 1), a CYP such as NtEAH (SEQ ID NO 2), SlEAH (SEQ ID NO 9), DsEAH (SEQ ID NO 11), CcEAH (SEQ ID NO 13) or CaEAH (SEQ ID NO 15), and a CPR, or a functional variant thereof having at least 65% identity, homology or similarity to any of the aforementioned.

In some embodiments, the yeast cell overexpresses mevalonate kinase, such as native mevalonate kinase, that can catalyse the conversion of mevalonic acid (mevalonate) to mevalonate-3-phosphate. In some embodiments, the yeast cell is *Y. lipolytica,* and the

cell overexpresses native mevalonate kinase such as *Y. lipolytica* ERG12 (YlERG12), for example YlERG12 as set forth in accession number: XP_500956 or a functional variant thereof having at least 70% identity, homology or similarity thereto. In some embodiments, the one or more modifications comprise increasing the activity of mevalonate kinase such as YlERG12, or functional variants thereof having at least 70% identity, homology or similarity thereto, respectively. Thus, in other embodiments, the yeast cell may be a *Y. lipolytica* cell overexpressing YlERG12, and further expressing a TPS such as LdTPS8 (SEQ ID NO 1), a CYP such as NtEAH (SEQ ID NO 2), SlEAH (SEQ ID NO 9), DsEAH (SEQ ID NO 11), CcEAH (SEQ ID NO 13) or CaEAH (SEQ ID NO 15), and a CPR, or a functional variant thereof having at least 65% identity, homology or similarity to any of the aforementioned.

In some embodiments, the yeast cell overexpresses isopentyl diphosphate isomerase (IDI), such as native IDI. In some embodiments, the yeast cell is *Y. lipolytica,* and the cell overexpresses native isopentyl diphosphate isomerase 1 such as *Y. lipolytica* IDI1 (YlIDI1), for example YlIDI1 as set forth in accession number: XP_504974 or a functional variant thereof having at least 70% identity, homology or similarity thereto. In some embodiments, the one or more modifications comprise increasing the activity of IDI such as IDI1 or YlIDI1, or functional variants thereof having at least 70% identity, homology or similarity thereto, respectively. Thus, in other embodiments, the yeast cell may be a *Y. lipolytica* cell overexpressing YlIDI1, and further expressing a TPS such as LdTPS8 (SEQ ID NO 1), a CYP such as NtEAH (SEQ ID NO 2), SlEAH (SEQ ID NO 9), DsEAH (SEQ ID NO 11), CcEAH (SEQ ID NO 13) or CaEAH (SEQ ID NO 15), and a CPR, or a functional variant thereof having at least 65% identity, homology or similarity to any of the aforementioned.

In some embodiments, the yeast cell overexpresses farnesyl diphosphate synthase (FPPS), such as ERG20. In some embodiments, the yeast cell is a *Y. lipolytica* cell, and the cell overexpresses native FPPS such as *Y. lipolytica* ERG20 (YlERG20) for example YlERG20 as set forth in accession number: XP_503599 or a functional variant thereof having at least 70% identity, homology or similarity thereto. In some embodiments, the one or more modifications comprise increasing the activity of FPPS such as ERG20 or YlERG20, or functional variants thereof having at least 70% identity, homology or similarity thereto, respectively. Thus, in other embodiments, the yeast cell may be a *Y. lipolytica* cell overexpressing YlERG20, and further expressing a TPS

such as LdTPS8 (SEQ ID NO 1), a CYP such as NtEAH (SEQ ID NO 2), SlEAH (SEQ ID NO 9), DsEAH (SEQ ID NO 11), CcEAH (SEQ ID NO 13) or CaEAH (SEQ ID NO 15), and a CPR, or a functional variant thereof having at least 65% identity, homology or similarity to any of the aforementioned.

In some embodiments, the one or more polypeptides with modified activity, such as increased activity and/or reduced activity, are native to the yeast cell or are non-native to the yeast cell or a combination of native and non-native. In some embodiments, the mutation resulting in reduced activity of a polypeptide comprises down-regulation of a gene encoding said polypeptide and/or mutation of said polypeptide such as a loss-of-function mutation. In some embodiments, the mutation resulting in increased activity of a polypeptide comprises overexpression.

In some embodiments, the activity of native farnesyl-diphosphate farnesyl transferase, also known as squalene synthase (SQS), for example ERG9, is reduced for the yeast cell of the present disclosure as compared to the activity of SQS and/or ERG9 in a non-modified/non-engineered yeast cell. In some embodiments, reducing the activity of SQS and/or ERG9 is obtained by downregulating the expression of SQS and/or ERG9 in a modified/engineered yeast cell of the present disclosure compared to the expression in a non-modified/non-engineered cell. In some embodiments, reducing the activity of SQS and/or ERG9 is obtained by modification of the native SQS and/or ERG9 promoter (PrSQS and/or PrERG9) such as by mutating PrSQS and/or PrERG9 and/or replacing PrSQS and/or PrERG9 with the nucleic acid sequence of another promoter, for example another native or heterologous promoter to the yeast cell of the present disclosure, whereby the activity of SQS and/or ERG9 is reduced. In some embodiments, the yeast cell of the present disclosure is a *Y. lipolytica* cell, and the promoter of ERG9, and/or squalene synthase 1 (SQS1) (PrERG9 and/or PrSQS1) of the *Y. lipolytica* cell is replaced with the native promoter of lanosterol 14-alpha demethylase (ERG11) (PrERG11) of the *Y. lipolytica* cell. Thus, in some embodiments PrSQS such as PrSQS1 and/or PrERG9 is exchanged for PrERG11, whereby the activity of SQS and/or ERG9 is reduced. Thus, in other embodiments, the yeast cell may be a *Y. lipolytica* cell, wherein PrSQS1 is replaced by PrERG11, and further expressing a TPS such as LdTPS8 (SEQ ID NO 1), a CYP such as NtEAH (SEQ ID NO 2), SlEAH (SEQ ID NO 9), DsEAH (SEQ ID NO 11), CcEAH (SEQ ID NO 13) or CaEAH (SEQ ID NO 15), and a CPR, or a functional variant thereof having at least

65% identity, homology or similarity to any of the aforementioned. Thus, in other embodiments, the yeast cell may be a *Y. lipolytica* cell, wherein PrERG9 is replaced by PrERG11, and further expressing a TPS such as LdTPS8 (SEQ ID NO 1), a CYP such as NtEAH (SEQ ID NO 2), SlEAH (SEQ ID NO 9), DsEAH (SEQ ID NO 11), CcEAH (SEQ ID NO 13) or CaEAH (SEQ ID NO 15), and a CPR, or a functional variant thereof having at least 65% identity, homology or similarity to any of the aforementioned.

Improved production of FPP may be assessed by LC coupled to tandem mass spectrometry (MS/MS) as described in Luo et al., 2020.

The above mentioned modifications may be encoded by one or more of the nucleic acids, nucleic acid constructs, polynucleotides, nucleic acid molecules and/or expression systems disclosed in “Expression systems” herein below.

Any of the above-mentioned modifications may be introduced in the yeast cell, such as a yeast cell belonging to the species of *Saccharomyces boulardi*, *Komagetella phaffi* (*Pichia pastoris*), *Kluyveromyces marxianus, Cryptococcus albidus, Lipomyces lipofera, Lipomyces starkeyi, Rhodosporidium toruloides, Rhodotorula glutinis* and *Trichosporon pullulan*, expressing any of the enzymes described herein.

In particular, any of the above-mentioned modifications may be introduced in a *Y. lipolytica* cell expressing a TPS such as LdTPS8 (SEQ ID NO 1) and a CYP such as NtEAH (SEQ ID NO 2), SlEAH (SEQ ID NO 9), DsEAH (SEQ ID NO 11), CcEAH (SEQ ID NO 13) or CaEAH (SEQ ID NO 15), or a functional variant thereof having at least 65% identity, homology or similarity to any of the aforementioned. Preferably the CYP is any of NtEAH (SEQ ID NO 2) and/or DsEAH (SEQ ID NO 11). For example, the yeast cell may be a *Y. lipolytica* cell expressing a TPS such as LdTPS8 (SEQ ID NO 1), a CYP such as NtEAH (SEQ ID NO 2), SlEAH (SEQ ID NO 9), DsEAH (SEQ ID NO 11), CcEAH (SEQ ID NO 13) or CaEAH (SEQ ID NO 15), a CPR and or a functional variant thereof having at least 65% identity, homology or similarity to any of the aforementioned

In other embodiments, any one or more of the above-mentioned modifications may be introduced in a *S. cerevisiae* cell expressing a TPS such as LdTPS8 (SEQ ID NO 1) and a CYP such as NtEAH (SEQ ID NO 2), SlEAH (SEQ ID NO 9), DsEAH (SEQ ID

NO 11), CcEAH (SEQ ID NO 13) or CaEAH (SEQ ID NO 15), or a functional variant thereof having at least 65% identity, homology or similarity to any of the aforementioned. Preferably the *Y. lipolytica* and/or the *S. cerevisiae* cell also expresses a CPR, such as a CPR disclosed herein, for example in the sections "Cytochrome P450 reductase" and Production of hernandulcin. The CYP is preferably any of NtEAH (SEQ ID NO 2) and/or DsEAH (SEQ ID NO 11).

Expression systems and nucleic acids

Provided herein are expression systems useful for expression in a yeast cell being capable of producing (+)-*epi*-α-bisabolol, hernandulcin and/or one or more derivatives thereof as disclosed herein. Provided are also nucleic acids and/or yeast cell comprising said nucleic acids useful for producing (+)-*epi*-α-bisabolol, hernandulcin and/or one or more derivatives thereof. The present nucleic acids may be provided as one or more nucleic acid molecules, nucleic acid constructs or polynucleotides, for example they may be comprised in one or more vectors and/or expression systems. Such nucleic acids may be introduced in the cell by methods known in the art. The term expression system, nucleic acid constructs and polynucleotides may be used interchangeably herein. Said nucleic acid constructs, polynucleotides and/or expression systems may be useful for expression in, engineering and/or modifying a yeast cell.

It will be understood that throughout the present disclosure, the term 'nucleic acid encoding a polypeptide" and/or "polynucleotide encoding a polypeptide" shall refer to a nucleic acid such as a nucleic acid molecule or nucleic acid construct capable of encoding a polypeptide, a protein or a fragment thereof. Such nucleic acid molecules be open reading frames or genes, or fragments thereof.

In one aspect, the present disclosure provides an expression system for expression in a yeast cell, comprising:

i. a nucleic acid encoding at least one (+)-epi-alpha-bisabolol synthase, preferably a heterologous (+)-epi-alpha-bisabolol synthase such as *Lippia dulcis* terpene synthase 8 (LdTPS8) (EC 4.2.3.138) as set forth in SEQ ID NO 1 or a functional variant thereof having at least 70% identity, homology or similarity thereto;

ii. a nucleic acid encoding at least one cytochrome P450 enzyme (EC), preferably a heterologous cytochrome P450 enzyme, for example a heterologous plant cytochrome P450 enzyme such as *Nicotiana tabacum* 5-epi-aristolocene dihydroxylase (NtEAH) as set forth in SEQ ID NO 2, DsEAH as set forth in SEQ ID NO 11), *Solanum lycopersicum* premnaspirodiene oxygenase-like protein (SlEAH) as set forth in SEQ ID NO 9, CcEAH as set forth in SEQ ID NO 13 and/or *Capsicum annuum* cytochrome P450 71D7-like protein (CaEAH) as set forth in SEQ ID NO 15, or a functional variant thereof having at least 65% identity, homology or similarity thereto; and

iii. optionally a nucleic acid encoding at least one cytochrome P450 reductase, preferably a heterologous cytochrome P450 reductase such as *Arabidopsis thaliana* cytochrome P450 reductase ATR2 (AtATR2) (EC 1.6.2.4) as set forth in SEQ ID NO 3 or a functional variant thereof having at least 70% identity, homology or similarity thereto.

In some embodiments, the expression system comprises a further nucleic acid encoding at least one further cytochrome P450 reductase (CPR), preferably a heterologous CPR such as *Lippia dulcis* CPR LdCPR1 as set forth in SEQ ID NO 7, or a functional variant thereof having at least 70% identity, homology or similarity thereto.

In some embodiments, the nucleic acid encoding at least one CPR and/or the nucleic acid encoding the at least one further CPR is a heterologous CPR, optionally a plant CPR. In some embodiments, said plant CPR is selected from a *Lippia dulcis* CPR such as LdCPR1 (SEQ ID NO 7), a *Arabidopsis thaliana* CPR such as AtATR2 (NP_194750.1) or AtATR1 (NP_194183.1), a *Medicago truncatula* CPR such as MtCPR1 (XP_003602898.1) or MtCPR2 (XP_003610109.1), a *Lotus japonicas* CPR such as LjCPR1 or LjCPR2 (BAG68945.1), or *Glycyrrhiza uralensis* CPR such as GuCPR1 (AUG98241.1) or GuCPR2 (QCZ35624.1), or a functional variant thereof having at least 70% identity, homology or similarity thereto, encoding a CPR.

Thus, in some embodiments, the present disclosure provides an expression system for expression in a yeast cell, said expression system comprising:

i. a nucleic acid encoding the at least one (+)-epi-alpha-bisabolol synthase, preferably said nucleic acid comprises or consists of *LdTPS8*

as set forth in SEQ ID NO 4 or a homologue thereof having at least 70% identity, homology or similarity thereto;

ii. a nucleic acid encoding the at least one cytochrome P450 enzyme, preferably said nucleic acid comprises or consists of:

i. *NtEAH* as set forth in SEQ ID NO 5,
ii. *SlEAH* as set forth in SEQ ID NO 10,
iii. *DsEAH* as set forth in SEQ ID NO 12,
iv. *CcEAH* as set forth in SEQ ID NO 14,
v. *CaEAH* as set forth in SEQ ID NO 16,

or a homologue thereof having at least 65% identity, homology or similarity thereto; and

iii. optionally a nucleic acid encoding the at least one cytochrome P450 reductase, preferably said nucleic acid comprises or consists of *AtATR2* as set forth in SEQ ID NO 6 or a homologue thereof having at least 70% identity, homology or similarity thereto; and

iv. optionally a nucleic acid encoding the at least one further cytochrome P450 reductase, preferably said nucleic acid comprises or consists of *LdCPR1* as set forth in SEQ ID NO 8 or a homologue thereof having at least 70% identity, homology or similarity thereto.

In some embodiments, the expression system comprises one or more nucleic acids comprising *LdTPS8* as set forth in SEQ ID NO 4, *NtEAH* as set forth in SEQ ID NO 5, *AtATR2* as set forth in SEQ ID NO 6, and/or *LdCPR1* as set forth in SEQ ID NO 8 or homologues thereof having at least 65% identity, homology or similarity to SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 6, and/or SEQ ID NO 8, respectively, such as at least 71%, such as at least 72%, such as at least 73%, such as at least 74%, such as at least 75%, such as at least 76%, such as at least 77%, such as at least 78%, such as at least 79%, such as at least 80%, such as at least 81%, such as at least 82%, such as at least 83%, such as at least 84%, such as at least 85%, such as at least 86%, such as at least 87%, such as at least 87%, such as at least 88%, such as at least 89%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% identity, similarity, or homology to SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 6, and/or SEQ ID NO 8, respectively.

In some embodiments, the expression system comprises one or more nucleic acids comprising *LdTPS8* as set forth in SEQ ID NO 4 and *NtEAH* as set forth in SEQ ID NO 5 or homologues thereof having at least 65% identity, homology or similarity to SEQ ID NO 4 and SEQ ID NO 5, respectively. In other embodiments, the expression system comprises one or more nucleic acids comprising *LdTPS8* as set forth in SEQ ID NO 4, *NtEAH* as set forth in SEQ ID NO 5 and *AtATR2* as set forth in SEQ ID NO 6 or homologues thereof having at least 65% identity, homology or similarity to SEQ ID NO 4, SEQ ID NO 5 and SEQ ID NO 6, respectively. In some embodiments, the expression system comprises one or more nucleic acids comprising *LdTPS8* as set forth in SEQ ID NO 4, *NtEAH* as set forth in SEQ ID NO 5, and *LdCPR1* as set forth in SEQ ID NO 8 or homologues thereof having at least 65% identity, homology or similarity to SEQ ID NO 4, SEQ ID NO 5 and SEQ ID NO 8, respectively. In other embodiments, the expression system comprises one or more nucleic acids comprising *LdTPS8* as set forth in SEQ ID NO 4, *NtEAH* as set forth in SEQ ID NO 5, *AtATR2* as set forth in SEQ ID NO 6, and *LdCPR1* as set forth in SEQ ID NO 8 or homologues thereof having at least 65% identity, homology or similarity to SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 6, and SEQ ID NO 8, respectively.

In some embodiments, the expression system comprises one or more nucleic acids comprising *LdTPS8* (SEQ ID NO 4), and *SlEAH* (SEQ ID NO 10), *DsEAH* (SEQ ID NO 12), *CcEAH* (SEQ ID NO 14), or *CaEAH* (SEQ ID NO 16), or homologues thereof having at least 65% identity, homology or similarity to any of the aforementioned. In other embodiments, the expression system comprises one or more nucleic acids comprising *LdTPS8* (SEQ ID NO 4), *SlEAH* (SEQ ID NO 10), *DsEAH* (SEQ ID NO 12), *CcEAH* (SEQ ID NO 14), or *CaEAH* (SEQ ID NO 16), and *AtATR2* (SEQ ID NO 6), or homologues thereof having at least 65% identity, homology or similarity to any of the aforementioned. In some embodiments, the expression system comprises one or more nucleic acids comprising *LdTPS8* (SEQ ID NO 4), *SlEAH* (SEQ ID NO 10), *DsEAH* (SEQ ID NO 12), *CcEAH* (SEQ ID NO 14), or *CaEAH* (SEQ ID NO 16), and *LdCPR1* (SEQ ID NO 8), or homologues thereof having at least 65% identity, homology or similarity to any of the aforementioned.

In other embodiments, the expression system further comprises one or more promoters such as PrTEF, PrGPD, or homologues thereof having at least 70% identity, homology or similarity thereto, respectively.

In some embodiments, one or more of the at least one (+)-epi-alpha-bisabolol synthase nucleic acid, the at least one cytochrome P450 enzyme nucleic acid, the at least one cytochrome P450 reductase nucleic acid and/or the at least one further P450 reductase nucleic acid is codon-optimised.

Preferably, the yeast cell is as defined herein above in “Yeast cell” and/or anywhere else herein.

In some embodiments, said yeast cell comprising the expression system disclosed herein, is capable of producing hernandulcin and/or one or more derivatives thereof.

In some embodiments, each of the nucleic acids encoding each of the present polynucleotides, i.e. a (+)-epi-alpha-bisabolol synthase, a cytochrome P450 enzyme, and a cytochrome P450 reductase may be designed to be integrated within the genome of the yeast cell and/or they may be within one or more vectors comprised within the yeast cell and/or a combination of both integrated within the genome and within one or more vectors comprised within the yeast cell. In some embodiments, the nucleic acids encoding each of the present polynucleotides, i.e. a (+)-epi-alpha-bisabolol synthase, a cytochrome P450 enzyme, and a cytochrome P450 reductase may be designed to be integrated within the genome of the yeast cell. In some embodiments, the expression system comprising one or more of the present polynucleotides, such as the at least one (+)-epi-alpha-bisabolol synthase, the at least one cytochrome P450 enzyme, the at least one cytochrome P450 reductase, and the at least one further cytochrome P450 reductase may be designed to be integrated within the genome of the yeast cell.

In some embodiments, one or more of the nucleic acids encoding each of the present polynucleotides may be integrated in the genome of said yeast cell. Methods for integrating a nucleic acid are well known in the art. Thus, in some embodiments the polynucleotide of interest is encoded by introduction of a heterologous nucleic acid in the yeast cell. The nucleic acid such as heterologous nucleic acid encoding said polynucleotide may be codon-optimised, and/or may comprise features that can help improve the polynucleotide. Such modifications include, but are not limited to, introduction of localisation signals, gain-of-function and/or loss-of-function mutations,

fusion of the polypeptide to a marker or a tag such as fluorescent tag, insertion of an inducible promoter, introduction of modifications conferring increased stability and/or half-life.

The introduction of the heterologous nucleic acid encoding the polynucleotide of interest can be performed by methods known in the art. The skilled person will recognise that such methods include, but are not limited to: cloning and homologous recombination-based methods. Cloning methods may involve the design and construction of a plasmid e.g. in an organism such as *Escherichia coli*. The plasmid may be an integrative or a non-integrative vector. Cloning-free methods comprise homologous recombination-based methods such as adaptamer-mediated PCR or gap repair. Such methods often result in integration of the heterologous nucleic acid in the genome of the yeast cell.

The nucleic acids encoding the polynucleotides of interest may be present in high copy number. The nucleic acids encoding the polynucleotides of interest may be present in low copy number.

In some embodiments, the nucleic acid, nucleic acid construct, polynucleotide and/or expression system further comprises or consists of one or more vectors, such as an integrative vector or a replicative vector. In some embodiments, the vector is a high copy replicative vector. In some embodiments, the vector is a low copy replicative vector. In some embodiments, the vector is an episomal plasmid.

Each of the nucleic acids comprised within the present expression system(s) may be present in multiple copies in the cell. In some embodiments, at least one of the nucleic acids is present in the cell in at least 2 copies, such as at least 3 copies, such as at least 4 copies, such as at least 5 copies, such as at least 10 copies, such as at least 20 copies, such as at least 30 copies, such as at least 50 copies, such as at least 70 copies, such as at least 80 copies, such as at least 100 copies, such as at least 200 copies, or more. In some embodiments, all of the nucleic acids are present in the cell at least 2 copies, such as at least 3 copies, such as at least 4 copies, such as at least 5 copies, such as at least 10 copies, such as at least 20 copies, such as at least 30 copies, such as at least 50 copies, such as at least 70 copies, such as at least 80

copies, such as at least 100 copies, such as at least 150 copies, such as at least 200 copies, or more.

The expression systems may, in addition to the at least one (+)-epi-alpha-bisabolol synthase nucleic acid, the at least one cytochrome P450 enzyme nucleic acid, the at least one cytochrome P450 reductase nucleic acid and/or the at least one further P450 reductase nucleic acid disclosed above, also comprise additional nucleic acids or polynucleotides useful for introducing additional modifications in the yeast cell, to obtain cells as disclosed in "Other modifications" above or anywhere herein. Designing such additional nucleic acids, nucleic acid constructs or polynucleotides can be performed as is known in the art.

The nucleic acid constructs may be one or more PCR products or one or more synthetic DNA molecules.

In a further aspect, the present disclosure provides a yeast cell comprising:

i. a nucleic acid encoding the at least one (+)-epi-alpha-bisabolol synthase, preferably wherein said nucleic acid comprises or consists of *LdTPS8* as set forth in SEQ ID NO 4, or a homologue thereof having at least 70% identity, homology or similarity thereto;
ii. a nucleic acid encoding the at least one cytochrome P450 enzyme, preferably wherein said nucleic acid comprises or consists of:
    i. *NtEAH* as set forth in SEQ ID NO 5,
    ii. *DsEAH* as set forth in SEQ ID NO 12,
    iii. *SlEAH* as set forth in SEQ ID NO 10,
    iv. *CcEAH* as set forth in SEQ ID NO 14,
    v. *CaEAH* as set forth in SEQ ID NO 16,
    or homologues thereof having at least 65% identity, homology or similarity thereto; and
iii. a nucleic acid encoding the at least one plant cytochrome P450 reductase, preferably wherein said nucleic acid comprises or consists of *AtATR2* as set forth in SEQ ID NO 6, or a homologue thereof having at least 70% identity, homology or similarity thereto; and
iv. optionally a nucleic acid encoding the at least one further cytochrome P450 reductase, preferably wherein said nucleic acid comprises or consists of

LdCPR1 as set forth in SEQ ID NO 8, or a homologue thereof having at least 70% identity, homology or similarity thereto.

The at least one (+)-epi-alpha-bisabolol synthase nucleic acid, the at least one plant cytochrome P450 enzyme nucleic acid, the at least one cytochrome P450 reductase nucleic acid and/or the at least one further P450 reductase nucleic acid may be native or codon-optimised.

The yeast cell may be a yeast cell that is useful for producing (+)-*epi*-α-bisabolol, hernandulcin and/or one or more derivatives thereof, such as a yeast cell disclosed herein and in particular in the section "Yeast cell".

Methods for producing hernandulcin

The present disclosure relates to methods for producing hernandulcin and/or one or more derivatives thereof. The yeast cells, expression systems and nucleic acids disclosed herein are useful for microbial-based production of hernandulcin and/or one or more derivatives thereof.

Throughout the present disclosure, it will be understood that the yeast cells disclosed herein can produce the compounds of interest listed herein such as hernandulcin when incubated in a cultivation medium under conditions that enable the cells to grow and produce the desired compound and/or product. From the description of the yeast cells, also sometimes referred to as cells, production host cells, production hosts or host cells, provided herein, and knowing the type of host cell used, the skilled person will not have difficulties in identifying suitable cultivation media and/or conditions to achieve production of said compounds and/or products.

In particular, the cultivation may be performed aerobically or anaerobically, at temperatures and at pH suitable for supporting growth of the cells. The cultivation medium should include the required nutrients, and may be supplemented with precursors as applicable. The time of cultivation will vary depending on which cell is used, but can easily be adapted by the skilled person.

In one aspect, the present disclosure provides a method for producing hernandulcin and/or one or more derivatives thereof in a yeast cell, said method comprising the steps of:

i. providing a yeast cell disclosed herein; and
ii. incubating said yeast cell in a medium,

whereby hernandulcin and/or one or more derivatives thereof is produced.

Also provided herein are yeast cells (+)-*epi*-α-bisabolol, hernandulcin and/or one or more derivatives thereof comprising an expression system disclosed herein above and anywhere else herein, in particular in the section “Expression systems”.

Indeed, yeast cells useful for producing (+)-*epi*-α-bisabolol, hernandulcin and/or one or more derivatives thereof are disclosed herein above and anywhere else herein, in particular in the section “Yeast cell”.

Also provided herein is (+)-*epi*-α-bisabolol, hernandulcin and/or one or more derivatives thereof obtainable by the methods disclosed herein.

Recovery

The present methods may comprise a further step of recovering and/or purifying the compounds and/or products such as hernandulcin and/or one or more derivatives obtained by the methods and/or yeast cells disclosed herein. Methods for recovering and/or purifying (+)-*epi*-α-bisabolol, hernandulcin and/or one or more derivatives thereof obtained by the present disclosure are known in the art, for example ethyl acetate extraction and silica column purification for (+)-*epi*-α-bisabolol, and supercritical fluid extraction for hernandulcin as described by Attia et al., 2012 or De Oliveira et al., 2012.

For example, the step of recovering the compound(s) such as hernandulcin and/or one or more derivatives may comprise separating the cell culture in a solid phase to obtain a cell phase and in a liquid phase to obtain a supernatant. The compounds may be present in the supernatant and/or the cell phase. The cell phase is also known as cell pellet. Supercritical fluid extraction may be used to recover one or more compounds of interest from the cell phase such as described by De Oliveira et al., 2012.

The present methods may further comprise a step of converting hernandulcin and/or one or more derivatives to one or more further derivatives thereof.

Thus, in one aspect the present methods may further comprise the steps of:

i. recovering the hernandulcin and/or the one or more derivatives thereof; and
ii. optionally converting said hernandulcin and/or the one or more derivatives thereof to one or more further derivatives thereof; and/or
iii. formulating said hernandulcin and/or the one or more derivatives thereof in a composition.

The composition may be a food composition and/or a beverage.

Step ii of the method above may be performed prior to or after step i. Thus, step i may be performed prior to or after step ii.

Yeast cells useful for producing (+)-*epi*-α-bisabolol, hernandulcin and/or one or more derivatives thereof are disclosed herein above and anywhere else herein, in particular in the section “Yeast cell”.

Also provided herein is (+)-*epi*-α-bisabolol, hernandulcin and/or one or more derivatives thereof obtainable by the methods disclosed herein.

In some embodiments, the method is for production of hernandulcin and/or one or more derivatives thereof and further comprises a step of recovering said hernandulcin and/or one or more derivatives thereof. In some embodiments, the method is for production of hernandulcin and further comprises a step of recovering said hernandulcin.

In some embodiments, the method is for production of hernandulcin and/or one or more derivatives thereof and further comprises a step of converting said hernandulcin and/or one or more derivatives thereof to one or more further derivatives thereof.

Conversion of hernandulcin and/or one or more derivatives into one or more further derivatives thereof may be performed by chemical synthesis, such as by contacting

said hernandulcin and/or one or more derivative with one or more chemical reagents to react the compounds and reagents, whereby hernandulcin and/or one or more derivatives are being converted into to one or more further derivatives thereof said one or more further derivatives are produced.

Thus, in some embodiments the method is for production of hernandulcin and/or one or more derivatives thereof and further comprises a step of converting said hernandulcin and/or one or more derivatives thereof to a derivative thereof. In some embodiments the method is for production of hernandulcin and/or one or more derivatives thereof and further comprises a step of converting said hernandulcin and/or one or more derivatives thereof to one or more further derivatives thereof. Further, in some embodiments the method is for production of hernandulcin and further comprises a step of converting said hernandulcin to one or more derivatives thereof. In some embodiments the method is for production of hernandulcin and further comprises a step of converting said hernandulcin to one or more derivatives thereof and further to one or more further derivatives thereof.

In some embodiments, the method is for production of hernandulcin and further comprise a step of recovering said hernandulcin as well as converting said hernandulcin into a derivative. In some embodiments, the method is for production of hernandulcin and further comprises a step of recovering said hernandulcin as well as converting said hernandulcin into one or more further derivatives thereof.

A derivative of hernandulcin is for example 4-hydroxy-hernandulcin. Thus, provided herein are yeast cells, methods and/or use of enzymes useful for production of 4-hydroxy-hernandulcin.

Products and compositions

Also provided herein is (+)-*epi*-α-bisabolol, hernandulcin and/or one or more derivatives thereof obtainable by the methods disclosed herein. Thus, disclosed herein is hernandulcin obtainable by the methods disclosed herein.

Provided herein is also a fermentation liquid comprising hernandulcin and/or one or more derivatives thereof obtained by the methods disclosed herein, such as in the section “Methods for producing hernandulcin”. Said fermentation liquid may be

processed further to obtain a processed fermentation liquid. In some embodiments, the fermentation liquid comprises at least 50% yeast cell debris. In other words, the fermentation liquid may comprise 50% yeast cell debris. In some embodiments, at least 50% of cellular material, such as yeast cell debris, is separated from the fermentation liquid. Thus, in other embodiments, the fermentation liquid comprises less than 50% yeast cell debris and/or cellular material.

Provided herein is also a composition comprising hernandulcin and/or one or more derivatives thereof obtained by the methods disclosed herein. Said composition may be a food composition and/or a beverage. Thus, provided herein is also a food composition and/or a beverage comprising hernandulcin and/or one or more derivatives thereof, preferably wherein said hernandulcin and/or one or more derivatives thereof is obtained by a method and/or a yeast cell disclosed herein.

Titer

The yeast cells and/or methods disclosed in the present disclosure are capable of producing (+)-*epi*-α-bisabolol, hernandulcin and/or one or more derivatives thereof. In preferred embodiments, the yeast cells are capable of producing hernandulcin.

A method for quantifying hernandulcin is provided in the section “Example 1 – Materials and Methods”, particularly in “Cultivation and sample preparation”.

In some embodiments, the yeast cells disclosed herein are capable of producing (+)-*epi*-α-bisabolol, hernandulcin and/or one or more derivatives thereof with a titer of at least 10 µg/L, such as at least 15 µg/L, such as at least 20 µg/L, such as at least 25 µg/L, such as at least 40 µg/L, such as at least 50 µg/L, such as at least 75 µg/L, such as at least 100 µg/L, such as at least 125 µg/L, such as at least 145 µg/L, such as at least 165 µg/L, such as at least 170 µg/L, such as at least 180 µg/L, such as at least 190 µg/L, such as at least 200 µg/L, such as at least 220 µg/L, such as at least 250 µg/L, such as at least 300 µg/L, such as at least 350 µg/L, such as at least 400 µg/L, such as at least 500 µg/L, such as at least 750 µg/L, such as at least 1 g/L, or more.

In some embodiments, the yeast cells disclosed herein are capable of producing hernandulcin and/or one or more derivatives thereof with a titer of at least 10 µg/L, such as at least 15 µg/L, such as at least 20 µg/L, such as at least 25 µg/L, such as at

least 40 µg/L, such as at least 50 µg/L, such as at least 75 µg/L, such as at least 100 µg/L, such as at least 125 µg/L, such as at least 145 µg/L, such as at least 165 µg/L, such as at least 170 µg/L, such as at least 180 µg/L, such as at least 190 µg/L, such as at least 200 µg/L, such as at least 220 µg/L, such as at least 250 µg/L, such as at least 300 µg/L, such as at least 350 µg/L, such as at least 400 µg/L, such as at least 500 µg/L, such as at least 750 µg/L, such as at least 1 g/L, or more.

In some embodiments, the yeast cells disclosed herein are capable of producing hernandulcin with a titer of at least 10 µg/L, such as at least 15 µg/L, such as at least 20 µg/L, such as at least 25 µg/L, such as at least 40 µg/L, such as at least 50 µg/L, such as at least 75 µg/L, such as at least 100 µg/L, such as at least 125 µg/L, such as at least 145 µg/L, such as at least 165 µg/L, such as at least 170 µg/L, such as at least 180 µg/L, such as at least 190 µg/L, such as at least 200 µg/L, such as at least 220 µg/L, such as at least 250 µg/L, such as at least 300 µg/L, such as at least 350 µg/L, such as at least 400 µg/L, such as at least 500 µg/L, such as at least 750 µg/L, such as at least 1 g/L, or more.

The methods disclosed herein are capable of yielding a titer of (+)-*epi*-α-bisabolol, hernandulcin and/or one or more derivatives thereof of at least 10 µg/L, such as at least 15 µg/L, such as at least 20 µg/L, such as at least 25 µg/L, such as at least 40 µg/L, such as at least 50 µg/L, such as at least 75 µg/L, such as at least 100 µg/L, such as at least 125 µg/L, such as at least 145 µg/L, such as at least 165 µg/L, such as at least 170 µg/L, such as at least 180 µg/L, such as at least 190 µg/L, such as at least 200 µg/L, such as at least 220 µg/L, such as at least 250 µg/L, such as at least 300 µg/L, such as at least 350 µg/L, such as at least 400 µg/L, such as at least 500 µg/L, such as at least 750 µg/L, such as at least 1 g/L, or more.

In some embodiments, the methods disclosed herein are capable of yielding a titer of hernandulcin and/or one or more derivatives thereof of at least 10 µg/L, such as at least 15 µg/L, such as at least 20 µg/L, such as at least 25 µg/L, such as at least 40 µg/L, such as at least 50 µg/L, such as at least 75 µg/L, such as at least 100 µg/L, such as at least 125 µg/L, such as at least 145 µg/L, such as at least 165 µg/L, such as at least 170 µg/L, such as at least 180 µg/L, such as at least 190 µg/L, such as at least 200 µg/L, such as at least 220 µg/L, such as at least 250 µg/L, such as at least

300 µg/L, such as at least 350 µg/L, such as at least 400 µg/L, such as at least 500 µg/L, such as at least 750 µg/L, such as at least 1 g/L, or more.

In some embodiments, the methods disclosed herein are capable of yielding a titer of hernandulcin of at least 10 µg/L, such as at least 15 µg/L, such as at least 20 µg/L, such as at least 25 µg/L, such as at least 40 µg/L, such as at least 50 µg/L, such as at least 75 µg/L, such as at least 100 µg/L, such as at least 125 µg/L, such as at least 145 µg/L, such as at least 165 µg/L, such as at least 170 µg/L, such as at least 180 µg/L, such as at least 190 µg/L, such as at least 200 µg/L, such as at least 220 µg/L, such as at least 250 µg/L, such as at least 300 µg/L, such as at least 350 µg/L, such as at least 400 µg/L, such as at least 500 µg/L, such as at least 750 µg/L, such as at least 1 g/L, or more.

Thus, the yeast cell producing hernandulcin with any of the above titers may be a yeast cell belonging to a genus selected from *Saccharomyces* such as *Saccharomyces cerevisiae* and *Saccharomyces boulardi*, *Pichia* such as *Komagetella phaffi* (*Pichia pastoris*), *Yarrowia* such as *Y. lipolytica*, *Kluyveromyces* such as *Kluyveromyces marxianus*, *Candida, Rhodotorula* such as *Rhodotorula glutinis, Rhodosporidium* such as *Rhodosporidium toruloides, Cryptococcus* such as *Cryptococcus albidus, Trichosporon* such as *Trichosporon pullulan* and *Lipomyces* such as *Lipomyces lipofera* or *Lipomyces starkeyi*.

In preferred embodiments, the yeast cell producing hernandulcin is a *Y. lipolytica* cell capable of producing hernandulcin and/or one of more derivatives thereof with a titer of at least 10 µg/L, such as at least 15 µg/L, such as at least 20 µg/L, such as at least 25 µg/L, such as at least 40 µg/L, such as at least 50 µg/L, such as at least 75 µg/L, such as at least 100 µg/L, such as at least 125 µg/L, such as at least 145 µg/L, such as at least 165 µg/L, such as at least 170 µg/L, such as at least 180 µg/L, such as at least 190 µg/L, such as at least 200 µg/L, such as at least 220 µg/L, such as at least 250 µg/L, such as at least 300 µg/L, such as at least 350 µg/L, such as at least 400 µg/L, such as at least 500 µg/L, such as at least 750 µg/L, such as at least 1 g/L, or more. Said *Y. lipolytica* cell may be used in a method of producing hernandulcin and/or one of more derivatives thereof yielding a titer of hernandulcin of at least 10 µg/L, such as at least 15 µg/L, such as at least 20 µg/L, such as at least 25 µg/L, such as at least 40

µg/L, such as at least 50 µg/L, such as at least 75 µg/L, such as at least 100 µg/L, such as at least 125 µg/L, such as at least 145 µg/L, such as at least 165 µg/L, such as at least 170 µg/L, such as at least 180 µg/L, such as at least 190 µg/L, such as at least 200 µg/L, such as at least 220 µg/L, such as at least 250 µg/L, such as at least 300 µg/L, such as at least 350 µg/L, such as at least 400 µg/L, such as at least 500 µg/L, such as at least 750 µg/L, such as at least 1 g/L, or more.

In other preferred embodiments, the yeast cell producing hernandulcin is a *S. cerevisiae* cell capable of producing hernandulcin and/or one of more derivatives thereof with a titer of at least 10 µg/L, such as at least 15 µg/L, such as at least 20 µg/L, such as at least 25 µg/L, such as at least 40 µg/L, such as at least 50 µg/L, such as at least 75 µg/L, such as at least 100 µg/L, such as at least 125 µg/L, such as at least 145 µg/L, such as at least 165 µg/L, such as at least 170 µg/L, such as at least 180 µg/L, such as at least 190 µg/L, such as at least 200 µg/L, such as at least 220 µg/L, such as at least 250 µg/L, such as at least 300 µg/L, such as at least 350 µg/L, such as at least 400 µg/L, such as at least 500 µg/L, such as at least 750 µg/L, such as at least 1 g/L, or more. Said *S. cerevisiae* cell may be used in a method of producing hernandulcin and/or one of more derivatives thereof yielding a titer of hernandulcin of at least 10 µg/L, such as at least 15 µg/L, such as at least 20 µg/L, such as at least 25 µg/L, such as at least 40 µg/L, such as at least 50 µg/L, such as at least 75 µg/L, such as at least 100 µg/L, such as at least 125 µg/L, such as at least 145 µg/L, such as at least 165 µg/L, such as at least 170 µg/L, such as at least 180 µg/L, such as at least 190 µg/L, such as at least 200 µg/L, such as at least 220 µg/L, such as at least 250 µg/L, such as at least 300 µg/L, such as at least 350 µg/L, such as at least 400 µg/L, such as at least 500 µg/L, such as at least 750 µg/L, such as at least 1 g/L, or more.

Uses

Further disclosed herein is the use of a *Nicotiana* cytochrome P450 enzyme, preferably a *Nicotiana tabacum* cytochrome P450 enzyme, in a method for producing hernandulcin and/or one or more derivatives, optionally wherein the *Nicotiana tabacum* cytochrome P450 enzyme is the polypeptide as set forth in SEQ ID NO 2, or a functional variant thereof having at least 65% identity, homology or similarity thereto. Disclosed herein is also the use of a *Datura* cytochrome P450 enzyme, preferably a

Datura stramonium cytochrome P450 enzyme, in a method for producing hernandulcin and/or one or more derivatives, optionally wherein the *Datura stramonium* cytochrome P450 enzyme is DsEAH as set forth in SEQ ID NO 11, or a functional variant thereof having at least 65% identity, homology or similarity thereto. Also disclosed is the use of a *Solanum* cytochrome P450 enzyme, preferably a *Solanum lycopersicum* cytochrome P450 enzyme, in a method for producing hernandulcin and/or one or more derivatives, optionally wherein the *Solanum lycopersicum* cytochrome P450 enzyme is SlEAH as set forth in SEQ ID NO 9, or a functional variant thereof having at least 65% identity, homology or similarity thereto. Further disclosed herein is use of a *Capsicum* cytochrome P450 enzyme, preferably a *Capsicum chinense* cytochrome P450 enzyme and/or a *Capsicum annuum* cytochrome P450 enzyme, in a method for producing hernandulcin and/or one or more derivatives, optionally wherein the *Capsicum chinense* cytochrome P450 enzyme is CcEAH as set forth in SEQ ID NO 13, the *Capsicum annuum* cytochrome P450 enzyme is CaEAH as set forth in SEQ ID NO 15, or a functional variants thereof having at least 65% identity, homology or similarity to any of the aforementioned.

In some embodiments, the polypeptide comprises the sequence as set forth in SEQ ID NO 2, SEQ ID NO 9, SEQ ID NO 11, SEQ ID NO 13 and/or SEQ ID NO 15, with the exception that at the most 54 residues are mutated, such as at the most 53 residues, such as at the most 52 residues, for example 51 residues are mutated, such as at the most 50 residues, for example at the most 49 residues, such as at the most 48 residues, for example at the most 45 residues, such as at the most 40 residues, for example at the most 35 residues, such as at the most 30 residues, for example at the most 25 residues, such as at the most 20 residues, for example at the most 15 residues, such as at the most 10 residues, for example at the most 5 residues, or less. Thus, in some embodiments. Thus, in some embodiments, the polypeptide comprises the sequence as set forth in SEQ ID NO 2, SEQ ID NO 9, SEQ ID NO 11, SEQ ID NO 13 and/or SEQ ID NO 15, with the exception that at the most 54 residues are mutated, for example between 54 and 51 residues are mutated, such as between 51 and 45 residues, such as between 35 and 45 residues, such as between 25 and 35 residues, such as between 15 and 25 residues, such as between 5 and 15 residues, or less.

In some embodiments, the use comprises expressing the *Nicotiana* cytochrome P450 enzyme, preferably a *Nicotiana tabacum* cytochrome P450 enzyme, optionally wherein

the *Nicotiana tabacum* cytochrome P450 enzyme is NtEAH as set forth in SEQ ID NO 2, or functional variants thereof having at least 65% identity, homology or similarity thereto in a yeast cell, wherein said yeast cell is as disclosed in "Yeast cell" herein above, or anywhere else herein.

In further some embodiments, the use comprises expressing the *Datura* cytochrome P450 enzyme, preferably a *Datura stramonium* cytochrome P450 enzyme, optionally wherein the *Datura stramonium* cytochrome P450 enzyme is DsEAH as set forth in SEQ ID NO 11, or functional variants thereof having at least 65% identity, homology or similarity thereto in a yeast cell, wherein said yeast cell is as disclosed in "Yeast cell" herein above, or anywhere else herein.

In other embodiments, the use comprises expressing the *Solanum* cytochrome P450 enzyme in a yeast cell, preferably a *Solanum lycopersicum* cytochrome P450 enzyme, optionally wherein the *Solanum lycopersicum* cytochrome P450 enzyme is SlEAH as set forth in SEQ ID NO 9, or functional variants thereof having at least 65% identity, homology or similarity thereto in a yeast cell, wherein said yeast cell is as disclosed in "Yeast cell" herein above, or anywhere else herein.

In further embodiments, the use comprises expressing the *Capsicum* cytochrome P450 enzyme in a yeast cell, preferably a *Capsicum chinense* cytochrome P450 enzyme and/or a *Capsicum annuum* cytochrome P450 enzyme, optionally wherein the *Capsicum chinense* cytochrome P450 enzyme is CcEAH as set forth in SEQ ID NO 13, the *Capsicum annuum* cytochrome P450 enzyme is CaEAH as set forth in SEQ ID NO 15, or functional variants thereof having at least 65% identity, homology or similarity to any of the aforementioned in a yeast cell, wherein said yeast cell is as disclosed in "Yeast cell" herein above, or anywhere else herein.

In some embodiments, the use of the *Nicotiana* cytochrome P450 enzyme, preferably a *Nicotiana tabacum* cytochrome P450 enzyme such as NtEAH as set forth in SEQ ID NO 2, or functional variants thereof having at least 65% identity, homology or similarity thereto, is in the method for producing hernandulcin and/or one or more derivatives as disclosed in "Methods for producing hernandulcin" herein above, or anywhere else herein. In other embodiments, the use of the *Datura* cytochrome P450 enzyme, preferably a *Datura stramonium* cytochrome P450 enzyme, optionally wherein the

Datura stramonium cytochrome P450 enzyme is DsEAH as set forth in SEQ ID NO 11, or functional variants thereof having at least 65% identity, homology or similarity thereto, is in the method for producing hernandulcin and/or one or more derivatives as disclosed in "Methods for producing hernandulcin" herein above, or anywhere else herein. In further other embodiments, the use of the *Solanum* cytochrome P450 enzyme in a yeast cell, preferably a *Solanum lycopersicum* cytochrome P450 enzyme, optionally wherein the *Solanum lycopersicum* cytochrome P450 enzyme is SlEAH as set forth in SEQ ID NO 9, or functional variants thereof having at least 65% identity, homology or similarity thereto, is in the method for producing hernandulcin and/or one or more derivatives as disclosed in "Methods for producing hernandulcin" herein above, or anywhere else herein. In further embodiments, the use of the *Capsicum* cytochrome P450 enzyme in a yeast cell, preferably a *Capsicum chinense* cytochrome P450 enzyme and/or a *Capsicum annuum* cytochrome P450 enzyme, optionally wherein the *Capsicum chinense* cytochrome P450 enzyme is CcEAH as set forth in SEQ ID NO 13, the *Capsicum annuum* cytochrome P450 enzyme is CaEAH as set forth in SEQ ID NO 15, or functional variants thereof having at least 65% identity, homology or similarity to any of the aforementioned, is in the method for producing hernandulcin and/or one or more derivatives as disclosed in "Methods for producing hernandulcin" herein above, or anywhere else herein.

Also provided herein is the use of nucleic acids, expression systems and/or yeast cells disclosed anywhere herein such as in "Expression systems" or "Yeast cell" herein above, for the production of hernandulcin and/or one or more derivatives thereof.

Examples

Example 1 – Materials and Methods

Strains and media

A strain previously engineered for high mevalonate (MVA) pathway flux (ST9149) or without engineering (ST6512) were used to construct the hernandulcin producing strains (Arnesen et al., 2020; Marella et al., 2020) (Table 3). Both strains were based on the W29-strain (Y-63746), received as a kind gift from the ARS Culture Collection, NCAUR, USA.

The DH5α *Escherichia coli* strain was used for plasmids construction. Lysogeny broth (LB) media with 100 mg/L ampicillin was used to cultivate *E. coli* cells at 300 rpm

shaking and 37°C. The *Y. lipolytica* cells were cultivated at 30°C on media containing 10 g/L yeast extract, 20 g/L peptone, and 20 or 80 g/L glucose (YPD or YPD80) with 20 g/L agar added for solid media. Hygromycin (400 mg/L) or nourseothricin (250 mg/L) was added to the media for yeast cell selection.

Strain construction

Biobricks, plasmids and strains are listed in Table 1, 2 and 3, respectively Phusion U polymerase (Thermo Scientific) was used to PCR-amplify the biobricks, which were assembled into EasyCloneYALI plasmids by USER cloning (Holkenbrink et al., 2018). The USER reactions were transformed into *E. coli* and correct assembly was verified by sequencing.

The genes encoding the *Lippia dulcis* terpene synthase 8 (LdTPS8), *L. dulcis* cytochrome P450 reductase (LdCPR1), *Nicotiana tabacum* 5-Epi-aristolochene dihydroxylase (EAH), and EAH-related enzymes from *Solanum lycopersicum* (SlEAH), *Datura stramonium* (DtEAH), *S. commersonii* (ScEAH, NCBI accession number for polypeptide: KAG5574796.1), *Capsicum chinense* (*CcEAH*), *C. annuum* (*CaEAH*), *C. baccatum* (*CbEAH,* NCBI accession number for polypeptide: PHT58219.1), and *Hyoscyamus muticus* (*HmHPO,* Uniprot accession number for polypeptide: A6YIH8 (C7D55_HYOMU)) were ordered as GeneArt String DNA fragments or full gene synthesis from Thermo Fischer Scientific. The *Arabidopsis thaliana* cytochrome P450 reductase 2 (AtATR2) gene was described in a previous publication (Kildegaard et al., 2021). The DNA-sequences were codon-optimized for *Y. lipolytica*.

The integration vectors were NotI-digested before lithium acetate transformation based on a previously described protocol (Holkenbrink et al., 2018). The genomic integration of the plasmids were confirmed by colony PCR with primers complementary to the genomic region and plasmid (Holkenbrink et al., 2018).

Table 1: Biobrick table

Biobrick number	Template	Reference
BB3865 (<-PrGPD_PrTEFint->)	See reference (therein BB3865 (<-PrGDP_Tefint->))	(Arnesen et al., 2020)
BB4645 (PrGPD_link_LdCPR1)	LdCPR1 synthetic gene	-
BB4646 (PrTEFint_link_LdTPS8)	LdTPS8 synthetic gene	-

BB5274 (pIntE_1_backbone_user)	pCfB6677 (pIntE_1-TPex20-TLip2)	-
BB5282 (AtATR2<-PrGPD-PrTEFint_new)	pCfB9247 (pIntF_3-AtATR2<-PrGPD-PrTEFInt->MtCYP716A12)	-
BB5284 (NtEAH)	NtEAH synthetic gene	-
BB5277 (IntE_3_backbone_PrTEFint_user)	pCfB10567 (pIntE_3_PrTEFint->MdOSC1_mut-YlSQE)	-
BB6038 (LdTPS8_User)	pCfB10064 (pIntE4_LdCPR1<-PrGPD_PrTEFint->LdTPS8)	-
BB5265 (PrTEFint_pcrUSER)	pCfB10567 (pIntE_3_PrTEFint->MdOSC1_mut-YlSQE)	-
BB5275 (IntE_3_backbone_user)	pCfB6681 (pIntE_3-TPex20-TLip2)	-
BB6096 (IntE_1_backbone_PrTEFint_user)	pCfB10924 (pIntE_1_pTEFint->BM3)	-
BB6077 (SlEAH)	SlEAH synthetic gene	-
BB6078 (DtEAH)	DtEAH synthetic gene	-
BB6079 (ScEAH)	ScEAH synthetic gene	-
BB6080 (CcEAH)	CcEAH synthetic gene	-
BB6081 (CaEAH)	CaEAH synthetic gene	-
BB6082 (CbEAH)	CbEAH synthetic gene	-
BB6083 (HmHPO)	pCfB10182 (intF3_PrTEFint->HmHPO)	-

Table 2: Plasmid table

Plasmid number	**Parental plasmid**	**Biobricks**	**Reference**
pCfB10064 (pIntE4_LdCPR1<-PrGPD_PrTEFint->LdTPS8)	pCfB6679 (pIntE_4-TPex20-TLip2)	BB4645 (PrGPD_link_LdCPR1):BB3865 (<-PrGPD_PrTEFint->):BB4646 (PrTEFint_link_LdTPS8)	-

pCfB6638 (pNat-YLgRNA2_IntE_4)	see reference	see reference	(Holkenbrink et al., 2018)
pCfB8858 (pHphM-YLgRNA2_IntE_1)	-	-	Laboratory collection
pCfB10742 (pIntE_1-AtATR2<-PrGPD-PrTEFint->NtEAH)	-	BB5274 (pIntE_1_backbone_user):BB5282 (AtATR2<-PrGPD-PrTEFint_new):BB5284 (NtEAH)	-
pCfB6679 (pIntE_4-TPex20-TLip2)	see reference	see reference	(Holkenbrink et al., 2018)
pCfB6677 (pIntE_1-TPex20-TLip2)	see reference	see reference	(Holkenbrink et al., 2018)
pCfB9247 (pIntF_3-AtATR2<-PrGPD-PrTEFInt->MtCYP716A12)	see reference	see reference (therein pCfB9247 (IntF_3-AtATR2<-GPD-tefInt->MtCYP716A12)	(Arnesen et al., 2020)
pCfB8860 (pHphM-YLgRNA2_IntE_3)	-	-	Laboratory collection
pCfB6633 (pNat-YLgRNA2_IntE_1)	-	See reference	(Holkenbrink et al., 2018)
pCfB6637 (pNat-YLgRNA3_IntE_3)	-	See reference	(Holkenbrink et

			al., 2018)
pCfB11702 (pIntE_3-PrTEFint>LdTPS8)	-	BB5277 (IntE_3_backbone_PrTEFint_user) :BB6038 (LdTPS8_User)	-
pCfB11703 (pIntE_1-PrTEFint->NtEAH)	-	BB5274 (IntE_1_backbone_user):BB5265 (PrTEFint_pcrUSER):BB5284 (NtEAH)	-
pCfB11765 (pIntE3-AtATR2-PrGPD-PrTEFint->LdTPS8)	-	BB5275 (IntE_3_backbone_user):BB5282 (AtATR2<-PrGPD-PrTEFint_new):BB6038 (LdTPS8_User)	-
pCfB11766 (pIntE_1-PrTEFint->SlEAH)	-	BB6096 (IntE_1_backbone_PrTEFint_user) :BB6077 (SlEAH)	-
pCfB11767 (pIntE_1-PrTEFint->DtEAH)	-	BB6096 (IntE_1_backbone_PrTEFint_user) :BB6078 (DtEAH)	-
pCfB11768 (pIntE_1-PrTEFint->ScEAH)	-	BB6096 (IntE_1_backbone_PrTEFint_user) :BB6079 (ScEAH)	-
pCfB11769 (pIntE_1-PrTEFint->CcEAH)	-	BB6096 (IntE_1_backbone_PrTEFint_user) :BB6080 (CcEAH)	-
pCfB11770 (pIntE_1-PrTEFint->CaEAH)	-	BB6096 (IntE_1_backbone_PrTEFint_user) :BB6081 (CaEAH)	-
pCfB11771 (pIntE_1-PrTEFint->CbEAH)	-	BB6096 (IntE_1_backbone_PrTEFint_user) :BB6082 (CbEAH)	-
pCfB11772 (pIntE_1-PrTEFint->HmHPO)	-	BB6096 (IntE_1_backbone_PrTEFint_user) :BB6083 (HmHPO)	-

pCfB10924 (pIntE_1_pTEFint->BM3)	-	-	Laboratory collection
pCfB10567 (pIntE_3_PrTEFint->MdOSC1_mut-YISQE)	-	-	(Arnesen et al., 2022)
pCfB10182 (intF3_PrTEFint->HmHPO)	-	-	Laboratory collection

Table 3: Strain table. All strains are *Yarrowia lipolytica*.

Strain	**Genotype**	**Parental strain**	**Vector elements**	**Reference**
ST10274	ST9149 IntE4_LdCPR1<-PrGPD_PrTEFint->LdTPS8	ST9149	pCfB6638 (pNat-YLgRNA2_IntE_4):pCfB10064 (pIntE4_LdCPR1<-PrGPD_PrTEFint->LdTPS8)	-
ST10275	ST6512 IntE4_LdCPR1<-PrGPD_PrTEFint->LdTPS8	ST6512	pCfB6638 (pNat-YLgRNA2_IntE_4):pCfB10064 (pIntE4_LdCPR1<-PrGPD_PrTEFint->LdTPS8)	-
ST11474 (HRN1-producer)	ST10274 IntE_1-AtATR2<-PrGPD-PrTEFint->NtEAH	ST10274	pCfB8858 (pHphM-YLgRNA2_IntE_1):pCfB10742 (pIntE_1-AtATR2<-PrGPD-PrTEFint->NtEAH)	-

ST11669 (HRN2-producer)	ST10275 IntE_1-AtATR2<-PrGPD-PrTEFint->NtEAH	ST10275	pCfB8858 (pHphM-YLgRNA2_IntE_1):pCfB 10742 (pIntE_1-AtATR2<-PrGPD-PrTEFint->NtEAH)	-
ST6512	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2	See reference	See reference	(Marella et al., 2020)
ST9149	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTEFint->ERG12 IntC_3-SeACS<-PrGPD-PrTEFint->YlACL1 IntD_1-IDI1<-PrGPD-PrTEFint->ERG20 pERG11::pSQS1	See reference	See reference	(Arnesen et al., 2020)
ST12430	ST6512 IntE3-PrTEFint->LdTPS8	ST6512	pCfB8860 (pHphM-YLgRNA2_IntE_3):pCfB 11702 (pIntE_3-PrTEFint->LdTPS8)	-
ST12433	ST12430 IntE_1-PrTEFint->NtEAH	ST12430	pCfB6633 (pNat-YLgRNA2_IntE_1):pCfB 11703 (pIntE_1-PrTEFint->NtEAH)	-
ST12439	ST12430 pIntE_1-	ST12430	pCfB6633 (pNat-YLgRNA2_IntE_1):pCfB	-

	AtATR2<-PrGPD-PrTEFint->NtEAH		10742 (pIntE_1-AtATR2<-PrGPD-PrTEFint->NtEAH)	
ST12475	ST9149 IntE3-AtATR2-PrGPD-PrTEFint->LdTPS8	ST9149	pCfB6637 (pNat-YLgRNA3_IntE_3):pCfB 11765 (pIntE3-AtATR2-PrGPD-PrTEFint->LdTPS8)	-
ST12476	ST12475 IntE_1-PrTEFint->NtEAH	ST12475	pCfB8858 (pHphM-YLgRNA2_IntE_1):pCfB 11703 (pIntE_1-PrTEFint->NtEAH)	-
ST12477	ST12475 IntE_1-PrTEFint->SlEAH	ST12475	pCfB8858 (pHphM-YLgRNA2_IntE_1):pCfB 11766 (pIntE_1-PrTEFint->SlEAH)	-
ST12478	ST12475 IntE_1-PrTEFint->DtEAH	ST12475	pCfB8858 (pHphM-YLgRNA2_IntE_1):pCfB 11767 (pIntE_1-PrTEFint->DtEAH)	-
ST12479	ST12475 IntE_1-PrTEFint->ScEAH	ST12475	pCfB8858 (pHphM-YLgRNA2_IntE_1):pCfB 11768 (pIntE_1-PrTEFint->ScEAH)	-
ST12480	ST12475 IntE_1-PrTEFint->CcEAH	ST12475	pCfB8858 (pHphM-YLgRNA2_IntE_1):pCfB 11769 (pIntE_1-PrTEFint->CcEAH)	-
ST12481	ST12475 IntE_1-PrTEFint->CaEAH	ST12475	pCfB8858 (pHphM-YLgRNA2_IntE_1):pCfB 11770 (pIntE_1-PrTEFint->CaEAH)	-
ST12482	ST12475 IntE_1-PrTEFint->CbEAH	ST12475	pCfB8858 (pHphM-YLgRNA2_IntE_1):pCfB	-

			11771 (pIntE_1-PrTEFint->CbEAH)	
ST12483	ST12475 IntE_1-PrTEFint->HmHPO	ST12475	pCfB8858 (pHphM-YLgRNA2_IntE_1):pCfB 11772 (pIntE_1-PrTEFint->HmHPO)	-

Cultivation and sample preparation

For relative and non-quantitative analysis of hernandulcin, 2.5 mL of YPD80 in 24-well plates with an air-penetrable lid (EnzyScreen, NL) was inoculated with single yeast clones from glycerol stocks or plates, and cultivated for 72 hours at 30°C and 300 rpm agitation. For quantitative hernandulcin analysis, precultures of 2.5 ml of YPD or YPD80 in 24-well plates were inoculated with single yeast clones from glycerol stock or plates and grown for 16-24 h at 30°C with shaking. The optical densities at 600 nm (OD600) of the precultures were then measured with a VWR NanoPhotometerTM 7122 and the precultures were used to inoculate 2.5 mL of YPD80 in 24-deep well plates were inoculated to a starting OD of 0.1, which were grown at 30°C with 300 rpm agitation for 72 hours. Alternatively, 10 µL of preculture was used to inoculate 2.5 mL of YPD80 in 24-deep well plates, which were grown at 30°C with 225 rpm agitation for 72 hours. The cultures for quantitative analysis were performed with three replicates for each strain.

Cultivation broth was collected and centrifuged, whereafter the supernatant fraction was sampled for analysis. For cell extract analysis, 1 mL of cultivation broth was transferred to a 2 ml microtube (Sarstedt), which was centrifuged, and the supernatant discarded. The cell pellets were washed twice with water, by addition of 1 mL water, centrifugation, and water removal. Then 500 µL of 0.212-0.3 mm acid-washed glass beads and 1 mL ethanol (99%) were added to the cell pellets. Thereafter, the cells were disrupted with a Precellys®24 homogenizer (Bertin Corp.) using three cycles at 5000 rpm for 30 seconds each. The samples were then centrifuged and the ethanolic phase was sampled for analysis.

Analytics

The LC-MS(MS) analysis was performed using a Dionex 3000 HPLC system connected to an Orbitrap Fusion Mass Spectrometer (Thermo Fisher Scientific, San

Jose, CA). The chromatographic separation was achieved using a Waters ACQUITY BEH C18 (10 cm × 2.1 mm, 1.7 µm) column equipped with an ACQUITY BEH C18 guard column kept at 40°C. The mobile phases consisted of MilliQ© water + O.1% formic acid (A) and acetonitrile + 0.1% formic acid (B). The initial composition was 2%B, held for 0.8 min, followed by a linear gradient till 5% in 3.3 min, and to 100%B in 10 min and held for 1 min before going back to initial conditions. Re-equilibration time was 2.7 min. Flow rate was kept constant at 0.35 mL/min and injection volume was 1uL. The MS(MS) measurement was done in positive-heated electrospray ionization (HESI) mode with a voltage of 2500 V acquiring in full MS/MS spectra (Data Dependent Acquisition-driven MS/MS) in the mass range of 70–1000 Da. The resolution was set at 120,000 for MS and to 30,000 for the MS2. Precursor ions were fragmented by High Energy Collision Dissociation (HCD) using collision energies of 20, 40, and 55.

Example 2 - Results

We attempted to find an enzyme capable of catalyzing the conversion of (+)-epi-α-bisabolol to hernandulcin. We searched the literature for potential plant cytochrome P450 candidates capable of oxygenating (+)-epi-α-bisabolol and tested the *Nicotiana tabacum* 5-Epi-aristolochene dihydroxylase (NtEAH). NtEAH has been shown to oxygenate the allylic position on cyclic sesquiterpenes, thus converting premnaspirodiene into solavetivol and solavetivone, and valencene into nootkatol and nootkatone as examples (Takahashi et al., 2007).

A *Y. lipolytica* platform strain (ST9149, Arnesen et al., 2020) was previously engineered for sesquiterpenoid production was further engineered for hernandulcin production. We reconstituted a biosynthetic pathway towards hernandulcin by expressing the genes encoding the *Lippia dulcis* terpene synthase 8 (LdTPS8), *L. dulcis* cytochrome P450 reductase (LdCPR1), *Arabidopsis thaliana* cytochrome P450 reductase 2 (AtATR2), and NtEAH in ST9149 generating the HRN1-producer (Table 3, Figure 1), *LdTPS8*, *LdCPR1*, *AtATR2* and *NtEAH*, respectively. The genes were codon-optimized for expression in *Y. lipolytica*.

The presence of hernandulcin was demonstrated in the cultivation supernatant and cell phase extract of the HRN1-producer by LC-MS analysis (Figure 2). Hernandulcin was quantified at 67.6 ± 41.2 µg/L in the cell phase based on three replicates and at 104.9 ±

56.3 µg/L in the supernatant (liquid phase) based on two replicates (Figure 3, Table 4). Hernandulcin could not be detected in the supernatant of the parental strain ST10274.

Table 4: Presence of hernandulcin in engineered yeast supernatant or cell phase. Averages and standard deviations are based on three replicates or two replicates (marked with *). nd: not detected.

Strain number	Genotype	Phase	Hernandulcin (µg/L)
ST10274	ST9149 + *LdTPS8* + *LdCPR1*	Supernatant	nd
ST11474 (HRN1-producer)	ST9149 + *LdTPS8* + *LdCPR1* + *AtATR2* + *NtEAH*	Supernatant	104.9336 ± 56.3073*
ST11474 (HRN1-producer)	ST9149 + *LdTPS8* + *LdCPR1* + *AtATR2* + *NtEAH*	Cell phase	67.5906 ± 41.2332

The genes *LdTPS8*, *LdCPR1*, *AtATR2* and *NtEAH* were co-expressed in a strain (ST6512) without improved mevalonate (MVA) pathway flux generating the HRN2-producer (Table 3).

Hernandulcin $[M+ H – H_2O]^+$ was detected in the supernatant of the HRN2-producer by LC-MS analysis (Figure 4, Table 5).

Table 5: Hernandulcin production by engineered yeast cells with or without MVA-pathway optimization. The supernatant samples were analyzed by LC-MS. Values are based on single replicates. nd: not detected.

Strain number	Genotype	Hernandulcin $[M+ H – H_2O]^+$ (AU)
ST10274	ST9149 + *LdTPS8* + *LdCPR1*	nd
ST10275	ST6512 + *LdTPS8* + *LdCPR1*	nd
ST11474 (HRN1-producer)	ST9149 + *LdTPS8* + *LdCPR1* + *AtATR2* + *NtEAH*	2464413.521
ST11669 (HRN2-producer)	ST6512 + *LdTPS8* + *LdCPR1* + *AtATR2* + *NtEAH*	745117.6919

The genes *LdTPS8* and *NtEAH* were co-expressed with or without *AtATR2* in ST6512 to test whether cytochrome P450 reductase was essential for hernandulcin production in *Y. lipolytica*. Hernandulcin could only be detected in supernatant samples by LC-MS analysis when *LdTPS8*, *AtATR2*, and *NtEAH* were co-expressed (Table 6).

Table 6: Hernandulcin production by engineered yeast cells with or without *AtATR2*-expression. The supernatant samples were analyzed by LC-MS. Averages and standard deviations are based on three replicates or two replicates (marked with *). nd: not detected.

Strain number	Genotype	Hernandulcin (µM)	Hernandulcin $[M+H]^+$ (AU)	Hernandulcin $[M+H-H_2O]^+$ (AU)	Hernandulcin C8H13 fragment (AU)
ST6512	Parental strain	nd	nd	nd	nd
ST12430	ST6512 + *LdTPS8*	nd	nd	nd	nd
ST12433	ST12430 + *NtEAH*	nd	nd	nd	nd
ST12439	ST12430 + *NtEAH* + *AtATR2*	0.3344 ± 0.0538*	147,917 ± 53,784*	937,610 ± 127,109	403,017 ± 48,359

Sequences related to NtEAH from *Solanum lycopersicum* (SlEAH, SEQ ID NO 9), *Datura stramonium* (DsEAH, SEQ ID NO 11), *S. commersonii* (ScEAH, NCBI accession number: KAG5574796.1), *Capsicum chinense* (CcEAH, SEQ ID NO 13), *C. annuum* (CaEAH, SEQ ID NO 15), *C. baccatum* (CbEAH, NCBI accession number: PHT58219.1), and *Hyoscyamus muticus* (HmHPO, Uniprot accession: A6YIH8 (C7D55_HYOMU)) were co-expressed with *LdTPS8* and *AtATR2* in ST9149 (Table 3). Expression of *DsEAH* led to hernandulcin production, and hernandulcin $[M+H-H_2O]^+$ was detected when *SlEAH* was expressed which suggested that small amounts of hernandulcin was produced (Table 7). However, since only the ion fragment $[C_8H_{13}]^+$ was detected for engineered yeast cells expressing *CcEAH* and *CaEAH*, suggesting that low amounts of hernandulcin were produced by these strains.

Table 7: Hernandulcin production by engineered yeast cells co-expressing *NtEAH*-related genes together with *LdTPS8* and *AtATR2*. The supernatant samples were analyzed by LC-MS. Averages and standard deviations are based on three replicates. nd; not detected.

Strain number	Genotype	Hernandulcin (µM)	Hernandulcin $[M+H]^+$ (AU)	Hernandulcin $[M+H-H_2O]^+$ (AU)	Hernandulcin $[C_8H_{13}]^+$ fragment (AU)
ST12475	ST9149 + *LdTPS8* + *AtATR2*	nd	nd	nd	nd
ST12476	ST12475 + *NtEAH*	0.6616 ± 0.0620	475,112 ± 62,044	3,095,388 ± 356,946	1,525,688 ± 160,149

ST12477	ST12475 + *SlEAH*	nd	nd	250,445 ± 40,250	678,875 ± 85,353
ST12478	ST12475 + *DsEAH*	0.4127 ± 0.0328	226,250 ± 32,848	1,502,119 ± 200,112	1,346,511 ± 258,926
ST12479	ST12475 + *ScEAH*	nd	nd	nd	nd
ST12480	ST12475 + *CcEAH*	nd	nd	nd	186,362 ± 15,365
ST12481	ST12475 + *CaEAH*	nd	nd	nd	87,027 ± 10,667
ST12482	ST12475 + *CbEAH*	nd	nd	nd	nd
ST12483	ST12475 + *HmHPO*	nd	nd	nd	nd

These results demonstrate that hernandulcin can be produced by engineered *Y. lipolytica* strains with or without prior engineering for precursor improvement.

Sequence overview

SEQ ID NO 1: LdTPS8 from *Lippia dulcis* (*Phyla scarberrima*), amino acid

SEQ ID NO 2: NtEAH from *Nicotiana tabacum*, CYP71D20, Uniprot accession: Q94FM7 (C71DK_TOBAC), amino acid

SEQ ID NO 3: AtATR2 from *Arabidopsis thaliana*, accession number: NP_194750.1, amino acid

SEQ ID NO 4: *LdTPS8* from *Lippia dulcis* (*Phyla scarberrima*), nucleic acid, codon-optimised for *Y. lipolytica*

SEQ ID NO 5: *NtEAH* from *Nicotiana tabacum*, nucleic acid, codon-optimised for *Y. lipolytica*

SEQ ID NO 6: *AtATR2* from *Arabidopsis thaliana*, nucleic acid, codon-optimised for *Y. lipolytica*

SEQ ID NO 7: LdCPR1 from *Lippia dulcis* (*Phyla scarberrima*), amino acid

SEQ ID NO 8: *LdCPR1* from *Lippia dulcis* (*Phyla scarberrima*), nucleic acid, codon-optimised for *Y. lipolytica*

SEQ ID NO 9: SlEAH from *Solanum lycopersicum*, amino acid. accession number: XP_004249520.2

SEQ ID NO 10: *SlEAH* from *Solanum lycopersicum*, nucleic acid, codon-optimised for *Y. lipolytica*

SEQ ID NO 11: DsEAH from *Datura stramonium*, amino acid, accession number: MCD7452234.1

SEQ ID NO 12: *DsEAH* from *Datura stramonium*, nucleic acid, codon-optimised for *Y. lipolytica*
SEQ ID NO 13: CcEAH from *Capsicum chinense*, amino acid, accession number: PHT99583.1
SEQ ID NO 14: *CcEAH* from *Capsicum chinense*, nucleic acid, codon-optimised for *Y. lipolytica*
SEQ ID NO 15: CaEAH from *Capsicum annuum*, amino acid, accession number: XP_016549742.1,
SEQ ID NO 16: *CaEAH* from *Capsicum annuum*, nucleic acid, codon-optimised for *Y. lipolytica*

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Items 1

1. A yeast cell capable of producing hernandulcin and/or one or more derivatives thereof, said yeast cell expressing:
   i. at least one (+)-epi-alpha-bisabolol synthase capable of converting farnesyl diphosphate into (+)-*epi*-α-bisabolol, preferably a heterologous (+)-epi-alpha-bisabolol synthase;

ii. at least one cytochrome P450 enzyme capable of converting (+)-*epi*-α-bisabolol into hernandulcin, preferably a heterologous cytochrome P450 enzyme such as *Nicotiana tabacum* 5-epi-aristolocene dihydroxylase (NtEAH) as set forth in SEQ ID NO 2 or a functional variant thereof having at least 65% identity, homology or similarity thereto; and

iii. optionally at least one cytochrome P450 reductase, preferably a heterologous cytochrome P450 reductase,

whereby said yeast cell is capable of producing hernandulcin and/or one or more derivatives thereof.

2. The yeast cell according to item 1, wherein:
   i. the at least one (+)-epi-alpha-bisabolol synthase is a (+)-epi-alpha-bisabolol synthase with EC 4.2.3.138;
   ii. the at least one cytochrome P450 enzyme is a cytochrome P450 oxygenase belonging to EC 1.14.14. -; and/or
   iii. the at least one cytochrome P450 reductase is a cytochrome P450 reductase with EC 1.6.2.4.

3. The yeast cell according to any one of the preceding items, wherein:
   i. the at least one (+)-epi-alpha-bisabolol synthase is a *Lippia* (+)-epi-alpha-bisabolol synthase, such as a *Lippia dulcis* (+)-epi-alpha-bisabolol synthase, preferably the *Lippia dulcis* terpene synthase 8 (LdTPS8) as set forth in SEQ ID NO 1 or a functional variant thereof having at least 70% identity, homology or similarity thereto;
   ii. the at least one cytochrome P450 enzyme is a 5-epi-aristolocene dihydroxylase, such as *Nicotiana* 5-epi-aristolocene dihydroxylase, preferably a *Nicotiana tabacum* 5-epi-aristolocene dihydroxylase, more preferably the *Nicotiana tabacum* 5-epi-aristolocene dihydroxylase (NtEAH) as set forth in SEQ ID NO 2 or a functional variant thereof having at least 65% identity, homology or similarity thereto; and/or
   iii. the at least one cytochrome P450 reductase is a plant cytochrome P450 reductase, such as an *Arabidopsis* cytochrome P450 reductase, such as an *Arabidopsis thaliana* cytochrome P450 reductase, such as the *Arabidopsis thaliana* cytochrome P450 reductase ATR2 (AtATR2) as set forth in SEQ ID

NO 3 or a functional variant thereof having at least 70% identity, homology or similarity thereto.

4. The yeast cell according to any one of the preceding items, wherein the yeast cell expresses at least one further cytochrome P450 reductase, preferably a heterologous cytochrome P450 reductase, optionally wherein said at least one further cytochrome P450 reductase is a plant cytochrome P450 reductase such as a *Lippia* cytochrome P450 reductase, such as a *Lippia dulcis* cytochrome P450 reductase, preferably the *Lippia dulcis* cytochrome P450 reductase LdCPR1 as set forth in SEQ ID NO 7, or a functional variant thereof having at least 70% identity, homology or similarity thereto.

5. The yeast cell according to any one of the preceding items, wherein the yeast cell is a non-pathogenic yeast cell.

6. The yeast cell according to any one of the preceding items, wherein said yeast cell belongs to a genus selected from *Saccharomyces*, *Pichia*, *Yarrowia*, *Kluyveromyces*, *Candida, Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon* and *Lipomyces,* optionally wherein the yeast cell belongs to a species selected from *Saccharomyces cerevisiae, Saccharomyces boulardi, Komagetella phaffi* (*Pichia pastoris*), *Kluyveromyces marxianus, Cryptococcus albidus, Lipomyces lipofera, Lipomyces starkeyi, Rhodosporidium toruloides, Rhodotorula glutinis, Trichosporon pullulan* and *Yarrowia lipolytica,* preferably the yeast cell is a *Yarrowia lipolytica* cell.

7. The yeast cell according to any one of the preceding items, wherein the yeast cell is further modified by:
    i. having a mutation resulting in increased activity of one or more of HMG, ERG12, ACL, IDI, and ERG20 or a functional variant thereof having at least 70% identity, homology or similarity to any of said polypeptides;
    ii. overexpressing *Salmonella enterica* ACS^{L641P} or a functional variant thereof having at least 70% identity, homology or similarity thereto; and/or

iii. having a mutation resulting in reduced activity of SQS such as ERG9 and/or SQS1 or a functional variant thereof having at least 70% identity, homology or similarity thereto;

whereby production of farnesyl diphosphate (FPP) is improved.

8. The yeast cell according to item 7, wherein the mutation resulting in increased activity of a polypeptide comprises overexpression of a gene encoding said polypeptide.

9. The yeast cell according to item 7, wherein the mutation resulting in reduced activity of SQS comprises modifying the promoter of SQS such as by replacing the SQS promoter sequence, fragments thereof or a homologue thereof having at least 70% identity, homology or similarity to the SQS promoter sequence, for the nucleic acid sequence of the ERG11 promoter or a homologue thereof having at least 70% identity, homology or similarity thereto.

10. The yeast cell according to items 7 to 9, wherein the mutation resulting in reduced activity of a polypeptide comprises down-regulation of a gene encoding said polypeptide and/or mutation of said polypeptide such as a loss-of-function mutation.

11. The yeast cell according to any one of items 7 to 10, wherein the polypeptides are native to the yeast cell or are non-native to the yeast cell or a combination of native and non-native.

12. The yeast cell according to any one of the preceding items, wherein hernandulcin is produced with at titer of at least 10 µg/L, such as at least 15 µg/L, such as at least 20 µg/L, such as at least 25 µg/L, such as at least 40 µg/L, such as at least 50 µg/L, such as at least 75 µg/L, such as at least 100 µg/L, such as at least 125 µg/L, such as at least 145 µg/L, such as at least 165 µg/L, such as at least 170 µg/L, such as at least 180 µg/L, such as at least 190 µg/L, such as at least 200 µg/L, such as at least 220 µg/L, such as at least 250 µg/L, such as at least 300 µg/L, such as at least 350 µg/L, such as at least 400 µg/L, such as at least 500 µg/L, such as at least 750 µg/L, such as at least 1 g/L, or more.

13. The yeast cell according to any one of the preceding items, wherein the yeast cell comprises:
    i. a nucleic acid encoding the at least one (+)-epi-alpha-bisabolol synthase, preferably wherein said nucleic acid comprises or consists of *LdTPS8* as set forth in SEQ ID NO 4, or a homologue thereof having at least 70% identity, homology or similarity thereto;
    ii. a nucleic acid encoding the at least one cytochrome P450 enzyme, preferably wherein said nucleic acid comprises or consists of *NtEAH* as set forth in SEQ ID NO 5, or a homologue thereof having at least 65% identity, homology or similarity thereto; and
    iii. optionally a nucleic acid encoding the at least one plant cytochrome P450 reductase, preferably wherein said nucleic acid comprises or consists of *AtATR2* as set forth in SEQ ID NO 6, or a homologue thereof having at least 70% identity, homology or similarity thereto; and
    iv. optionally a nucleic acid encoding the at least one further cytochrome P450 reductase, preferably wherein said nucleic acid comprises or consists of *LdCPR1* as set forth in SEQ ID NO 8, or a homologue thereof having at least 70% identity, homology or similarity thereto.

14. The yeast cell according to any one of the preceding items, wherein one or more of the at least one (+)-epi-alpha-bisabolol synthase nucleic acid, the at least one cytochrome P450 enzyme nucleic acid, the at least one cytochrome P450 reductase nucleic acid and/or the at least one further P450 reductase nucleic acid is codon-optimised.

15. An expression system for expression in a yeast cell, comprising:
    i. a nucleic acid encoding at least one (+)-epi-alpha-bisabolol synthase (EC 4.2.3.138), preferably a heterologous (+)-epi-alpha-bisabolol synthase such as *Lippia dulcis* terpene synthase 8 (LdTPS8) as set forth in SEQ ID NO 1 or a functional variant thereof having at least 70% identity, homology or similarity thereto;
    ii. a nucleic acid encoding at least one cytochrome P450 enzyme, preferably a heterologous cytochrome P450 enzyme such as *Nicotiana tabacum* 5-epi-aristolocene dihydroxylase (NtEAH) as set forth in SEQ ID NO 2 or a

functional variant thereof having at least 65% identity, homology or similarity thereto; and

iii. optionally a nucleic acid encoding at least one cytochrome P450 reductase (EC 1.6.2.4), preferably a heterologous cytochrome P450 reductase such as *Arabidopsis thaliana* cytochrome P450 reductase ATR2 (AtATR2) (EC 1.6.2.4) as set forth in SEQ ID NO 3 or a functional variant thereof having at least 70% identity, homology or similarity thereto; and

iv. optionally a nucleic acid encoding at least one further cytochrome P450 reductase, preferably a heterologous cytochrome P450 reductase such as *Lippia dulcis* cytochrome P450 reductase LdCPR1 (EC 1.6.2.4) as set forth in SEQ ID NO 7 or a functional variant thereof having at least 70% identity, homology or similarity thereto.

16. The expression system according to item 15, wherein:

i. the nucleic acid encoding the at least one (+)-epi-alpha-bisabolol synthase comprises or consists of *LdTPS8* as set forth in SEQ ID NO 4 or a homologue thereof having at least 70% identity, homology or similarity thereto;

ii. the nucleic acid encoding the at least one cytochrome P450 enzyme comprises or consists of *NtEAH* as set forth in SEQ ID NO 5 or a homologue thereof having at least 65% identity, homology or similarity thereto; and

iii. optionally the nucleic acid encoding the at least one cytochrome P450 reductase comprises or consists of *AtATR2* as set forth in SEQ ID NO 6 or a homologue thereof having at least 70% identity, homology or similarity thereto; and

iv. optionally the nucleic acid encoding the at least one further cytochrome P450 reductase comprises or consists of *LdCPR1* as set forth in SEQ ID NO 8 or a homologue thereof having at least 70% identity, homology or similarity thereto.

17. The expression system according to any one of items 15 to 16, wherein the nucleic acids further comprises one or more promoters.

18. The expression system according to any one of items 15 to 17, wherein one or more of the at least one (+)-epi-alpha-bisabolol synthase nucleic acid, the at least one cytochrome P450 enzyme nucleic acid, the at least one cytochrome P450 reductase nucleic acid and/or the at least one further P450 reductase nucleic acid is codon-optimised.

19. The expression system according to any one of items 15 to 19, wherein the yeast cell is as defined in any one of items 1 to 14.

20. The yeast cell according to any one of items 1 to 14, said yeast cell comprising the expression system according to any one of items 15 to 19, whereby said yeast cell is capable of producing hernandulcin and/or one or more derivatives thereof.

21. A method for producing hernandulcin and/or one or more derivatives thereof in a yeast cell, said method comprising the steps of:
    i. providing a yeast cell according to any one of items 1 to 14 or 20; and
    ii. incubating said yeast cell in a medium,
    whereby hernandulcin and/or one or more derivatives thereof is produced.

22. The method according to item 21, wherein the yeast cell is as defined in anyone of items 1 to 14 or 20.

23. The method according to any one of items 21 to 22, wherein the yeast cell comprises an expression system as defined in any one of items 15 to 19.

24. The method according to any of items one of the preceding items, wherein hernandulcin and/or the one or more derivatives thereof is produced with at titer of at least 10 µg/L, such as at least 15 µg/L, such as at least 20 µg/L, such as at least 25 µg/L, such as at least 40 µg/L, such as at least 50 µg/L, such as at least 75 µg/L, such as at least 100 µg/L, such as at least 125 µg/L, such as at least 145 µg/L, such as at least 165 µg/L, such as at least 170 µg/L, such as at least 180 µg/L, such as at least 190 µg/L, such as at least 200 µg/L, such as at least 220 µg/L, such as at least 250 µg/L, such as at least 300 µg/L, such as at

least 350 µg/L, such as at least 400 µg/L, such as at least 500 µg/L, such as at least 750 µg/L, such as at least 1 g/L, or more.

25. The method according to any one of the preceding claims 9 to 10, further comprising the steps of:
    i. recovering the hernandulcin and/or the one or more derivatives thereof; and
    ii. optionally converting said hernandulcin and/or the one or more derivatives thereof to one or more further derivatives thereof; and/or
    iii. formulating said hernandulcin and/or the one or more derivatives thereof in a composition.

26. A composition comprising hernandulcin and/or one or more derivatives thereof obtained by the method according to any one of items 21 to 25.

27. Hernandulcin and/or one or more derivatives thereof obtained by the method according to any one of items 21 to 25.

28. Use of a *Nicotiana* cytochrome P450 enzyme, preferably a *Nicotiana tabacum* cytochrome P450 enzyme, in a method for producing hernandulcin and/or one or more derivatives, optionally wherein the *Nicotiana tabacum* cytochrome P450 enzyme is NtEAH as set forth in SEQ ID NO 2, or a functional variant thereof having at least 65% identity, homology or similarity thereto.

29. The use according to item 28, wherein the polypeptide comprises the sequence as set forth in SEQ ID NO 2, with the exception that at the most 51 residues are mutated.

30. The use according to any one of items 28 to 29, said use comprising expressing the *Nicotiana* cytochrome P450 enzyme, preferably the *Nicotiana tabacum* cytochrome P450 enzyme, optionally wherein the *Nicotiana tabacum* cytochrome P450 enzyme is NtEAH as set forth in SEQ ID NO 2, or functional variants thereof having at least 65% identity, homology or similarity thereto in a yeast cell, preferably wherein said yeast cell is as defined in any one of items 1 to 14 or 20.

31. The use according to any one of items 28 to 30, wherein said method is as defined in any one of items 21 to 25.

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Items 2

1. A yeast cell capable of producing hernandulcin and/or one or more derivatives thereof, said yeast cell expressing:
   i. at least one (+)-epi-alpha-bisabolol synthase capable of converting farnesyl diphosphate into (+)-*epi*-α-bisabolol, preferably a heterologous (+)-epi-alpha-bisabolol synthase;
   ii. at least one cytochrome P450 enzyme capable of converting (+)-*epi*-α-bisabolol into hernandulcin, preferably a heterologous cytochrome P450 enzyme such as *Nicotiana tabacum* 5-epi-aristolocene dihydroxylase (NtEAH) as set forth in SEQ ID NO 2 or a functional variant thereof having at least 65% identity, homology or similarity thereto; and
   iii. optionally at least one cytochrome P450 reductase, preferably a heterologous cytochrome P450 reductase,

   whereby said yeast cell is capable of producing hernandulcin and/or one or more derivatives thereof.

2. The yeast cell according to any one of the preceding items, wherein:
   i. the at least one (+)-epi-alpha-bisabolol synthase is a *Lippia* (+)-epi-alpha-bisabolol synthase, such as a *Lippia dulcis* (+)-epi-alpha-bisabolol synthase, preferably the *Lippia dulcis* terpene synthase 8 (LdTPS8) as set forth in SEQ ID NO 1 or a functional variant thereof having at least 70% identity, homology or similarity thereto;
   ii. the at least one cytochrome P450 enzyme is a 5-epi-aristolocene dihydroxylase, such as *Nicotiana* 5-epi-aristolocene dihydroxylase, preferably a *Nicotiana tabacum* 5-epi-aristolocene dihydroxylase, more preferably the *Nicotiana tabacum* 5-epi-aristolocene dihydroxylase (NtEAH) as set forth in SEQ ID NO 2 or a functional variant thereof having at least 65% identity, homology or similarity thereto; and
   iii. the at least one cytochrome P450 reductase is a plant cytochrome P450 reductase, such as an *Arabidopsis* cytochrome P450 reductase, such as an *Arabidopsis thaliana* cytochrome P450 reductase, such as the *Arabidopsis thaliana* cytochrome P450 reductase ATR2 (AtATR2) as set forth in SEQ ID NO 3 or a functional variant thereof having at least 70% identity, homology or similarity thereto.

3. The yeast cell according to any one of the preceding items, wherein the yeast cell expresses at least one further cytochrome P450 reductase, preferably a heterologous cytochrome P450 reductase, optionally wherein said at least one further cytochrome P450 reductase is a plant cytochrome P450 reductase such as a *Lippia* cytochrome P450 reductase, such as a *Lippia dulcis* cytochrome P450 reductase, preferably the *Lippia dulcis* cytochrome P450 reductase LdCPR1 (EC 1.6.2.4) as set forth in SEQ ID NO 7, or a functional variant thereof having at least 70% identity, homology or similarity thereto.

4. The yeast cell according to any one of the preceding items, wherein said yeast cell belongs to a genus selected from *Saccharomyces*, *Pichia*, *Yarrowia*, *Kluyveromyces*, *Candida, Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon* and *Lipomyces,* optionally wherein the yeast cell belongs to a species selected from *Saccharomyces cerevisiae, Saccharomyces boulardi, Komagetella phaffi* (*Pichia pastoris*), *Kluyveromyces marxianus, Cryptococcus albidus, Lipomyces lipofera, Lipomyces starkeyi, Rhodosporidium toruloides, Rhodotorula glutinis, Trichosporon pullulan* and *Yarrowia lipolytica,* preferably the yeast cell is a *Yarrowia lipolytica* cell.

5. The yeast cell according to any one of the preceding items, wherein the yeast cell is further modified by;
   iv. having a mutation resulting in increased activity of one or more of HMG, ERG12, ACL, IDI, and ERG20 or a functional variant thereof having at least 70% identity, homology or similarity to any of said polypeptides;
   v. overexpressing *Salmonella enterica* ACS^{L641P} or a functional variant thereof having at least 70% identity, homology or similarity thereto; and/or
   vi. having a mutation resulting in reduced activity of SQS such as ERG9 and/or SQS1 or a functional variant thereof having at least 70% identity, homology or similarity thereto, optionally the mutation resulting in reduced activity of SQS comprises modifying the promoter of SQS such as by replacing the SQS promoter sequence, fragments thereof or a homologue thereof having at least 70% identity, homology or similarity to the SQS promoter sequence, for the nucleic acid sequence of the

ERG11 promoter or a homologue thereof having at least 70% identity, homology or similarity thereto,

whereby production of farnesyl diphosphate (FPP) is improved.

6. The yeast cell according to any one of the preceding items, wherein hernandulcin is produced with at titer of at least 10 µg/L, such as at least 15 µg/L, such as at least 20 µg/L, such as at least 25 µg/L, such as at least 40 µg/L, such as at least 50 µg/L, such as at least 75 µg/L, such as at least 100 µg/L, such as at least 125 µg/L, such as at least 145 µg/L, such as at least 165 µg/L, such as at least 170 µg/L, such as at least 180 µg/L, such as at least 190 µg/L, such as at least 200 µg/L, such as at least 220 µg/L, such as at least 250 µg/L, such as at least 300 µg/L, such as at least 350 µg/L, such as at least 400 µg/L, such as at least 500 µg/L, such as at least 750 µg/L, such as at least 1 g/L, or more.

7. An expression system for expression in a yeast cell, comprising:
   i. a nucleic acid encoding at least one (+)-epi-alpha-bisabolol synthase (EC 4.2.3.138), preferably a heterologous (+)-epi-alpha-bisabolol synthase such as *Lippia dulcis* terpene synthase 8 (LdTPS8) as set forth in SEQ ID NO 1 or a functional variant thereof having at least 70% identity, homology or similarity thereto;
   ii. a nucleic acid encoding at least one cytochrome P450 enzyme, preferably a heterologous cytochrome P450 enzyme such as *Nicotiana tabacum* 5-epi-aristolocene dihydroxylase (NtEAH) as set forth in SEQ ID NO 2 or a functional variant thereof having at least 65% identity, homology or similarity thereto; and
   iii. optionally a nucleic acid encoding at least one cytochrome P450 reductase (EC 1.6.2.4), preferably a heterologous cytochrome P450 reductase such as *Arabidopsis thaliana* cytochrome P450 reductase ATR2 (AtATR2) (EC 1.6.2.4) as set forth in SEQ ID NO 3 or a functional variant thereof having at least 70% identity, homology or similarity thereto; and
   iv. optionally a nucleic acid encoding at least one further cytochrome P450 reductase, preferably a heterologous cytochrome P450 reductase such as *Lippia dulcis* cytochrome P450 reductase LdCPR1 as set forth in SEQ ID

NO 7 or a functional variant thereof having at least 70% identity, homology or similarity thereto.

8. The yeast cell according to any one of items 1 to 6, said yeast cell comprising the expression system according to item 7, whereby said yeast cell is capable of producing hernandulcin and/or one or more derivatives thereof.

9. A method for producing hernandulcin and/or one or more derivatives thereof in a yeast cell, said method comprising the steps of:
   i. providing a yeast cell according to any one of items 1 to 6 or 8; and
   ii. incubating said yeast cell in a medium,

   whereby hernandulcin and/or one or more derivatives thereof is produced.

10. The method according to item 9, wherein hernandulcin and/or the one or more derivatives thereof is produced with at titer of at least 10 µg/L, such as at least 15 µg/L, such as at least 20 µg/L, such as at least 25 µg/L, such as at least 40 µg/L, such as at least 50 µg/L, such as at least 75 µg/L, such as at least 100 µg/L, such as at least 125 µg/L, such as at least 145 µg/L, such as at least 165 µg/L, such as at least 170 µg/L, such as at least 180 µg/L, such as at least 190 µg/L, such as at least 200 µg/L, such as at least 220 µg/L, such as at least 250 µg/L, such as at least 300 µg/L, such as at least 350 µg/L, such as at least 400 µg/L, such as at least 500 µg/L, such as at least 750 µg/L, such as at least 1 g/L, or more.

11. The method according to any one of the preceding items 9 to 10, further comprising the steps of:
   i. recovering the hernandulcin and/or the one or more derivatives thereof; and
   ii. optionally converting said hernandulcin and/or the one or more derivatives thereof to one or more further derivatives thereof; and/or
   iii. formulating said hernandulcin and/or the one or more derivatives thereof in a composition.

12. A composition comprising hernandulcin and/or one or more derivatives thereof obtained by the method according to any one of items 9 to 11.

13. Use of a *Nicotiana* cytochrome P450 enzyme, preferably a *Nicotiana tabacum* cytochrome P450 enzyme, in a method for producing hernandulcin and/or one or more derivatives, optionally wherein the *Nicotiana tabacum* cytochrome P450 enzyme is NtEAH as set forth in SEQ ID NO 2, or a functional variant thereof having at least 65% identity, homology or similarity thereto.

14. The use according to item 13, said use comprising expressing the *Nicotiana* cytochrome P450 enzyme, preferably the *Nicotiana tabacum* cytochrome P450 enzyme, optionally wherein the *Nicotiana tabacum* cytochrome P450 enzyme is NtEAH as set forth in SEQ ID NO 2, or functional variants thereof having at least 65% identity, homology or similarity thereto in a yeast cell, preferably wherein said yeast cell is as defined in any one of items 1 to 6 or 8.

15. The use according to any one of items 13 to 14, wherein said method is as defined in any one of items 9 to 11.

Claims

1. A yeast cell capable of producing hernandulcin and/or one or more derivatives thereof, said yeast cell expressing:
   i. at least one (+)-epi-alpha-bisabolol synthase capable of converting farnesyl diphosphate into (+)-*epi*-α-bisabolol, preferably a heterologous (+)-epi-alpha-bisabolol synthase;
   ii. at least one plant cytochrome P450 enzyme capable of converting (+)-*epi*-α-bisabolol into hernandulcin, preferably a heterologous plant cytochrome P450 enzyme such as *Nicotiana tabacum* 5-epi-aristolocene dihydroxylase (NtEAH) as set forth in SEQ ID NO 2, DsEAH as set forth in SEQ ID NO 11, or a functional variant thereof having at least 65% identity, homology or similarity to any of the aforementioned; and
   iii. at least one cytochrome P450 reductase, preferably a heterologous cytochrome P450 reductase,
   whereby said yeast cell is capable of producing hernandulcin and/or one or more derivatives thereof.

2. The yeast cell according to claim 1, wherein:
   i. the at least one (+)-epi-alpha-bisabolol synthase is a (+)-epi-alpha-bisabolol synthase with EC 4.2.3.138;
   ii. the at least one cytochrome P450 enzyme is a cytochrome P450 oxygenase belonging to EC 1.14.14. -; and/or
   iii. the at least one cytochrome P450 reductase is a cytochrome P450 reductase with EC 1.6.2.4.

3. The yeast cell according to any one of the preceding claims, wherein the at least one (+)-epi-alpha-bisabolol synthase is a *Lippia* (+)-epi-alpha-bisabolol synthase, such as a *Lippia dulcis* (+)-epi-alpha-bisabolol synthase, preferably the *Lippia dulcis* terpene synthase 8 (LdTPS8) as set forth in SEQ ID NO 1 or a functional variant thereof having at least 70% identity, homology or similarity thereto.

4. The yeast cell according to any one of the preceding claims, wherein the at least one plant cytochrome P450 enzyme is native to an organism of a genus selected from *Nicotiana, Solanum*, *Datura*, and *Capsicum*, such as *Nicotiana*

tabacum, Solanum lycopersicum, *Datura stramonium*, *Capsicum chinense* and *C. annuum*, optionally wherein the plant cytochrome P450 enzyme is NtEAH (SEQ ID NO 2), SlEAH (SEQ ID NO 9), DsEAH (SEQ ID NO 11), CcEAH (SEQ ID NO 13) or CaEAH (SEQ ID NO 15), or a functional variant thereof having at least 65% identity, homology or similarity to any of the aforementioned.

5. The yeast cell according to any one of the preceding claims, wherein the at least one plant cytochrome P450 enzyme is a 5-epi-aristolocene dihydroxylase, such as *Nicotiana* 5-epi-aristolocene dihydroxylase, preferably a *Nicotiana tabacum* 5-epi-aristolocene dihydroxylase, more preferably the *Nicotiana tabacum* 5-epi-aristolocene dihydroxylase (NtEAH) as set forth in SEQ ID NO 2 or a functional variant thereof having at least 65% identity, homology or similarity thereto.

6. The yeast cell according to any one of the preceding claims, wherein the at least one plant cytochrome P450 enzyme is a *Datura* cytochrome P450 enzyme, preferably a *Datura stramonium* cytochrome P450 enzyme, more preferably DsEAH as set forth in SEQ ID NO 11, or a functional variant thereof having at least 65% identity, homology or similarity thereto.

7. The yeast cell according to any one of the preceding claims, wherein the at least one plant cytochrome P450 enzyme is a premnaspirodiene oxygenase-like protein, such as a *Solanum* premnaspirodiene oxygenase-like protein, preferably a *Solanum lycopersicum* premnaspirodiene oxygenase-like protein, more preferably the *Solanum lycopersicum* premnaspirodiene oxygenase-like protein (SlEAH) as set forth in SEQ ID NO 9, or a functional variant thereof having at least 65% identity, homology or similarity thereto.

8. The yeast cell according to any one of the preceding claims, wherein the at least one plant cytochrome P450 enzyme is a cytochrome P450 71D7-like protein, such as a *Capsicum* cytochrome P450 71D7-like protein, preferably a *Capsicum annuum* cytochrome P450 71D7-like protein, more preferably the *Capsicum annuum* cytochrome P450 71D7-like protein (CaEAH) as set forth in SEQ ID NO 15, or a functional variant thereof having at least 65% identity, homology or similarity thereto.

9. The yeast cell according to any one of the preceding claims, wherein the at least one plant cytochrome P450 enzyme is a *Capsicum* cytochrome P450 enzyme, preferably a *Capsicum chinense* cytochrome P450 enzyme, more preferably the CcEAH as set forth in SEQ ID NO 13, or a functional variant thereof having at least 65% identity, homology or similarity thereto.

10. The yeast cell according to any one of the preceding claims, wherein the at least one cytochrome P450 reductase is a plant cytochrome P450 reductase, such as an *Arabidopsis* cytochrome P450 reductase, such as an *Arabidopsis thaliana* cytochrome P450 reductase, such as the *Arabidopsis thaliana* cytochrome P450 reductase ATR2 (AtATR2) as set forth in SEQ ID NO 3 or a functional variant thereof having at least 70% identity, homology or similarity thereto.

11. The yeast cell according to any one of the preceding claims, wherein the yeast cell expresses at least one further cytochrome P450 reductase, preferably a heterologous cytochrome P450 reductase, optionally wherein said at least one further cytochrome P450 reductase is a plant cytochrome P450 reductase such as a *Lippia* cytochrome P450 reductase, such as a *Lippia dulcis* cytochrome P450 reductase, preferably the *Lippia dulcis* cytochrome P450 reductase LdCPR1 as set forth in SEQ ID NO 7, or a functional variant thereof having at least 70% identity, homology or similarity thereto.

12. The yeast cell according to any one of the preceding claims, wherein the yeast cell is a non-pathogenic yeast cell.

13. The yeast cell according to any one of the preceding claims, wherein said yeast cell belongs to a genus selected from *Saccharomyces*, *Pichia*, *Yarrowia*, *Kluyveromyces*, *Candida, Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon* and *Lipomyces,* optionally wherein the yeast cell belongs to a species selected from *Saccharomyces cerevisiae, Saccharomyces boulardi, Komagetella phaffi* (*Pichia pastoris*), *Kluyveromyces marxianus, Cryptococcus albidus, Lipomyces lipofera, Lipomyces starkeyi, Rhodosporidium toruloides,*

Rhodotorula glutinis, Trichosporon pullulan and *Yarrowia lipolytica,* preferably the yeast cell is a *Yarrowia lipolytica* cell.

14. The yeast cell according to any one of the preceding claims, wherein the yeast cell is further modified by:
    i. having a mutation resulting in increased activity of one or more of HMG, ERG12, ACL, IDI, and ERG20 or a functional variant thereof having at least 70% identity, homology or similarity to any of said polypeptides;
    ii. overexpressing *Salmonella enterica* ACS^{L641P} or a functional variant thereof having at least 70% identity, homology or similarity thereto; and/or
    iii. having a mutation resulting in reduced activity of SQS such as ERG9 and/or SQS1 or a functional variant thereof having at least 70% identity, homology or similarity thereto;
    whereby production of farnesyl diphosphate (FPP) is improved.

15. The yeast cell according to claim 14, wherein the mutation resulting in increased activity of a polypeptide comprises overexpression of a gene encoding said polypeptide.

16. The yeast cell according to claim 14, wherein the mutation resulting in reduced activity of SQS comprises modifying the promoter of SQS such as by replacing the SQS promoter sequence, fragments thereof or a homologue thereof having at least 70% identity, homology or similarity to the SQS promoter sequence, for the nucleic acid sequence of the ERG11 promoter or a homologue thereof having at least 70% identity, homology or similarity thereto.

17. The yeast cell according to claims 14 to 16, wherein the mutation resulting in reduced activity of a polypeptide comprises down-regulation of a gene encoding said polypeptide and/or mutation of said polypeptide such as a loss-of-function mutation.

18. The yeast cell according to any one of claims 14 to 17, wherein the polypeptides are native to the yeast cell or are non-native to the yeast cell or a combination of native and non-native.

19. The yeast cell according to any one of the preceding claims, wherein hernandulcin is produced with at titer of at least 10 µg/L, such as at least 15 µg/L, such as at least 20 µg/L, such as at least 25 µg/L, such as at least 40 µg/L, such as at least 50 µg/L, such as at least 75 µg/L, such as at least 100 µg/L, such as at least 125 µg/L, such as at least 145 µg/L, such as at least 165 µg/L, such as at least 170 µg/L, such as at least 180 µg/L, such as at least 190 µg/L, such as at least 200 µg/L, such as at least 220 µg/L, such as at least 250 µg/L, such as at least 300 µg/L, such as at least 350 µg/L, such as at least 400 µg/L, such as at least 500 µg/L, such as at least 750 µg/L, such as at least 1 g/L, or more.

20. The yeast cell according to any one of the preceding claims, wherein the yeast cell comprises:
    i. a nucleic acid encoding the at least one (+)-epi-alpha-bisabolol synthase, preferably wherein said nucleic acid comprises or consists of *LdTPS8* as set forth in SEQ ID NO 4, or a homologue thereof having at least 70% identity, homology or similarity thereto;
    ii. a nucleic acid encoding the at least one cytochrome P450 enzyme, preferably wherein said nucleic acid comprises or consists of *NtEAH* as set forth in SEQ ID NO 5, *DsEAH* as set forth in SEQ ID NO 12, or homologues thereof having at least 65% identity, homology or similarity thereto; and
    iii. a nucleic acid encoding the at least one plant cytochrome P450 reductase, preferably wherein said nucleic acid comprises or consists of *AtATR2* as set forth in SEQ ID NO 6, or a homologue thereof having at least 70% identity, homology or similarity thereto; and
    iv. optionally a nucleic acid encoding the at least one further cytochrome P450 reductase, preferably wherein said nucleic acid comprises or consists of *LdCPR1* as set forth in SEQ ID NO 8, or a homologue thereof having at least 70% identity, homology or similarity thereto.

21. The yeast cell according to claim 20, wherein:
    i. the nucleic acid encoding the at least one (+)-epi-alpha-bisabolol synthase comprises or consists of *LdTPS8* as set forth in SEQ ID NO 4

or a homologue thereof having at least 70% identity, homology or similarity thereto;

ii. the nucleic acid encoding the at least one plant cytochrome P450 enzyme comprises or consists of:

i. *NtEAH* as set forth in SEQ ID NO 5,
ii. *SlEAH* as set forth in SEQ ID NO 10,
iii. *DsEAH* as set forth in SEQ ID NO 12,
iv. *CcEAH* as set forth in SEQ ID NO 14,
v. *CaEAH* as set forth in SEQ ID NO 16,

or a homologue thereof having at least 65% identity, homology or similarity to any of the aforementioned; and

iii. the nucleic acid encoding the at least one cytochrome P450 reductase comprises or consists of *AtATR2* as set forth in SEQ ID NO 6 or a homologue thereof having at least 70% identity, homology or similarity thereto.

22. The yeast cell according to any one of the preceding claims, wherein one or more of the at least one (+)-epi-alpha-bisabolol synthase nucleic acid, the at least one plant cytochrome P450 enzyme nucleic acid, the at least one cytochrome P450 reductase nucleic acid and/or the at least one further P450 reductase nucleic acid is codon-optimised.

23. An expression system for expression in a yeast cell, comprising:

i. a nucleic acid encoding at least one (+)-epi-alpha-bisabolol synthase (EC 4.2.3.138), preferably a heterologous (+)-epi-alpha-bisabolol synthase such as *Lippia dulcis* terpene synthase 8 (LdTPS8) as set forth in SEQ ID NO 1 or a functional variant thereof having at least 70% identity, homology or similarity thereto;

ii. a nucleic acid encoding at least one plant cytochrome P450 enzyme, preferably a heterologous plant cytochrome P450 enzyme such as *Nicotiana tabacum* 5-epi-aristolocene dihydroxylase (NtEAH) as set forth in SEQ ID NO 2, DsEAH as set forth in SEQ ID NO 11, or a functional variant thereof having at least 65% identity, homology or similarity to any of the aforementioned; and

iii. a nucleic acid encoding at least one cytochrome P450 reductase (EC 1.6.2.4), preferably a heterologous cytochrome P450 reductase, for example a plant cytochrome P450 reductase such as *Arabidopsis thaliana* cytochrome P450 reductase ATR2 (AtATR2) (EC 1.6.2.4) as set forth in SEQ ID NO 3 or a functional variant thereof having at least 70% identity, homology or similarity thereto.

24. The expression system according to claim 23, wherein:
    i. the nucleic acid encoding the at least one (+)-epi-alpha-bisabolol synthase comprises or consists of *LdTPS8* as set forth in SEQ ID NO 4 or a homologue thereof having at least 70% identity, homology or similarity thereto;
    ii. the nucleic acid encoding the at least one plant cytochrome P450 enzyme comprises or consists of:
        i. *NtEAH* as set forth in SEQ ID NO 5,
        ii. *SlEAH* as set forth in SEQ ID NO 10,
        iii. *DsEAH* as set forth in SEQ ID NO 12,
        iv. *CcEAH* as set forth in SEQ ID NO 14,
        v. *CaEAH* as set forth in SEQ ID NO 16,

    or a homologue thereof having at least 65% identity, homology or similarity to any of the aforementioned; and
    iii. the nucleic acid encoding the at least one cytochrome P450 reductase comprises or consists of *AtATR2* as set forth in SEQ ID NO 6 or a homologue thereof having at least 70% identity, homology or similarity thereto.

25. The expression system according to any one of claims 23 to 24, further comprising a nucleic acid encoding at least one further cytochrome P450 reductase, preferably a heterologous cytochrome P450 reductase, optionally a plant cytochrome P450 reductase, such as *Lippia dulcis* cytochrome P450 reductase LdCPR1 (EC 1.6.2.4) as set forth in SEQ ID NO 7 or a functional variant thereof having at least 70% identity, homology or similarity thereto.

26. The expression system according to any one of claims 23 to 25, wherein the nucleic acids further comprises one or more promoters.

27. The expression system according to any one of claims 23 to 26, wherein one or more of the at least one (+)-epi-alpha-bisabolol synthase nucleic acid, the at least one plant cytochrome P450 enzyme nucleic acid, the at least one cytochrome P450 reductase nucleic acid and/or the at least one further cytochrome P450 reductase nucleic acid is codon-optimised.

28. The expression system according to any one of claims 23 to 27, wherein the yeast cell is as defined in any one of claims 1 to 22.

29. The yeast cell according to any one of claims 1 to 22, said yeast cell comprising the expression system according to any one of claims 23 to 28, whereby said yeast cell is capable of producing hernandulcin and/or one or more derivatives thereof.

30. A method for producing hernandulcin and/or one or more derivatives thereof in a yeast cell, said method comprising the steps of:
    i. providing a yeast cell according to any one of claims 1 to 22 or 29; and
    ii. incubating said yeast cell in a medium,
whereby hernandulcin and/or one or more derivatives thereof is produced.

31. The method according to claim 30, wherein the yeast cell is as defined in anyone of claims 1 to 22 or 29.

32. The method according to any one of claims 30 to 31, wherein the yeast cell comprises an expression system as defined in any one of claims 23 to 28.

33. The method according to any of claims 30 to 32, wherein hernandulcin and/or the one or more derivatives thereof is produced with at titer of at least 10 µg/L, such as at least 15 µg/L, such as at least 20 µg/L, such as at least 25 µg/L, such as at least 40 µg/L, such as at least 50 µg/L, such as at least 75 µg/L, such as at least 100 µg/L, such as at least 125 µg/L, such as at least 145 µg/L, such as at least 165 µg/L, such as at least 170 µg/L, such as at least 180 µg/L, such as at least 190 µg/L, such as at least 200 µg/L, such as at least 220 µg/L, such as at least 250 µg/L, such as at least 300 µg/L, such as at least 350 µg/L,

such as at least 400 µg/L, such as at least 500 µg/L, such as at least 750 µg/L, such as at least 1 g/L, or more.

34. The method according to any one of 30 to 33, further comprising the steps of:
    i. recovering the hernandulcin and/or the one or more derivatives thereof; and
    ii. optionally converting said hernandulcin and/or the one or more derivatives thereof to one or more further derivatives thereof; and/or
    iii. formulating said hernandulcin and/or the one or more derivatives thereof in a composition.

35. A composition, preferably a food composition and/or a beverage, comprising hernandulcin and/or one or more derivatives thereof obtained by the method according to any one of claims 30 to 34.

36. The composistion according to claim 35, wherein the composition is a food composition and/or a beverage.

37. Hernandulcin and/or one or more derivatives thereof obtained by the method according to any one of claims 30 to 34.

38. Use of a *Nicotiana* cytochrome P450 enzyme, preferably a *Nicotiana tabacum* cytochrome P450 enzyme, in a method for producing hernandulcin and/or one or more derivatives, optionally wherein the *Nicotiana tabacum* cytochrome P450 enzyme is NtEAH as set forth in SEQ ID NO 2, or a functional variant thereof having at least 65% identity, homology or similarity thereto.

39. The use according to claim 38, wherein the polypeptide comprises the sequence as set forth in SEQ ID NO 2, with the exception that at the most 51 residues are mutated.

40. The use according to any one of claims 38 to 40, said use comprising expressing the *Nicotiana* cytochrome P450 enzyme in a yeast cell, preferably a *Nicotiana tabacum* cytochrome P450 enzyme, optionally wherein the *Nicotiana tabacum* cytochrome P450 enzyme is NtEAH as set forth in SEQ ID NO 2, or

functional variants thereof having at least 65% identity, homology or similarity thereto, preferably wherein said yeast cell is as defined in any one of claims 1 to 22 or 29.

41. Use of a *Datura* cytochrome P450 enzyme, preferably a *Datura stramonium* cytochrome P450 enzyme, in a method for producing hernandulcin and/or one or more derivatives, optionally wherein the *Datura stramonium* cytochrome P450 enzyme is DsEAH as set forth in SEQ ID NO 11, or a functional variant thereof having at least 65% identity, homology or similarity thereto.

42. The use according to claim 41, wherein the polypeptide comprises the sequence as set forth in SEQ ID NO 11, with the exception that at the most 51 residues are mutated.

43. The use according to any one of claims 41 to 42, said use comprising expressing the *Datura* cytochrome P450 enzyme, preferably a *Datura stramonium* cytochrome P450 enzyme, optionally wherein the *Datura stramonium* cytochrome P450 enzyme is DsEAH as set forth in SEQ ID NO 11, or functional variants thereof having at least 65% identity, homology or similarity thereto in a yeast cell, preferably wherein said yeast cell is as defined in any one of claims 1 to 22 or 29.

44. Use of a *Solanum* cytochrome P450 enzyme, preferably a *Solanum lycopersicum* cytochrome P450 enzyme, in a method for producing hernandulcin and/or one or more derivatives, optionally wherein the *Solanum lycopersicum* cytochrome P450 enzyme is SlEAH as set forth in SEQ ID NO 9, or a functional variant thereof having at least 65% identity, homology or similarity thereto.

45. The use according to claim 44, wherein the polypeptide comprises the sequence as set forth in SEQ ID NO 9, with the exception that at the most 54 residues are mutated.

46. The use according to any one of claims 44 to 45, said use comprising expressing the *Solanum* cytochrome P450 enzyme in a yeast cell, preferably a

Solanum lycopersicum cytochrome P450 enzyme, optionally wherein the *Solanum lycopersicum* cytochrome P450 enzyme is SlEAH as set forth in SEQ ID NO 9, or functional variants thereof having at least 65% identity, homology or similarity thereto, preferably wherein said yeast cell is as defined in any one of claims 1 to 22 or 29.

47. Use of a *Capsicum* cytochrome P450 enzyme, preferably a *Capsicum chinense* cytochrome P450 enzyme and/or a *Capsicum annuum* cytochrome P450 enzyme, in a method for producing hernandulcin and/or one or more derivatives, optionally wherein the *Capsicum chinense* cytochrome P450 enzyme is CcEAH as set forth in SEQ ID NO 13, the *Capsicum annuum* cytochrome P450 enzyme is CaEAH as set forth in SEQ ID NO 15, or functional variants thereof having at least 65% identity, homology or similarity to any of the aforementioned.

48. The use according to claim 47, wherein the polypeptide comprises the sequence as set forth in SEQ ID NO 13 and/or SEQ ID NO 15, with the exception that at the most 51 residues are mutated.

49. The use according to any one of claims 47 to 48, said use comprising expressing the *Capsicum* cytochrome P450 enzyme in a yeast cell, preferably a *Capsicum chinense* cytochrome P450 enzyme and/or the *Capsicum annuum* cytochrome P450 enzyme, optionally wherein the *Capsicum chinense* cytochrome P450 enzyme is CcEAH as set forth in SEQ ID NO 13, the *Capsicum annuum* cytochrome P450 enzyme is CaEAH as set forth in SEQ ID NO 15, or functional variants thereof having at least 65% identity, homology or similarity to any of the aforementioned, preferably wherein said yeast cell is as defined in any one of claims 1 to 22 or 29.

50. The use according to any one of claims 38 to 49, wherein said method is as defined in any one of claims 30 to 34.

51. A fermentation liquid comprising hernandulcin and/or one or more derivatives thereof, wherein said fermentation liquid is obtained by a method according to any one of claims 30 to 34, optionally wherein at least 50% of the yeast cells

are disrupted and/or wherein at least 50% of cellular material, such as yeast cell debris, is separated from the fermentation liquid.

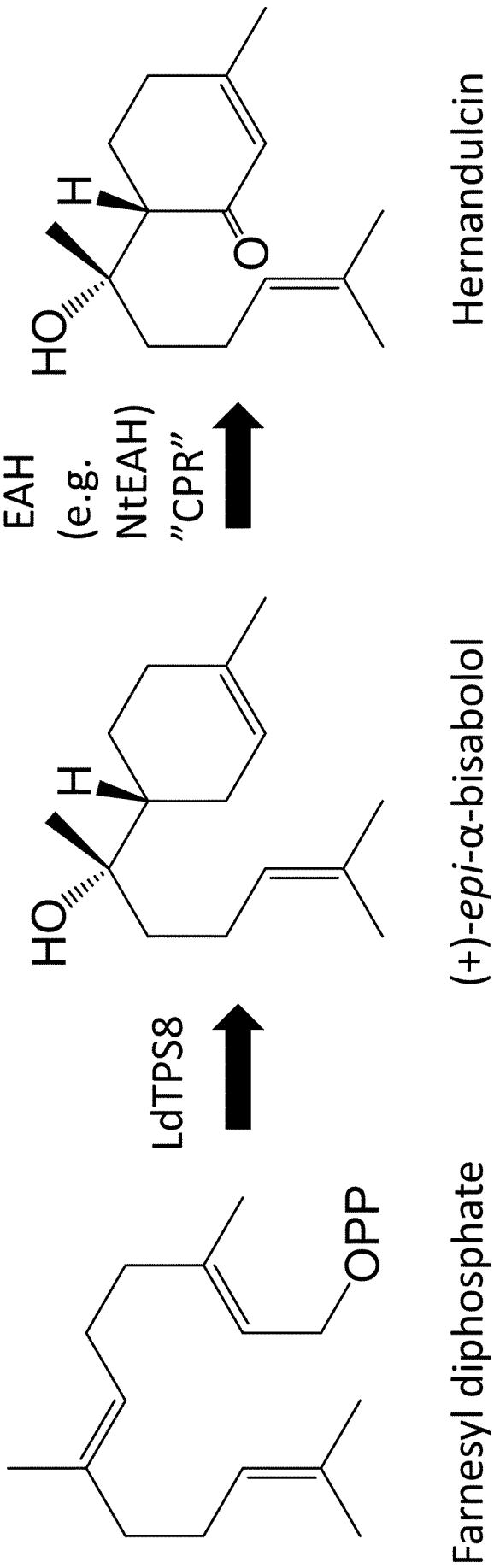
OPP
LdTPS8
HO
H
EAH
(e.g.
NtEAH)
"CPR"
HO
H
O
Farnesyl diphosphate
(+)-epi-α-bisabolol
Hernandulcin

Figure 1

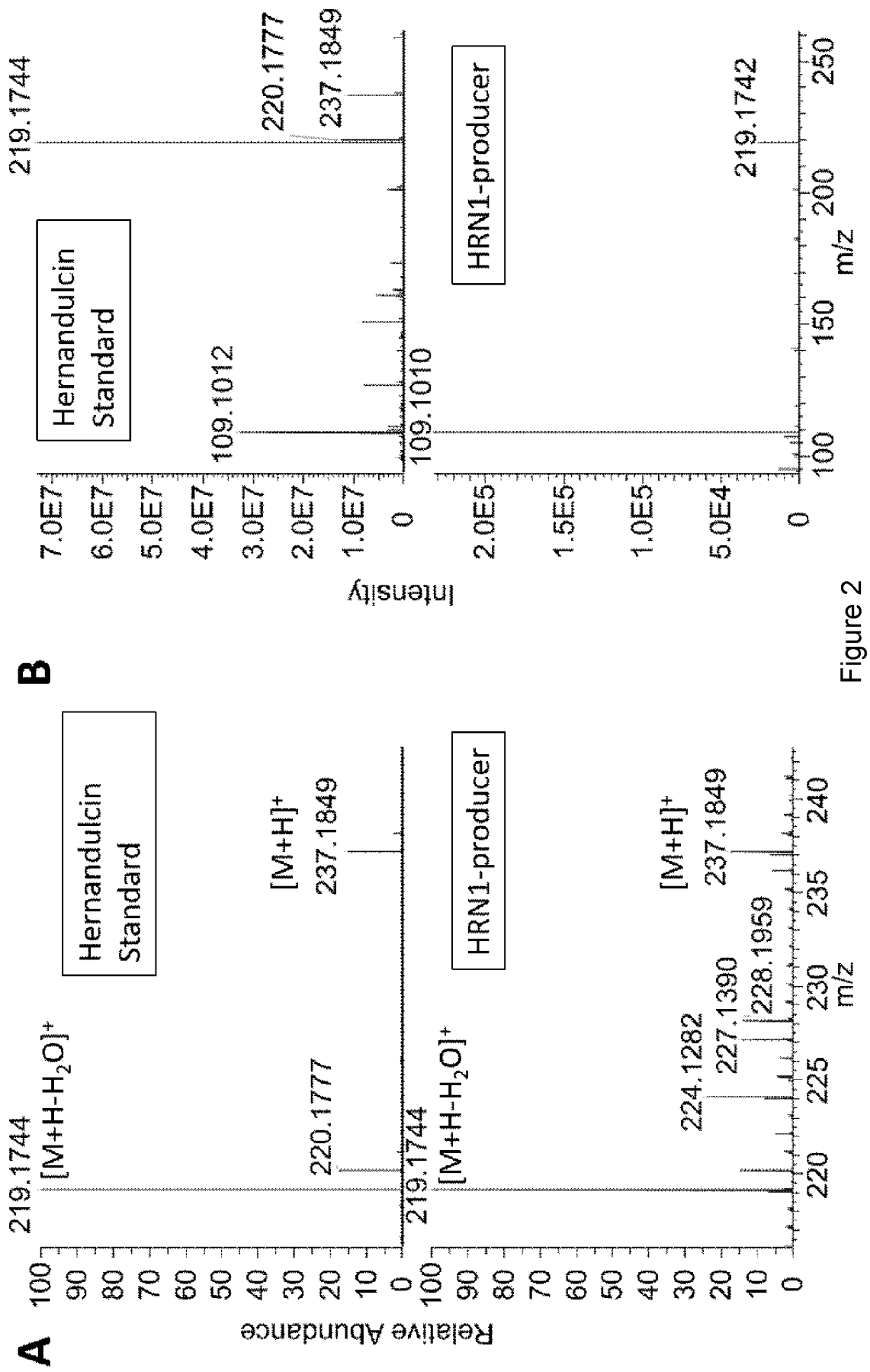
A
Hernandulcin Standard
HRN1-producer
219.1744
[M+H-H2O]+
220.1777
[M+H]+
237.1849
224.1282
227.1390
228.1959
Relative Abundance
m/z
B
Hernandulcin Standard
HRN1-producer
109.1012
109.1010
220.1777
237.1849
219.1744
219.1742
Intensity
m/z

Figure 2

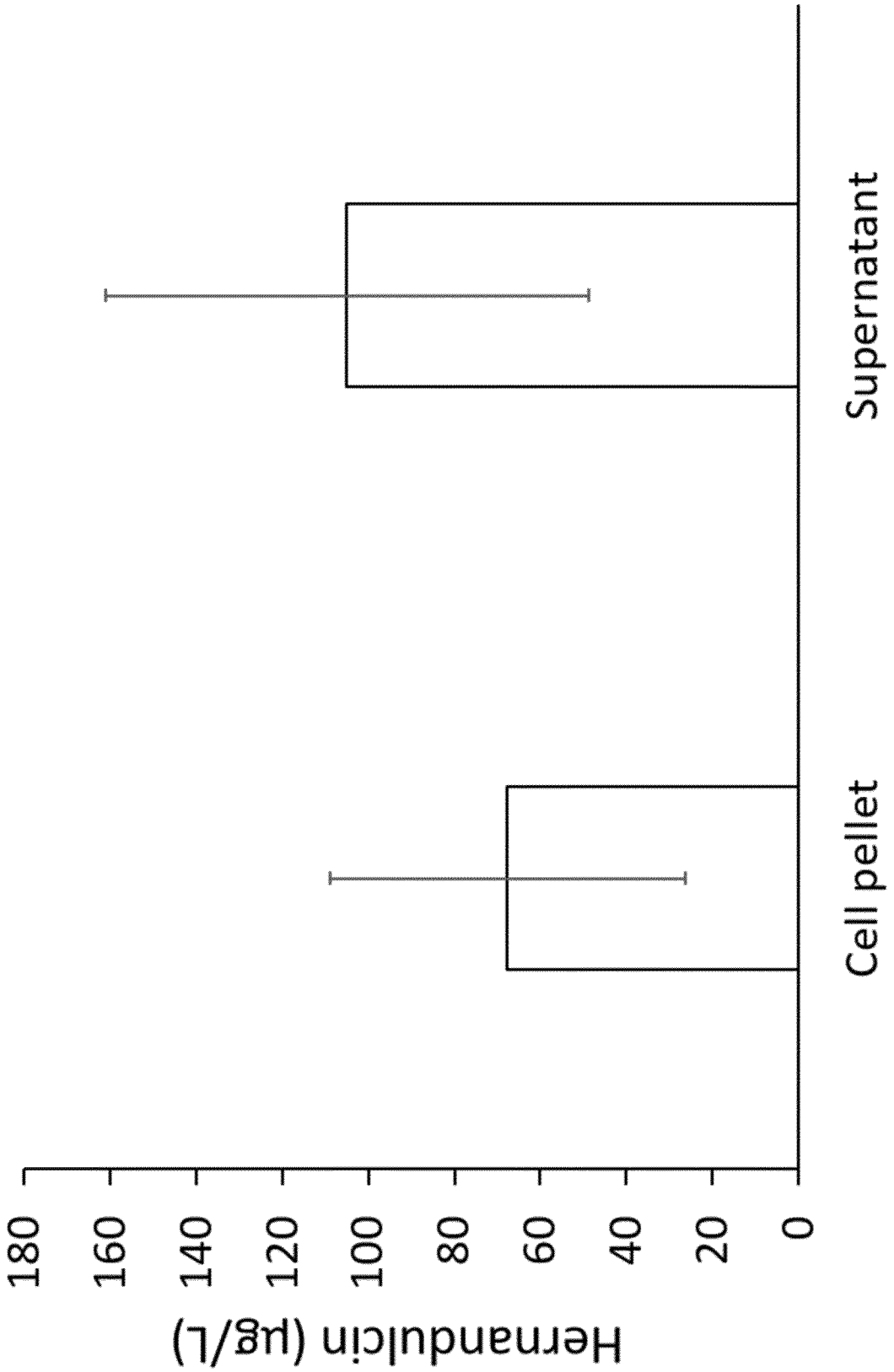
Hernandulcin (µg/L)
0
20
40
60
80
100
120
140
160
180
Cell pellet
Supernatant

Figure 3

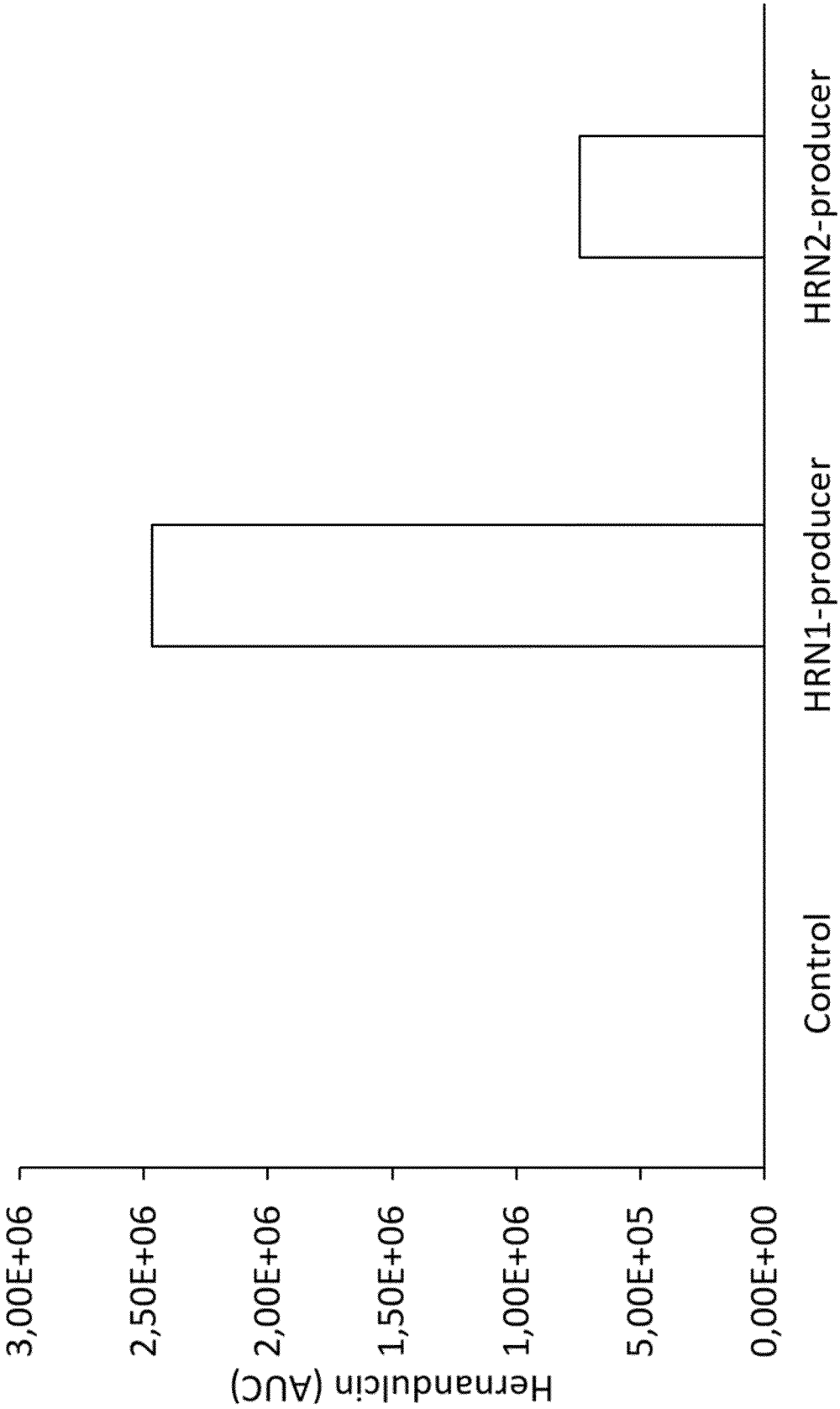
Hernandulcin (AUC)
3,00E+06
2,50E+06
2,00E+06
1,50E+06
1,00E+06
5,00E+05
0,00E+00
Control
HRN1-producer
HRN2-producer

Figure 4

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2023/066249

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/88 C12N9/02 C12N15/81 C12P5/00
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 C40B 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, CHEM ABS Data, COMPENDEX, 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	ATTIA MOHAMED ET AL: "Molecular cloning and characterization of (+)-epi-[alpha]-bisabolol synthase, catalyzing the first step in the biosynthesis of the natural sweetener, hernandulcin, inLippia dulcis", ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, vol. 527, no. 1, 31 July 2012 (2012-07-31) , pages 37-44, XP028941544, ISSN: 0003-9861, DOI: 10.1016/J.ABB.2012.07.010 cited in the application	35-37,51
A	the whole document in particular: abstract; materials and methods; results; figures 1-5; table 1	1-34, 38-50

-/--

[x] Further documents are listed in the continuation of Box C.

[x] 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	Date of mailing of the international search report
4 October 2023	13/10/2023

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

3

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

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2018/015512 A1 (EVOLVA SA [CH]; UNIV COPENHAGEN [DK]) 25 January 2018 (2018-01-25) the whole document	1-51
A	KR 101 958 113 B1 (KOREA RES INST BIOSCIENCE & BIOTECHNOLOGY [KR] ET AL.) 14 March 2019 (2019-03-14) the whole document	1-51
A	US 2016/213039 A1 (KUMAR MANOJ [NL] ET AL) 28 July 2016 (2016-07-28) the whole document	1-51
A	WO 2015/053510 A1 (SNU R&DB FOUNDATION [KR]) 16 April 2015 (2015-04-16) the whole document	1-51
A	KR 2021 0068662 A (KOREA RES INST BIOSCIENCE & BIOTECHNOLOGY [KR]) 10 June 2021 (2021-06-10) the whole document	1-51
A	CN 108 300 726 B (UNIV SUZHOU) 31 January 2020 (2020-01-31) the whole document	1-51
A	SON YOUNG-JIN ET AL: "Molecular cloning and characterization of drimenol synthase from valerian plant (Valeriana officinalis)", BIOCHEMICAL JOURNAL, vol. 463, no. 2, 22 July 2014 (2014-07-22), pages 239-248, XP055846319, GB ISSN: 0264-6021, DOI: 10.1042/BJ20140306 the whole document	1-51
A	MOONHYUK KWON ET AL: "Molecular cloning and characterization of drimenol synthase from valerian plant (Valeriana officinalis)", FEBS LETTERS, ELSEVIER, AMSTERDAM, NL, vol. 588, no. 24, 4 November 2014 (2014-11-04), pages 4597-4603, XP071254660, ISSN: 0014-5793, DOI: 10.1016/J.FEBSLET.2014.10.031 the whole document	1-51

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2023/066249

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [ ] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2023/066249

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2018015512 A1	25-01-2018	NONE	
KR 101958113 B1	14-03-2019	NONE	
US 2016213039 A1	28-07-2016	AU 2014298430 A1	11-02-2016
		BR 112016001950 A2	29-08-2017
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		CN 105658081 A	08-06-2016
		EP 3027048 A1	08-06-2016
		US 2016213039 A1	28-07-2016
		WO 2015014969 A1	05-02-2015
WO 2015053510 A1	16-04-2015	NONE	
KR 20210068662 A	10-06-2021	NONE	
CN 108300726 B	31-01-2020	NONE	