



(86) Date de dépôt PCT/PCT Filing Date: 2013/08/19
(87) Date publication PCT/PCT Publication Date: 2014/02/20
(85) Entrée phase nationale/National Entry: 2015/02/03
(86) N° demande PCT/PCT Application No.: EP 2013/067262
(87) N° publication PCT/PCT Publication No.: 2014/027118
(30) Priorités/Priorities: 2012/08/17 (US61/684,422);
2012/12/21 (US61/745,164)

(51) Cl.Int./Int.Cl. *C12P 23/00* (2006.01),
C12N 15/63 (2006.01), *C12N 9/10* (2006.01),
C12P 7/00 (2006.01)

(71) Demandeur/Applicant:
EVOLVA SA, CH

(72) Inventeurs/Inventors:
TANGE, THOMAS OESTERGAARD, CH;
NAESBY, MICHAEL, FR;
FOLLY, CHRISTOPHE, CH;
DELEGRANGE, FANNY, FR;
HOUGHTON-LARSEN, JENS, DK;
CARLSEN, SIMON, DK

(74) Agent: BENOIT & COTE INC.

(54) Titre : PRODUCTION AUGMENTEE DE TERPENES ET DE TERPENOIDES
(54) Title: INCREASED PRODUCTION OF TERPENES AND TERPENOIDS

(57) Abrégé/Abstract:

This invention provides recombinant cells and methods for producing terpenes and terpenoids by increasing production or accumulation or both of isoprenoid precursors thereof.



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

(19) World Intellectual Property
Organization
International Bureau



WIPO | PCT



(10) International Publication Number

WO 2014/027118 A1

(43) International Publication Date
20 February 2014 (20.02.2014)

(51) International Patent Classification:

C12P 23/00 (2006.01) C12N 15/63 (2006.01)
C12P 7/00 (2006.01) C12N 9/10 (2006.01)

(21) International Application Number:

PCT/EP2013/067262

(22) International Filing Date:

19 August 2013 (19.08.2013)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

61/684,422 17 August 2012 (17.08.2012) US
61/745,164 21 December 2012 (21.12.2012) US

(71) Applicant: EVOLVA SA [CH/CH]; Duggingerstrasse 23,
CH-4153 Reinach (CH).

(72) Inventors: TANGE, Thomas Oestergaard; Hor-
burgstrasse 24, CH-4057 Basel (CH). NAESBY, Michael;
2, rue de l'Hotel de Ville, F-68330 Huingue (FR).
FOLLY, Christophe; Claramattweg 10, CH-4057 Basel
(CH). DELEGRANGE, Fanny; 17 Rue Alphonse Studer,
F-68220 Hesingue (FR). HOUGHTON, Jens; Dugginger-
strasse 23, CH-4153 Reinach (CH). CARLSEN, Simon;
Veggervej 7, DK-4600 Køge (DK).

(74) Agent: WP THOMPSON; 55 Drury Lane, London WC2B
5SQ (GB).

(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, CZ, DE, DK, DM,
DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KN, KP, KR,
KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME,
MG, MK, MN, 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, SM, ST, SV, SY, TH, TJ, TM,
TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM,
ZW.

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, 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, 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).

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))

(54) Title: INCREASED PRODUCTION OF TERPENES AND TERPENOIDS

(57) Abstract: This invention provides recombinant cells and methods for producing terpenes and terpenoids by increasing produc-
tion or accumulation or both of isoprenoid precursors thereof.



WO 2014/027118 A1

INCREASED PRODUCTION OF TERPENES AND TERPENOIDS

BACKGROUND OF INVENTION

5 Field of the invention

The invention set forth herein relates to genetic engineering and recombinant cells useful in producing terpenes and terpenoids by increasing production or accumulation or both of isoprenoid precursors thereof. The invention provides recombinant cells and methods for using such cells having reduced enzymatic activity for farnesyl
10 diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activities, and methods of use thereof. The recombinant cells provided by the invention generally have higher metabolic flux through the mevalonate biochemical pathway, and can also comprise additional recombinant expression constructs encoding enzymes useful for
15 increasing products of the mevalonate pathway, particularly isoprenoids.

Background of the related art

Terpenes and the related terpenoids comprise a large class of biologically derived organic molecules. Terpenes and terpenoids are derived from five-carbon isoprene
20 units and are accordingly also referred to as isoprenoids. They are produced from isoprenoid pyrophosphates which are organic molecules that serve as precursors in the biosynthesis of a number of biologically and commercially important molecules.

Terpenoids can be found in all classes of living organisms, and comprises the largest
25 group of natural products. Plant terpenoids are used extensively for their aromatic qualities and play a role in traditional herbal remedies and are under investigation for antibacterial, antineoplastic, and other pharmaceutical functions. Terpenoids contribute to the scent of eucalyptus, the flavors of cinnamon, cloves, and ginger, and the color of yellow flowers. Well-known terpenoids include citral, menthol, camphor,
30 Salvinorin A in the plant *Salvia divinorum*, and cannabinoids.

While the biosynthetic steps leading from isopentenylpyrophosphate (IPP) and/or dimethylallylpyrophosphate (DMAPP) to terpenoids are universal, two different pathways leading to IPP and DMAPP exist - the mevalonic acid pathway and the non-
35 mevalonic, 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate (MEP/DOXP) pathway. The mevalonate pathway is responsible for the production of

isoprenoid-derived molecules in numerous organisms. Many isoprenoid molecules have high commercial value and production of some of these molecules in genetically engineered hosts rather than in the natural host is highly desirable for economical and sustainability reasons.

5

The part of the mevalonate pathway that generates the basic C5 isoprenoid pyrophosphates, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) comprises seven enzymatic steps. The seven *S. cerevisiae* genes involved in these steps are (in consecutive order in the pathway): *ERG10*, *ERG13*, *HMGR*,
10 *ERG12*, *ERG8*, *ERG19* and *IDI1*. IPP and DMAPP are the isoprene units that form the basis for synthesis of higher order isoprenoid pyrophosphate precursors containing any number of isoprene units between two and ten. The most important ones are geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP).

15

SUMMARY OF INVENTION

The present invention comprises methods for increased production of terpenes and
20 terpenoids, advantageously in recombinant cells resulting from increasing production of isoprenoid pyrophosphate precursors. In particular, the invention relates to methods for increasing the production or accumulation or both of isopentenyl pyrophosphate (IPP), dimethylallyl pyrophosphate (DMAPP), geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) in said recombinant
25 cells.

In one aspect, the invention relates to a method for producing a terpene or terpenoid in a recombinant cell, the method comprising the steps of culturing the cell under conditions wherein the terpene or terpenoid is produced in a genetically engineered cell
30 having reduced expression of endogenous prenyl diphosphate synthase, such as farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity, and further comprising one or more recombinant expression constructs encoding heterologous enzymes for producing said terpene or terpenoid.

35

In an embodiment of the invention, the cell is genetically engineered to reduce expression of farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity.

5

In another embodiment, reduced expression of endogenous farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is produced in the recombinant cell by introducing into the cell a recombinant genetic construct wherein nucleic acid encoding farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is operably linked in the construct to a promoter sequence that directs expression of said farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity at levels that are less than the levels of the promoter for the endogenous gene encoding farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity.

In a further embodiment, the reduced expression of endogenous farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is produced in the recombinant cell by introducing into the cell a recombinant genetic construct wherein nucleic acid encoding farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is operably linked in the construct to a promoter sequence that directs expression of said farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity, wherein between said promoter and nucleic acid sequences encoding farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is a heterologous insert sequence having the formula:



wherein X_2 comprises at least 4 consecutive nucleotides being complementary to, and forming a hairpin secondary structure element with at least 4 consecutive nucleotides of X_4 , and

35

wherein X_3 either comprises zero nucleotides or one or more unpaired nucleotides forming a hairpin loop between X_2 and X_4 , and X_4 comprises at least 4 consecutive nucleotides being complementary to, and forming a hairpin secondary structure element with at least 4 consecutive nucleotides of X_2 ; and
5 wherein X_1 and X_5 comprises zero, one or more nucleotides.

In certain embodiments, the reduced expression of endogenous farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is produced in the cell
10 by introducing into the cell a recombinant genetic construct wherein nucleic acid farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is operably linked in the construct to a messenger RNA destabilizing motif.

15 In another embodiment, the invention further or alternatively comprises introducing into the cell a recombinant expression construct encoding a truncated version of 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMGR) comprising the catalytically active carboxyl terminal portion thereof. In additional embodiments, the invention further or alternatively comprises introducing into the cell a recombinant expression construct
20 encoding a heterologous nucleic acid sequence encoding a dual function enzyme, wherein said dual function enzyme is an acetoacetyl-CoA thiolase and a HMG-CoA reductase. In a non-limiting example, the dual function enzyme is the *mvaE* gene encoded by *E. faecalis* or a functional homologue thereof.

25 In other embodiments, the host cell is a eukaryotic cell or a prokaryotic cell. In an embodiment, the host cell is a eukaryotic cell and is a mammalian cell, a plant cell, a fungal cell or a yeast cell. In a further embodiment, the eukaryotic cell is a yeast cell and the yeast cell is a yeast of species *Saccharomyces cerevisiae*,
Schizosaccharomyces pombe, *Yarrowia lipolytica*, *Candida glabrata*, *Candida boidinii*,
30 *Hansenula polymorpha*, *Kluyveromyces lactis*, *Pichia pastoris*, *Ashbya gossypii*, *Arxula adenivorans*, *Cyberlindnera jadinii*, or *Candida albicans*. In a particular embodiment, the yeast cell is *Saccharomyces cerevisiae* and the prenyl diphosphate synthase is ERG20, ERG9 or BTS1.

In an embodiment of the invention, the terpene or terpenoid is a monoterpene, a diterpene, a sesquiterpene, a triperpenoid or a tetraterpenoid. Non-limiting embodiments of a monoterpene produced by the methods of the invention are pinene, myrcene or geraniol. Non-limiting embodiments of a diterpene produced by the methods of the invention are geranylgeranyl pyrophosphate, retinol, retinal, phytol, taxol, forskolin or aphidicolin. Non-limiting embodiments of a sesquiterpene produced by the methods of the invention are amorphadiene, patchoulol, santalol, longifolene or thujopsene. Non-limiting embodiments of a triterpenoid produced by the methods of the invention are squalene and the tetraterpenoid is carotenoid.

10

In a second aspect, the invention relates to a recombinant cell for producing a terpene or terpenoid genetically engineered to have reduced expression of endogenous farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity, and further comprising one or more recombinant expression constructs encoding heterologous enzymes for producing said terpene or terpenoid.

15

In an embodiment of the invention, the reduced expression of endogenous farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is produced in the recombinant cell by introducing into the cell a recombinant genetic construct wherein nucleic acid encoding farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is operably linked in the construct to a promoter sequence that directs expression of said farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity at levels that are less than the levels of the promoter for the endogenous gene encoding farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity.

20

25

30

In additional embodiments of the invention, the reduced expression of endogenous farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is produced in the recombinant cell by introducing into the cell a recombinant genetic

35

construct wherein nucleic acid encoding farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is operably linked in the construct to a promoter sequence that directs expression of said farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity, wherein between said promoter and nucleic acid sequences encoding farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is a heterologous insert sequence having the formula:

10
$$-X_1-X_2-X_3-X_4-X_5-$$

wherein X_2 comprises at least 4 consecutive nucleotides being complementary to, and forming a hairpin secondary structure element with at least 4 consecutive nucleotides of X_4 , and

wherein X_3 either comprises zero nucleotides or one or more unpaired nucleotides forming a hairpin loop between X_2 and X_4 , and
 15 X_4 comprises at least 4 consecutive nucleotides being complementary to, and forming a hairpin secondary structure element with at least 4 consecutive nucleotides of X_2 ; and wherein X_1 and X_5 comprises zero, one or more nucleotides.

20 In another embodiment, the reduced expression of endogenous farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is produced in the cell by introducing into the cell a recombinant genetic construct wherein nucleic acid farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having
 25 both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is operably linked in the construct to a messenger RNA destabilizing motif.

Further or alternative embodiments of the recombinant cells provided by this invention in addition comprise a recombinant expression construct encoding a truncated version
 30 of 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMGR) comprising the catalytically active carboxyl terminal portion thereof. In other additional or alternative embodiments, the recombinant cell comprises a recombinant expression construct encoding a heterologous nucleic acid sequence encoding a dual function enzyme, wherein said dual function enzyme is an acetoacetyl-CoA thiolase and a HMG-CoA

reductase. In a non-limiting example, the dual function enzyme is the *mvaE* gene encoded by *E. faecalis* or a functional homologue thereof.

5 In certain embodiments, the host cell is a eukaryotic cell or a prokaryotic cell. In particular embodiments, the host cell is a eukaryotic cell that is a mammalian cell, a plant cell, a fungal cell or a yeast cell. In a further embodiment, the eukaryotic cell is a yeast cell and the yeast cell is a yeast of species *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Yarrowia lipolytica*, *Candida glabrata*, *Candida boidinii*, *Hansenula polymorpha*, *Kluyveromyces lactis*, *Pichia pastoris*, *Ashbya gossypii*, *Arxula*
10 *adeninivorans*, *Cyberlindnera jadinii*, or *Candida albicans*. In an embodiment yeast cell is *Saccharomyces cerevisiae* and the prenyl diphosphate synthase is ERG20, ERG9 or BTS1.

The invention described here relates to recombinant cells genetically engineered to
15 have increased mevalonate production and/or have higher metabolic flux through the mevalonate biochemical pathway, and can also comprise additional recombinant expression constructs encoding enzymes useful for increasing products of the mevalonate pathway, particularly isoprenoids. In some embodiments the genetically engineered recombinant cells express a phenotype of increased mevalonate
20 production or accumulation or both.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

25

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows an overview of a construct for homologous recombination useful for inserting (A) the weak KEX2 promoter or (B) the CYC1+SL in front of the ORF
30 encoding farnesyl diphosphate synthase. (C) shows CYC1+SL refers to the CYC1 promoter linked to the heterologous insert sequence of SEQ ID NO:2.

Figure 2A shows part of the endogenous mevalonate pathway including the pathway to various alkaloids and terpenoids (left panel). In addition various reactions to yield said
35 alkaloids and terpenoids and the enzymes involved are shown (right panel). Figure 2B shows an overview of the mevalonate pathway together by a modified pathway. The

middle column starting with ERG10 shows the endogenous pathway of *S. cerevisiae*. The right column shows an example of a modified pathway according to the invention. The plasmids used in the methods described in Example 4 are also outlined.

5 Figure 3 shows the reactions catalysed by CpDmaW and FgaMT.

Figure 4 DMAT and Me-DMAT production in yeast cells containing CYC1(5%)-ERG20 or KEX2-ERG20 compared to the wildtype strain.

10 Figure 5 shows levels of limonene expression determined in isopropyl myristate in the KEX2-ERG20 compared to the wildtype strain.

Figure 6 shows levels of mevalonate (upper panel) and amorphaadiene (lower panel) produced in yeast cells containing the ADS plasmid as well as a control plasmid, a truncated version of *S. cerevisiae* HMGR1 or *mvaE* and *mvaS* of *E. faecalis*.
15

Figure 7 shows growth of yeast cells having a deletion of *ERG13* (left) and yeast cells having a deletion of *ERG13*, but also containing *mvaE* and *mvaS* of *E. faecalis* (right).

20 Figure 8 shows the final OD₆₀₀ of the wild-type strain and the KEX2-ERG20+tGPPS+tLIMS strain. It shows that the modified strain grows well and to an OD₆₀₀ greater than 10.

Figure 9 shows levels of kaurene production determined in the wild-type strain and the
25 KEX2- ERG20+FPPS+GPPS+CDPS+KS strain.

DETAILED DESCRIPTION

30 All publications, patents and patent applications cited herein are hereby expressly incorporated by reference for all purposes.

Methods well known to those skilled in the art can be used to construct genetic expression constructs and recombinant cells according to this invention. These
35 methods include in vitro recombinant DNA techniques, synthetic techniques, in vivo

recombination techniques, and PCR techniques. See, for example, techniques as described in Maniatis *et al.*, 1989, MOLECULAR CLONING: A LABORATORY MANUAL, Cold Spring Harbor Laboratory, New York; Ausubel *et al.*, 1989, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Greene Publishing Associates and Wiley Interscience, New York, and PCR Protocols: A Guide to Methods and Applications (Innis *et al.*, 1990, Academic Press, San Diego, CA).

Before describing the present invention in detail, a number of terms will be defined. As used herein, the singular forms “a”, “an”, and “the” include plural referents unless the context clearly dictates otherwise. For example, reference to a “nucleic acid” means one or more nucleic acids.

It is noted that terms like “preferably”, “commonly”, and “typically” are not utilized herein to limit the scope of the claimed invention or to imply that certain features are critical, essential, or even important to the structure or function of the claimed invention. Rather, these terms are merely intended to highlight alternative or additional features that can or cannot be utilized in a particular embodiment of the present invention.

For the purposes of describing and defining the present invention it is noted that the term “substantially” is utilized herein to represent the inherent degree of uncertainty that can be attributed to any quantitative comparison, value, measurement, or other representation. The term “substantially” is also utilized herein to represent the degree by which a quantitative representation can vary from a stated reference without resulting in a change in the basic function of the subject matter at issue.

As used herein, the terms “polynucleotide”, “nucleotide”, “oligonucleotide”, and “nucleic acid” can be used interchangeably to refer to nucleic acid comprising DNA, RNA, derivatives thereof, or combinations thereof.

As used herein, the term “terpenoid” shall be taken to include molecules in which at least part of the molecule is derived from a prenyl pyrophosphate, such as IPP, DMAPP, *etc.*

It is noted that the terms “pyrophosphate” and “diphosphate” are used interchangeably herein.

Regarding sequence identity between nucleotide and amino acid sequences as set forth herein, and as would be understood by the skilled worker, a high level of sequence identity indicates likelihood that a first sequence is derived from a second sequence. Amino acid sequence identity requires identical amino acid sequences between two aligned sequences. Thus, a candidate sequence sharing 70% amino acid identity with a reference sequence requires that, following alignment, 70% of the amino acids in the candidate sequence are identical to the corresponding amino acids in the reference sequence. Identity according to the present invention is determined by aid of computer analysis, such as, without limitations, the ClustalW computer alignment program (Higgins *et al.*, 1994, Nucleic Acids Res. 22: 4673-4680), and the default parameters suggested therein. The ClustalW software is available from as a ClustalW WWW Service at the European Bioinformatics Institute <http://www.ebi.ac.uk/clustalw>. Using this program with its default settings, the mature (bioactive) part of a query and a reference polypeptide are aligned. The number of fully conserved residues are counted and divided by the length of the reference polypeptide. The ClustalW algorithm can similarly be used to align nucleotide sequences. Sequence identities can be calculated in a similar way as indicated for amino acid sequences. In certain embodiments, the cell of the present invention comprises a nucleic acid sequence encoding modified, heterologous and additional enzymatic components of terpene and terpenoid biosynthetic pathways, as defined herein.

In one aspect, the invention relates to a method for producing a terpene or terpenoid in a recombinant host cell, the method comprising the steps of culturing under conditions wherein the terpene or terpenoid is produced in a genetically engineered cell having reduced expression of endogenous farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity, and further comprising one or more recombinant expression constructs encoding heterologous enzymes for producing said terpene or terpenoid .

The methods of the invention can be used, for example, for large-scale production of a terpene and/or a terpenoid and/or an isoprenoid by a recombinant host cell, as described for the methods of the invention. As shown in the examples that follow, the methods of the invention can be used to produce recombinant host cells with increased

metabolic flux through the pathway of interest and efficient production of a terpene and/or a terpenoid and/or an isoprenoid of interest at unexpectedly higher levels in a recombinant host cell.

- 5 The increased metabolic flux described herein means at least 2-fold increase in the terpene and/or a terpenoid and/or an isoprenoid of interest flux in a recombinant host cell compared with flux towards a terpene and/or a terpenoid and/or an isoprenoid of interest in a reference host cell.

10 **Downregulation of Farnesyl diphosphate synthase and/or geranyl diphosphate synthase**

In one aspect, the invention relates to host cells having reduced activity or expression of endogenous farnesyl diphosphate synthase and/or geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate
15 synthase activity. In particular embodiments, when a wild type host cell expresses an enzyme with both farnesyl diphosphate synthase and geranyl diphosphate synthase activity, then the host cells of the invention preferably have reduced activity of said enzyme with both farnesyl diphosphate synthase and geranyl diphosphate synthase activity. A non-limiting example of this is the host cell is *S. cerevisiae* and the
20 endogenous enzyme encoded by the ERG20 gene.

In some embodiments of the invention, the wild type host cells do not express any enzyme with both farnesyl diphosphate synthase and geranyl diphosphate synthase activity. In such an embodiment, the host cells preferably have reduced activity of
25 farnesyl diphosphate synthase and/or geranyl diphosphate synthase.

Said reduced activity results in production or accumulation or both of IPP and DMAPP and thus the host cells of the invention are useful in methods for accumulating and producing IPP, DMAPP as well as compounds having IPP or DMAPP as precursors,
30 and for producing increased amounts of terpenes or terpenoids produced from said isoprenoid precursors.

The farnesyl diphosphate synthase can be any of the farnesyl pyrophosphate synthases described herein. In general the host cell carries an endogenous gene
35 encoding farnesyl diphosphate synthase, where the recombinant cell as provided by

the invention has been genetically engineered in order to reduce the activity of farnesyl diphosphate synthase.

5 The geranyl diphosphate synthase can be any of the geranyl pyrophosphate synthases described herein. In general the recombinant cell as provided by the invention has been genetically engineered in order to reduce the activity of geranyl diphosphate synthase.

10 Some host cells comprise a geranyl diphosphate synthase which also has some GGPP synthase activity. In embodiments of the invention using such host cells, then the geranyl diphosphate synthase can be an enzyme having both geranyl diphosphate synthase and GGPP synthase activity

15 When the host cell carries an endogenous gene encoding an enzyme with both farnesyl diphosphate synthase and geranyl diphosphate synthase activity, then the recombinant cell as provided by the invention has been genetically engineered to reduce the activity of said enzyme.

20 A recombinant cell having reduced activity of farnesyl diphosphate synthase activity according to the invention can have an activity of farnesyl diphosphate synthase, which is about 80%, about 50%, about 30%, for example in the range of 10 to 50% of the activity of farnesyl diphosphate synthase in a similar cell having wild type farnesyl diphosphate synthase activity. It is in general important that the recombinant cell retains at least some farnesyl diphosphate synthase activity, since this is essential for
25 most cells. As shown herein, farnesyl diphosphate synthase activity can be greatly reduced without significantly impairing cell viability. Recombinant cells with greatly reduced farnesyl diphosphate synthase activity can have a somewhat slower growth rate than corresponding wild type cells. Thus it is preferred that recombinant cells of the invention have a growth rate which is at least 50% of the growth of a similar cell
30 having wild type farnesyl diphosphate synthase activity.

In certain embodiments of the invention the host cell having reduced activity of an enzyme with both farnesyl diphosphate synthase and geranyl diphosphate synthase activity according to the invention has an activity of said enzyme, which is at the most
35 80%, preferably at the most 50%, such as at the most 30%, for example in the range of

10 to 50% of the activity of said enzyme in a similar host cell having a wild type enzyme with both farnesyl diphosphate synthase and geranyl diphosphate synthase activity. It is in general important that recombinant cells retain at least some farnesyl diphosphate synthase and at least some geranyl diphosphate synthase activity, since this is
5 essential for most host cells. As shown herein, both the farnesyl diphosphate synthase and geranyl diphosphate synthase activity can be greatly reduced without significantly impairing cell viability. Recombinant cells with greatly reduced activity can have a somewhat slower growth rate than corresponding wild type cells. Thus it is preferred that the recombinant cells of the invention have a growth rate which is at least 50% of
10 the growth of a similar cell having a wild enzyme with both farnesyl diphosphate synthase and geranyl diphosphate synthase activity.

In other embodiments of the invention, recombinant cells having reduced activity of geranyl diphosphate synthase activity according to the invention has an activity of
15 geranyl diphosphate synthase, which is at the most 80%, preferably at the most 50%, such as at the most 30%, for example in the range of 10 to 50% of the activity of geranyl diphosphate synthase in a similar host cell having wild type geranyl diphosphate synthase activity. It is in general important that the recombinant cell retains at least some geranyl diphosphate synthase activity, since this is essential for
20 most host cells. As shown herein, geranyl diphosphate synthase activity can be greatly reduced without significantly impairing viability. Recombinant cells with greatly reduced geranyl diphosphate synthase activity can have a somewhat slower growth rate than corresponding wild type cells. However, it is preferred that recombinant cells of the invention have a growth rate which is at least 50% of the growth of a similar host cell
25 having wild type geranyl diphosphate synthase activity.

The activity of farnesyl diphosphate synthase can be reduced in a number of different ways. In certain embodiments, the wild type promoter of a gene encoding farnesyl diphosphate synthase can be exchanged for a weak promoter, such as any of the weak
30 promoters described herein below in the section "Promoter sequence". The endogenous gene can therefore be inactivated by introduction of a construct including a weak promoter, either by homologous recombination or by deletion and insertion. Accordingly, the recombinant cell can comprise an ORF encoding farnesyl diphosphate synthase under the control of a weak promoter, which for example can be any of the
35 weak promoters described in the section "Promoter sequence". In general, cells of the

invention only contain one ORF encoding the farnesyl diphosphate synthase endogenous to the host cell, ensuring that the overall level of the endogenous farnesyl diphosphate synthase activity is reduced.

5 In other embodiments, alternatively or simultaneously, the recombinant cell can comprise a heterologous insert sequence, which reduces the expression of mRNA encoding farnesyl diphosphate synthase. In particular embodiments, the heterologous nucleic acid insert sequence can be positioned between the promoter sequence and the ORF encoding farnesyl diphosphate synthase. Said heterologous insert sequence
10 can be any of the heterologous insert sequences described herein below in the section "Heterologous insert sequence".

In further embodiments, farnesyl diphosphate synthase activity can be reduced using a motif that de-stabilizes mRNA transcripts. Thus, recombinant cells of this invention can
15 comprise a nucleic acid comprising a promoter sequence operably linked to an open reading frame (ORF) encoding farnesyl diphosphate synthase, and a nucleotide sequence comprising a motif that de-stabilizes mRNA transcripts. Said motif can be any of the motif that de-stabilize mRNA transcripts described herein below in the section "Motif that de-stabilize mRNA transcripts".

20 Similarly, the activity of an enzyme with both farnesyl diphosphate synthase and geranyl diphosphate activity or an enzyme with geranyl diphosphate synthase activity can be reduced using the same or similar methods.

25 In particular embodiments of the invention, the recombinant cell can also have inactivated and/or no endogenous farnesyl diphosphate synthase activity and/or no endogenous geranyl diphosphate synthase activity. This can for example be accomplished by:

- 30 a) deletion of the entire gene encoding endogenous farnesyl diphosphate synthase; or
b) deletion of the entire coding region encoding endogenous farnesyl diphosphate synthase; or
c) deletion of part of the gene encoding farnesyl diphosphate synthase leading to a total loss of endogenous farnesyl diphosphate synthase activity; or

- d) deletion of the entire gene encoding endogenous geranyl diphosphate synthase; or
- e) deletion of the entire coding region encoding endogenous geranyl diphosphate synthase; or
- 5 f) deletion of part of the gene encoding endogenous geranyl diphosphate synthase leading to a total loss of farnesyl diphosphate synthase activity; or
- g) deletion of the entire gene encoding an endogenous enzyme with both farnesyl diphosphate synthase and geranyl diphosphate synthase activity; or
- h) deletion of the entire coding region encoding an endogenous enzyme with both
- 10 farnesyl diphosphate synthase and geranyl diphosphate synthase activity; or
- i) deletion of part of the gene encoding an endogenous enzyme with both farnesyl diphosphate synthase and geranyl diphosphate synthase activity leading to a total loss of activity of said enzyme.
- 15 Farnesyl diphosphate synthase activity and geranyl synthase activity are generally essential for host cells, since FPP and GPP are precursors for essential cellular constituents, *e.g.* ergosterol. Accordingly, in embodiments of the invention where the host cell or recombinant cell have no endogenous farnesyl diphosphate synthase activity:
- 20 a) cells are cultivated in the presence of ergosterol; or
- b) cells comprise a heterologous nucleic acid encoding an enzyme with farnesyl diphosphate activity.
- Similarly, in embodiments of the invention where the host cell or recombinant have no
- 25 endogenous geranyl diphosphate synthase activity, in advantageous embodiments
- a) cells are cultivated in the presence of ergosterol; or
- b) cells comprise a heterologous nucleic acid encoding an enzyme with geranyl diphosphate and farnesyl diphosphate activity.
- 30 In a second aspect, the invention provides recombinant cells for producing a terpene or terpenoid that are genetically engineered to have reduced expression of endogenous farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity, and further comprising one or more recombinant expression constructs encoding
- 35 heterologous enzymes for producing said terpene or terpenoid.

Host and recombinant cells

Host and recombinant cells provided herein can be any cell suitable for protein expression (*i.e.*, expression of heterologous genes) including, but not limited to, eukaryotic cells, prokaryotic cells, yeast cells, fungal cells, mammalian cells, plant cells, microbial cells and bacterial cells. Furthermore, cells according to the invention meet one or more of the following criteria: said cells should be able grow rapidly in large fermentors, should produce small organic molecules in an efficient way, should be safe and, in case of pharmaceutical embodiments, should produce and modify the products to be as similar to "human" as possible. Furthermore, a host cell is a cell that can be genetically engineered according to the invention to produce a recombinant cell, which is a cell wherein a nucleic acid has been disabled (by deletion or otherwise), or substituted (for example, by homologous recombination at a genetic locus to change the phenotype of the cell, *inter alia*, to produce reduced expression of a cellular enzyme or any gene of interest), or a heterologous nucleic acid, *inter alia*, encoding an enzyme or enzymes to confer a novel or enhanced phenotype on the cell has been introduced.

In further and particular embodiments, recombinant cells are yeast cells that are of yeast species *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Yarrowia lipolytica*, *Candida glabrata*, *Ashbya gossypii*, *Cyberlindnera jadinii*, *Candida albicans*, *Arxula adeninivorans*, *Candida boidinii*, *Hansenula polymorpha*, *Kluyveromyces lacti* and *Pichia pastoris*. Yeasts are known in the art to be useful as host cells for genetic engineering and recombinant protein expression. Yeast of different species differ in productivity and with respect to their capabilities to process and modify proteins and to secrete metabolic products thereof. The different 'platforms' of types of yeast make them better suited for different industrial applications. In general, yeasts and fungi are excellent host cells to be used with the present invention. They offer a desired ease of genetic manipulation and rapid growth to high cell densities on inexpensive media. As eukaryotes, they are able to perform protein modifications like glycosylation (addition of sugars), thus producing even complex foreign proteins that are identical or very similar to native products from plant or mammalian sources.

In other embodiments, the host cell for genetic engineering as set forth herein is a microalgal cell such as a cell from *Chlorella* or *Prototheca* species. In other

embodiment, the host cell is a cell of a filamentous fungus, for example *Aspergillus* species. In other embodiments, the host cell is a plant cell. In yet additional embodiments, the host cell is a mammalian cell, such as a human, feline, porcine, simian, canine, murine, rat, mouse or rabbit cell. The host cell can also be a CHO, CHO-K1, HEI193T, HEK293, COS, PC12, HiB5, RN33b, BHK cell. In other
5 CHO-K1, HEI193T, HEK293, COS, PC12, HiB5, RN33b, BHK cell. In other
embodiments, the host cell can be a prokaryotic cell, such as a bacterial cell, including, but not limited to *E. coli* or cells of *Corynebacterium*, *Bacillus*, *Pseudomonas* and *Streptomyces* species.

- 10 In certain embodiments, the host cell is a cell that, in its nonrecombinant form comprises a gene encoding at least one of the following:
- a) farnesyl diphosphate synthase
 - b) geranyl diphosphate synthase
 - c) an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate
15 synthase activity

In other embodiments, the host cell is a cell that in its nonrecombinant form comprises a gene encoding an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity. For example, the host cell can be *S. cerevisiae* that
20 comprises non-recombinant, endogenous ERG20, and which according to this invention can be recombinantly manipulated for reduced expression of the ERG20 gene.

Additional Aspects of Recombinant Cells

- 25 In addition to the genetic engineering performed as set forth herein to reduce expression of farnesyl diphosphate synthase, geranyl diphosphate synthase, or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activities, the invention provides recombinant cells, in particular embodiments recombinant prokaryotic or eukaryotic cells, having increased levels of mevalonate. In
30 certain embodiments, the invention provides recombinant cells comprising a heterologous nucleic acid sequence encoding a dual function enzyme, wherein the dual function enzyme is an acetoacetyl-CoA thiolase and a HMG-CoA reductase, including, but not limited to, the *mvaE* gene encoded by *E. faecalis* or a functional homologue thereof. In addition to the heterologous nucleic acid sequence encoding a
35 dual function enzyme, the recombinant cell also can also comprise a heterologous

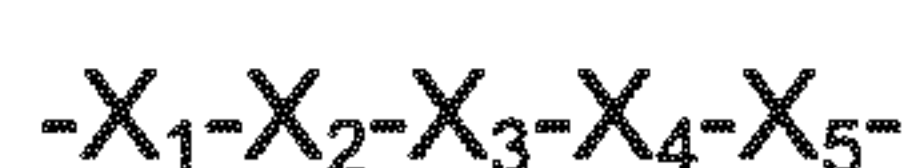
nucleic acid sequence encoding a 3-hydroxy-3-methyl-glutaryl coenzyme A synthase (HMGS), including but not limited to, *mvaS* gene encoded by *E. faecalis* or a functional homologue thereof.

5 In yet further embodiments, the invention provides recombinant cells comprising a recombinant expression construct encoding a truncated version of 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMGR) comprising the catalytically active carboxyl terminal portion thereof.

10 **Heterologous insert sequence**

In some embodiments the recombinant cells of the invention comprise a heterologous nucleic acid insert sequence positioned between the promoter sequence and the ORF encoding farnesyl diphosphate synthase, geranyl diphosphate synthase, or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activities. In these embodiments of the invention the promoter can be any promoter directing expression of said ORF in the host cell, such as any of the promoters described herein in the section "Promoter sequence". Thus, the promoter can be a weak promoter wherein the promoter activity is less than the promoter activity of the wild type promoter in strength. In a non-limiting example, said weak promoter has decreased promoter activity compared to the ERG20 promoter in *S. cerevisiae*. Thus, in embodiments of the invention wherein the nucleic acid comprises a heterologous nucleic acid insert sequence between the promoter sequence and the ORF encoding farnesyl diphosphate synthase, geranyl diphosphate synthase, or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activities, then the promoter sequence can be a promoter directing expression of said ORF in a wild type host cell, e.g. the wild type ERG20 promoter. The heterologous nucleic acid insert sequence can be any nucleic acid sequence that adapts the secondary structure element of a hairpin.

30 In particular embodiments, the heterologous insert sequence can be a nucleic acid sequence having the general formula (I):



35 wherein X_2 comprises at least 4 consecutive nucleotides being complementary to, and forming a hairpin secondary structure element with at least 4 consecutive nucleotides

of X_4 , and

wherein X_3 either comprises zero nucleotides or one or more unpaired nucleotides forming a hairpin loop between X_2 and X_4 , and

5

wherein X_4 comprises or comprises at least 4 consecutive nucleotides being complementary to, and forming a hairpin secondary structure element with at least 4 consecutive nucleotides of X_2 , and

10

wherein X_1 and X_5 comprises zero, one or more nucleotides.

15

X_2 and X_4 in general comprises a sequence of nucleotides. Preferably the heterologous nucleic acid insert sequence comprises sections X_2 and X_4 which are complementary and hybridizes to one another, thereby forming a hairpin. Sections X_2 and X_4 can be directly connected to each other. In other embodiments X_2 and X_4 can flank section X_3 , which forms a loop – the hairpin loop. In general X_3 comprises unpaired nucleic acids.

20

Advantageously, the heterologous insert sequence is long enough to allow a loop to be completed, but short enough to allow a limited translation rate of the ORF following the heterologous insert sequence. In general the longer the stem of the insert stem loop sequence, the lower the translation rate. Thus, in embodiments of the invention, where a very low translation rate of the ORF is desired, then a long heterologous insert sequence should be selected and in particular a heterologous insert sequence with long X_2 and X_4 sequences complementary to each other should be selected. Thus, in certain embodiments of the present invention the heterologous nucleic acid insert sequence comprises in the range of 10 to 50 nucleotides, preferably in the range of 10 to 30 nucleotides, more preferably in the range of 15 to 25 nucleotides, more preferably in the range of 17 to 23 nucleotides, more preferably in the range of 18 to 22 nucleotides, for example in the range of 18 to 21 nucleotides, such as 19 to 20 nucleotides.

25

30

35

X_2 and X_4 can individually comprise any suitable number of nucleotides, so long as a consecutive sequence of at least 4 nucleotides of X_2 is complementary to a consecutive sequence of at least 4 nucleotides of X_4 . In a preferred embodiment X_2

and X_4 comprise the same number of nucleotides. It is preferred that a consecutive sequence of at least 6 nucleotides, more preferably at least 8 nucleotides, even more preferably at least 10 nucleotides, such as in the range of 8 to 20 nucleotides of X_2 is complementary to a consecutive sequence of the same amount of nucleotides of X_4 .

5

X_2 can for example comprise in the range of 4 to 25, such as in the range of 4 to 20, for example of in the range of 4 to 15, such as in the range of 6 to 12, for example in the range of 8 to 12, such as in the range of 9 to 11 nucleotides.

10

X_4 can for example comprise in the range of 4 to 25, such as in the range of 4 to 20, for example of in the range of 4 to 15, such as in the range of 6 to 12, for example in the range of 8 to 12, such as in the range of 9 to 11 nucleotides.

15

In one preferred embodiment X_2 comprises a nucleotide sequence, which is complementary to the nucleotide sequence of X_4 , *i.e.*, it is preferred that all nucleotides of X_2 are complementary to the nucleotide sequence of X_4 .

20

In one preferred embodiment X_4 comprises a nucleotide sequence, which is complementary to the nucleotide sequence of X_2 , *i.e.*, it is preferred that all nucleotides of X_4 are complementary to the nucleotide sequence of X_2 . Very preferably, X_2 and X_4 comprises the same number of nucleotides, wherein X_2 is complementary to X_4 over the entire length of X_2 and X_4 .

25

X_3 can be absent, *i.e.*, X_3 can comprise zero nucleotides. It is also possible that X_3 comprises in the range of 1 to 5, such as in the range of 1 to 3 nucleotides. As mentioned above, then it is preferred that X_3 does not hybridise with either X_2 or X_4 .

30

X_1 can be absent, *i.e.*, X_1 can comprise zero nucleotides. It is also possible that X_1 comprises in the range of 1 to 25, such as in the range of 1 to 20, for example in the range of 1 to 15, such as in the range of 1 to 10, for example in the range of 1 to 5, such as in the range of 1 to 3 nucleotides.

35

X_5 can be absent, *i.e.*, X_5 can comprise zero nucleotides. It is also possible that X_5 can comprise in the range 1 to 5, such as in the range of 1 to 3 nucleotides.

The sequence can be any suitable sequence fulfilling the requirements defined herein above. In one non-limiting example the heterologous insert sequence comprises or comprises SEQ ID NO: 2.

5 **Farnesyl diphosphate synthase and geranyl diphosphate synthase**

Recombinant cells of the invention in general comprise an open reading frame (ORF) encoding farnesyl diphosphate synthase, geranyl diphosphate synthase, or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase. Said farnesyl diphosphate synthase, geranyl diphosphate synthase, or an enzyme having
10 both farnesyl diphosphate synthase and geranyl diphosphate synthase can be any farnesyl diphosphate synthase, geranyl diphosphate synthase, or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase. Frequently it will be a farnesyl diphosphate synthase, geranyl diphosphate synthase, or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase
15 endogenous to the host cell. Thus, by way of example, in embodiments of the invention wherein the host cell is *S. cerevisiae*, then preferably the ORF encoding farnesyl diphosphate synthase encodes an *S. cerevisiae* farnesyl diphosphate synthase.

20 The farnesyl diphosphate synthase can be any enzyme, which is capable of catalysing the following chemical reaction:

Geranyl diphosphate + Isopentenyl diphosphate $\rightleftharpoons$ Diphosphate + trans,trans-Farnesyl diphosphate

25

It is preferred that the farnesyl diphosphate synthase according to the present invention is an enzyme categorised under EC 2.5.1.10.

The geranyl diphosphate synthase can be any enzyme, which is capable of catalysing
30 the following chemical reaction:

Dimethylallyl diphosphate + Isopentenyl diphosphate $\rightleftharpoons$ Diphosphate + Geranyl diphosphate

It is preferred that the farnesyl diphosphate synthase and/or a geranyl diphosphate synthase according to the present invention is an enzyme categorised under EC 2.5.1.1.

5 An enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is capable of catalysing both of the afore-mentioned reactions is particularly advantageous, and that said enzyme thus is an enzyme categorised under both EC 2.5.1.1 and EC 2.5.1.10.

10 Farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity can be from a variety of sources, such as from bacteria, fungi, plants or mammals. Farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity can be wild
15 type embodiments thereof or a functional homologue thereof.

For example, an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity can be an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity of *S. cerevisiae*. Thus, said
20 enzyme can be an enzyme of SEQ ID NO:4 or a functional homologue thereof sharing at least 70%, for example 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 88%, such as
25 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 at least 99.5%, such as at least 99.6%, such as at least 99.7%, such as at least 99.8%, such as at least 99.9%, such as 100% sequence identity therewith. The sequence identity is
30 preferably calculated as described herein.

A functional homologue of an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is also capable of catalysing one or both of the following chemical reactions:

35

Dimethylallyl diphosphate + Isopentenyl diphosphate $\rightleftharpoons$ Diphosphate + Geranyl diphosphate

and/or

5

Geranyl diphosphate + Isopentenyl diphosphate $\rightleftharpoons$ Diphosphate + trans,trans-Farnesyl diphosphate

Embodiments comprising such a homolog are advantageous as set forth further herein.

10

Promoter sequence

15

In certain embodiments, this invention provides recombinant host cells comprising a nucleic acid comprising a promoter sequence operably linked to an ORF encoding farnesyl diphosphate synthase, geranyl diphosphate synthase, or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activities, wherein said ORF preferably is endogenous to said host cell. The invention also relates to recombinant cells comprising a nucleic acid comprising a promoter sequence operably linked to an ORF, wherein said ORF encodes farnesyl diphosphate synthase, geranyl diphosphate synthase, or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activities. In these embodiments, a promoter sequence can be any sequence capable of directing expression of said ORF in the particular host cell.

20

25

As used herein, the term "promoter" is intended to mean a region of DNA that facilitates transcription of a particular gene. Promoters are generally located in close proximity to the genes they regulate, being encoded on the same strand as the transcribed ORF and typically upstream (towards the 5' region of the sense strand). In order for transcription to take place, the enzyme that synthesizes RNA, known as RNA polymerase, must attach to the DNA 5' to the beginning of the ORF. Promoters contain specific DNA sequences and response elements that provide an initial binding site for RNA polymerase and for proteins called transcription factors that recruit RNA polymerase. These transcription factors have specific activator or repressor sequences of corresponding nucleotides that attach to specific promoters and regulate gene expressions.

30

35

The promoter sequence can in general be positioned immediately adjacent to the open reading frame (ORF), or a heterologous nucleic acid insert sequence can be positioned between the promoter sequence and the ORF. Positions in the promoter are in general designated relative to the transcriptional start site, where transcription of RNA begins for a particular gene (*i.e.*, positions upstream are negative numbers counting back from -1, for example -100 is a position 100 base pairs upstream).

The promoter sequence according to the present invention in general comprises at least a core promoter, which is the minimal portion of the promoter required to properly initiate transcription. In addition the promoter sequence can comprise one or more of the following promoter elements:

- transcription start site (TSS)
- a binding site for RNA polymerase
- general transcription factor binding sites
- proximal promoter sequence upstream of the gene that tends to contain primary regulatory elements
- specific transcription factor binding sites
- distal promoter sequence upstream of the gene that can contain additional regulatory elements, often with a weaker influence than the proximal promoter
- binding sites for repressor proteins

Prokaryotic promoters

In prokaryotes, the promoter comprises two short sequences at -10 and -35 positions upstream from the transcription start site. Sigma factors not only help in enhancing RNA polymerase binding to the promoter, but also help RNAP target specific genes to transcribe. The sequence at -10 is called the Pribnow box, or the -10 element, and usually comprises the six nucleotides TATAAT. The other sequence at -35 (the -35 element) usually comprises the seven nucleotides TTGACAT. Both of the above consensus sequences, while conserved on average, are not found intact in most promoters. On average only 3 of the 6 base pairs in each consensus sequence is found in any given promoter. No naturally occurring promoters have been identified to date having an intact consensus sequences at both the -10 and -35; artificial promoters with complete conservation of the -10/-35 hexamers has been found to promote RNA chain initiation at very high efficiencies.

Some promoters also contain a UP element (consensus sequence 5'-AAAWWTWTTTTNNNAAANN-3'; W = A or T; N = any base) centered at -50; the presence of the -35 element appears to be unimportant for transcription from the UP element-containing promoters.

Eukaryotic promoters

Eukaryotic promoters are also typically located upstream of the ORF and can have regulatory elements several kilobases (kb) away from the transcriptional start site. In eukaryotes, the transcriptional complex can cause the DNA to fold back on itself, which allows for placement of regulatory sequences far from the actual site of transcription. Many eukaryotic promoters contain a TATA box (sequence TATAAA), which in turn binds a TATA binding protein which assists in the formation of the RNA polymerase transcriptional complex. The TATA box typically lies very close to the transcriptional start site (often within 50 bases).

Host and recombinant cells of the present invention comprise recombinant expression constructs having a promoter sequence operably linked to a nucleic acid sequence encoding a protein including *inter alia*, farnesyl diphosphate synthase, geranyl diphosphate synthase, or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activities. The promoter sequence is not limiting for the invention and can be any promoter suitable for the host cell of choice.

In certain embodiments of the present invention the promoter is a constitutive or inducible promoter. The promoter sequence can also be a synthetic promoter.

In a further embodiment of the invention, the promoter is, in non-limiting examples, an endogenous promoter, KEX2, PGK-1, GPD1, ADH1, ADH2, PYK1, TPI1, PDC1, TEF1, TEF2, FBA1, GAL1-10, CUP1, MET2, MET14, MET25, CYC1, GAL1-S, GAL1-L, TEF1, ADH1, CAG, CMV, human UbiC, RSV, EF-1alpha, SV40, Mt1, Tet-On, Tet-Off, Mo-MLV-LTR, Mx1, progesterone, RU486 or Rapamycin-inducible promoter.

In particular embodiments of the invention, the recombinant cell comprises a heterologous insert sequence between the promoter sequence and the ORF encoding farnesyl diphosphate synthase, geranyl diphosphate synthase, or an enzyme having

both farnesyl diphosphate synthase and geranyl diphosphate synthase activities. Promoter sequences can comprise a wild type promoter, for example the promoter sequence can be the promoter directing expression of said ORF in a wild type host cell. Thus, the promoter sequence can for example be the wild type ERG20 promoter.

5

In another embodiment of the invention, the promoter sequence is a weak promoter. In particular, in embodiments of the invention wherein the nucleic acid does not contain a heterologous nucleic acid insert sequence, then the promoter sequence is preferably a weak promoter. A weak promoter according to the present invention is a promoter, which directs a lower level of transcription in the host cell. In particular it is preferred that the promoter sequence directs expression of an ORF encoding farnesyl diphosphate synthase, geranyl diphosphate synthase, or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activities at an expression level significantly lower than the expression level obtained with the wild type promoter (*e.g.*, in yeast an ERG20 promoter). Said ORF is preferably an ORF encoding native farnesyl diphosphate synthase, native geranyl diphosphate synthase, or a native enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activities, and accordingly the ORF is preferably endogenous to the host or recombinant cell.

10
15
20

It can be determined whether a promoter sequence is a weak promoter or directs a lower level of transcription in the host cell, by determining the expression level of mRNA encoding farnesyl diphosphate synthase, geranyl diphosphate synthase, or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activities in a host cell, comprising an ORF encoding farnesyl diphosphate synthase, geranyl diphosphate synthase, or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activities operably linked to the potential weak promoter, and by determining the expression level of mRNA encoding farnesyl diphosphate synthase, geranyl diphosphate synthase, or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activities in a second reference cell comprising an ORF encoding farnesyl diphosphate synthase, geranyl diphosphate synthase, or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activities operably linked to the wild type ERG20 promoter. The second reference cell can be a wild type cell and preferably the tested recombinant cell is of the same species as the second cell. The expression level of

25
30
35

mRNA encoding farnesyl diphosphate synthase, geranyl diphosphate synthase, or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activities can be determined using any useful method known to the skilled person such as by quantitative PCR. If the expression level of said mRNA in the host cell
5 comprising the potential weak promoter is significantly lower than in the second reference cell, then the promoter is a weak promoter.

It is preferred that the promoter sequence to be used with the present invention directs expression of the ORF encoding farnesyl diphosphate synthase, geranyl diphosphate
10 synthase, or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activities at an expression level, which is at the most 70%, such as at the most 60%, for example at the most 50%, such as at the most 40% of the expression level obtained with the wild type ERG20 promoter. The expression level is preferably determined as described above.

15

Thus, in certain embodiments it is preferred that the promoter sequence to be used with the present invention, when contained in a host cell and operably linked to an ORF encoding farnesyl diphosphate synthase, geranyl diphosphate synthase, or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase
20 activities, directs expression of said ORF in said host cell so the level of mRNA encoding farnesyl diphosphate synthase in said host cell is at the most 70%, such as at the most 60%, for example at the most 50%, such as at the most 40%, preferably in the range of 10 to 50% of the level of mRNA encoding farnesyl diphosphate synthase, geranyl diphosphate synthase, or an enzyme having both farnesyl diphosphate
25 synthase and geranyl diphosphate synthase activities present in a second cell containing a wild type ERG20 gene, wherein the host cell and the second cell is of the same species.

Thus, in certain embodiments it is preferred that the heterologous promoter sequence
30 to be used with the present invention, when contained in a host cell and operably linked to an ORF encoding farnesyl diphosphate synthase, geranyl diphosphate synthase, or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activities, directs expression of said ORF in said host cell so the level of mRNA encoding farnesyl diphosphate synthase, geranyl diphosphate synthase, or an
35 enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase

activities in said recombinant cell is at the most 70%, preferably at the most 60%, even more preferably at the most 50%, such as at the most 40%, preferably is in the range of 10 to 50% of the level of mRNA encoding farnesyl diphosphate synthase, geranyl diphosphate synthase, or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activities present in a second cell containing a wild type gene encoding farnesyl diphosphate synthase, geranyl diphosphate synthase, or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activities, wherein the recombinant cell and the second cell is of the same species.

It can also be determined whether a promoter sequence is a weak promoter or directs a lower level of transcription in the host cell, by determining the expression level of any test protein, including but not limited to a reporter gene (a non-limiting example of a reporter gene is green fluorescent protein, GFP) in a recombinant cell, comprising an ORF encoding said test protein operably linked to the potential weak promoter, and by determining the expression level of the same test protein in a second cell comprising an ORF encoding said test protein operably linked to the wild type ERG20 promoter. The second cell can be a wild type cell and preferably the tested recombinant cell is of the same species as the second cell. The expression level of test protein can be determined using any useful method known to the skilled person. For example the test protein can be a fluorescent protein and the expression level can be assessed by determining the level of fluorescence.

Thus, in a preferred embodiment of the invention the heterologous promoter sequence to be used with the present invention, when contained in a recombinant cell and operably linked to an ORF encoding a test protein, directs expression of said ORF in said recombinant cell so the level of the test protein in said recombinant cell is at the most 70%, such as at the most 60%, for example at the most 50%, such as at the most 40%, preferably in the range of 10 to 50% of the level of the test protein present in a second cell containing an ORF encoding the test protein operably linked to a wild type ERG20 promoter, wherein the host cell and the second cell is of the same species. The test protein is preferably a fluorescent protein, *e.g.* GFP.

Non-limiting examples of weak promoters useful with the present include the CYC-1 promoter or the KEX-2 promoter; in particular the promoter sequence can be the KEX-

2 promoter. Thus in certain embodiments of the invention the heterologous promoter sequence comprises or comprises the KEX-2 promoter.

Thus, in embodiments of the invention where the ORF encodes a farnesyl diphosphate synthase, then preferably said farnesyl diphosphate synthase is a farnesyl diphosphate synthase native to the host or recombinant cell, and the heterologous promoter sequence is a weak promoter directing expression of said native farnesyl diphosphate synthase at a level, which is significantly lower than the native expression level.

In embodiments of the invention where the ORF encodes a geranyl diphosphate synthase, then preferably said geranyl diphosphate synthase is a geranyl diphosphate synthase native to the host or recombinant cell, and the heterologous promoter sequence is a weak promoter directing expression of said native geranyl diphosphate synthase at a level, which is significantly lower than the native expression level.

The term “significantly lower” as used herein preferably means at the most 70%, preferably at the most 60%, even more preferably at the most 50%, such as at the most 40%. In particular the term “significantly lower” can be used to mean in the range of 10 to 50%.

Motifs that de-stabilize mRNA transcripts

In certain embodiments the recombinant cells of the invention comprises a nucleic acid comprising a promoter sequence operably linked to an open reading frame (ORF) encoding farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity, and a nucleotide sequence comprising a motif that de-stabilizes mRNA transcripts.

In this embodiment the promoter can be any of the promoters described herein in the section “Promoter sequence”, for example the promoter can be the wild type ERG20 promoter. Thus, the host cell can comprise the native farnesyl diphosphate gene, geranyl diphosphate synthase gene or a gene encoding an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity, which has been further modified to contain, downstream of its ORF, a DNA sequence motif that reduces the half-life of the mRNA produced from this gene, such as a motif that de-stabilize mRNA transcripts. The motif that de-stabilizes mRNA transcripts can be any

motif, which when positioned in the 3'-UTR of a mRNA transcript can de-stabilize the mRNA transcript and lead to reduced half-life of the transcript (see e.g. Shalgi *et al.*, 2005 *Genome Biology* 6:R86). Thus, to further reduce the activity of the farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity, a nucleotide sequence containing a motif that de-stabilizes mRNA transcripts can be inserted into the native farnesyl diphosphate gene, geranyl diphosphate synthase gene or a gene encoding an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity, downstream of the ORF. Examples of such destabilizing sequences in yeast include, but are not limited to the M1 motif consensus sequence of TATATATATATAT (SEQ ID NO: 28) and the M24 motif consensus sequence of TGTATAATA (SEQ ID NO: 29).

Additional heterologous nucleic acid

Recombinant cells of the invention can in addition to the nucleic acid comprising an ORF encoding farnesyl diphosphate synthase and/or a geranyl diphosphate synthase operably linked to a promoter sequence also comprise one or more additional heterologous nucleic acids. In alternative embodiments, said recombinant cells can comprise additional recombinant expression constructs that direct expression in the cell of enzymes, *inter alia*, for producing terpenes or terpenoids as described herein.

In particular embodiments, said additional heterologous nucleic acid can contain a nucleic acid encoding an enzyme useful in the biosynthesis of a compound, which is desirable to synthesize from mevalonate

The heterologous nucleic acid preferably contains a nucleic acid encoding an enzyme useful in the biosynthesis of a compound, which is desirable to synthesize from either IPP or DMAPP or from both IPP and DMAPP. Thus, the additional heterologous nucleic acid can encode an enzyme useful in the biosynthesis of a terpene, a terpenoid or an alkaloid from IPP or DMAPP.

Thus, the additional heterologous nucleic acid can encode any enzyme using IPP or DMAPP as a substrate. Such enzymes can be any enzyme classified under EC 2.5.1.- using IPP or DMAPP as a substrate. Examples of such enzymes include GPP synthases, FPP synthases, GGPP synthases, synthases capable of catalysing

incorporation of longer isoprenoid chains (e.g. chains of up to around 10 isoprenoids) and prenyl transferases.

5 In particular, the additional heterologous nucleic acid can be selected according to the particular isoprenoid compound or terpene or terpenoid to be produced by the recombinant cell. Thus, if the recombinant cell is to be used in the production of a particular isoprenoid compound or terpene or terpenoid, then the cell can comprise one or more additional heterologous nucleic acid sequences encoding one or more enzymes of the biosynthesis pathway of that particular isoprenoid compound or
10 terpene or terpenoid.

Thus, the additional heterologous nucleic acid can in certain embodiments of the invention encode a terpene synthase. In particular, in embodiments of the invention wherein the recombinant cell is to be employed in methods for production of a terpene,
15 then it is preferred that the recombinant cell comprises an additional heterologous nucleic acid encoding a terpene synthase. Said terpene can for example be any of the terpenes described herein below in the section "Terpenoids and terpenes". Examples of useful terpene synthases to be used with the present invention are described in Degenhardt *et al.*, 2009, *Phytochemistry* 70:1621-1637. Thus, the additional
20 heterologous nucleic acid can for example encode any of the terpene synthases described Degenhardt *et al.*, 2009.

In certain embodiments of the invention one additional heterologous nucleic acid can encode a monoterpene synthase. In particular, in embodiments of the invention
25 wherein the host cell is to be employed in methods for production of a monoterpene, then it is preferred that the host cell comprise a heterologous nucleic encoding a monoterpene synthase. Said monoterpene can for example be any of the monoterpenes described herein below in the section "Terpenoids and terpenes". Said monoterpene synthase can be any monoterpene synthase, for example any of the
30 monoterpene synthases described in Table 1 of Degenhardt *et al.*, 2009. Said table also outlines for synthesis of which particular monoterpene each monoterpene synthase is useful.

In certain embodiments of the invention, an additional heterologous nucleic acid can
35 encode a monoterpenoid synthase. In particular, in embodiments of the invention

wherein the recombinant cell is to be employed in methods for production of a monoterpenoid, then it is preferred that the cell comprise a heterologous nucleic encoding a monoterpenoid synthase. Said monoterpenoid can for example be any of the monoterpenoids described herein below in the section "Terpenoids and terpenes".

5 Thus, the monoterpenoid can for example be limonene, in which case the cell can comprise an additional nucleic acid encoding limonene synthase. A limonene synthase according to the invention is an enzyme capable of catalysing the following reaction:



10

In particular the limonene synthase can be an enzyme classified under EC 4.2.3.16. Limonene synthase can for example be limonene synthase 1 from *Citrus limon* or a functional homologue thereof. In particular the limonene synthase can be a polypeptide comprising or consisting of SEQ ID NO: 13 or a functional homologue thereof sharing
15 at least 70%, 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 88%, such as at least 89%, such as at least 90%, such as at least 91%, such as at least 92%, such as
20 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% sequence identity with SEQ ID NO:13.

In another embodiment of the invention an additional heterologous nucleic acid can
25 encode a sesquiterpene synthase. In particular, in embodiments of the invention wherein the host cell is to be employed in methods for production of a sesquiterpene, then it is preferred that the host cell comprise a heterologous nucleic encoding a sesquiterpene synthase. Said sesquiterpene can for example be any of the sesquiterpenes described herein below in the section "Terpenoids and terpenes". Said
30 sesquiterpene synthase can be any sesquiterpene synthase, for example any of the sesquiterpene synthases described in Table 2 of Degenhardt *et al.*, 2009, *Id.* Said table also outlines for synthesis of which particular sequiterpene each sesquiterpene synthase is useful.

In certain embodiments of the invention, the additional heterologous nucleic acid can encode an amorphadiene synthase, for example an amorphadiene synthase. Said amorphadiene synthase can be any enzyme capable of catalysing the following reaction:

5



In particular the amorphadiene synthase to be used with the present invention can be any enzyme classified under E.C. 4.2.3.24.

10

In a particular embodiment, the amorphadiene synthase is amorphadiene synthase of SEQ ID NO: 8 or a functional homologue thereof, wherein said functional homologue shares at least 70%, 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 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% sequence identity SEQ ID NO:8. The sequence identity is preferably determined as described herein. In addition to the aforementioned sequence identity, a functional homologue of amorphadiene synthase should also be capable of catalysing above-mentioned reaction.

25

In yet another embodiment of the invention, the additional heterologous nucleic acid can encode a GPP synthase. In particular, in embodiments of the invention wherein the recombinant cell is to be employed in methods for production of a GPP, then it is preferred that the cell comprise a heterologous nucleic encoding a GPP synthase. In addition, in embodiments of the invention wherein the cell is to be employed in methods for preparing monoterpenes, for example pinenes, myrcene and/or geraniol, said cell advantageously comprises a heterologous nucleic encoding a GPP synthase. Said GPP synthase can be any GPP synthase. Preferably, the GPP synthase is an enzyme capable of catalysing the following reaction:

35

dimethylallyl diphosphate + isopentenyl diphosphate -> diphosphate + geranyl
diphosphate

- Preferably, the GPP synthase is an enzyme classified under EC 2.5.1.1. An example of a useful GPP synthase is *Humulus lupulus* GPP synthase, such as the *H. lupulus* GPP synthase described in Wang and Dixon, 2009, *Proc. Natl. Acad. Sci. USA* 106: 9914-9919. Other examples of useful GPP synthases are described in Orlova *et al.*, 2009, *Plant Cell*, Vol. 21, 4002-4017 and in Chang *et al.*, 2010, *Plant Cell*, Vol. 22, 454-467. The GPP synthase can also be a functional homologue of the *H. lupulus* synthase described in Wang and Dixon 2009, wherein said functional homologue shares at least 70%, 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 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% sequence identity with *H. lupulus* GPP synthase.
- Another example of a useful GPP synthase, which can be used with the present invention is GPP synthase 2 from *Abies grandis*. Thus, the GPP synthase can be GPP synthase 2 of *Abies grandis* or a fragment thereof or a functional homologue thereof retaining GPP synthase activity. Yet another example of a useful GPP synthase, which can be used with the present invention is GDPS of *Picea abies*. In particular the GPP synthase can be a polypeptide comprising or consisting of SEQ ID NO: 12 or a functional homologue thereof sharing at least 70%, 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 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% sequence identity with SEQ ID NO:12.

In yet another embodiment of the invention, the additional heterologous nucleic acid can encode a FPP synthase, which is not endogenous to the host cell. In particular, in embodiments of the invention wherein the recombinant cell is to be employed in methods for production of a FPP, then it is preferred that the cell comprise a
5 heterologous nucleic encoding a FPP synthase, which is not endogenous to the host cell. In addition, in embodiments of the invention wherein the recombinant cell is to be employed in methods for preparing sesquiterpenes, for example patchoulol, santalol, longiferolene or thujopsene, then it is preferred that the cell comprises a heterologous nucleic encoding a FPP synthase not endogenous to said host cell. Said FPP synthase
10 can be any FPP synthase not endogenous to the host cell. In particular the FPP synthase can be an enzyme capable of catalysing production of FPP from DMAPP and IPP.

Examples of useful FPP synthases include *A. tridentate* FPPS-1 or *A. tridentate* FPPS-
15 2. The FPP synthase can also be a functional homologue of *A. tridentate* FPPS-1 or *A. tridentate* FPPS-2, wherein said functional homologue shares at least 70%, 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%,
20 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% sequence identity with *A. tridentate* FPPS-1 or *A. tridentate* FPPS-2.

25

The FDPS can be any FDPS, but it is preferred with the present invention that the FDPS is an enzyme, which is capable of catalyzing at least one of the following reactions:

- 1) Synthesis of FPP from one DMAPP and 2 IPP
- 30 2) Synthesis of FPP from one GPP and one IPP

Other examples of FPP synthases, which can be used with the present invention include, but are not limited to FDPS(WH5701) and FDPS(CB101) from *Synechococcus*. Thus, the FPP synthase can be the polypeptide of SEQ ID NO: 14.
35 The FPP synthase can also be the polypeptide of SEQ ID NO: 15. The FPP synthase

can also be a functional homologue of SEQ ID NO: 14 or SEQ ID NO: 15, wherein said functional homologue shares at least 70%, 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 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% sequence identity with SEQ ID NO:14 or SEQ ID NO:15.

10

Recombinant cells of the present invention can in certain embodiments contain an additional heterologous nucleic acid sequence encoding Geranylgeranyl Pyrophosphate Synthase (GGPPS). In particular, in embodiments of the invention wherein the cell is to be employed in methods for production of GGPP, then it is preferred that the recombinant cell comprise a heterologous nucleic encoding a GGPP synthase. In addition, in embodiments of the invention wherein the recombinant cell is to be employed in methods for preparing diterpenes or tetraterpenoids, for example carotenoids, then it is preferred that the cell comprise a heterologous nucleic encoding a GGPP synthase. GGPPS can be any GGPPS, but advantageously the GGPPS is an enzyme, which is capable of catalyzing at least one of the following reactions:

15

20

- 3) Synthesis of GGPP from one DMAPP and 3 IPP
- 4) Synthesis of GGPP from one GPP and 2 IPP
- 5) Synthesis of GGPP from one FPP and 1 IPP

25

In particular the GGPPS can be capable of catalysing synthesis of GGPP from one DMAPP and 3 IPP. In particular embodiments, the GGPP synthase is an enzyme classified under EC 2.5.1.1 or EC 2.5.1.10 or, even more preferably under EC 2.5.1.29.

30

The GGPPS can be GGPPS from a variety of sources, such as from bacteria, fungi or mammals. In particular, the GGPPS can be an enzyme from *S. alcidocaldarius* GGPP synthase and *H. lupulus* GGPP synthase or a functional homologue thereof sharing at least 70%, 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 88%, such as at least 89%, such as at least 90%, such as at least 91%, such as at least 92%, such as at

35

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% sequence identity with *S. acidocaldarius* GGPP synthase or with *H. lupulus* GGPP synthase.

5 In particular the GGPPS can be the GGPPS of SEQ ID NO:7 or a functional
homologue thereof sharing at least 70%, 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
10 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%
sequence identity therewith.

The additional heterologous nucleic acid can also encode a GGPP synthase, which is
15 includes but is not limited to GGPP synthases from *S. cerevisiae*. Thus, the GGPP
synthase can be the GGPP synthase of SEQ ID NO: 23 or a functional homologue
sharing at least 70%, 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
20 at least 85%, such as at least 86%, 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% sequence
identity with SEQ ID NO: 23.

25 The additional heterologous nucleic acid can also encode an enzyme involved in the
biosynthesis of a diterpene. For example the additional heterologous nucleic acid can
also encode a diterpene synthase. Examples of diterpene synthases include but are
not limited to ent-kaurene synthase. An example of ent-kaurene synthase is the
polypeptide of SEQ ID NO: 17 or a functional homologue thereof sharing at least 70%,
30 preferably at least 80%, yet more preferably at least 85%, yet more preferably at least
90%, yet more preferably at least 95% sequence identity therewith. The heterologous
nucleic acid can also encode an ent-copalyl-diphosphate synthase, such as the
polypeptide of SEQ ID NO: 18 or a functional homologue thereof sharing at least 70%,

preferably at least 80%, yet more preferably at least 85%, yet more preferably at least 90%, yet more preferably at least 95% sequence identity therewith.

The additional heterologous nucleic acid can also encode an isoprene synthase. Said isoprene synthase can be any enzyme capable of catalyzing the following reaction:



In particular, the isoprene synthase can be any isoprene synthase classified under EC 4.2.3.27.

- 10 The additional heterologous nucleic acid sequence can also encode any enzyme used in the process of preparing the target product terpenoid or terpene. Said enzyme can for example be any enzyme "located downstream" of the isopentenyl-pyrophosphate or dimethylallyl-pyrophosphate, which is intended to indicate that the enzyme or enzymes catalyse production in the recombinant cell of metabolites produced from IPP or
- 15 DMAPP. Said enzyme can thus for example can be dimethylallyltransferase (EC 2.5.1.1), and geranyltranstransferase (EC 2.5.1.10).

- Recombinant cells of the invention can furthermore comprise one or more additional heterologous nucleic acids encoding one or more enzymes, for example,
- 20 phosphomevalonate kinase (EC 2.7.4.2), diphosphomevalonate decarboxylase (EC 4.1.1.33), 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (EC 1.17.7.1), 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (EC 1.17.1.2), isopentenyl-diphosphate Delta-isomerase 1 (EC 5.3.3.2), short-chain Z-isoprenyl diphosphate synthase (EC 2.5.1.68), dimethylallyltransferase (EC 2.5.1.1), geranyltranstransferase
- 25 (EC 2.5.1.10) or geranylgeranyl pyrophosphate synthetase (EC 2.5.1.29).

- Additionally and in some embodiments alternatively, recombinant cells of the invention can also comprise one or more additional heterologous nucleic acids encoding one or more enzymes, for example, acetoacetyl CoA thiolase, HMG-CoA reductase or the
- 30 catalytic domain thereof, HMG-CoA synthase, mevalonate kinase, phosphomevalonate kinase, phosphomevalonate decarboxylase, isopentenyl pyrophosphate isomerase, farnesyl pyrophosphate synthase, D-1-deoxyxylulose 5-phosphate synthase, and 1-deoxy-D-xylulose 5-phosphate reductoisomerase and farnesyl pyrophosphate

synthase, wherein in said alternative embodiments the cells express a phenotype of increased mevalonate production or accumulation or both.

5 The invention described here relates to recombinant cells genetically engineered to have increased mevalonate production and/or have higher metabolic flux through the mevalonate biochemical pathway, and can also comprise additional recombinant expression constructs encoding enzymes useful for increasing products of the mevalonate pathway, particularly isoprenoids. In some embodiments the genetically engineered recombinant cells express a phenotype of increased mevalonate
10 production or accumulation or both

Said additional heterologous nucleic acid sequences encoding a terpene synthase can be generally provided operably linked to a nucleic acid sequence directing expression of said terpene synthase in the recombinant cell. The nucleic acid sequence directing
15 expression of terpene synthase in the recombinant cell can be and generally is a promoter sequence, and preferably said promoter sequence is selected according the particular host cell. The promoter can for example be any of the promoters described herein above in the section "Promoter sequence".

20 In another embodiment the recombinant cell can comprise an additional heterologous nucleic acid encoding a dimethylallyltyrosine synthase. Such cells are for example useful for production of DMAT. Said dimethylallyltyrosine synthase is preferably an enzyme classified under EC 2.5.1.34. For example the dimethylallyltyrosine synthase can be the protein of SEQ ID NO: 5 or a functional homologue thereof sharing at least
25 70%, preferably at least 80%, such as at least 85%, for example at least 90%, such as at least 95% sequence identity therewith.

The host cell can comprise an additional heterologous nucleic acid encoding a prenyl transferase. Said prenyl transferase may be any enzyme capable of catalysing transfer
30 of an allylic prenyl group to an acceptor molecule. For example, the prenyltransferase may be a prenyl diphosphate synthase. Examples of useful prenyltransferases can be found in Bonitz *et al.*, 2011 *PLoS One* 6(11):E27336.

35 An important goal of engineering of eukaryotic cells, such as yeast for production of isoprenoid molecules is to find ways to circumvent the extensive regulation of the

mevalonate pathway (see Figure 2A) to boost production. In particular, the *HMGR* step, which is a rate-limiting step, is subject to feedback inhibition by different intermediates and derivatives from the mevalonate pathway. In particular, *S. cerevisiae* encodes two *HMGR* paralogs, *HMGR1* and *HMGR2* that both are controlled by feedback inhibition, although in different ways. Eukaryotic HMGRs are typically endoplasmic reticulum (ER)-resident integral membrane proteins consisting of two distinct domains: a hydrophobic NH₂-terminal membrane anchor consisting of 2 – 8 transmembrane segments, and a COOH-terminal catalytic domain that extends into the cytoplasm. The COOH-terminal catalytic domain of Class I HMGRs forms a dimer that comprises the active enzyme and each monomer contributes catalytic residues to form the active site. The budding yeast *S. cerevisiae* encodes two HMGR genes, designated HMG1 and HMG2. *HMGR1* is the primary source of *HMGR* activity during aerobic growth (Burg *et al.*, 2011 *Prog Lipid Res.* 50(4):403-410). It has been found that overexpression of a truncated version of the *S. cerevisiae HMGR1* consisting of the catalytically active C-terminus (region from amino acids 619-1025) can stimulate mevalonate levels and increase production of heterologous isoprenoid derived molecules (Rico *et al.*, 2010 *Appl Environ Microbiol.* Oct;76(19):6449-54). Accordingly, in certain additional or alternative embodiments of the invention the recombinant cell comprises an additional heterologous nucleic acid sequence encoding a truncated version of HMGR. Said truncated version of HMGR most advantageously comprises a catalytically active C-terminus, for example it can comprise the catalytically active C-terminus of HMGR1 of *S. cerevisiae* where, for example, amino acids 2-530 have been deleted from the N-terminus. For example said truncated version of HMGR can be truncated HMGR1 described in Rico *et al.*, 2010. In particular, the truncated HMGR is truncated HMGR derived from SEQ ID NO: 8 or a functional homologue thereof sharing at least 70%, 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 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% sequence identity therewith over the entire length. Said functional homologue preferably comprises at the most 550 amino acids, such as at the most 527 amino acids and is capable of catalysing reduction of HMG CoA to form mevanolate.

Recombinant cells of the invention can also have been modulated to reduce activity of squalene synthase. Squalene synthase according to the invention is preferably an enzyme classified under EC 2.5.1.21. In particular, if the host cell is a yeast cell, then said yeast cell can have been modulated to reduced expression of the ERG-9 gene. This can for example be accomplished by placing the ORF encoding squalene synthase under the control of a weak promoter, such as any of the weak promoters described herein in the section "Promoter sequence". This can be accomplished, for example, by replacing the entire wild type gene encoding squalene synthase or by replacing the wild type promoter. Optionally, the cell is a recombinant cell that comprises a heterologous sequence that reduces expression of mRNA encoding squalene synthase. In particular embodiments, the heterologous nucleic acid insert sequence can be positioned between the promoter sequence and the ORF encoding squalene synthase. Said heterologous insert sequence can be any of the heterologous insert sequences described herein below in the section "Heterologous insert sequence".

The invention also provides methods and recombinant cells wherein squalene synthase activity is reduced with using a motif that de-stabilizes mRNA transcripts. Thus, recombinant cells of the present invention can comprise a nucleic acid comprising a promoter sequence operably linked to an open reading frame (ORF) encoding squalene synthase, and a nucleotide sequence comprising a motif that de-stabilizes mRNA transcripts. Said motif, can be any of the motif that de-stabilize mRNA transcripts described herein below in the section "Motif that de-stabilize mRNA transcripts".

Dual function enzyme

Recombinant cells according to the invention can also comprise a heterologous nucleic acid sequence encoding a dual function enzyme, wherein said dual function enzyme is an acetoacetyl-CoA thiolase and a HMG-CoA reductase. Similarly, recombinant eukaryotic cells of the invention can comprise a heterologous nucleic acid sequence encoding a dual function enzyme, wherein said dual function enzyme is an acetoacetyl-CoA thiolase and a HMG-CoA reductase.

Thus, a dual function enzyme according to the invention is preferably an enzyme, which is capable of catalysing both of the following reactions:

- i) $2 \text{ acetyl-CoA} \rightleftharpoons \text{CoA} + \text{acetoacetyl-CoA}$
- 5 ii) $(R)\text{-mevalonate} + \text{CoA} + 2 \text{ NADP}^+ \rightleftharpoons (S)\text{-3-hydroxy-3-methylglutaryl-CoA} + 2 \text{ NADPH} + 2 \text{ H}^+$

Enzymes capable of catalysing reaction i) are classified under E.C: 2.3.19, whereas enzymes capable of catalysing reaction ii) are classified under E.C. 1.1.1.34. Thus
10 preferred dual function enzymes to be used with the present invention can be classified either under E.C. 2.3.19 or under E.C. 1.1.1.34.

Said dual function enzyme can be derived from any useful source. In particular, the dual function enzyme can be of prokaryotic origin.

15

In a particular embodiment, the dual function enzyme is the enzyme encoded by *E. faecalis* gene *mvaE* or a functional homologue thereof. Thus the dual function enzyme can be the polypeptide of SEQ ID NO: 9 or a functional homologue thereof, wherein said functional homologue shares at least 70%, such as at least 75%, such as at least
20 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 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
25 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99% sequence identity SEQ ID NO: 9. The sequence identity is preferably determined as described herein.

In addition to the aforementioned sequence identity, a functional homologue of the enzyme encoding by *E. faecalis* gene *mvaE* should also be capable of catalysing
30 reactions i) and ii) outlined herein above in this section.

HMGS

Recombinant cells useful according to this invention can also comprise a heterologous
35 nucleic acid sequence encoding a 3-hydroxy-3-methyl-glutaryl coenzyme A synthase

(HMGS). Thus, the recombinant eukaryotic cells of the present invention can in preferred embodiment comprise a heterologous nucleic acid sequence encoding a 3-hydroxy-3-methyl-glutaryl coenzyme A synthase (HMGS).

- 5 The HMGS to be used with the present invention is preferably enzyme, which is capable of catalysing the following reaction:



- 10 In particular the HMGS to be used with the present invention can be any enzyme classified under E.C. 2.3.3.10.

Said HMGS can be derived from any useful source. In particular, the HMGS can be of prokaryotic origin.

15

- In one preferred embodiment the HMGS is the enzyme encoded by *E. faecalis* gene *mvaS* or a functional homologue thereof. Thus the HMGS can be the polypeptide of SEQ ID NO: 10 or a functional homologue thereof, wherein said functional homologue shares at least 70%, such as at least 75%, such as at least 76%, such as at least 77%,
 20 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 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%,
 25 such as at least 97%, such as at least 98%, such as at least 99% sequence identity SEQ ID NO: 10. The sequence identity is preferably determined as described herein.

- In addition to the aforementioned sequence identity, then a functional homologue of the enzyme encoding by *E. faecalis* gene *mvaS* should also be capable of catalysing
 30 above-mentioned reaction.

Methods for producing terpenes or terpenoids

As mentioned herein above, recombinant cells of this invention are useful in enhancing yield of isoprenoid pyrophosphates and/or terpenes and/or terpenoids.

35

Specific particular embodiments of the recombinant cells of the invention can therefore be genetically engineered in order to increase accumulation of isoprenoid pyrophosphate precursors and thus to increase yield of terpenoid or terpene products resulting from enzymatic conversion of said isoprenoids pyrophosphates.

5

Accordingly, in one aspect the present invention relates to a method of producing a terpene or a terpenoid, said method comprising the steps of cultivating a recombinant cell as described herein under conditions in which a terpene or terpenoid product is produced by the cell, and isolating said terpene or terpenoid.

10

In a particular example using a recombinant yeast cell embodiment, said cell having reduced activity of the ERG20 gene results in enhanced accumulation of IPP and DMAPP. DMAPP and IPP accumulation can be exploited for increased production of GPP, FPP and GGPP when combined with a heterologous GGP synthase, or heterologous FPP synthase or heterologous GGPP synthase.

15

Thus, in another aspect, the invention provides methods for producing isoprenoid pyrophosphate that include but are not limited to farnesyl-pyrophosphate (FPP), isopentenyl-pyrophosphate (IPP), dimethylallyl-pyrophosphate (DMAPP), geranyl-pyrophosphate (GPP) and/or geranylgeranyl-pyrophosphate (GGPP), by culturing a recombinant cell according to the invention under conditions where said isoprenoid pyrophosphates are produced and then isolating said isoprenoic pyrophosphate.

20

In certain additional or alternative embodiments, mevalonate accumulation is enhanced in a recombinant cell, e.g. a eukaryotic cell that comprises a heterologous nucleic acid sequence encoding a dual function enzyme, wherein said dual function enzyme is an acetoacetyl-CoA thiolase and a HMG-CoA reductase and optionally further comprising a heterologous nucleic acid sequence encoding a 3-hydroxy-3-methyl-glutaryl coenzyme A synthase (HMGS), accumulation of mevalonate. In further additional or alternative embodiments, compounds having mevalonate as a metabolic precursor also accumulate in said recombinant cells when mevalonate production, accumulation or both is enhanced as described herein. Such cells are advantageously employed for producing IPP and DMAPP, and for enhanced production of GPP, FPP and GGPP, when said recombinant cell comprises a heterologous GGP synthase, or heterologous FPP synthase or heterologous GGPP synthase.

25

30

35

Thus, it is also an aspect of the invention to provide methods for producing an isoprenoid pyrophosphate that is farnesyl-pyrophosphate (FPP), isopentenyl-pyrophosphate (IPP), dimethylallyl-pyrophosphate (DMAPP), geranyl-pyrophosphate (GPP) and/or geranylgeranyl-pyrophosphate (GGPP), by culturing said recombinant cell comprising a heterologous nucleic acid sequence encoding a dual function enzyme, wherein said dual function enzyme is an acetoacetyl-CoA thiolase and a HMG-CoA reductase and optionally further comprising a heterologous nucleic acid sequence encoding a 3-hydroxy-3-methyl-glutaryl coenzyme A synthase (HMGS), and optionally further comprising one or more of the additional heterologous nucleic acid sequences described herein above in the section "Additional heterologous nucleic acids", under conditions wherein said FPP, IPP, DMAPP, GPP or GGPP is produced, and then isolating said FPP, IPP, DMAPP, GPP or GGPP.

The invention provides methods and recombinant cells for producing terpenes or terpenoids, particularly having increased yields thereof. In certain embodiments the terpenoid or the terpene to be produced by the methods of the invention is a hemiterpenoid, monoterpene, sesquiterpenoid, diterpenoid, sesterpene, triterpenoid, tetraterpenoid or polyterpenoid.

More specifically, the terpenoid or terpene is farnesyl phosphate, farnesol, geranylgeranyl, geranylgeraniol, isoprene, prenol, isovaleric acid, geranyl pyrophosphate, eucalyptol, limonene, pinene, farnesyl pyrophosphate, artemisinin, bisabolol, geranylgeranyl pyrophosphate, retinol, retinal, phytol, taxol, forskolin, aphidicolin, lanosterol, lycopene or carotene.

Recombinant cells according to the invention useful for producing said terpenes and terpenoids have been genetically engineered to exhibit reduced farnesyl diphosphate production according to the methods set forth herein. In said embodiments, the phenotype of the recombinant cell includes decreasing turnover of IPP to FPP and/or of DMAPP to FPP. Recombinant cells according to the invention also exhibit a phenotype wherein FPP, IPP, DMAPP, GPP and GGPP accumulation is enhanced, by genetically engineering said cells as set forth herein. In certain additional embodiments, the invention provides recombinant cells useful in the disclosed inventive methods for producing and recovering FPP, IPP, DMAPP, GPP or GGPP from said cell, wherein

said recombinant cells are cultured under conditions wherein FPP, IPP, DMAPP, GPP and GGPP are produced by the cell, advantageously in enhanced yield.

5 In further embodiments, the recombinant cells further comprise, endogenously or as the result of introducing additional heterologous recombinant expression constructs, enzyme or enzymes comprising a metabolic pathway for producing terpenes or terpenoids according to the invention. In said embodiments, terpene or terpenoid production is enhanced as the result of reduced expression of FPP, GPP or an enzyme having both FPP and GPP synthase activities, or in addition or alternatively increased
10 accumulation of mevalonate precursors using recombinant cells and methods as set forth herein.

Alternatively, said IPP, FPP, GPP, DMAPP, or GGPP precursors can be recovered from said recombinant cells and used in further processes for producing the desired
15 terpenoid product compound. The further process can take place in the same cell culture as the process performed and defined herein above, such as the accumulation of the terpenoid precursors by the cell of the present invention. Alternatively, the recovered precursors can be added to another cell culture, or a cell free system, to produce the desired products.

20 As the isoprenoids pyrophosphates can serve as intermediates, endogenous production of terpenoids or terpenes can occur based on the isoprenoid pyrophosphates. Also, the recombinant cells of the invention can have additional genetic modifications such that they are capable of performing both the accumulation
25 of the isoprenoids pyrophosphates and whole or substantially the whole subsequent biosynthesis process to a desired terpenoid or terpene product.

Thus, in certain embodiments the method of the invention further comprises recovering a compound being biosynthesised from said IPP, FPP, DMAPP, GPP or GGPP
30 precursors in the recombinant cells provided by this invention.

In alternative embodiments, the invention provides methods and genetically engineered recombinant cells wherein production or accumulation of IPP, DMAPP or both is enhanced, comprising culturing recombinant cells of the invention wherein metabolic
35 activity farnesyl diphosphate synthase activity, geranyl diphosphate synthase activity

and/or the activity of an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity, is downregulated as set forth herein.

5 In additional or alternative embodiments, said recombinant cell comprises a heterologous nucleic acid sequence encoding a dual function enzyme as set forth herein, wherein said cell produced or accumulates or both enhanced metabolites in the mevalonate pathway, particular mevalonate, including *inter alia* expression of heterologous HMGS. In further additional or alternative embodiments, said recombinant cell is a yeast cell that is genetically engineered for reduced ERG9
10 expression or activity.

In additional specific embodiments, the invention provides methods and recombinant cells for producing GPP, particular wherein production, accumulation or both of GPP is enhanced, wherein GPP is obtained in advantageously greater yields by culturing a
15 recombinant cell that has been genetically engineered for reduced expression of farnesyl diphosphate synthase activity, geranyl diphosphate synthase activity and/or the activity of an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity, and wherein said recombinant cell further comprises a recombinant expression construct encoding a heterologous GPP synthase. In
20 additional or alternative embodiments, the recombinant cell comprises a heterologous nucleic acid sequence encoding a dual function enzyme as set forth herein, wherein said cell produced or accumulates or both enhanced metabolites in the mevalonate pathway, particular mevalonate, including *inter alia* expression of heterologous HMGS. In further additional or alternative embodiments, said recombinant cell is a yeast cell
25 that is genetically engineered for reduced ERG9 expression or activity.

In additional specific embodiments, the invention provides methods and recombinant cells for producing FPP, particular wherein production, accumulation or both of FPP is enhanced, wherein FPP is obtained in advantageously greater yields by culturing a
30 recombinant cell that has been genetically engineered for reduced expression of farnesyl diphosphate synthase activity, geranyl diphosphate synthase activity and/or the activity of an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity, and wherein said recombinant cell further comprises a recombinant expression construct encoding a heterologous FPP synthase. In
35 additional or alternative embodiments, the recombinant cell comprises a heterologous

nucleic acid sequence encoding a dual function enzyme as set forth herein, wherein said cell produced or accumulates or both enhanced metabolites in the mevalonate pathway, particular mevalonate, including *inter alia* expression of heterologous HMGS. In further additional or alternative embodiments, said recombinant cell is a yeast cell
5 that is genetically engineered for reduced ERG9 expression or activity.

In additional specific embodiments, the invention provides methods and recombinant cells for producing GGPP, particular wherein production, accumulation or both of GGPP is enhanced, wherein GGPP is obtained in advantageously greater yields by
10 culturing a recombinant cell that has been genetically engineered for reduced expression of farnesyl diphosphate synthase activity, geranyl diphosphate synthase activity and/or the activity of an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity, and wherein said recombinant cell further comprises a recombinant expression construct encoding a heterologous GGPP
15 synthase. In additional or alternative embodiments, the recombinant cell comprises a heterologous nucleic acid sequence encoding a dual function enzyme as set forth herein, wherein said cell produced or accumulates or both enhanced metabolites in the mevalonate pathway, particular mevalonate, including *inter alia* expression of heterologous HMGS. In further additional or alternative embodiments, said
20 recombinant cell is a yeast cell that is genetically engineered for reduced ERG9 expression or activity.

In additional specific embodiments, the invention provides methods and recombinant cells for producing isoprene, particular wherein production, accumulation or both of
25 isoprene is enhanced, wherein isoprene is obtained in advantageously greater yields by culturing a recombinant cell that has been genetically engineered for reduced expression of farnesyl diphosphate synthase activity, geranyl diphosphate synthase activity and/or the activity of an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity, and wherein said recombinant cell further
30 comprises a recombinant expression construct encoding a heterologous isoprene synthase. In additional or alternative embodiments, the recombinant cell comprises a heterologous nucleic acid sequence encoding a dual function enzyme as set forth herein, wherein said cell produced or accumulates or both enhanced metabolites in the mevalonate pathway, particular mevalonate, including *inter alia* expression of
35 heterologous HMGS. In further additional or alternative embodiments, said

recombinant cell is a yeast cell that is genetically engineered for reduced ERG9 expression or activity. In certain specific embodiments, said isoprene is isolated and further polymerized to produce isoprene rubber.

5 The invention specifically provides methods and recombinant cells for producing terpenes and terpenoids. In particular embodiments, the recombinant cells provide herein are used to produce a monoterpenoid, including but not limited to the monoterpenoids described herein in the section "Terpenoids and terpenes". As provided herein, said monoterpenoids are produced by culturing a recombinant cell that
10 has been genetically engineered for reduced expression of farnesyl diphosphate synthase activity, geranyl diphosphate synthase activity and/or the activity of an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity, and wherein said recombinant cell further comprises a recombinant expression construct encoding a heterologous GPP synthase and one or more additional
15 heterologous nucleic acids each encoding an enzyme of the biosynthetic pathway to produce said monoterpenoid from GPP, for example said heterologous nucleic acids can encode any of the monoterpenoid synthases described herein in the section "Additional heterologous nucleic acids. In additional or alternative embodiments, the recombinant cell comprises a heterologous nucleic acid sequence encoding a dual
20 function enzyme as set forth herein, wherein said cell produced or accumulates or both enhanced metabolites in the mevalonate pathway, particular mevalonate, including inter alia expression of heterologous HMGS. In further additional or alternative embodiments, said recombinant cell is a yeast cell that is genetically engineered for reduced ERG9 expression or activity. Exemplary monoterpenoids include but are not
25 limited to limonene, in which case said monoterpenoid synthase can be any of the limonene synthases described herein above in the section "Additional heterologous nucleic acid".

In additional particular embodiments, the recombinant cells provide herein are used to
30 produce sesquiterpenoids or triterpenoids, including but not limited to the sesquiterpenoids or triterpenoids described herein in the section "Terpenoids and terpenes". As provided herein, said sesquiterpenoids or triterpenoids are produced by culturing a recombinant cell that has been genetically engineered for reduced expression of farnesyl diphosphate synthase activity, geranyl diphosphate synthase
35 activity and/or the activity of an enzyme having both farnesyl diphosphate synthase and

geranyl diphosphate synthase activity, and wherein said recombinant cell further comprises a recombinant expression construct encoding a heterologous FPP synthase and one or more additional heterologous nucleic acids each encoding an enzyme of the biosynthetic pathway to produce said sesquiterpenoid or triterpenoid from FPP, for example said heterologous nucleic acids can encode any of the sesquiterpenoid or triterpenoid synthases described herein in the section "Additional heterologous nucleic acids. In additional or alternative embodiments, the recombinant cell comprises a heterologous nucleic acid sequence encoding a dual function enzyme as set forth herein, wherein said cell produced or accumulates or both enhanced metabolites in the mevalonate pathway, particular mevalonate, including inter alia expression of heterologous HMGS. In further additional or alternative embodiments, said recombinant cell is a yeast cell that is genetically engineered for reduced ERG9 expression or activity. Exemplary sesquiterpenoids include but are not limited to amorphadiene or artemisinin, in which case one sesquiterpenoid synthase can be amorphadiene synthase, such as any of the amorphadiene synthases described herein above in the section "Additional heterologous nucleic acids". Exemplary triterpenoids include but are not limited to cycloartenol, curcubitacin E, azadirachtin A, lupeol, beta-amyrin and saponins, in which case said triterpenoids synthase can be any of the EC 2.5.1.21 (squalene synthase) synthases described herein above in the section "Additional heterologous nucleic acid".

In additional particular embodiments, the recombinant cells provide herein are used to produce diterpenoids or tetraterpenoids, including but not limited to the diterpenoids or tetraterpenoids described herein in the section "Terpenoids and terpenes". As provided herein, said diterpenoids or tetraterpenoids are produced by culturing a recombinant cell that has been genetically engineered for reduced expression of farnesyl diphosphate synthase activity, geranyl diphosphate synthase activity and/or the activity of an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity, and wherein said recombinant cell further comprises a recombinant expression construct encoding a heterologous GPP synthase, a heterologous FPP synthase and one or more additional heterologous nucleic acids each encoding an enzyme of the biosynthetic pathway to produce said diterpenoid or tetraterpenoid from GPP, FPP and/or GPP synthase, for example said heterologous nucleic acids can encode any of the diterpenoid or tetraterpenoid synthases described herein in the section "Additional heterologous nucleic acids. In additional or alternative

embodiments, the recombinant cell comprises a heterologous nucleic acid sequence encoding a dual function enzyme as set forth herein, wherein said cell produced or accumulates or both enhanced metabolites in the mevalonate pathway, particular mevalonate, including inter alia expression of heterologous HMGS. In further
 5 additional or alternative embodiments, said recombinant cell is a yeast cell that is genetically engineered for reduced ERG9 expression or activity. Exemplary diterpenoids include but are not limited to casbene, taxadiene, abietadiene, paclitaxel, and incensole, in which case said diterpenoid synthase can be any GGPP synthase, described herein above in the section "Additional heterologous nucleic acids".

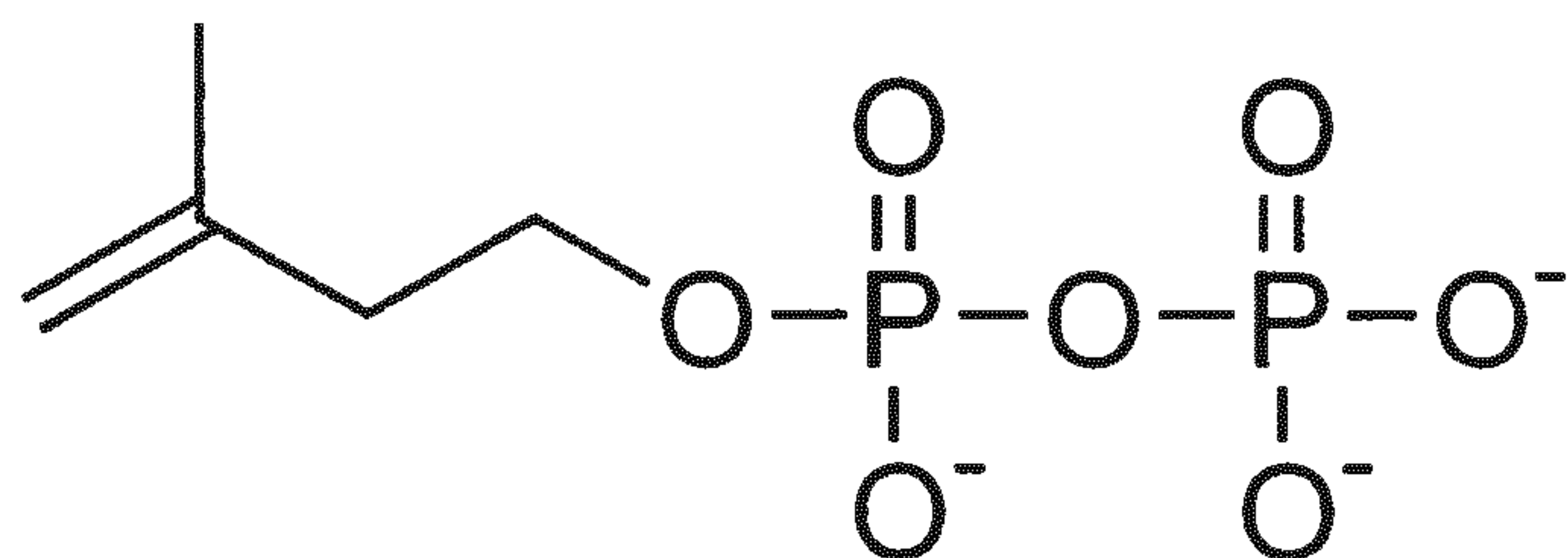
10 Exemplary tetraterpenoids include but are not limited to lutein, beta-caroten, zeaxanthin, astaxanthin, and apo-carotenoids like retinol, beta-ionone, abscissic acid and bixin, in which case said tetraterpenoid synthase can be any of the EC 2.5.1.32 synthases described herein above in the section "Additional heterologous nucleic acids".

15

Terpenoids and terpenes

The invention provides methods and recombinant cells for producing terpenoids, terpenes or isoprenoids (terpenoids are also commonly referred to as isoprenoids) using the recombinant cells of the invention characterised by reduced farnesyl
 20 diphosphate synthase activity, geranyl diphosphate synthase activity and/or the activity of an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity, wherein in particular embodiments the recombinant cell is a yeast cell expressing reduced ERG20 activity..

25 Terpenoids are classified according to the number of isoprene units (depicted below) used.



30 The classification thus comprises the following classes:

- Hemiterpenoids, 1 isoprene unit (5 carbons)

- Monoterpenoids, 2 isoprene units (10C)
- Sesquiterpenoids, 3 isoprene units (15C)
- Diterpenoids, 4 isoprene units (20C) (e.g. ginkgolides)
- Sesterterpenoids, 5 isoprene units (25C)
- 5 • Triterpenoids, 6 isoprene units (30C)
- Tetraterpenoids, 8 isoprene units (40C) (e.g. carotenoids)
- Polyterpenoid with a larger number of isoprene units.

Hemiterpenoids include isoprene, prenol and isovaleric acid.

10

Monoterpenoids include Geranyl pyrophosphate, Eucalyptol, Limonene and Pinene.

Sesquiterpenoids include Farnesyl pyrophosphate, amorphadiene, Artemisinin and Bisabolol.

15

Diterpenoids include Geranylgeranyl pyrophosphate, Retinol, Retinal, Phytol, Taxol, Forskolin and Aphidicolin. Another non-limiting example of a diterpene is ent-kaurene.

Triterpenoids include Squalene and Lanosterol.

20

Tetraterpenoids include Lycopene and Carotene and carotenoids.

Terpenes are hydrocarbons resulting from the combination of several isoprene units. Terpenoids can be thought of as terpene derivatives. The term terpene is sometimes used broadly to include the terpenoids. Just like terpenes, the terpenoids can be classified according to the number of isoprene units used. The present invention is focussed on terpenoids and in particular terpenoids derived from the isoprenoid pyrophosphates farnesyl-pyrophosphate (FPP), isopentenyl-pyrophosphate (IPP), dimethylallyl-pyrophosphate (DMAPP), geranyl-pyrophosphate (GPP) and/or geranylgeranyl-pyrophosphate (GGPP).

25

30

By terpenoids is understood terpenoids of the *Hemiterpenoid* class such as but not limited to isoprene, prenol and isovaleric acid; terpenoids of the *Monoterpenoid* class such as but not limited to geranyl pyrophosphate, eucalyptol, limonene and pinene; terpenoids of the *Sesquiterpenoids* class such as but not limited to farnesyl pyro-

35

phosphate, artemisinin and bisabolol; terpenoids of the *diterpenoid class* such as but not limited to geranylgeranyl pyrophosphate, retinol, retinal, phytol, taxol, forskolin and aphidicolin; terpenoids of the *Triterpenoid class* such as but not limited to lanosterol; terpenoids of the *Tetraterpenoid class* such as but not limited to lycopene and carotene.

The invention also relates to methods for production of other prenylated compounds. Thus the invention relates to methods for production of any compound, which has been prenylated to contain isoprenoid side-chains.

Table 1. Nucleic acid and amino acid sequences.

SEQ ID NO: 1	KEX2 promoter sequence
SEQ ID NO: 2	Example of heterologous insert sequence
SEQ ID NO: 3	CYC1 promoter sequence
SEQ ID NO: 4	Protein sequence of farnesyl diphosphate synthase (ERG20 gene) from <i>S. cerevisiae</i>
SEQ ID NO: 5	Protein sequence of DmaW from <i>Claviceps purpurea</i> (CpDmaW)
SEQ ID NO: 6	Protein sequence of FgaMT of <i>Aspergillus fumigatus</i>
SEQ ID NO: 7	Protein sequence of GGPPS of <i>S. acidocaldarius</i>
SEQ ID NO: 8	Protein sequence of HMGR1 (tHMGR1) from <i>S. cerevisiae</i> (YML075C).
SEQ ID NO: 9	Protein sequence of <i>E. faecalis</i> mvaE
SEQ ID NO: 10	Protein sequence of <i>E. faecalis</i> mvaS
SEQ ID NO: 11	<i>Artemisia annua</i> amorpho-4,11-diene synthase (ADS)
SEQ ID NO: 12	Protein sequence of GPPS2 from <i>Abies grandis</i> (AAN01134)
SEQ ID NO: 13	Protein sequence of LIMS1 from <i>Citrus limon</i> (Q8L5K3)
SEQ ID NO: 14	Protein sequence of FDPS (WH5701) from <i>Synechococcus</i>
SEQ ID NO: 17	Protein sequence of Ent-Kaurene synthase from <i>A. thaliana</i>
SEQ ID NO: 18	ent-Copalyl-diphosphate Synthase (CDPS) from <i>A. thaliana</i> (NP_192187)
SEQ ID NO: 19	ERG20 <i>S. cerevisiae</i> (NP_012368)
SEQ ID NO: 20	FPPS1 from <i>A. tridentata</i> (Q7XYS9)

SEQ ID NO: 21	FPPS2 from <i>A. tridentata</i> (Q7XYT0)
SEQ ID NO: 22	FPPS2 from <i>A. thaliana</i> (NP_974565)
SEQ ID NO: 23	GGPPS (BTS1) <i>S. cerevisiae</i> (NP_015256)
SEQ ID NO: 25	GGPPS <i>S. acidocaldarius</i> (YP_254812)
SEQ ID NO: 26	GPPS(IDS2)from <i>Picea abies</i> (ACA21458)
SEQ ID NO: 27	<i>S. cerevisiae</i> ERG9 gene for squalene synthetase (X59959.1)

EXAMPLES

5 Example 1

Substitution of the native ERG20 promoter with a weak KEX2 promoter

The wildtype ERG20 promoter region was replaced by a KEX2 promoter sequence by homologous recombination. A DNA fragment encompassing an ERG20 promoter upstream sequence (for homologous recombination), an expression cassette for the
10 gene (NatR) that confers resistance to nourseothricin, a KEX2 promoter, and an ERG20 ORF sequence (for homologous recombination) were generated by PCR. An overview of the PCR fragment and the homologous recombination is provided in figure 1A. The sequence of the KEX2 promoter is provided as SEQ ID NO: 1. The PCR DNA fragment was transformed in an *S. cerevisiae* host strain that subsequently was
15 selected on nourseothricin-containing growth plates. Clones with successful exchange of the native ERG20 promoter by the KEX2 promoter were identified. Such yeast strains are also referred to as KEX2-ERG20 strains herein.

20 **Substitution of the native ERG20 promoter with a CYC1 promoter and a short sequence that creates a stem-loop structure in 5'UTR of the ERG20 gene**

The wildtype ERG20 promoter region was replaced by the CYC1 promoter sequence and a heterologous 5'UTR sequence by homologous recombination. The 5'UTR region contains a sequence that folds up as a stem-loop structure, which partially blocks the 5'->3' directed ribosomal scanning for the AUG and thus reduces the translation of the
25 transcript. The sequence of the 5'UTR region is provided as SEQ ID NO: 2. A DNA fragment encompassing an ERG20 promoter upstream sequence (for homologous recombination), an expression cassette for the gene (NatR) that confers resistance to nourseothricin, a CYC1 promoter with a 5'UTR sequence containing a stem-loop structure sequence, and an ERG20 ORF sequence (for homologous recombination)

were generated by PCR. An overview of the PCR fragment and the homologous recombination is provided in Figure 1B and a detailed figure showing the 5'UTR region is provided in Figure 1C. The sequence of the CYC1 promoter is provided as SEQ ID NO:3. The DNA fragment was transformed in an *S. cerevisiae* host strain that subsequently was selected on nourseothricin-containing growth plates. Clones with successful exchange of the native ERG20 promoter by the CYC1 promoter with the stem-loop containing 5'UTR sequence were identified. Such yeast strains are also referred to as CYC1(5%)-ERG20 herein.

10

Example 2. Assessment of DMAPP accumulation

The first part of the Mevalonate pathway produces the isoprenoid pyrophosphates isopentenyl pyrophosphate/isopentenyl diphosphate (IPP) and dimethylallyl pyrophosphate/ dimethylallyl diphosphate (DMAPP). An overview of the pathway is provided in Figure 2. The isopentenyl-diphosphate delta isomerase 1 (IDI1) catalyzes the interconversion between IPP and DMAPP molecules and this ratio is normally 5:1 in *S. cerevisiae*. The present invention describes that accumulation of IPP and DMAPP creates a potential for making more geranyl pyrophosphate (GPP) (joining one DMAPP and one IPP), farnesyl pyrophosphate (FPP) (joining one DMAPP and two IPPs), and geranylgeranyl pyrophosphate (GGPP) (joining one DMAPP and three IPPs) when combined with expression of either a heterologous GPP synthase (GPPS), or a heterologous FPP synthase (FPPS) or a heterologous GGPP synthase (GGPPS).

20

The two first steps of the biosynthetic pathway for the ergot alkaloid chanoclavine can be catalyzed by the two enzymes, *Claviceps purpurea* CpDmaW (SEQ ID NO: 5) and *Apergillus fumigatus* FgaMT (SEQ ID NO: 6) that both are active in *S. cerevisiae*. The first enzyme, CpDmaW catalyses the joining of a Tryptophan and a DMAPP molecule to produce DMAT, and the second enzyme, FgaMT catalyses the subsequent methylation step that leads to Me-DMAT (see Figure 3).

25

30

Measurements of DMAT and/or Me-DMAT were used to indirectly assess the accumulation of DMAPP in yeast strains that had a wild type ERG20 gene, or the KEX2 promoter in front of the ERG20 ORF or has the CYC1 promoter with stem-loop structure in the heterologous 5'UTR in front of the ERG20 ORF. The CpDmaW and FgaMT genes were cloned on a multicopy double expression plasmid (2 μ) with

35

CpDmaW under the control of the TEF1 promoter and the FgaMT under the control of the PGK1 promoter. This plasmid was transformed in wild type and the ERG20 engineered *S. cerevisiae* strains.

5 Yeast cultures were grown at 30°C overnight and then used to inoculate 250 ml culture flasks containing 25ml synthetic complete (SC) 2% medium at an OD600 of 0.1. The main cultures were grown for 72 hours at 30°C. The yeast culture supernatant was extracted with ethyl acetate and the extract used for quantification of DMAT and Met-DMAT by LC-MS.

10

The CYC1(5%)-ERG20 and the KEX2-ERG20 strain showed approximately 2-fold and 3-fold increase of DMAT accumulation after 72 hours compared to the unmodified control (see Figure 4A). This represents an approximately 2-fold and 3-fold boosting of DMAPP levels. In all likelihood, this also reflects a similar accumulation of IPP since
15 the isopentenyl-diphosphate delta isomerase 1 (IDI1) catalyses both the forward and reverse reaction between IPP and DMAPP.

20

The CYC1(5%)-ERG20 and the KEX2-ERG20 strain showed approximately 2-fold and 2.5-fold increase of Me-DMAT accumulation after 72 hours compared to the unmodified control strain (see Figure 4B). This represents an approximately 2-fold and 2.5-fold boosting of DMAPP, and probably also a similar accumulation of IPP. The amount of DMAT and Me-DMAT was calculated per OD₆₀₀, thus providing an indication of the production per cell.

25

These measurements demonstrate that the DMAPP level can be increased several fold by exchanging the native ERG20 promoter for either a weak KEX2 promoter or a CYC1 promoter that introduces a stem-loop structure in the 5'UTR of the ERG20 transcript. The DMAPP and IPP accumulation can be exploited for increased production of GPP, FPP and GGPP when combined with a heterologous GPP synthase, or heterologous
30 FPP synthase or heterologous GGPP synthase.

Example 3. Production of GPP

35

GPP production was indirectly determined by determining the level of Limonene in yeast strains expressing Limonene synthase 1. Limonene synthase 1 catalyses

generation of Limonene from GPP and thus the level of limonene can in such yeast strains be used as an indirect measure of the level of GPP.

The yeast strains used in this example was the following:

5 A nucleic acid encoding truncated GPP synthase 2 from *Abies grandis* (derived from GPPS2 *Abies grandis*; SEQ ID NO: 12) under the control of the TEF1 promoter and a nucleic acid encoding truncated Limonene synthase 1 from *Citrus limon* (derived from LIMS1 *Citrus limon*; SEQ ID NO: 13) under the control of the PGK1p promoter were cloned on a single copy vector (ARS-CEN). The truncated GPPS2 sequence is
10 derived from GPPS2 of *Abies grandis* (coded by GenBank accession number AF513112) from which amino acids 2-86 have been deleted to make the truncated tGPPS2. The truncated LIMS1 sequence is derived from LIMS from *Citrus limon* (coded by GenBank accession number Q8L5K3), from which amino acids 2-52 have been deleted to make the truncated tLIMS1

15

This plasmid was transformed into wild type *S. cerevisiae* (referred to as "WT+tGPPS+tLIMS") as well as into the KEX2-ERG20 *S. cerevisiae* strain prepared as described in Example 1 (referred to as "KEX2-ERG20+tGPPS+tLIMS") and into the CYC1(5%)-ERG20 *S. cerevisiae* strain prepared as described in Example 1 (referred to
20 as "CYC1(5%)-ERG20+tGPPS+tLIMS").

Yeasts cultures were grown at 30°C overnight and then used to inoculate 250 ml culture flasks containing 25ml SC 2% medium at an OD₆₀₀ of 0.1 supplemented with 10% Isopropyl myristate. The main cultures were grown for 72 hours at 30°C. The
25 modified strain grows well and to an OD₆₀₀ greater than 10 (see Figure 8). The limonene accumulated in the isopropyl myristate was quantified by GC-MS. The amount of limonene was calculated per OD₆₀₀, thus providing an indication of the production of limonene per cell.

30 The KEX2-ERG20+tGPPS+tLIMS strain showed a surprising 80-100 fold increase of the limonene levels compared to the WT+tGPPS+tLIMS strain as shown in Figure 5, which indicates a similar level of boosting the GPP level.

Boosting of the GPP levels was also obtained in CYC1(5%)-ERG20+tGPPS+tLIMS,
35 however to a lower level than in KEX2-ERG20+tGPPS+tLIMS.

Example 4

Native *E. faecalis mvaE* and *mvaS* sequences were synthesized and cloned as two independent expression cassettes under the control of the constitutive *PGK1* and *TEF1* promoters, respectively, on a single copy vector (ARS-CEN) to produce the *mvaE/mvaS* plasmid. The native *E. faecalis mvaE* encodes a polypeptide of SEQ ID NO: 9 and the native *E. faecalis mvaS* encodes a polypeptide of SEQ ID NO: 10. A nucleic acid encoding a truncated version of *S. cerevisiae HMGR1 (tHMGR)*; derived from SEQ ID NO: 8) was PCR amplified from *S. cerevisiae* genomic DNA and cloned as an expression cassette under the control of the constitutive *GPD1* promoter on a single copy vector (ARS-CEN) to produce the *tHMGR* plasmid. A yeast codon optimized *Artemisia annua* amorpho-4,11-diene synthase gene encoding the polypeptide of SEQ ID NO: 11 was synthesized and cloned as an expression cassette under the control of the constitutive *PGK1* promoter on a multi copy vector (2 μ) to produce the *ADS* plasmid. The *ADS* plasmid was transformed in yeast *S. cerevisiae* with either *mvaE/mvaS*, *HMGR*, or an empty control plasmid. The yeast strain that was used for the experiment has an *ERG9* gene that is translationally downregulated by a stem-loop structure in the 5'UTR.

Two ml yeast starter-cultures were grown at 30°C overnight and used to inoculate 25 ml SC 2% glucose medium containing 10% dodecane in a 250 ml shake flask. Dodecane acts as a trapping agent for amorpho-4,11-diene. The cultures were grown for 72 hours at 30°C. The dodecane was separated from the yeast cells and culture supernatant by centrifugation and used directly for analysis in a gas chromatography–mass spectrometry system (GC-MS) to assess amorpho-4,11-diene production. To measure mevalonate levels, a small fraction of the yeast culture was treated with 2M HCl to convert mevalonate to mevanololactone. Next, the sample was extracted with ethylacetate followed by GC-MS analysis. The results are shown in Figure 6.

mvaS* can rescue a defective mevalonate pathway in *S. cerevisiae

Deletion of *ERG13* in *S. cerevisiae* leads to a defective mevalonate pathway. A Δ *ERG13* strain was produced by replacing the *ERG13* gene with an expression cassette for the *NatR* gene that confers resistance to nourseothricin by homologous recombination. The deletion strain can only grow if the growth media is supplemented with mevalonate (10 mg/ml mevalonate). After transformation of the deletion strain

with the *mvaE/mvaS* plasmid, the strain can grow without mevalonate supplement in the growth media, which demonstrates that the *mvaS* can functionally rescue the ERG13 deletion in *S. cerevisiae*. The results are shown in Figure 7.

5

Example 5. Production of GGPP

GGPP production was indirectly determined by determining the level of ent-kaurene in yeast strains expressing FPPS, GGPPS, ent-Copalyl-diphosphate synthase (CDPS) and ent-Kaurene synthase (KS). ent-Copalyl-diphosphate synthase from *A. thaliana* was used (CDPS) the sequence of which is provided as (SEQ ID NO: 18). The ent-kaurene synthase of *A. thaliana* was used the sequence of which is provided as (SEQ ID NO: 17). ent-Copalyl-diphosphate synthase catalyses formation of ent-copalyl-PP from GGPP and ent-Kaurene synthase catalyses formation of ent-kaurene from ent-copalyl-pp. Thus, the level of ent-Kaurene can in such yeast strains be used as an indirect measure of the level of GGPP.

The yeast strains used in this example were the following:
A nucleic acid encoding truncated GPP synthase 2 from *S. cerevisiae* (BTS1; SEQ ID NO:23) under the control of the TEF1 promoter and a nucleic acid encoding FPP synthase from *Synechococcus* (SEQ ID NO:14) and a nucleic acid encoding CDPS from *A. thaliana* of SEQ ID NO: 18 under the control of the PGK1 promoter and KS of SEQ ID NO: 17 under the control of the TEF1 promoter were transformed into wild type *S. cerevisiae* (referred to as "WT+FPPS+BTS1+CDPS+KS ") as well as into the KEX2-ERG20 *S. cerevisiae* strain prepared as described in Example 1 (referred to as "KEX2-ERG20+FPPS+BTS1+CDPS+KS").

The presence of ent-kaurene was analysed in a gas chromatography–mass spectrometry system (GC-MS). The results are shown in Figure 9.

30

35

References

Hedl *et al.*, J Bacteriol. **2002** Apr;184(8):2116-22.

Suterlin *et al.*, J Bacteriol. **2002** Aug;184(15):4065-70.

Burg *et al.*, Prog Lipid Res. **2011** Oct;50(4):403-10. Epub **2011** Jul 23.

5 Shalgi *et al.*, Genome Biology. **2005** Sep; 6(10): Article R86.

Degenhardt *et al.*, Phytochemistry **2009** 70:1621-1637.

Wang & Dixon, Proc. Natl. Acad. Sci. USA **2009** 106(24):9914-19.

Bonitz *et al.*, PLOS One **2011** 6(11):E27336 pages 1-8.

Rico *et al.*, *Appl Environ Microbiol.* **2010** Oct;76(19):6449-54.

Claims

1. A method for producing a terpene or terpenoid in a recombinant host cell, the method comprising the steps of culturing under conditions wherein the terpene or terpenoid is produced in a genetically engineered cell having reduced expression of endogenous farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity, and further comprising one or more recombinant expression constructs encoding heterologous enzymes for producing said terpene or terpenoid.
2. The method of claim 1, wherein the cell is genetically engineered to reduce expression of farnesyl diphosphate synthase.
3. The method of claim 1, wherein the cell is genetically engineered to reduce expression of geranyl diphosphate synthase.
4. The method of claim 1, wherein the cell is genetically engineered to reduce expression of an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity.
5. The method of any of claims 1 through 4, wherein reduced expression of endogenous farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is produced in the cell by introducing into the cell a recombinant genetic construct wherein nucleic acid encoding farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is operably linked in the construct to a promoter sequence that directs expression of said farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity at levels that are less than the levels of the promoter for the endogenous gene encoding farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity.
6. The method of any of claims 1 through 4, wherein reduced expression of endogenous farnesyl diphosphate synthase, geranyl diphosphate synthase or an

enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is produced in the cell by introducing into the cell a recombinant genetic construct wherein nucleic acid encoding farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is operably linked in the construct to a promoter sequence that directs expression of said farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity, wherein between said promoter and nucleic acid sequences encoding farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is a heterologous insert sequence having the formula:



wherein X_2 comprises at least 4 consecutive nucleotides being complementary to, and forming a hairpin secondary structure element with at least 4 consecutive nucleotides of X_4 , and

wherein X_3 either comprises zero nucleotides or one or more unpaired nucleotides forming a hairpin loop between X_2 and X_4 , and

X_4 comprises at least 4 consecutive nucleotides being complementary to, and forming a hairpin secondary structure element with at least 4 consecutive nucleotides of X_2 ; and wherein X_1 and X_5 comprises zero, one or more nucleotides.

7. The method of any of claims 1 through 4, wherein reduced expression of endogenous farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is produced in the cell by introducing into the cell a recombinant genetic construct wherein nucleic acid farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is operably linked in the construct to a messenger RNA destabilizing motif.

8. The method of any of claims 1 through 4, wherein reduced expression of endogenous farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is produced in the cell by inactivating the endogenous farnesyl diphosphate

synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity in the cell, and introducing into the cell a heterologous genetic construct encoding farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity regulated by a weak promoter.

9. The method of any of claims 1 through 8, further comprising introducing into the cell a recombinant expression construct encoding a truncated version of 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMGR) comprising the catalytically active carboxyl terminal portion thereof.

10. The method of any of claims 1 through 9 further comprising a heterologous nucleic acid sequence encoding a dual function enzyme, wherein said dual function enzyme is an acetoacetyl-CoA thiolase and a HMG-CoA reductase.

11. The method of claim 10 wherein the dual function enzyme is an *mvaE* gene encoded by *E. faecalis* or a functional homologue thereof

12. The method of any of claims 1 through 11, wherein the host cell is a eukaryotic cell or a prokaryotic cell.

13. The method of any of claims 1 through 12, wherein the host cell is a eukaryotic cell is a mammalian cell, a plant cell, a fungal cell or a yeast cell.

14. The method of claim 13, wherein the eukaryotic cell is a yeast cell.

15. The method of claim 14, wherein the yeast cell is a yeast of species *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Yarrowia lipolytica*, *Candida glabrata*, *Candida boidinii*, *Hansenula polymorpha*, *Kluyveromyces lactis*, *Pichia pastoris*, *Ashbya gossypii*, *Arxula adeninivorans*, *Cyberlindnera jadinii*, or *Candida albicans*.

16. The method of claim 15, wherein the yeast cell is a *Saccharomyces cerevisiae* cell.

17. The method of claim 2, wherein the farnesyl diphosphate synthase is ERG20.
- 5 18. The method of claim 10, wherein the heterologous nucleic acid sequence encodes a truncated version of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR).
- 10 19. The method of any of claims 1 through 18 wherein the terpene or terpenoid is a monoterpene, a diterpene, a sesquiterpene, a triperpenoid or a tetraterpenoid.
- 15 20. The method of any of claims 1 through 19 wherein the terpene or terpenoid is a molecule comprising an isoprene moiety.
21. The method of claim 20 wherein the monoterpene is pinenes, myrcene or geraniol.
- 20 22. The method of claim 19 wherein the diterpene is geranylgeranyl pyrophosphate, retinol, retinal, phytol, taxol, forskolin or aphidicolin.
23. The method of claim 19 wherein the sesquiterpene is amorphadiene, patchoulol, santalol, longifolene or thujopsene.
- 25 24. The method of claim 19 wherein the triterpenoid is squalene.
25. The method of claim 19 wherein the tetraterpenoid is carotenoid.
- 30 26. A recombinant cell for producing a terpene or terpenoid genetically engineered to have reduced expression of endogenous farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity, and further comprising one or more recombinant expression constructs encoding heterologous enzymes for producing said terpene or terpenoid.

27. The recombinant cell of claim 26, wherein the cell is genetically engineered to reduce expression of farnesyl diphosphate synthase.

28. The recombinant cell of claim 26, wherein the cell is genetically engineered to reduce expression of geranyl diphosphate synthase.

29. The recombinant cell of claim 26, wherein the cell is genetically engineered to reduce expression of an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity.

10

30. The recombinant cell of any of claims 26 through 29, wherein reduced expression of endogenous farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is produced in the cell by introducing into the cell a recombinant genetic construct wherein nucleic acid encoding farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is operably linked in the construct to a promoter sequence that directs expression of said farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity at levels that are less than the levels of the promoter for the endogenous gene encoding farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity.

15

20

31. The recombinant cell of any of claims 26 through 29, wherein reduced expression of endogenous farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is produced in the cell by introducing into the cell a recombinant genetic construct wherein nucleic acid encoding farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is operably linked in the construct to a promoter sequence that directs expression of said farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity, wherein between said promoter and nucleic acid sequences encoding farnesyl diphosphate

25

30

35

synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is a heterologous insert sequence having the formula:

5 $-X_1-X_2-X_3-X_4-X_5-$

wherein X_2 comprises at least 4 consecutive nucleotides being complementary to, and forming a hairpin secondary structure element with at least 4 consecutive nucleotides of X_4 , and

wherein X_3 either comprises zero nucleotides or one or more unpaired nucleotides
10 forming a hairpin loop between X_2 and X_4 , and
 X_4 comprises at least 4 consecutive nucleotides being complementary to, and forming a hairpin secondary structure element with at least 4 consecutive nucleotides of X_2 ; and
wherein X_1 and X_5 comprises zero, one or more nucleotides.

15 32. The recombinant cell of any of claims 26 through 29, wherein reduced expression of endogenous farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is produced in the cell by introducing into the cell a recombinant genetic construct wherein nucleic acid farnesyl diphosphate synthase,
20 geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is operably linked in the construct to a messenger RNA destabilizing motif.

25 33. The recombinant cell of any of claims 26 through 29, wherein reduced expression of endogenous farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity is produced in the cell by inactivating the endogenous farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity in the
30 cell, and introducing into the cell a heterologous genetic construct encoding farnesyl diphosphate synthase, geranyl diphosphate synthase or an enzyme having both farnesyl diphosphate synthase and geranyl diphosphate synthase activity regulated by a weak promoter.

34. The recombinant cell of any of claims 26 through 33, further comprising introducing into the cell a recombinant expression construct encoding a truncated version of 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMGR) comprising the catalytically active carboxyl terminal portion thereof.

5

35. The recombinant cell of any of claims 26 through 34 further comprising a heterologous nucleic acid sequence encoding a dual function enzyme, wherein said dual function enzyme is an acetoacetyl-CoA thiolase and a HMG-CoA reductase.

10

36. The recombinant cell of any of claims 26 through 35, wherein the host cell is a eukaryotic cell or a prokaryotic cell.

37. The recombinant cell of any of claims 26 through 36, wherein the host cell is a eukaryotic cell that is a mammalian cell, a plant cell, a fungal cell or a yeast cell.

15

38. The recombinant cell of claim 37, wherein the eukaryotic cell is a yeast cell.

20

39. The recombinant cell of claim 38, wherein the yeast cell is from a yeast species that is *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Yarrowia lipolytica*, *Candida glabrata*, *Candida boidinii*, *Hansenula polymorpha*, *Kluyveromyces lactis*, *Pichia pastoris*, *Ashbya gossypii*, *Arxula adeninivorans*, *Cyberlindnera jadinii*, or *Candida albicans*.

25

40. The recombinant cell of claim 39, wherein the yeast cell is a *Saccharomyces cerevisiae* cell.

41. The recombinant cell of claim 27, wherein the farnesyl diphosphate synthase is yeast ERG20.

30

42. The recombinant cell of claim 35, wherein the heterologous nucleic acid sequence encodes a truncated version of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR).

43. The recombinant of any of claims 26 through 42 wherein the terpene or terpenoid is a monoterpene, a diterpene, a sesquiterpene, a triperpenoid or a tetraterpenoid.

5 44. The recombinant of any of claims 26 through 43 wherein the terpene or terpenoid is a molecule comprising an isoprene moiety.

45. The recombinant of claim 43 wherein the monoterpene is pinenes, myrcene or geraniol.

10

46. The recombinant of claim 43 wherein the diterpene is geranylgeranyl pyrophosphate, retinol, retinal, phytol, taxol, forskolin or aphidicolin.

15

47. The recombinant of claim 43 wherein the sesquiterpene is amorphadiene, patchoulol, santalol, longifolene or thujopsene.

48. The recombinant of claim 43 wherein the triterpenoid is squalene.

20

49. The recombinant of claim 43 wherein the tetraterpenoid is carotenoid.

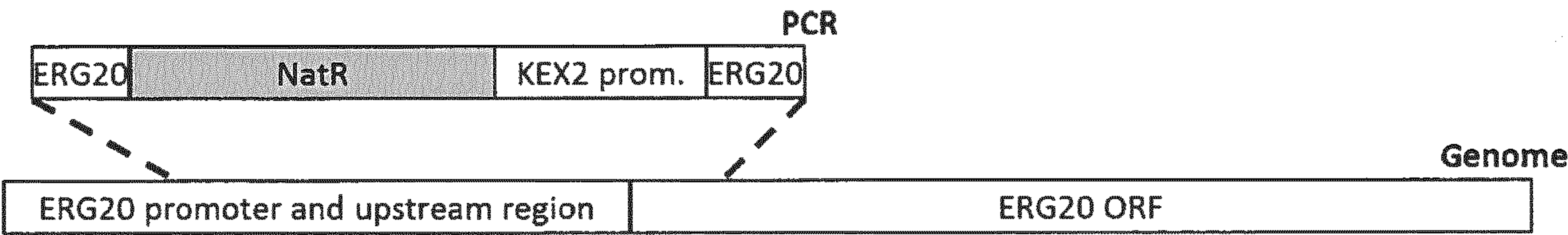


Fig. 1A

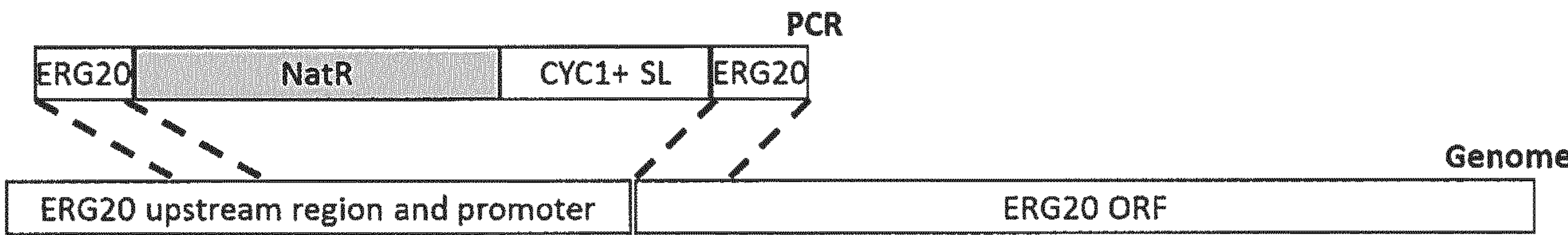


Fig. 1B

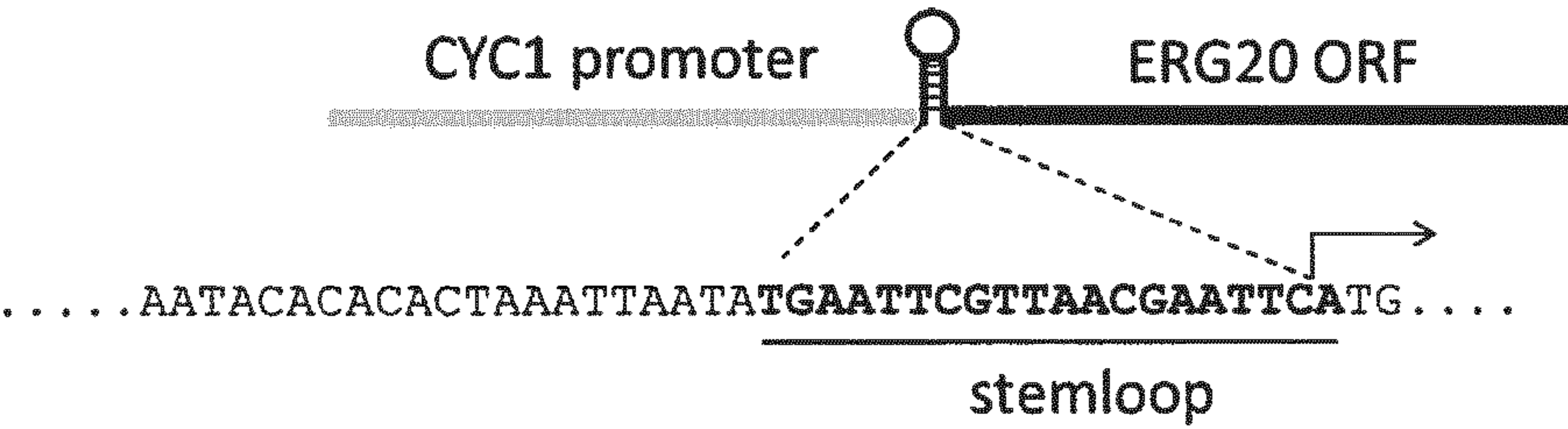
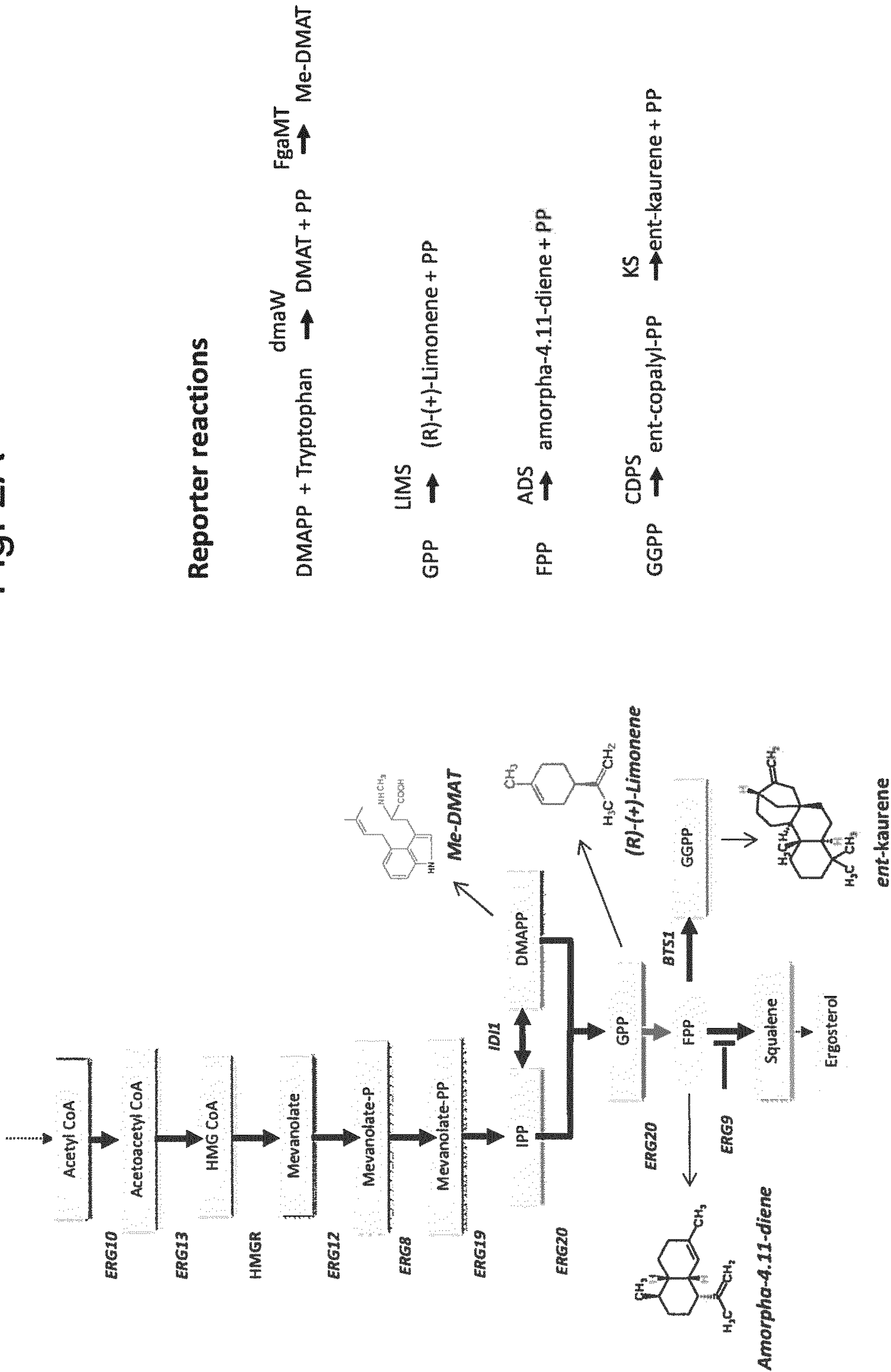


Fig. 1C

Fig. 2A



3/10

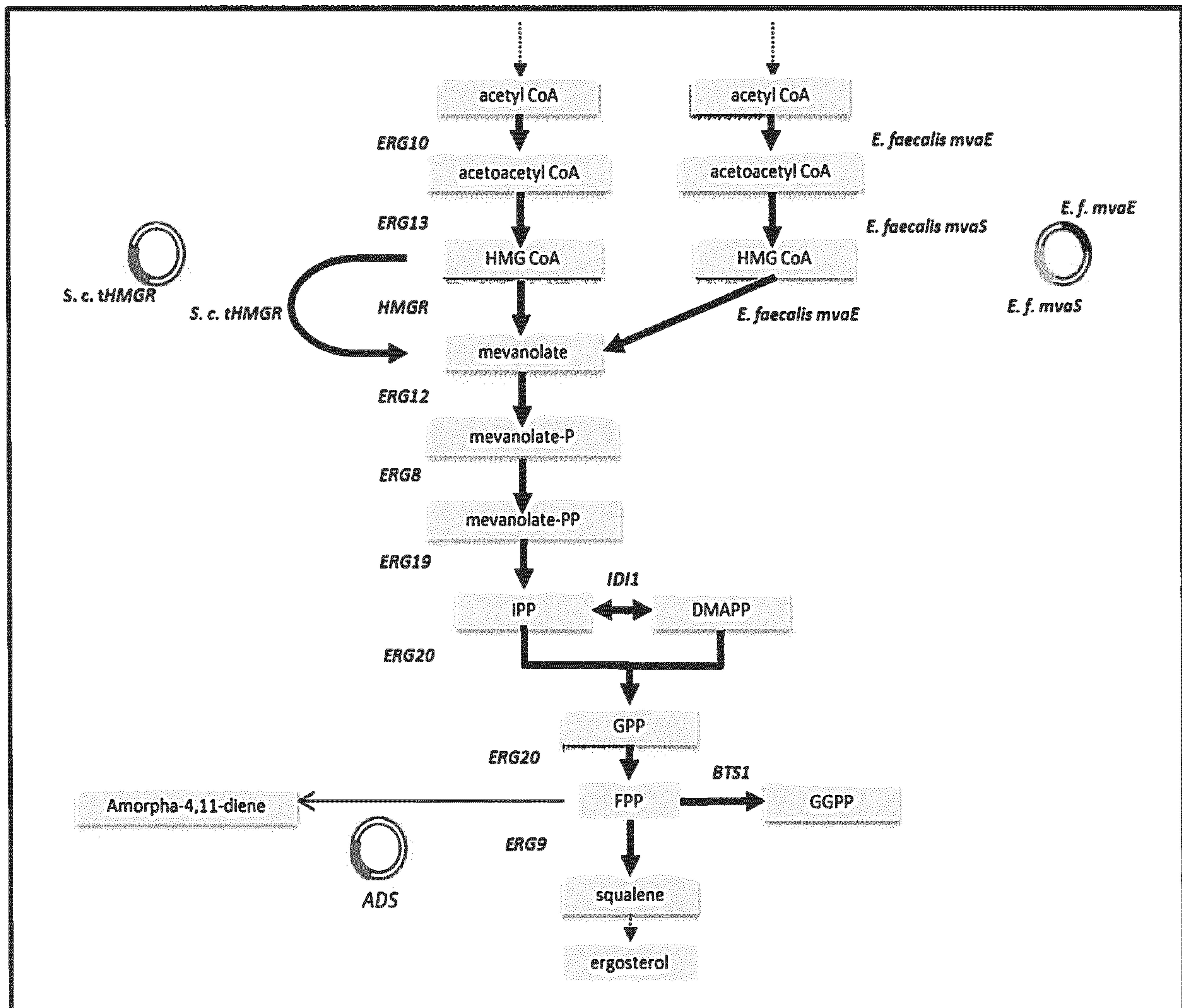


Fig. 2B

4/10

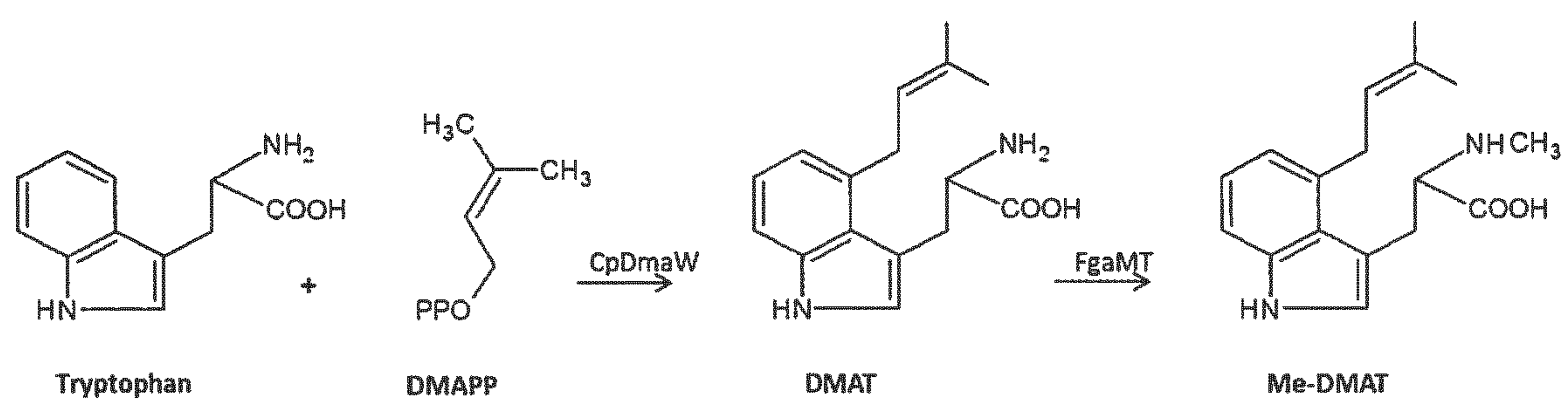


Fig. 3

5/10

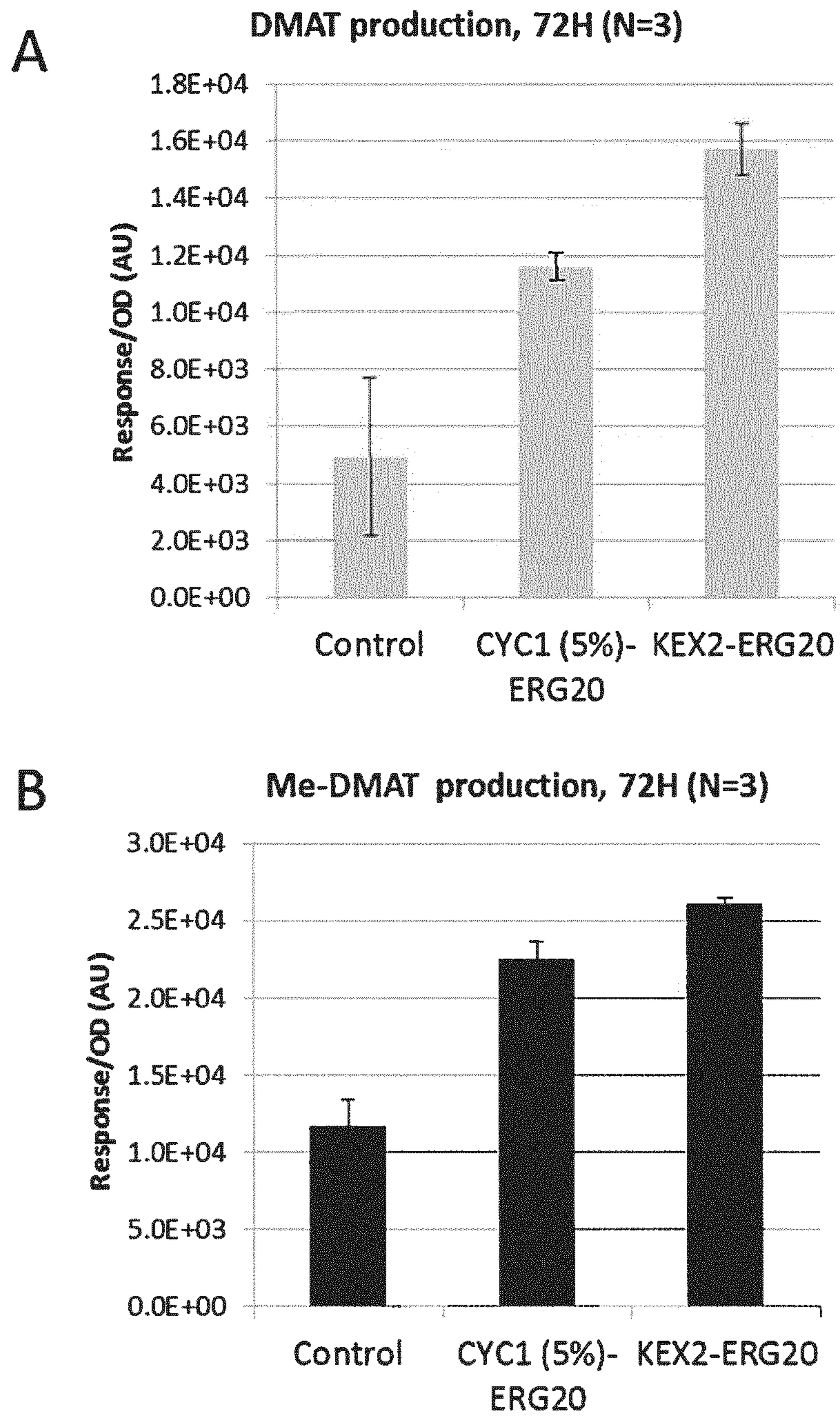


Fig. 4

6/10

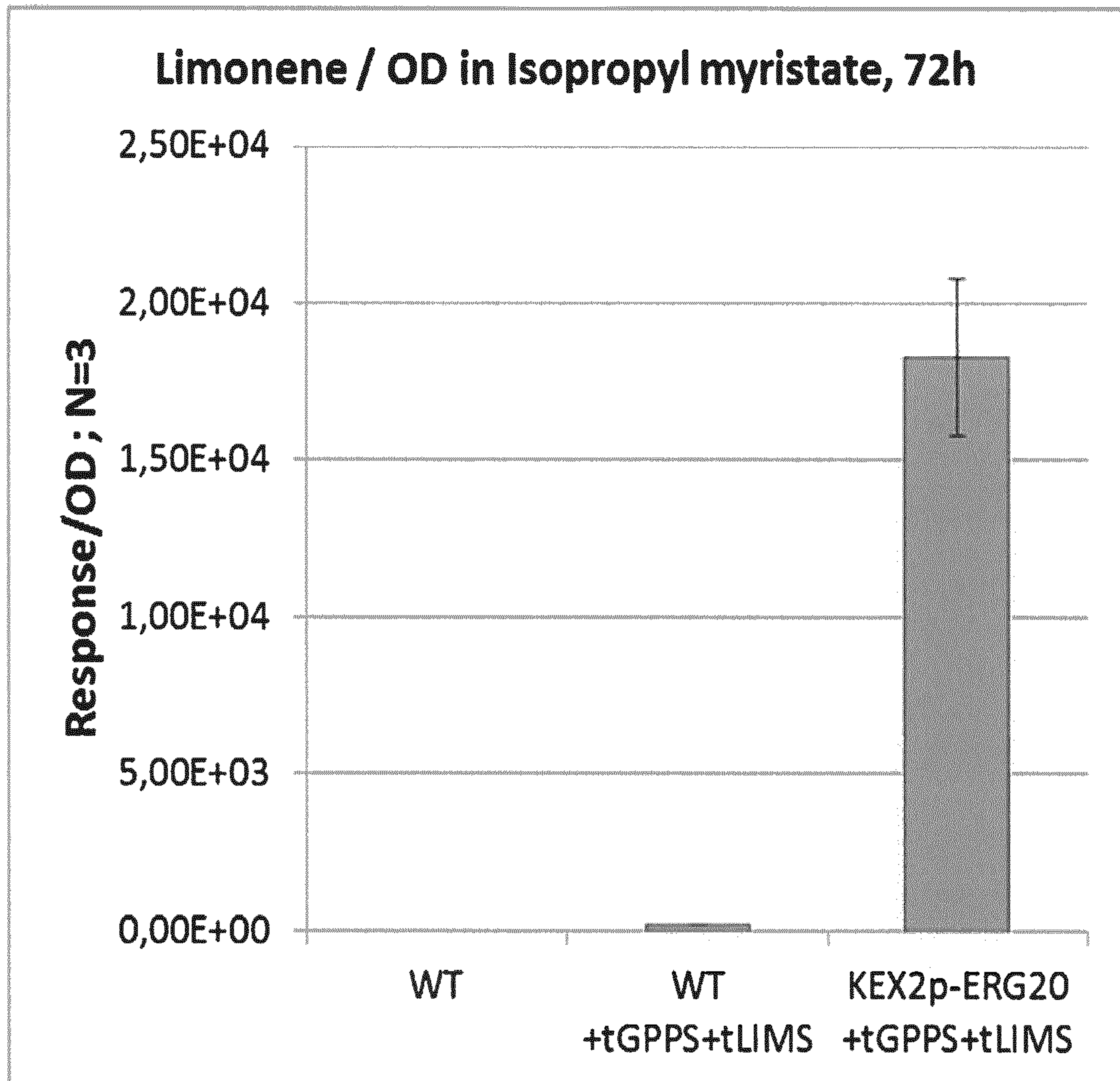


Fig. 5

7/10

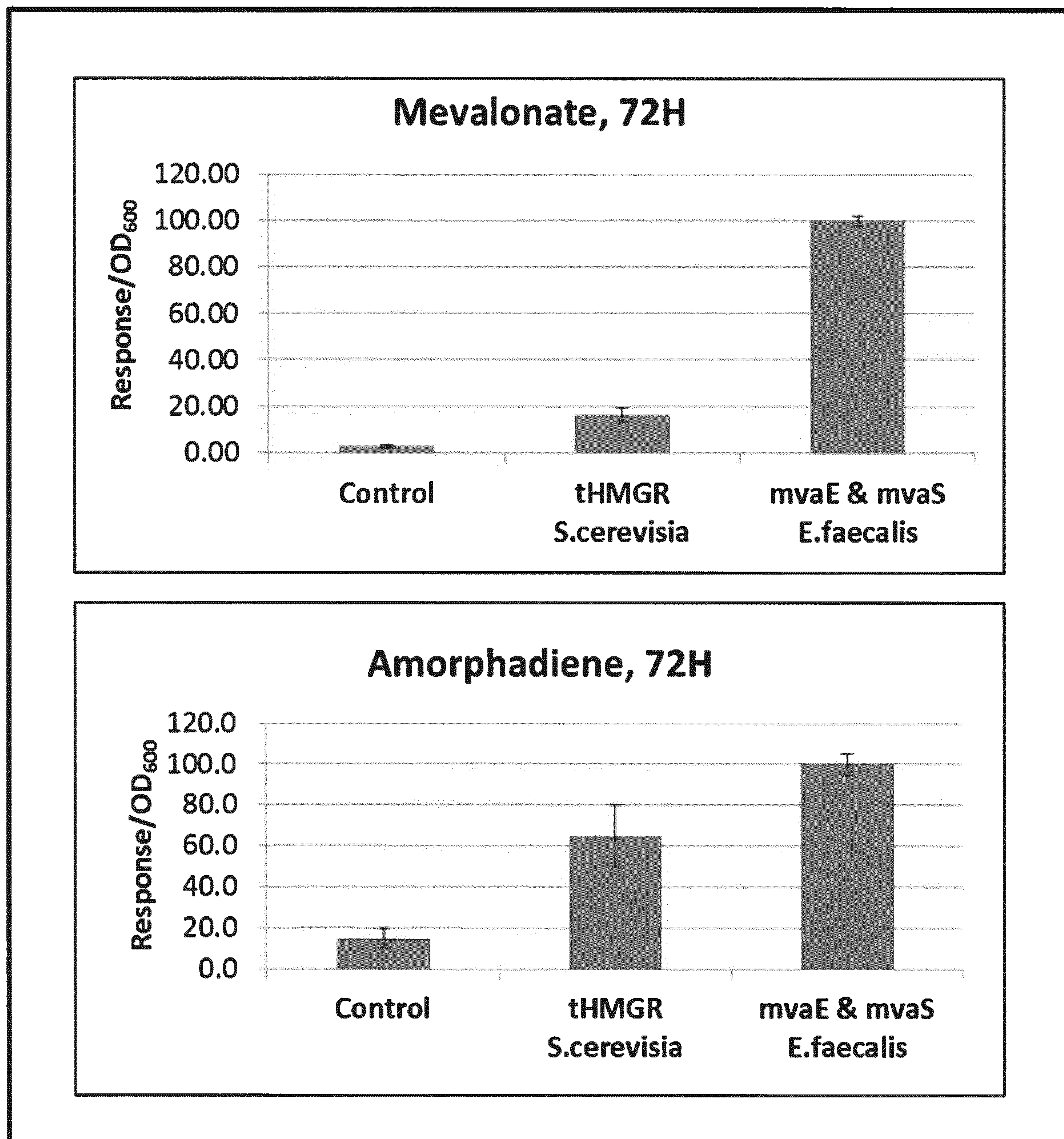


Fig. 6

8/10

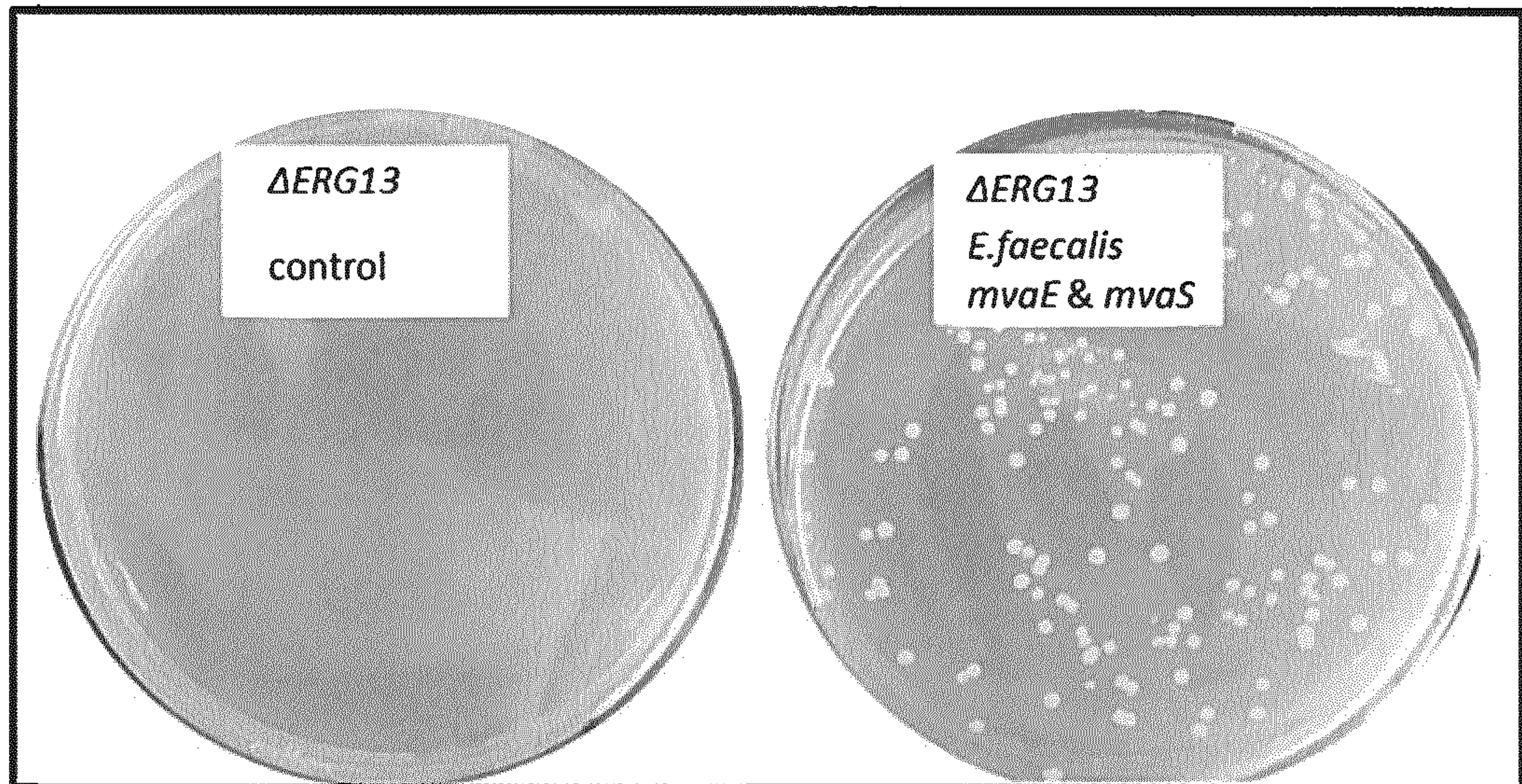


Fig. 7

9/10

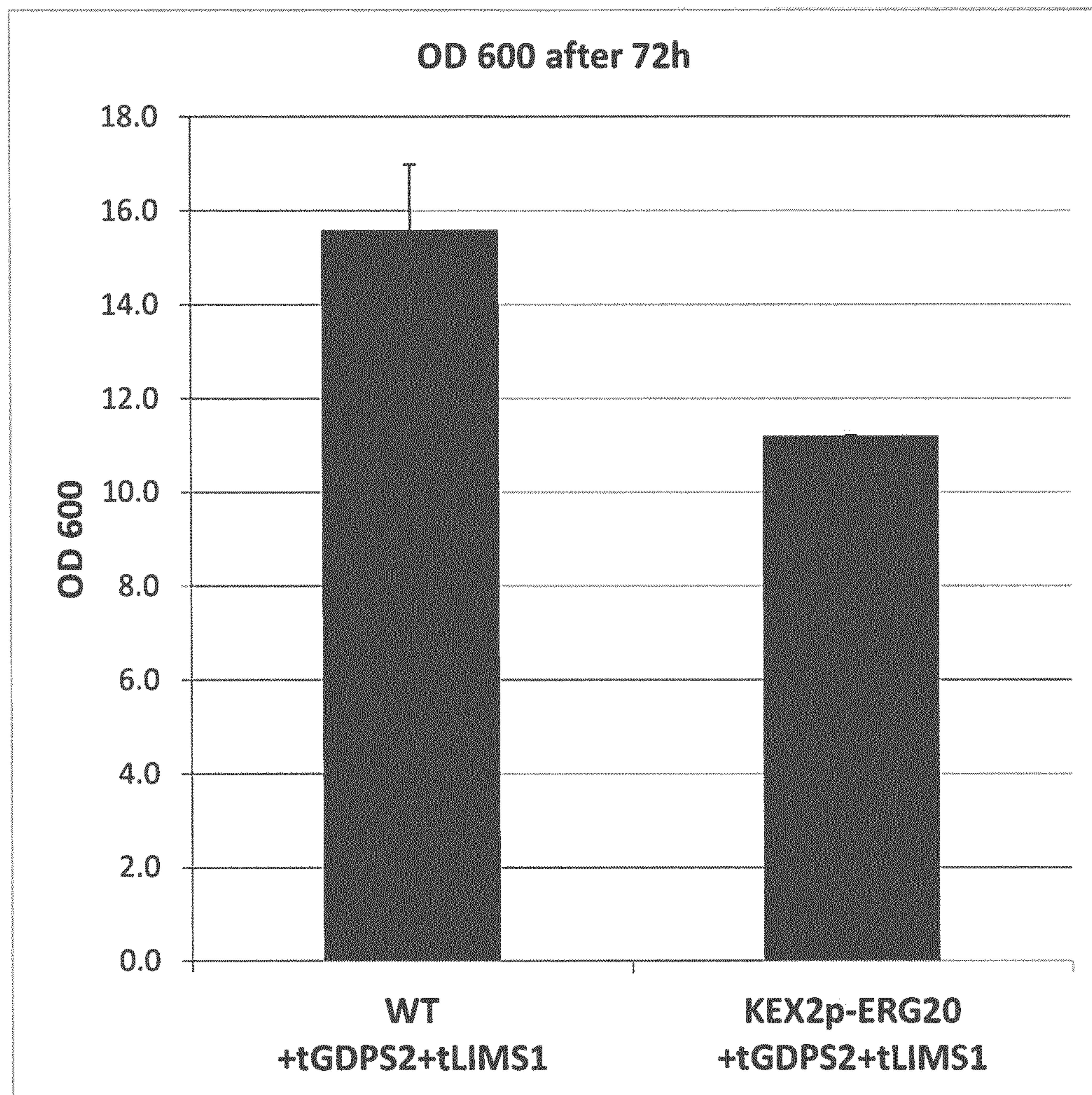


Fig. 8

10/10

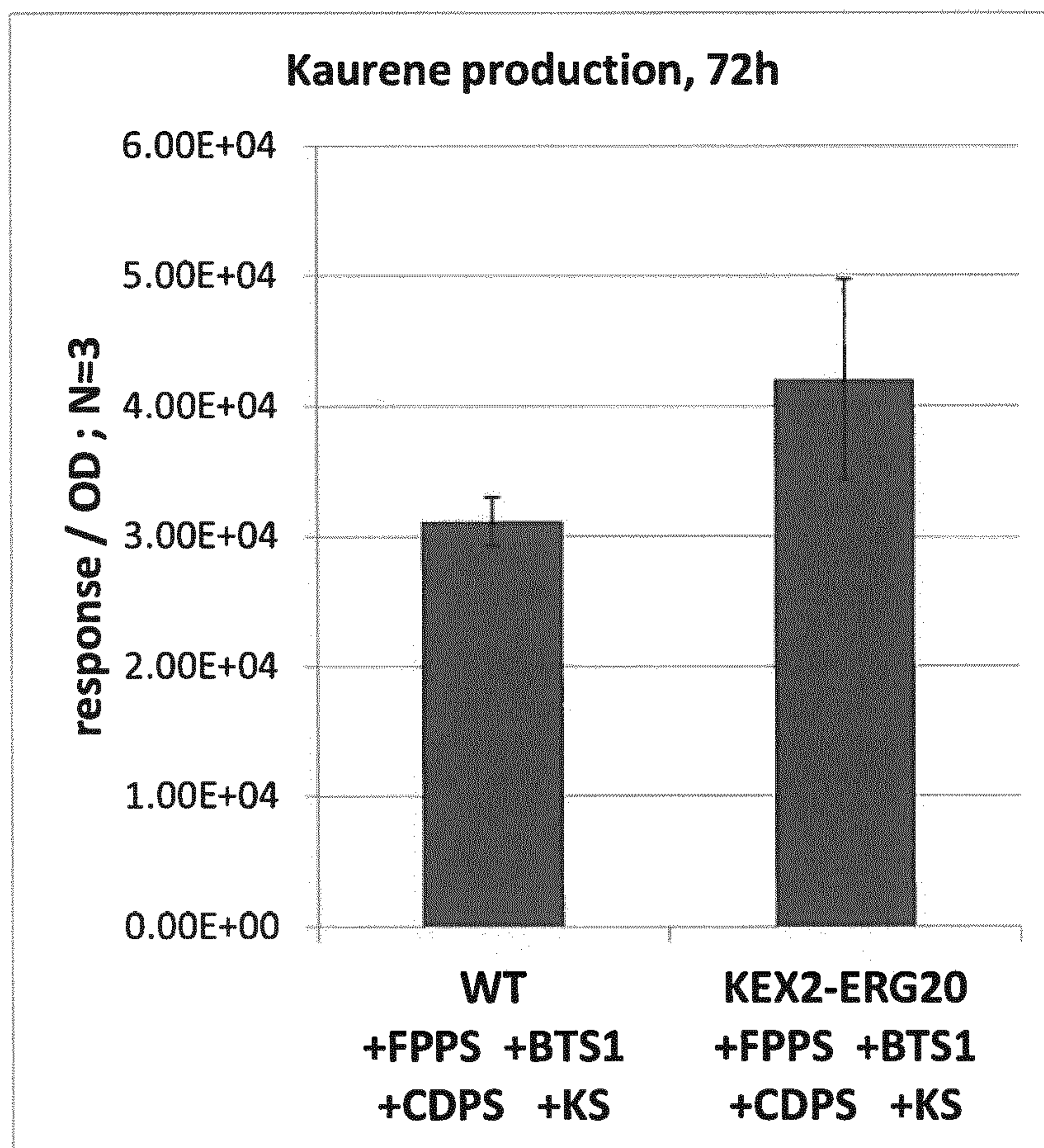


Fig. 9