

Production of a flavour compound in a host cell

Field of the invention

The present invention relates to the field of biotechnology; specifically the production of a flavor
5 compound (raspberry ketone) in a host cell.

Background of the invention

Raspberry ketone is one of key flavour compounds with typical raspberry characteristics and a low odour threshold. It is an expensive flavour compound which is extensively used in the food industry.
10 Raspberry ketone is naturally found in, of course, raspberry (*Rubus idaeus*), but also in other fruits, such as peaches, grapes, apples, several species of berries, in vegetables such as rhubarb and in the bark of some trees, such as yew, maple and pine.

Raspberry ketone is used for various purposes such as in the formulation of multiple aroma's, such as strawberry-, kiwi- and cherry-aroma as well as in cosmetics and as an anti-obesity agent.

15 It is not straightforward to obtain raspberry ketone in a commercially relevant way from its natural sources since the low content of the raspberry ketone containing fruits and other sources render the extraction and purification process non-profitable (less than 4 mg of raspberry ketone can be obtained per kg of raspberries). To date, raspberry ketone is produced chemically via the condensation of p-hydroxybenzaldehyde with acetone, an environmentally unfriendly process. In
20 addition, the chemical synthesis of flavour compounds often leads to non-desirable racemic-variants and –mixtures (Vandamme and Soetaert 2002; J Chem Techno Biotechnol 77:1323-1332). In raspberries, the synthesis of raspberry ketone is a two-enzyme part of the phenylpropanoid pathway. This pathway has been described by Borejsza-Wysocki and Hrazdina (1994). In the first step, coumaroyl-CoA (which is present in many plant tissues) is condensed with one malonyl CoA
25 into benzalacetone (p-hydroxyphenylbut-3-ene-2-one). The enzyme catalysing this step is called benzalacetone synthase (BAS). In the second step, the double bond in benzalacetone is reduced, resulting in raspberry ketone (p-hydroxyphenyl-2-butanone). The enzyme catalysing this step is called benzalacetone reductase (BAR), this enzyme requires the presence of NADPH.

Benzalacetone synthase (BAS), EC 2.3.1.-., is a member of the polyketide synthase family.
30 Benzalacetone synthase condenses one acetone unit of malonyl-CoA with one p-coumaric acid to form benzalacetone. The polyketide synthase family is described in detail in Schroder (1999). Abe et al (2001) teach the cloning of a BAS gene from rhubarb. Koeduka et al (2011) teach the characterization of raspberry BAS.

Soluble enzymes catalysing the reduction of a double bond using NADPH, for example
35 benzalacetone reductase (BAR), are classified by the International Union of Biochemistry and Molecular Biology as belonging to enzymatic class EC 1.3.1.X. For instance, an enzyme annotated as EC 1.3.1.11 from *Arthrobacter* sp. was reported to remove a double bond from coumarate (Levi and Weinstein, 1964), but no gene has been identified in connection to this enzymatic activity. Other enzymes in the enzymatic class EC 1.3.1.X are orotate reductase, 2-hexadecenal reductase,
40 cholestenone 5 alpha-reductase etc. for which genes are known. However, none of these enzymes

was reported to have benzalacetone reductase (BAR) activity. It is known from literature that 4-hydroxybenzalacetone can be transformed to raspberry ketone by fungi or yeasts like *Pichia*, *Saccharomyces*, *Beauveria*, *Kloeckera*, *Aureobasidium*, *Cladosporium*, *Geotrichum*, *Mucor*, and *Candida* spp. (Fuganti and Zucchi, 1998). Beekwilder et al. (2007) report that BAR activity is present
5 in *E. coli*. However, no gene has been identified in connection to this enzymatic activity.

Attempts to biosynthesise raspberry ketone have been described. Hugueny et al (1995, Bioflavour 95 pp 269-273) teach a biotechnological method for producing raspberry ketone. This method comprises culturing a microorganism which has a secondary alcoholdehydrogenase (ADH), such as *Candida boidinii*, and adding the precursor betuloside to the culture medium. In this cellular
10 environment the secondary ADH dehydrogenates betuligenol into raspberry ketone. Abe et al (2001) teach the cloning of rhubarb BAS, expression of the gene in *E. coli*, purification of the recombinant BAS protein and the in vitro synthesis of benzalacetone. However, this study does not teach the *in vivo* synthesis of benzalacetone or raspberry ketone.

EP1226265 teaches the bioproduction of para hydroxycinnamic acid, also known as p-coumaric acid, which can be used as precursor for raspberry ketone. EP1226265 describes a phenyl-ammonia-lyase enzyme (PAL)(EC4.3.1.5); it does not teach the *in vivo* synthesis of raspberry ketone. GB2416769 and GB2416770 describe the bioproduction of raspberry ketone in *E. coli* from the precursor p-coumaric acid that is added to the fermentation. Likewise in Beekwilder et al (2007), raspberry ketone is produced in *E. coli* from the precursor p-coumaric acid that is added to the
20 fermentation. In 2016, Lee et al published their results of production of raspberry ketone in *Saccharomyces cerevisiae* from the precursor p-coumaric acid that is added to the fermentation. In addition, they produced tiny amounts of raspberry ketone (0.49mg/L) by *Saccharomyces cerevisiae* using anaerobic fermentation. The addition of a precursor to be able to produce raspberry ketone in a microbial host cell is not efficient. In addition, the use of eukaryotic cells in anaerobic
25 fermentation is laborious and inefficient, certainly at industrial scale. Hence there is a need for the *de novo* bioproduction of raspberry ketone in a prokaryotic microbial host cell using straightforward fermentation conditions. To date, no *de novo* bioproduction of raspberry ketone by such prokaryotic host cells using such straightforward aerobic fermentation has been presented.

30 **Summary of the invention**

The invention relates to a cell capable of producing raspberry ketone, and to methods wherein such a cell is used. In a first aspect the invention provides a prokaryotic microbial cell capable of expressing, preferably expressing, a functional heterologous enzyme with tyrosine ammonium lyase (TAL) activity and further capable of expressing, preferably expressing, at least one functional
35 enzyme selected from the group consisting of a 4-coumarate-CoA ligase (4CL) and a benzalacetone synthase (BAS) and optionally further capable of expressing, preferably expressing, a heterologous benzalacetone reductase (BAR). In embodiments of this aspect the cell is provided wherein the functional heterologous enzyme with TAL activity is from *Rhodobacter capsulatus*, *Saccharothrix espanaensis*, or *Flavobacterium johnsoniae*; wherein the 4CL is from *Nicotiana tabacum*, *Arabidopsis thaliana*, *Physcomitrella patens* or *Streptomyces coelicolor*; wherein the BAS
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is from *Rubus idaeus* or *Rheum palmatum*; and wherein the optional BAR is from *Rubus idaeus*. In particular embodiments this aspect provides the cell, wherein the polynucleotide sequence of at least one of said enzymes is codon optimized. In further embodiments, this aspect provides the cell, wherein the cell is a Gram positive prokaryotic microbial cell, preferably a *Corynebacterium*, more preferably a *Corynebacterium glutamicum*, even more preferably *Corynebacterium glutamicum* ATCC13032, even more preferably a *Corynebacterium* capable of producing at least twice as much L-Tyrosine as compared to *Corynebacterium glutamicum* ATCC13032. In preferred embodiments of this aspect is provided the cell, wherein at least two of the enzymes are encoded by a single recombinant polynucleotide construct. In further preferred embodiments of this aspect is provided the cell, capable of producing at least 5mg/l raspberry ketone.

In a second aspect, the invention provides a method for the production of a cell according to the first aspect, the method comprising contacting a prokaryotic cell with an expression construct encoding a functional heterologous enzyme with tyrosine ammonium lyase (TAL) activity, and contacting that prokaryotic cell with an expression construct encoding at least one functional enzyme selected from the group consisting of a 4-coumarate-CoA ligase (4CL) and a benzalacetone synthase (BAS), and optionally contacting that prokaryotic cell with an expression construct encoding a heterologous benzalacetone reductase (BAR).

In a third aspect, the invention provides a method for the production of raspberry ketone, comprising culturing a cell according to the first aspect of the invention under conditions conducive to the production of raspberry ketone, and, optionally, isolating and/or purifying the raspberry ketone from the cell and/or the culture medium.

In a fourth aspect, the invention provides the use of a functional heterologous enzyme with tyrosine ammonium lyase (TAL) activity as described in the first aspect of the invention, for the production of raspberry ketone in a prokaryotic host cell, preferably a Gram positive prokaryotic host cell.

In a fifth aspect, the invention provides an expression vector comprising particular operon sequences. In a sixth aspect, the invention provides a polypeptide product expressed from the expression vector according to the fifth aspect.

Brief description of the drawings

Figure 1 - Biosynthetic pathway for the production of raspberry ketone from L-tyrosine; TAL = tyrosine ammonia lyase; 4CL = 4-coumarate-CoA ligase; BAS = benzalacetone synthase; BAR = benzalacetone reductase.

Figure 2 - Plasmid map of plasmid pECXK_P; RpBAS = *Rheum palmatum* benzalacetone synthase, Pp4CL = *Physcomitrella patens* 4-coumarate-CoA ligase, RcTAL = *Rhodobacter capsulatus* tyrosine ammonia lyase, LacIq = repressor gene, Ptrc = IPTG-inducible Ptrc promoter; T1 & T2 = rrnB transcriptional terminators T1 and T2; KanR = kanamycine resistance; repA = replication origin pGA1; per = positive effector of plasmid replication.

Figure 3 - Plasmid map of plasmid pECXK_PB; RpBAS = *Rheum palmatum* benzalacetone synthase; Pp4CL = *Physcomitrella patens* 4-coumarate-CoA ligase; RcTAL = *Rhodobacter capsulatus* tyrosine ammonia lyase; RiBAR = *Rubus idaeus* benzalacetone reductase; LacIq =

repressor gene; Ptrc = IPTG-inducible Ptrc promoter; T1 & T2 = rrnB transcriptional terminators T1 and T2; KanR = kanamycine resistance; repA = replication origin pGA1; per = positive effector of plasmid replication.

Figure 4 - *De novo* production of 4-coumaric acid in *E. coli*

5 HPLC chromatograms are shown for strains A.) Ec_RK_EV, B.) Ec_RK_P and C.) Ec_RK_PB and the D.) 4-coumaric acid standard. The peak of 4-coumaric acid is observed at the retention time of 17.9 min

Figure 5 - *De novo* production of hydroxyphenylbutenone in *E. coli*

HPLC chromatograms are shown for strains A.) Ec_RK_EV, B.) Ec_RK_P and C.) Ec_RK_PB and
10 the D.) hydroxyphenylbutenone standard. The peak of hydroxyphenylbutenone is observed at the retention time of 23.1 min and is indicated by an arrow.

Figure 6 - HPLC analysis of *de novo* production of hydroxyphenylbutenone in *C. glutamicum*.

HPLC chromatograms are shown for strains A.) Cg_RK_EV, B.) Cg_RK_P and C.) Cg_RK_PB and
15 the D.) hydroxyphenylbutenone standard. The peak of hydroxyphenylbutenone is observed at the retention time of 23.1 min and is indicated by an arrow.

Figure 7 - GC-MS analysis of raspberry ketone production in *C. glutamicum*.

The GC-MS chromatograms in panel A are shown for strains Cg_RK_EV, Cg_RK_P and Cg_RK_PB. The raspberry ketone peak is observed at the retention time of 14.2 min. In panel B
20 the mass spectrum of raspberry ketone produced by strain Cg_RK_PB is shown. In panel C the mass spectrum of the authentic raspberry ketone standard is shown.

Description of the invention

It has been established by the inventors that, surprisingly, raspberry ketone can be synthesized *de novo* from glucose by a recombinant prokaryotic microbial host cell using straightforward aerobic
25 fermentation conditions.

Accordingly, in a first aspect the invention provides for a prokaryotic microbial cell capable of expressing, preferably expressing, a functional heterologous enzyme with tyrosine ammonium lyase (TAL) activity and further capable of expressing, preferably expressing, at least one functional enzyme selected from the group consisting of a 4-coumarate-CoA ligase (4CL) and a
30 benzalacetone synthase (BAS) and optionally further capable of expressing, preferably expressing, a heterologous benzalacetone reductase (BAR). Such a prokaryotic microbial cell is referred to hereinafter as a cell according to the invention.

In preferred embodiments, this aspect provides for a prokaryotic microbial cell expressing a functional heterologous enzyme with tyrosine ammonium lyase (TAL) activity and further expressing
35 at least one functional enzyme selected from the group consisting of a 4-coumarate-CoA ligase (4CL) and a benzalacetone synthase (BAS) and optionally further expressing a heterologous benzalacetone reductase (BAR).

In particular embodiments, this aspect provides a prokaryotic microbial cell expressing a functional heterologous enzyme with tyrosine ammonium lyase (TAL) activity and further expressing a

functional 4-coumarate-CoA ligase (4CL) enzyme and optionally further expressing a heterologous benzalacetone reductase (BAR).

In particular embodiments, this aspect provides a prokaryotic microbial cell expressing a functional heterologous enzyme with tyrosine ammonium lyase (TAL) activity and further expressing a functional benzalacetone synthase (BAS) enzyme and optionally further expressing a heterologous benzalacetone reductase (BAR).

In particular embodiments, this aspect provides a prokaryotic microbial cell expressing a functional heterologous enzyme with tyrosine ammonium lyase (TAL) activity and further expressing a functional 4-coumarate-CoA ligase (4CL) enzyme and a functional benzalacetone synthase (BAS) enzyme and optionally further expressing a heterologous benzalacetone reductase (BAR).

In particular embodiments, this aspect provides a prokaryotic microbial cell expressing a functional heterologous enzyme with tyrosine ammonium lyase (TAL) activity and further expressing a functional 4-coumarate-CoA ligase (4CL) enzyme and a functional benzalacetone synthase (BAS) enzyme and further expressing a heterologous benzalacetone reductase (BAR).

Prokaryotic microbial cells are well known in the art. A prokaryote is a unicellular organism that lacks a membrane-bound nucleus, mitochondria, and other membrane-bound organelles. Examples of prokaryotes are bacteria and archaea. Preferred prokaryotic cells are bacteria such as *Escherichia coli*, or preferably Gram positive bacteria such as *Corynebacterium*, more preferably such as *Corynebacterium glutamicum*, because their culturing is well-established. In preferred embodiments, this aspect provides a cell according to the invention, wherein the cell is a Gram positive prokaryotic microbial cell, preferably a *Corynebacterium*, more preferably a *Corynebacterium glutamicum*, even more preferably *Corynebacterium glutamicum* ATCC13032, even more preferably a *Corynebacterium* capable of producing at least twice as much L-Tyrosine as compared to *Corynebacterium glutamicum* ATCC13032. Strain ATCC13032 is known to the person skilled in art; this strain is also known as DSM 20300, JCM 1318, LMG 3730 and NCIMB 10025.

Throughout this application, expression is considered to be the transcription of a gene into functional mRNA, leading to a functional polypeptide such as an enzyme. An enzyme is a polypeptide that can catalyse a reaction. In this context, increased expression of an enzyme can be considered an increased level of mRNA encoding said enzyme, an increased level of enzyme polypeptide, or an increased total activity of said enzyme. Preferably, an increased expression of an enzyme results in an increased activity of said enzyme, which can be caused by increased levels of enzyme polypeptide. An enzyme that shows activity in catalysing its associated reaction is a functional enzyme. Associated reactions for TAL, 4CL, BAS, and BAR are described elsewhere herein. Activity can be determined by monitoring for the increase of product concentration using chromatographic techniques, such as gas chromatography (GC), high performance liquid chromatography (HPLC), or GC or liquid chromatography with advantageously coupled mass spectrometric methods, such as LCMS or GCMS. A preferred method for detection of intermediates is GCMS. A preferred method for the detection of raspberry ketone is GCMS.

In the context of this application, a cell capable of expressing an enzyme is a cell that comprises the genetic information required for expressing said enzyme, preferably a nucleic acid encoding said enzyme. This does not mean that the enzyme is necessarily expressed. As a non-limiting example, a cell comprising a nucleic acid encoding an enzyme wherein the nucleic acid is under
5 the control of a promotor that responds to a particular induction, the promotor is not necessarily induced and thus the enzyme is not necessarily expressed, whereas the cell is in fact capable of such expression. In preferred embodiments of the invention, a preferred cell capable of expressing a functional enzyme is a cell that expresses said functional enzyme.

A heterologous enzyme is an enzyme derived from a different organism. A heterologous
10 polynucleotide is a polynucleotide derived from a different organism. A heterologous polynucleotide can be a synthetic polynucleotide. In preferred embodiments, the cell according to the invention is provided, wherein the polynucleotide sequence encoding at least one of said enzymes is codon optimized. A codon optimized polynucleotide sequence is a sequence wherein the codon usage bias has been mitigated through selection of alternative codons without altering the encoded
15 polypeptide. For example, rare codons can be replaced by more common codons, or regions comprising many identical codons can be interrupted by substitution of synonymic codons to reduce demand for the many identical codons. Codon optimization is known in the art, and bioinformatics tools for codon optimization are freely available on the internet from various providers.

20 Tyrosine ammonium lyase, referred to hereinafter as TAL, is also known as tyrosine ammonia lyase, L-tyrosine ammonia-lyase, and tyrase. It is an enzyme (EC 4.3.1.23) in the natural phenols biosynthesis pathway, which transforms L-tyrosine into p-coumaric acid, releasing ammonium. P-coumaric acid is also known as para coumaric acid, 4-coumaric acid, 4-hydroxycinnamic acid, and (E)-3-(4-hydroxyphenyl)-2-propenoic acid. A preferred TAL or a preferred enzyme with TAL activity
25 is a heterologous TAL or is a heterologous enzyme with TAL activity, which is a TAL or an enzyme with TAL activity that is derived from a different organism than the cell according to the invention. Preferred organisms from which TAL or enzymes with TAL activity can be derived are *Rhodobacter capsulatus* (for example RcTAL, represented by SEQ ID NO: 1 and encoded by SEQ ID NO: 2, see Kyndt et al. 2002 FEBS Lett. 512: 240-244), *Saccharothrix espanaensis* (for example SeSam8,
30 represented by SEQ ID NO: 3 and encoded by SEQ ID NO: 4, see Berner et al. 2006 J Bacteriol 188: 2666-2673), and *Flavobacterium johnsoniae* (for example FjTAL, represented by SEQ ID NO: 5 and encoded by SEQ ID NO: 6, see Jendersen et al. 2015 Appl Environ Microbiol 81:4458-4476). A preferred TAL or enzyme with TAL activity has at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, preferably at least 80%, 85%, 90%,
35 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, more preferably at least 90%, most preferably 100% sequence identity with SEQ ID NO: 1, SEQ ID NO:3, or SEQ ID NO: 5, preferably with SEQ ID NO: 1, or is encoded by a polynucleotide that has at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, preferably at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, more preferably at least
40 90%, most preferably 100% sequence identity with SEQ ID NO: 2, SEQ ID NO: 4, or SEQ ID NO:

6, preferably with SEQ ID NO: 2. Preferred polynucleotides that encode TAL or that encode enzymes with TAL activity are codon optimized. TAL activity of an enzyme can be assayed by chromatographic monitoring of p-coumaric acid production or of ammonia evolution when an enzyme that is to be tested for TAL activity is brought into contact with L-tyrosine in a suitable buffer.

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4-coumarate-CoA ligase, hereinafter referred to as 4CL, is also known as 4CL, 4-coumaroyl-CoA synthetase, p-coumaroyl CoA ligase, p-coumaroyl coenzyme A synthetase, p-coumaroyl-CoA synthetase, p-coumaroyl-CoA ligase, feruloyl CoA ligase, hydroxycinnamoyl CoA synthetase, and various other names. It is an enzyme (EC 6.2.1.12) that catalyzes the conjugation of coenzyme A (CoA) to 4-coumarate, forming 4-coumaroyl-CoA under consumption of ATP. 4-coumaroyl-CoA is also known as coumaroyl-CoA. A preferred 4CL is a heterologous 4CL, which is a 4CL that is derived from a different organism than the cell according to the invention. Preferred organisms from which 4CL can be derived are *Nicotiana tabacum* (for example Nt4CL, represented by SEQ ID NO: 7 and encoded by SEQ ID NO: 8, see Lee & Douglas. 1996 Plant Physiol. 112: 193-205), *Arabidopsis thaliana* (for example At4CL, represented by SEQ ID NO: 9 and encoded by SEQ ID NO: 10, see Ehrling et al. 1999 Plant. J. 19: 9-20), *Physcomitrella patens* (for example Pp4CL, represented by SEQ ID NO: 11 and encoded by SEQ ID NO: 12, see Silber et al. 2008 Phytochem. 69: 2449 - 2456), and *Streptomyces coelicolor* (for example Sc4CL, represented by SEQ ID NO: 13 and encoded by SEQ ID NO: 14). A preferred 4CL has at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, preferably at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, more preferably at least 90%, most preferably 100% sequence identity with SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, or SEQ ID NO: 13, preferably with SEQ ID NO: 11, or is encoded by a polynucleotide that has at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, preferably at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, more preferably at least 90%, most preferably 100% sequence identity with SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, or SEQ ID NO: 14, preferably with SEQ ID NO: 12. Preferred polynucleotides that encode 4CL are codon optimized.

30 Benzalacetone synthase, hereinafter referred to as BAS, is a plant-specific type III polyketide synthase (PKS). It is an enzyme (EC 2.3.1.212) that catalyzes the conversion of 4-coumaroyl-CoA into 4-hydroxybenzalacetone. 4-hydroxybenzalacetone is also known as hydroxyphenylbutenone, p-hydroxybenzalacetone, 1-(4-Hydroxybenzylidene)acetone, and (3E)-4-(4-Hydroxyphenyl)-3-buten-2-one. A preferred BAS is a heterologous BAS, which is a BAS that is derived from a different organism than the cell according to the invention. Preferred organisms from which BAS can be derived are *Rubus idaeus* (for example RiPKS, represented by SEQ ID NO: 15 and encoded by SEQ ID NO: 16, see Zheng & Hrazdina 2008 Arch Biochem Biophys 470: 139-145), and *Rheum palmatum* (for example RpBAS, represented by SEQ ID NO: 17 and encoded by SEQ ID NO: 18, see Abe et al. 2001 Eur J Biochem 268: 3354-3359). A preferred BAS has at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, preferably at

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least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, more preferably at least 90%, most preferably 100% sequence identity with SEQ ID NO: 15 or with SEQ ID NO: 17. Preferably with SEQ ID NO: 17, or is encoded by a polynucleotide that has at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, preferably at
5 least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, more preferably at least 90%, most preferably 100% sequence identity with SEQ ID NO: 16 or with SEQ ID NO: 18, preferably with SEQ ID NO: 18. Preferred polynucleotides that encode BAS are codon optimized.

Benzalacetone reductase is hereinafter referred to as BAR. It is an enzyme (EC 1.3.1.-) that
10 catalyses the conversion of 4-hydroxybenzalacetone into raspberry ketone. Raspberry ketone is also known as 4-(4-hydroxyphenyl)butan-2-one, p-hydroxybenzyl acetone, 4-(p-hydroxyphenyl)-2-butanone, frambinone, oxyphenylon, rheosmin, and rasketone. A preferred BAR is a heterologous BAR, which is a BAR that is derived from a different organism than the cell according to the invention. A preferred organism from which BAR can be derived is *Rubus idaeus* (for example
15 RiBAR, represented by SEQ ID NO: 19 and encoded by SEQ ID NO: 20, see Koeduka et al. 2011 Biochem Biophys Res Commun 412: 104–108). A preferred BAR has at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, preferably at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, more preferably at least 90%, most preferably 100% sequence identity with SEQ ID NO: 19, or is encoded by a
20 polynucleotide that has at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, preferably at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%, more preferably at least 90%, most preferably 100% sequence identity with SEQ ID NO: 20. Preferred polynucleotides that encode BAR are codon optimized.

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Within this aspect, a preferred cell according to the invention is provided wherein

- the functional heterologous enzyme with TAL activity is from *Rhodobacter capsulatus*, *Saccharothrix espanaensis*, or *Flavobacterium johnsoniae*; preferably, it is from *Rhodobacter capsulatus*;
- 30 - the at least one functional enzyme selected from the group consisting of a 4CL and a BAS is 4CL from *Nicotiana tabacum*, *Arabidopsis thaliana*, *Physcomitrella patens* or *Streptomyces coelicolor*, or is BAS from *Rubus idaeus* or *Rheum palmatum*; preferably, BAS is from *Rheum palmatum*; preferably, 4CL is from *Physcomitrella patens*; and
- the optional BAR is from *Rubus idaeus*.

35 In more preferred embodiments, a cell according to the invention is provided wherein

- the functional heterologous enzyme with TAL activity is from *Rhodobacter capsulatus*, *Saccharothrix espanaensis*, or *Flavobacterium johnsoniae*; preferably, it is from *Rhodobacter capsulatus*;
- the at least one functional enzyme selected from the group consisting of a 4CL and a BAS
40 is 4CL from *Nicotiana tabacum*, *Arabidopsis thaliana*, *Physcomitrella patens* or

Streptomyces coelicolor, or is BAS from *Rubus idaeus* or *Rheum palmatum*; preferably, BAS is from *Rheum palmatum*; preferably, 4CL is from *Physcomitrella patens*; and
- the BAR is from *Rubus idaeus*.

In even more preferred embodiments, a cell according to the invention is provided wherein:

- 5 - the functional heterologous enzyme with TAL activity is from *Rhodobacter capsulatus*;
- the at least one functional enzyme selected from the group consisting of a 4CL and a BAS is selected from the group consisting of a 4CL from *Physcomitrella patens* and a BAS from *Rheum palmatum*; and
- the optional BAR is from *Rubus idaeus*.

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In particular embodiments, both a BAS and a 4CL are expressed in the cell according to the invention. In such embodiments, a cell according to the invention is provided wherein:

- the functional heterologous enzyme with TAL activity is from *Rhodobacter capsulatus*, *Saccharothrix espanaensis*, or *Flavobacterium johnsoniae*;
- 15 - the 4CL is from *Nicotiana tabacum*, *Arabidopsis thaliana*, *Physcomitrella patens* or *Streptomyces coelicolor*;
- the BAS is from *Rubus idaeus* or *Rheum palmatum*; and
- the optional BAR is from *Rubus idaeus*.

In more preferred such embodiments, a cell according to the invention is provided wherein:

- 20 - the functional heterologous enzyme with TAL activity is from *Rhodobacter capsulatus*;
- the 4CL is from *Physcomitrella patens*;
- the BAS is from *Rheum palmatum*; and
- the optional BAR is from *Rubus idaeus*.

In preferred embodiments within this aspect, the invention provides a cell according to the invention, wherein:

- 25 - the functional heterologous enzyme with TAL activity has at least 60%, preferably at least 80%, more preferably at least 90%, most preferably 100% sequence identity with SEQ ID NO: 1, SEQ ID NO: 3, or SEQ ID NO: 5, preferably with SEQ ID NO: 1, or is encoded by a polynucleotide which has at least 60%, preferably at least 80%, more preferably at least 90%, most preferably 100% sequence identity with SEQ ID NO: 2, SEQ ID NO: 4, or SEQ ID NO: 6, preferably with SEQ ID NO: 2;
- 30 - the 4CL has at least 60%, preferably at least 80%, more preferably at least 90%, most preferably 100% sequence identity with SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, or SEQ ID NO: 13, preferably with SEQ ID NO: 11, or is encoded by a polynucleotide which has at least 60%, preferably at least 80%, more preferably at least 90%, most preferably 100% sequence identity with SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, or SEQ ID NO: 14, preferably with SEQ ID NO 12;
- 35 - the BAS has at least 60%, preferably at least 80%, more preferably at least 90%, most preferably 100% sequence identity with SEQ ID NO: 15 or SEQ ID NO: 17, preferably with
- 40 SEQ ID NO: 17, or is encoded by a polynucleotide which has at least 60%, preferably at

least 80%, more preferably at least 90%, most preferably 100% sequence identity with SEQ ID NO 16 or SEQ ID NO: 18, preferably with SEQ ID NO: 18;

- the BAR has at least 60%, preferably at least 80%, more preferably at least 90%, most preferably 100% sequence identity with SEQ ID NO: 19 or is encoded by a polynucleotide which has at least 60%, preferably at least 80%, more preferably at least 90%, most preferably 100% sequence identity with SEQ ID NO: 20.

In this aspect, it can be advantageous to use polynucleotide constructs that encode more than one enzyme. For example, a polycistronic construct can be used, or an operon wherein more than one enzyme is under the control of a single promotor. Such constructs can be created using recombinant DNA techniques that are well known in the art.

Accordingly in preferred embodiments this aspect provides a cell according to the invention, wherein at least two of the enzymes are encoded by a single recombinant polynucleotide construct. Such constructs can be comprised in an expression vector, which may be a plasmid. The two enzymes may also be a single fusion polypeptide.

Raspberry ketone is 4-(4-hydroxyphenyl)butan-2-one, and is also known as p-hydroxybenzyl acetone, 4-(p-hydroxyphenyl)-2-butanone, frambinone, oxyphenylon, rheosmin, and rasketone. It is a natural phenolic compound that is the primary aroma compound of red raspberries. It occurs in a variety of fruits, also including cranberries and blackberries. Cells according to the invention are capable of producing raspberry ketone and produce raspberry ketone when cultured. Preferred cells according to the invention are capable of producing, preferably producing, at least 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, or 50 mg/L raspberry ketone, preferably at least 5 mg/L. More preferably, cells according to the invention are capable of producing, preferably producing, at least 100mg/L, 200mg/L, 300mg/L, 400mg/L, 500mg/L, 600mg/L, 700mg/L, 800mg/L, 900mg/L, 1g/L, 2 g/L, 3 g/L, 4 g/L, 5 g/L, 6 g/L, 7 g/L, 8 g/L, 9 g/L, 10 g/L, 11 g/L, 12 g/L, 13 g/L, 14 g/L, 15 g/L, 16 g/L, 17 g/L, 18 g/L, 19 g/L, 20 g/L, 21 g/L, 22 g/L, 23 g/L, 24 g/L, or 25g/L raspberry ketone. Preferably, the raspberry ketone is detected in a cell according to the invention and/or in its culture broth or headspace wherein said cell is cultured.

Accordingly, this aspect provides a cell according to the invention, capable of producing at least 5 mg/L raspberry ketone. Preferred embodiments provide a cell according to the invention, capable of producing at least 1 g/L raspberry ketone. More Preferred embodiments provide a cell according to the invention, capable of producing at least 5 g/L raspberry ketone. Still more preferred embodiments provide a cell according to the invention, capable of producing at least 10 g/L raspberry ketone, most preferably at least 20 g/L raspberry ketone. The production of raspberry ketone can be monitored using chromatographic techniques on samples obtained from the production medium. Such amount is preferably obtained in at most 2 weeks, 1 week, 6 days, 5 days, 4 days, 3 days, 2 days, 1 day, 15 hours, 10 hours, 5 hours, 4 hours, 3 hours, 2 hours or 1 hour of culture, preferably after 24 hours of culture.

Production of a cell

In a second aspect, the invention provides a method for the production of a cell according to the invention. The features of this aspect are preferably those of the first aspect of the invention. In
5 embodiments of this aspect is provided a method for the production of a cell according to the invention, comprising

- contacting a prokaryotic cell with an expression construct encoding a functional heterologous enzyme with tyrosine ammonium lyase (TAL) activity, and
- contacting that prokaryotic cell with an expression construct encoding at least one
10 functional enzyme selected from the group consisting of a 4-coumarate-CoA ligase (4CL) and a benzalacetone synthase (BAS), and
- optionally contacting that prokaryotic cell with an expression construct encoding a heterologous benzalacetone reductase (BAR).

In preferred embodiments of this aspect the invention provides a method for the production of a cell
15 according to the invention, comprising

- contacting a prokaryotic cell with an expression construct encoding a functional heterologous enzyme with tyrosine ammonium lyase (TAL) activity, and
- contacting that prokaryotic cell with an expression construct encoding a functional 4-coumarate-CoA ligase (4CL) and a functional benzalacetone synthase (BAS), and
20 - optionally contacting that prokaryotic cell with an expression construct encoding a heterologous benzalacetone reductase (BAR).

In preferred embodiments of this aspect the invention provides a method for the production of a cell according to the invention, comprising

- contacting a prokaryotic cell with an expression construct encoding a functional
25 heterologous enzyme with tyrosine ammonium lyase (TAL) activity, and
- contacting that prokaryotic cell with an expression construct encoding a functional 4-coumarate-CoA ligase (4CL), and
- optionally contacting that prokaryotic cell with an expression construct encoding a heterologous benzalacetone reductase (BAR).

30 In preferred embodiments of this aspect the invention provides a method for the production of a cell according to the invention, comprising

- contacting a prokaryotic cell with an expression construct encoding a functional heterologous enzyme with tyrosine ammonium lyase (TAL) activity, and
- contacting that prokaryotic cell with an expression construct encoding a functional
35 benzalacetone synthase (BAS), and
- optionally contacting that prokaryotic cell with an expression construct encoding a heterologous benzalacetone reductase (BAR).

Corynebacterium, more preferably a *Corynebacterium glutamicum*

Suitable cell types were defined earlier herein. A preferred prokaryotic cell is a Gram positive cell,
40 more preferably a *Corynebacterium*, even more preferably a *Corynebacterium glutamicum*.

Accordingly, in preferred embodiments of this aspect the invention provides a method for the production of a cell according to the invention, comprising

- contacting a *Corynebacterium* cell, preferably a *Corynebacterium glutamicum* cell, with an expression construct encoding a functional heterologous enzyme with tyrosine ammonium lyase (TAL) activity, and
- contacting that *Corynebacterium* cell with an expression construct encoding at least one functional enzyme selected from the group consisting of a 4-coumarate-CoA ligase (4CL) and a benzalacetone synthase (BAS), and
- optionally contacting that *Corynebacterium* cell with an expression construct encoding a heterologous benzalacetone reductase (BAR).

For expression of an enzyme in a prokaryotic cell according to the inventions, as well as for additional genetic modification of a prokaryotic cell according to the invention, the cell can be transformed with a nucleic acid or nucleic acid construct described herein by any method known to the person skilled in the art. Such methods are e.g. known from standard handbooks, such as Sambrook and Russel (2001) "Molecular Cloning: A Laboratory Manual (3rd edition), Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, or F. Ausubel et al, eds., "Current protocols in molecular biology", Green Publishing and Wiley Interscience, New York (1987). Methods for transformation and genetic modification of bacterial cells are known from e.g. U.S. Pat. No. 6,699,696 or U.S. Pat. No. 4,778,759. For *Corynebacteria*, reference is made to Eggeling & Reyes 2005 Experiments. In: Eggeling, L., Bott, M. (Eds.), Handbook of *Corynebacterium glutamicum*. CRC Press, Boca Raton, FL, pp. 3535–3566. Examples are transformation using competent or supercompetent cells, electroporation, use of transfection lipids, use of transfection polymers, or gymnotic transformation. A preferred method is electroporation.

When a nucleic acid construct is used for expression of an enzyme in a prokaryotic cell according to the invention, a selectable marker may be present in the nucleic acid construct comprising a polynucleotide encoding the enzyme. The term "marker" refers herein to a gene encoding a trait or a phenotype which permits the selection of, or the screening for, a cell containing the marker. A marker gene may be an antibiotic resistance gene whereby the appropriate antibiotic can be used to select for transformed cells from among cells that are not transformed. A preferred selection marker is kanamycin and its corresponding resistance gene. Preferably however, a non-antibiotic resistance marker is used, such as an auxotrophic marker (URA3, TRP1, LEU2). A preferred cell according to the invention, e.g. transformed with a nucleic acid construct, is marker gene free. Methods for constructing recombinant marker gene free microbial host cells are described in (Cheah et al., 2013) and are based on the use of bidirectional markers. Alternatively, a screenable marker such as Green Fluorescent Protein, lacZ, luciferase, chloramphenicol acetyltransferase, beta-glucuronidase may be incorporated into a nucleic acid construct according to the invention allowing to screen for transformed cells.

Method for production of raspberry ketone

The prokaryotic cells according to the invention are useful for *de novo* bioproduction of raspberry ketone using straightforward aerobic fermentation methods. Accordingly, in a third aspect the invention provides a method for the production of raspberry ketone, comprising:

- 5 - culturing a cell according to the invention under conditions conducive to the production of raspberry ketone, and, optionally,
 - isolating and/or purifying the raspberry ketone from the cell and/or the culture medium.

In preferred embodiment, this aspect provides a method for the production of raspberry ketone, comprising:

- 10 - culturing a cell according to the invention under conditions conducive to the production of raspberry ketone, and
 - isolating and/or purifying the raspberry ketone from the cell and/or the culture medium.

The features of this aspect are preferably those of the first and second aspect of the invention.

This method is referred to hereinafter as a process according to the invention. Preferably, a process
15 according to the present invention for producing raspberry ketone comprises culturing a cell according to the present invention, preferably a Gram positive cell as defined in the first aspect of the present invention and more preferably a *Corynebacterium* cell as defined om the first aspect, wherein the culture conditions comprise culturing the cells at about 30 °C in LB medium or YT medium or CgXII medium, preferably in CgXII medium, wherein the medium is preferably
20 supplemented with about 20 g/L D-glucose, optionally supplemented with kanamycin (preferably about 50 µg/mL). A culture is preferably shaken or agitated at about 250 rpm. Culturing can involve induction using Isopropyl β-D-1-thiogalactopyranoside (IPTG) or arabinose or another suitable inductor, as will be apparent to the skilled person. Culturing can entail use of a starting culture. Further examples of conducive conditions are provided in the examples.

25 Usually, a single colony is inoculated in 5 mL LB medium supplemented with 50 µg/ml kanamycin and 1% glucose. The starter culture can be grown overnight at 37 °C and 230 rpm. About 200 µl of the starting culture can be used to inoculate about 20 ml of 2xYT medium (16 g/L tryptone, 10 g/L yeast extract, 10 g/L NaCl) supplemented with about 50 µg/ml kanamycin in a 100 mL erlenmeyer flask and incubated at 37 °C, 230 rpm until the optical density at 600 nm (OD600 or A600) of 0.4 –
30 0.6. Subsequently, 1mM IPTG can be added to the medium and cultures can be incubated at 30 °C at 250 rpm. Optionally, cultures can be supplemented with 4-coumaric acid, preferably with about 3 mM 4-coumaric acid. Total bacterial culture is preferably collected 24h after induction by IPTG and can be stored at -20 °C. This method is especially suitable for *E. coli*.

Alternately, starting cultures can be grown for 48h in 25 mL LB medium supplemented with 50 µg/ml
35 kanamycin and 1% glucose at 250 rpm and 30 °C. Starter cultures can then be centrifuged for about 10 min at about 5000 rpm and the bacterial pellet can be resuspended in about 1.5 ml CgXII minimal medium. Subsequently, the cultures can be transferred to about 100 mL Erlenmeyer flasks containing about 25 mL of CgXII minimal medium supplemented with about 50 µg/ml kanamycin and about 20 g/L D-glucose and can then directly be induced with about 1mM of IPTG. Bacterial
40 cultures can be cultivated at about 30 °C and about 250 rpm for about 30 hours, up to about 4 days.

Optionally, cultures can be supplemented with 4-coumaric acid, preferably with about 3mM 4-coumaric acid. After fermentation the total bacterial culture can be collected and stored at -20 °C. This method is especially suitable for *C. glutamicum*.

5 In a preferred process, the raspberry ketone is separated from the culture broth. This may be realized continuously with the production process or subsequently to it. Separation may be based on any separation method known to the person skilled in the art. Chromatography or liquid/liquid extraction are attractive techniques.

The raspberry ketone produced by a cell according to the invention and by a process according to
10 the invention has specific properties, such as being free of trace impurities that remain after chemical synthesis. Accordingly, there is provided for a raspberry ketone obtainable by a process according to the invention.

A raspberry ketone obtained using a process according to the invention can conveniently be used in a product. Accordingly, there is provided for a pharmaceutical composition, a flavor composition,
15 a fragrance composition, a cosmetic composition, or a food composition comprising a raspberry ketone obtainable by a process according to the invention.

A process according to the invention is preferably a *de novo* process, i.e. a method for the *de novo* production of raspberry ketone. In a *de novo* process, no dedicated precursor is added to a culture,
20 which is then to be converted via fermentation. In other words, in a *de novo* process the intended product is formed from metabolites that are conventionally present in a culture broth. As a non-limiting example, the addition of 4-coumaric acid to a culture broth in a process for obtaining raspberry ketone could be seen as not being a *de novo* process, as the raspberry ketone will be formed, at least in part, from the exogenously added 4-coumaric acid, and 4-coumaric acid is not a
25 conventional ingredient for a culture broth. As a non-limiting example, the formation of raspberry ketone out of L-tyrosine can be seen as a *de novo* production, as L-tyrosine is routinely present in culture broths.

Use of TAL

30 The functionality of TAL is an important feature of the present invention. Accordingly, in a fourth aspect the invention provides the use of a functional enzyme with tyrosine ammonium lyase (TAL) activity as defined earlier herein, for the production of raspberry ketone in a prokaryotic host cell, preferably a Gram positive prokaryotic host cell. The features of this aspect are preferably those of the first, second and third aspect of the invention. In preferred embodiments of this aspect, the
35 invention provides the use of a functional heterologous enzyme with tyrosine ammonium lyase (TAL) activity as defined earlier herein, for the production of raspberry ketone in a prokaryotic host cell, preferably a Gram positive prokaryotic host cell. In more preferred embodiments of this aspect, the invention provides the use of a functional heterologous enzyme with tyrosine ammonium lyase (TAL) activity as defined earlier herein, for the production of raspberry ketone in a Gram positive

prokaryotic host cell, preferably a *Corynebacterium* host cell, more preferably a *Corynebacterium glutamicum* host cell. Features and definitions are provided elsewhere herein.

Expression vectors

5 In a fifth aspect of the invention operons and expression vectors as defined earlier herein are provided. The features of this aspect are preferably those of the first, second, third and fourth aspect of the invention. Such expression vectors are referred to herein as expression vectors according to the invention. Such operons are referred to herein as operons according to the invention. Accordingly, preferred embodiments of this fifth aspect provide an expression vector comprising a
10 first polynucleotide that has at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity with SEQ ID NO: 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, or 70, more preferably with SEQ ID NO: 52 or with SEQ ID NO: 70, most preferably with SEQ ID NO: 70, or wherein the expression vector consists of a polynucleotide that has at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%,
15 99%, or 100% sequence identity with SEQ ID NO: 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, or 69, more preferably with SEQ ID NO: 51 or with SEQ ID NO: 69, most preferably with SEQ ID NO: 69.

More preferred embodiments of this fifth aspect provide an expression vector comprising a first polynucleotide that has at least 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity,
20 preferably at least 90% sequence identity with SEQ ID NO: 52 or with SEQ ID NO: 70, most preferably with SEQ ID NO: 70, or wherein the expression vector consists of a polynucleotide that has at least 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, preferably at least 90% sequence identity with SEQ ID NO: 51 or with SEQ ID NO: 69, most preferably with SEQ ID NO: 69.

25 Further preferred embodiments of this fifth aspect provide an expression vector comprising a first polynucleotide that has at least 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, preferably at least 90% sequence identity with SEQ ID NOs: 46, 48, 52, 54, or 70 most preferably with SEQ ID NOs: 46, 48, 52, or 54, or wherein the expression vector consists of a polynucleotide that has at least 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, preferably at
30 least 90% sequence identity with SEQ ID NOs: 45, 47, 51, 53, or 69, most preferably with SEQ ID NOs: 45, 47, 51, or 53.

A polynucleotide is represented by a nucleotide sequence. A polypeptide is represented by an amino acid sequence. A nucleic acid construct is defined as a polynucleotide which is isolated from
35 a naturally occurring gene or which has been modified to contain segments of polynucleotides which are combined or juxtaposed in a manner which would not otherwise exist in nature. Optionally, a polynucleotide present in a nucleic acid construct is operably linked to one or more control sequences, which direct the production or expression of said peptide or polypeptide in a cell or in a subject.

40 Polynucleotides described herein may be native or may be codon optimized. Codon optimization

adapts the codon usage for an encoded polypeptide towards the codon bias of the organism where the polypeptide is to be produced in. Codon optimization generally helps to increase the production level of the encoded polypeptide in the host cell, such as in the preferred host herein: *Corynebacterium*. Many algorithms are available to the person skilled in the art for codon optimization. A preferred method is the "guided random method based on a Monte Carlo algorithm available via the internet at genomes.urv.es/OPTIMIZER/ (P. Puigbò, E. Guzmán, A. Romeu, and S. Garcia-Vallve. Nucleic Acids Res. 2007 July; 35(Web Server issue): W126–W131).

As used herein the term "heterologous sequence" or "heterologous nucleic acid" is one that is not naturally found operably linked as neighboring sequence of said first nucleotide sequence. As used herein, the term "heterologous" may mean "recombinant". "Recombinant" refers to a genetic entity distinct from that generally found in nature. As applied to a nucleotide sequence or nucleic acid molecule, this means that said nucleotide sequence or nucleic acid molecule is the product of various combinations of cloning, restriction and/or ligation steps, and other procedures that result in the production of a construct that is distinct from a sequence or molecule found in nature. A recombinant oligonucleotide can be an oligonucleotide that comprises sequences from more than one single source.

"Operably linked" is defined herein as a configuration in which a control sequence is appropriately placed at a position relative to the nucleotide sequence coding for the polypeptide of the invention such that the control sequence directs the production/expression of the peptide or polypeptide of the invention in a cell and/or in a subject.

"Operably linked" may also be used for defining a configuration in which a sequence is appropriately placed at a position relative to another sequence coding for a functional domain such that a chimeric polypeptide is encoded in a cell and/or in a subject. Throughout this application, any nucleic acid sequence coding for an enzyme is preferably operably linked to another such sequence, or to a promoter.

As used herein, the term "promoter" refers to a nucleic acid fragment that functions to control the transcription of one or more nucleic acid molecules, located upstream with respect to the direction of transcription of the transcription initiation site of the nucleic acid molecule, and is structurally identified by the presence of a binding site for DNA-dependent RNA polymerase, transcription initiation sites and any other DNA sequences, including, but not limited to transcription factor binding sites, repressor and activator protein binding sites, and any other sequences of nucleotides known to one of skill in the art to act directly or indirectly to regulate the amount of transcription from the promoter. A "constitutive" promoter is a promoter that is active under most environmental and developmental conditions. An "inducible" promoter is a promoter that is active under environmental or developmental regulation.

Optional further elements that may be present in a nucleic acid construct according to the invention include, but are not limited to, one or more leader sequences, enhancers, integration factors, and/or reporter genes, intron sequences, centromeres, telomeres and/or matrix attachment (MAR) sequences. A nucleic acid construct according to the invention can be provided in a manner known per se, which generally involves techniques such as restricting and linking nucleic acids/nucleic

acid sequences, for which reference is made to the standard handbooks, such as Sambrook and Russel (2001) "Molecular Cloning: A Laboratory Manual (3rd edition), Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press.

5 Polypeptides

In a sixth aspect, the invention provides a polypeptide product expressed from the expression vector according to the invention. Features and definitions are provided elsewhere herein and are preferably those of the first, second, third, fourth and fifth aspect of the invention.

10 **Definitions**

"Sequence identity" or "identity" in the context of amino acid- or nucleic acid-sequence is herein defined as a relationship between two or more amino acid (peptide, polypeptide, or protein) sequences or two or more nucleic acid (nucleotide, polynucleotide) sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness
15 between amino acid or nucleotide sequences, as the case may be, as determined by the match between strings of such sequences. Within the present invention, sequence identity with a particular sequence preferably means sequence identity over the entire length of said particular polypeptide or polynucleotide sequence. The sequence information as provided herein should not be so narrowly construed as to require inclusion of erroneously identified bases. The skilled person is
20 capable of identifying such erroneously identified bases and knows how to correct for such errors. Preferred methods to determine identity are designed to give the largest match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Preferred computer program methods to determine identity and similarity between two sequences include e.g. the GCG program package (Devereux, J., et al., Nucleic Acids
25 Research 12 (1): 387 (1984)), BestFit, BLASTP, BLASTN, and FASTA (Altschul, S. F. et al., J. Mol. Biol. 215:403-410 (1990)). The BLAST X program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, MD 20894; Altschul, S., et al., J. Mol. Biol. 215:403-410 (1990)). The well-known Smith Waterman algorithm may also be used to determine identity.

30 Preferred parameters for polypeptide sequence comparison include the following: Algorithm: Needleman and Wunsch, J. Mol. Biol. 48:443-453 (1970); Comparison matrix: BLOSUM62 from Hentikoff and Hentikoff, Proc. Natl. Acad. Sci. USA. 89:10915-10919 (1992); Gap Penalty: 12; and Gap Length Penalty: 4. A program useful with these parameters is publicly available as the "Ogap" program from Genetics Computer Group, located in Madison, WI. The aforementioned parameters
35 are the default parameters for amino acid comparisons (along with no penalty for end gaps).

Preferred parameters for nucleic acid comparison include the following: Algorithm: Needleman and Wunsch, J. Mol. Biol. 48:443-453 (1970); Comparison matrix: matches=+10, mismatch=0; Gap Penalty: 50; Gap Length Penalty: 3. Available as the Gap program from Genetics Computer Group, located in Madison, Wis. Given above are the default parameters for nucleic acid comparisons.

40 Moderate conditions are herein defined as conditions that allow a nucleic acid sequences of at least

50 nucleotides, preferably of about 200 or more nucleotides, to hybridize at a temperature of about 45° C in a solution comprising about 1 M salt, preferably 6×SSC or any other solution having a comparable ionic strength, and washing at room temperature in a solution comprising about 1 M salt, preferably 6×SSC or any other solution having a comparable ionic strength. Preferably, the hybridization is performed overnight, i.e. at least for 10 hours, and preferably washing is performed for at least one hour with at least two changes of the washing solution. These conditions will usually allow the specific hybridization of sequences having up to 50% sequence identity. The person skilled in the art will be able to modify these hybridization conditions in order to specifically identify sequences varying in identity between 50% and 90%.

Expression will be understood to include any step involved in the production of the peptide or polypeptide including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification, and secretion.

In this document and in its claims, the verb "to comprise" and its conjugations is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. In addition, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one of the element is present, unless the context clearly requires that there be one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one".

The word "about" or "approximately" when used in association with a numerical value (e.g. about 10) preferably means that the value may be the given value (of 10) more or less 10% of the value. The sequence information as provided herein should not be so narrowly construed as to require inclusion of erroneously identified bases. The skilled person is capable of identifying such erroneously identified bases and knows how to correct for such errors. In case of sequence errors, the sequence of the enzymes obtainable by expression of the genes as represented by SEQ ID NO's 1, 3, 5, 7, 9, 11, 13, 15, 17, and 19 containing the enzyme encoding polynucleotide sequences should prevail.

All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

Examples

Example 1 - Enzymes and synthetic operons for the production of raspberry ketone

The inventors have introduced a specific four-enzyme pathway into *Escherichia coli* and *Corynebacterium glutamicum* to produce raspberry ketone. A biosynthetic pathway was designed to produce raspberry ketone (4-(4-Hydroxyphenyl)-2-butanone) from L-tyrosine in four steps (figure 1). The first dedicated step to the biosynthesis of raspberry ketone is the conversion of L-tyrosine to 4-coumaric acid (4-hydroxycinnamic acid) by the tyrosine ammonia lyase (TAL). In a second step the 4-coumarate-CoA ligase (4CL) catalyses activation of 4-coumaric acid to its CoA ester, 4-coumaroyl-CoA. In a third step a benzalacetone synthase (BAS) catalyses one-step decarboxylative condensation of 4-coumaroyl-CoA and malonyl-CoA to produce hydroxyphenylbutenone ((E)-4-(4-Hydroxyphenyl)-3-buten-2-one). In a final step for raspberry

ketone biosynthesis an enzyme with benzalacetone reductase enzyme activity (BAR) activity produces raspberry ketone from hydroxyphenylbutenone.

To produce 4-coumaric acid in *E. coli* or in *C. glutamicum* the following three TAL enzymes were used: TAL from *Rhodobacter capsulatus* (RcTAL; SEQ ID NO: 1, 2), TAL from *Saccharothrix*
5 *espanaensis* (SeSam8, SEQ ID NO: 3, 4), or TAL from *Flavobacterium johnsoniae* (FjTAL; SEQ ID NO: 5, 6). The following 4CL enzymes were used to produce 4-coumaroyl-CoA in *E. coli* or *C. glutamicum*: 4CL from *N. tabacum* (Nt4CL; SEQ ID NO: 7, 8), 4CL from *Arabidopsis thaliana* (At4CL; SEQ ID NO: 9, 10), 4CL from *Physcomitrella patens* (Pp4CL; SEQ ID NO: 11, 12) or 4CL from *Streptomyces coelicolor* (Sc4CL; SEQ ID NO: 13, 14). Two benzalacetone synthases were
10 used to produce hydroxyphenylbutenone in *E. coli* or *C. glutamicum*: BAS from *Rubus idaeus* (RiPKS; SEQ ID NO: 15, 16) or BAS from *Rheum palmatum* (RpBAS; SEQ ID NO: 17, 18). The benzalacetone reductase (BAR) enzyme from *Rubus idaeus* was used (RiBAR; SEQ ID NO: 19, 20). All genes except Nt4CL were codon optimised for expression in *C. glutamicum* and obtained through chemical synthesis. Nt4CL gene was obtained in plasmid pAC-4CL-STS (see Beekwilder
15 et al. 2006 Appl Environ Microbiol 72: 5670–5672) and was not codon optimised.

Subsequently, biosynthetic genes were assembled into 3-gene operons containing a BAS gene, a 4CL gene, and a TAL gene, in this respective order, using the Gibson assembly method. A combinatorial scheme was followed where the two BAS genes, four 4CL genes and three TAL genes were combined in a total of 24 possible combinations (Table 1).

20 Each of the biosynthetic genes was amplified by PCR. The amplification primers were designed to span the junction region between the vector and the insert or to span the junction between two adjacent genes. By this design overlapping regions were created in the PCR products that allow the plasmid assembly to proceed. Additionally, an intergenic spacer of 20 nucleotides was added containing a ribosomal binding site AGGAGG. The primers for the amplification of the genes are
25 listed in the sequence table (RiPKS: SEQ ID NOs: 71, 72, 73, 74, 75; RpBAS: SEQ ID NOs: 76, 77, 78, 79, 80; At4CL: SEQ ID NOs: 81, 82, 83, 84, 85; Pp4CL: SEQ ID NOs: 86, 87, 88, 89, 90; Sc4CL: SEQ ID NOs: 91, 92, 93, 94, 95; Nt4CL: SEQ ID NOs: 96, 97, 98, 99, 100; RcTAL: SEQ ID NOs: 101, 102, 103, 104, 105; FjTAL: SEQ ID NOs: 106, 107, 108, 109, 110; SeSam8: SEQ ID NOs: 111, 112, 113, 114, 115). This resulted in the generation of four different PCR products for
30 each BAS gene, 6 different PCR products for each 4CL gene and 4 different PCR products for each TAL gene.

The genes were amplified with a proof-reading Q5 High-Fidelity DNA Polymerase (from New England Biolabs, (NEB)). The PCR conditions were as follows: initial denaturation of 30 s at 98 °C was followed by thirty-five PCR cycles of 10 s at 98 °C, 20 s at 52 °C and 1 min at 72 °C and a final
35 extension of 2 minutes at 72 °C. The final concentration of PCR reagents was 1x Q5 reaction buffer (NEB), 400 µM dNTPs, 400 nM primers and 0.5 µL Q5 DNA polymerase (NEB) in a total reaction volume of 50 µL. The obtained PCR fragment was electrophoresed and subsequently purified from the agarose gel.

The *Escherichia coli*-*Corynebacterium glutamicum* shuttle vector pEC-XK99E (see Kirchner &
40 Tauch 2003 J Biotechnol 104:287–299) carrying the pGA1 origin of replication and the kanamycin

resistance marker (GenBank accession number: AY219683.1) was linearized by restriction with SacI and BamHI restriction enzymes (NEB). The linearized vector was electrophoresed and subsequently purified from the agarose gel. To create a circular plasmid containing the BAS-4CL-TAL operon 100 ng of the linearized vector was added to a reaction mix containing purified PCR
5 products of BAS, 4CL, and TAL synthetic genes, in 24 possible combinations, with a vector-insert ratio maintained at 1:3. Next, 1 x Gibson assembly mix (NEB) was added to the reactions and the total reaction volume was maintained at 20 µl. The reactions were incubated for 4 hours at 50 °C and subsequently transformed into chemically competent *E. coli* DH5α cells. Recombinant bacteria were selected on LB plates supplemented with 50 µg/ml kanamycin and 1% glucose. The
10 recombinant cells containing a BAS-4CL-TAL operon assembly of correct size were identified by colony PCR. A single bacterial colony was inoculated into 5 ml liquid LB medium supplemented by 50 µg/ml kanamycin and 1% glucose and grown overnight at 37 °C at 250 rpm. Plasmid DNA was isolated and the junction sites between the operon and the vector and the junction sites between the genes within the operon were sequenced by Sanger sequencing using a selection of vector and
15 gene specific primers. Strains of *E. coli* DH5α obtained by this procedure (Table 1) were maintained as glycerol stocks and were stored at -80 °C. Additionally, a strain of *E. coli* DH5α was generated containing the empty plasmid pEC-XK99E. This strain was named Ec_RK_EV.

Subsequently, the benzalacetone reductase from *R. ideaus* (RiBAR) was added to the operon. For
20 this purpose, plasmid pECXK_P (containing the RpBAS-Pp4CL-RcTAL operon, see figure 2) was linearized using Sall restriction enzyme (NEB). The linearized vector was electrophoresed and subsequently purified from the agarose gel. The RiBAR synthetic gene was amplified with a proof-reading Q5 High-Fidelity DNA Polymerase (NEB). The PCR conditions were as follows: initial denaturation of 30 s at 98 °C was followed by thirty-five PCR cycles of 10 s at 98 °C, 20 s at 52 °C
25 and 1 min at 72 °C and a final extension of 2 minutes at 72 °C. The final concentration of PCR reagents was 1x Q5 reaction buffer (NEB), 400 µM dNTPs, 400 nM primers and 0.5 µL Q5 DNA polymerase (NEB) in a total reaction volume of 50 µL. The obtained PCR fragment was electrophoresed and subsequently purified from the agarose gel. The amplification primers were designed to overlap with the adjacent RcTAL gene and the pEC-XK99E plasmid (RiBAR: SEQ ID
30 NO: 116, SEQ ID NO: 117). Additionally, a 20 bp intergenic region with a ribosomal binding site AGGAGG was added to the forward primer (SEQ ID NO: 116). To create a circular plasmid, 100 ng of the linearized plasmid pECXK_P was added to a reaction containing the purified RiBAR PCR product. Next, 1 x Gibson assembly mix (NEB) was added to the reaction and the total reaction volume was set at 20 µl. The reaction was incubated for 4 hours at 50 °C and subsequently
35 transformed into chemically competent *E. coli* DH5α cells. Recombinant bacteria were selected on LB plates supplemented with 50 µg/ml kanamycin and 1% glucose. The recombinant cells containing a plasmid comprising the RpBAS-Pp4CL-RcTAL-RiBAR operon assembly of correct size were identified by colony PCR. A single colony of transformed bacteria was inoculated into 5 ml liquid LB medium containing 50 µg/ml kanamycin and 1% glucose and grown overnight at 37 °C
40 at 250 rpm, and plasmid was isolated. The junction sites between the RiBAR gene and the RcTAL

gene and the RiBAR gene and the pEC-XK99E vector were confirmed by Sanger sequencing using vector and gene specific primers. The construct obtained by this procedure was named plasmid pECXK_PB (RpBAS-Pp4CL-RcTAL-RiBAR-pECXK; SEQ ID NO: 69, 70), see figure 3. The strain of *E. coli* DH5 α obtained by this procedure was named Ec_RK_PB and was maintained as glycerol stock and stored at -80 °C.

Table 1 - operons and vectors and strains for the production of raspberry ketone

Vector name (SEQ ID NO) ^a	Operon (SEQ ID NO) ^a	Corresponding strains	
		<i>E. coli</i>	<i>C. glutamicum</i>
pECXK_A (21)	RiPKS-At4CL-RcTAL (22)	Ec_RK_A	Cg_RK_A
pECXK_B (23)	RiPKS-At4CL-FjTAL (24)	Ec_RK_B	Cg_RK_B
pECXK_C (25)	RiPKS-At4CL-SeSam8 (26)	Ec_RK_C	Cg_RK_C
pECXK_D (27)	RiPKS-Pp4CL-RcTAL (28)	Ec_RK_D	Cg_RK_D
pECXK_E (29)	RiPKS-Pp4CL-FjTAL (30)	Ec_RK_E	Cg_RK_E
pECXK_F (31)	RiPKS-Pp4CL-SeSam8 (32)	Ec_RK_F	Cg_RK_F
pECXK_G (33)	RiPKS-Sc4CL-RcTAL (34)	Ec_RK_G	Cg_RK_G
pECXK_H (35)	RiPKS-Sc4CL-FjTAL (36)	Ec_RK_H	Cg_RK_H
pECXK_I (37)	RiPKS-Sc4CL-SeSam8 (38)	Ec_RK_I	Cg_RK_I
pECXK_J (39)	RiPKS-Nt4CL-RcTAL (40)	Ec_RK_J	Cg_RK_J
pECXK_K (41)	RiPKS-Nt4CL-FjTAL (42)	Ec_RK_K	Cg_RK_K
pECXK_L (43)	RiPKS-Nt4CL-SeSam8 (44)	Ec_RK_L	Cg_RK_L
pECXK_M (45)	RpBAS-At4CL-RcTAL (46)	Ec_RK_M	Cg_RK_M
pECXK_N (47)	RpBAS-At4CL-FjTAL (48)	Ec_RK_N	Cg_RK_N
pECXK_O (49)	RpBAS-At4CL-SeSam8 (50)	Ec_RK_O	Cg_RK_O
pECXK_P (51)	RpBAS-Pp4CL-RcTAL (52)	Ec_RK_P	Cg_RK_P
pECXK_Q (53)	RpBAS-Pp4CL-FjTAL (54)	Ec_RK_Q	Cg_RK_Q
pECXK_R (55)	RpBAS-Pp4CL-SeSam8 (56)	Ec_RK_R	Cg_RK_R
pECXK_S (57)	RpBAS-Sc4CL-RcTAL (58)	Ec_RK_S	Cg_RK_S
pECXK_T (59)	RpBAS-Sc4CL-FjTAL (60)	Ec_RK_T	Cg_RK_T
pECXK_U (61)	RpBAS-Sc4CL-SeSam8 (62)	Ec_RK_U	Cg_RK_U
pECXK_V (63)	RpBAS-Nt4CL-RcTAL (64)	Ec_RK_V	Cg_RK_V
pECXK_W (65)	RpBAS-Nt4CL-FjTAL (66)	Ec_RK_W	Cg_RK_W
pECXK_X (67)	RpBAS-Nt4CL-SeSam8 (68)	Ec_RK_X	Cg_RK_X
pECXK_PB (69)	RpBAS-Pp4CL-RcTAL-RiBAR (70)	Ec_RK_PB	Cg_RK_PB

a: the number in parentheses refers to the SEQ ID NO representing the vector or the operon

10 **Example 2 - Production of hydroxyphenylbutenone and raspberry ketone in *E. coli***

A single colony of strains Ec_RK_P and Ec_RK_PB was inoculated in 5 mL LB medium supplemented with 50 μ g/ml kanamycin and 1% glucose. The starter culture was grown overnight at 37 °C and 230 rpm. 200 μ l of the starting culture was used to inoculate 20 ml of 2xYT medium (16 g/L tryptone, 10 g/L yeast extract, 10 g/L NaCl) supplemented with 50 μ g/ml kanamycin in a 100 mL erlenmeyer flask and incubated at 37 °C and 230 rpm until the optical density at 600 nm (OD600 or A600) of 0.4 – 0.6 was reached. Subsequently, 1mM IPTG was added to the medium

and cultures were incubated at 30 °C at 250 rpm. Four fermentation flasks were set up in parallel for each bacterial strain, from which two were supplemented with 3mM 4-coumaric acid and, thus, in two no 4-coumaric acid was added. A fermentation experiment with a control strain Ec_RK_EV was set up in the same manner. Total bacterial culture was collected 24h after induction (IPTG) and
5 stored at -20 °C.

For the HPLC analysis 500 µl of 100% methanol was added to 500 µl of the bacterial culture. The mixture was mixed by vortexing for 20 s and subsequently sonicated for 10 min. Next, the extract was centrifuged for 15 min at 13.000 rpm in a table top centrifuge and the supernatant was filtered using a Minisart SRP4 syringe filter (Sartorius). 5 µl of the extract was used for HPLC analysis.

10 The HPLC system comprised a Waters e2695 HPLC, a Waters 2996 photodiodearray (PDA) detector, and a column incubator at 40 °C. The HPLC column used was a Luna 3u C18 (2) 100 A 150x2 mm (Phenomenex, CA). The eluents used were 0.1% formic acid in MQ water and 0.1% formic acid in acetonitrile. Separation of compounds in the extracts was conducted in a 40 min run during which a linear acetonitrile gradient was applied from 5 to 35% with a flow of 0.19 mL/min.
15 Compounds eluting from the column passed through a PDA detector (set at an absorbance range of 240-600 nm). The retention time and the maximum absorbance wavelength of the peaks in the fermentation samples was compared to authentic standards of 4-coumaric acid (Sigma), hydroxyphenylbutenone (Pfaltz & Bauer) and raspberry ketone (Apin Chemical Limited). The peak intensity of eluted compounds was analysed at the wavelengths of 280 nm for the detection of
20 raspberry ketone at the retention time of 21.9 min. The peak intensity of eluted compounds was also analysed at the wavelength of 312 nm for the detection of hydroxyphenylbutenone at retention time of 23.1 min and 4-coumaric acid at the retention time of 17.9 min.

In the cultures of strain Ec_RK_P where no 4-coumaric acid was added to the medium, *de novo* production of 4-coumaric acid was observed (Figure 4). 4-Coumaric acid was accumulated at the
25 54 ± 3 mg/L culture. Additionally, production of hydroxyphenylbutenone was observed at the concentration of 0.2 ± 0.0 mg/L bacterial culture (Figure 5). No raspberry ketone was observed in the HPLC chromatogram of the Ec_RK_P strain. For strain Ec_RK_PB accumulation of 4-coumaric acid at 49 ± 1 mg/L culture and hydroxyphenylbutenone at 0.1 ± 0.0 mg/L was observed. No raspberry ketone was detected by HPLC for strain Ec_RK_PB. For strain Ec_RK_EV, transformed
30 with the empty pEC-XK99E plasmid, no 4-coumaric acid, hydroxyphenylbutenone or raspberry ketone accumulation was observed, as expected.

In the *E. coli* cultures supplemented with 3mM 4-coumaric acid accumulation of 4-coumaric acid was observed for cultures Ec_RK_P, Ec_RK_PB and Ec_RK_EV at 328 ± 16 mg/L, 345 ± 26 mg/L and 303 ± 3 mg/L bacterial culture, respectively. Production of hydroxyphenylbutenone of 4.0 ± 0.1
35 mg/L and 3.9 ± 0.1 mg/L was observed for bacterial strains Ec_RK_P and Ec_RK_PB supplemented with 3mM 4-coumaric acid, respectively. No raspberry ketone production was observed for strains Ec_RK_P and Ec_RK_PB. No hydroxyphenylbutenone or raspberry ketone production was observed for strain Ec_RK_EV when supplemented with 4-coumaric acid, as expected.

From this experiment it was concluded that 4-coumaric acid accumulates in *E. coli* fermentation. The production of hydroxyphenylbutenone in *E. coli* was successful. To further investigate raspberry ketone production a more sensitive GC-MS analysis was employed.

For the GC-MS analysis 16 ml of bacterial culture of strains Ec_RK_P, Ec_RK_PB and Ec_RK_EV (not supplemented with 4-coumaric acid) was extracted with 4 ml ethyl acetate. The cultures and the solvent were mixed by vortexing for 20 s and were subsequently sonicated for 10 minutes. The cultures were centrifuged at 1200 rpm for 10 min and EtAc was collected in a new glass vial. The samples were dried using a Na₂SO₄ column. Analytes from 1 µL samples were separated using a gas chromatograph (5890 series II, Hewlett-Packard) equipped with a 30 m x 0.25 mm, 0.25 mm film thickness column (ZB-5, Phenomenex) using helium as carrier gas at flow rate of 1 ml/min. The injector was used in splitless mode with the inlet temperature set to 250 °C. The initial oven temperature of 45 °C was increased after 1 min to 300 °C at a rate of 10 °C/min and held for 5 min at 300 °C. The GC was coupled to a mass-selective detector (model 5972A, Hewlett-Packard). Raspberry ketone was identified by comparison of mass spectra and retention times (rt) with those of the authentic standard of raspberry ketone (Apin Chemical Limited). A trace amount of raspberry ketone was observed in the extract of strains Ec_RK_P and Ec_RK_PB. No raspberry ketone production was observed for strain Ec_RK_EV.

Example 3 - *C. glutamicum* strains for the production of raspberry ketone

Plasmids for raspberry ketone production were next transformed into electrocompetent cells of wild-type *C. glutamicum* strain ATCC13032. By this procedure 25 bacterial strains were created, which are listed in Table 1. Recombinant bacteria were selected on LB plates supplemented with 50 µg/ml kanamycin and 1% glucose after an incubation at 30 °C for two days. Additionally, the empty pEC-XK99E vector was transformed into *C. glutamicum* ATCC13032 and the obtained strain was named Cg_RK_EV. Glycerol stocks of strains obtained by this procedure were maintained at -80 °C.

Example 4 - Production of raspberry ketone in *C. glutamicum*

Starting cultures of *C. glutamicum* strains Cg_RK_P, Cg_RK_PB and Cg_RK_EV were grown for 48h in 25 mL LB medium supplemented with 50 µg/ml kanamycin and 1% glucose at 250 rpm and 30 °C. Starter cultures were centrifuged for 10 min at 5000 rpm and the bacterial pellet was resuspended in 1.5 ml CgXII minimal medium (see Eggeling & Reyes 2005). Subsequently, the cultures were transferred to 100 mL Erlenmeyer flasks containing 25 mL of CgXII minimal medium supplemented with 50 µg/ml kanamycin and 20 g/L D-glucose and were directly induced with 1mM of IPTG. Four fermentation flasks were set up in parallel for each bacterial strain, from which two were supplemented with 3mM 4-coumaric acid and in two no 4-coumaric acid was added. Bacterial cultures were cultivated at 30 °C and 250 rpm for 30 hours. After the fermentation the total bacterial culture was collected and stored at -20 °C until analysis.

Extraction of metabolites and HPLC analysis was conducted as described in Example 2. No accumulation of 4-coumaric acid was observed for *C. glutamicum* extracts for strains Cg_RK_P, Cg_RK_PB and Cg_RK_EV, both in cultures that were supplemented with 3mM 4-coumaric acid

and cultures where no 4-coumaric acid was added. This indicates that 4-coumaric acid was metabolised by *C. glutamicum*. For strain Cg_RK_P *de novo* production of hydroxyphenylbutenone was observed at 13.2 ± 0.0 mg/L when no 4-coumaric acid was added to the fermentation (figure 6). Production of hydroxyphenylbutenone at 3.7 ± 0.3 mg/L was observed for Cg_RK_P cultures supplemented with 3mM 4-coumaric acid. No hydroxyphenylbutenone was observed in *C. glutamicum* cultures of strain Cg_RK_EV. No raspberry ketone production was detected by HPLC for bacterial strains Cg_RK_P and Cg_RK_EV, neither for cultures supplemented with 3 mM 4-coumaric acid nor cultures with no added substrate.

For strain Cg_RK_PB, the production of hydroxyphenylbutenone was 7.1 ± 0.3 and 2.5 ± 0.2 mg/L for cultures with no added substrate and cultures supplemented with 4-coumaric acid, respectively. This corresponds to a reduction of hydroxyphenylbutenone production of 46% and 33% compared to strain Cg_RK_P. In addition, a clear peak of raspberry ketone was observed in HPLC for strain Cg_RK_PB. This indicates that addition of the BAR gene significantly increased the conversion of hydroxyphenylbutenone to raspberry ketone in *C. glutamicum*. The raspberry ketone production was further analysed by GC-MS using the protocol described in Example 2.

In the GC-MS analysis a predominant peak of raspberry ketone was observed in the extracts of Cg_RK_PB strains (figure 7). The production was quantified at 19 mg/L. No raspberry ketone was observed in the GC-MS analysis of strains Cg_RK_P and the strain Cg_RK_EV.

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Claims

[Claim 1]. A *Corynebacterium glutamicum* cell expressing a functional heterologous enzyme with tyrosine ammonium lyase (TAL) activity and further expressing, a functional 4-coumarate-CoA ligase (4CL) and a functional benzalacetone synthase (BAS), and further expressing a heterologous benzalacetone reductase (BAR), wherein the cell produces at least 5mg/L raspberry ketone when cultures in the absence of p-coumaric acid.

[Claim2]. The cell according to claim 1, wherein:

- the functional heterologous enzyme with TAL activity is from *Rhodobacter capsulatus*, *Saccharothrix espanaensis*, or *Flavobacterium johnsoniae*;
- the functional 4CL is 4CL from *Nicotiana tabacum*, *Arabidopsis thaliana*, *Physcomitrella patens* or *Streptomyces coelicolor*, or the functional BAS is BAS from *Rubus idaeus* or *Rheum palmatum*; and
- the BAR is from *Rubus idaeus*.

[Claim 3]. The cell according to claim 1, wherein:

- the functional heterologous enzyme with TAL activity is from *Rhodobacter capsulatus*;
- the functional 4CL is 4CL from *Physcomitrella patens* and the functional BAS is BAS from *Rheum palmatum*; and
- the BAR is from *Rubus idaeus*.

[Claim 4]. The cell according to claim 1, wherein:

- the functional heterologous enzyme with TAL activity has at least 60% sequence identity with SEQ ID NO: 1, SEQ ID NO: 3, or SEQ ID NO: 5, or is encoded by a polynucleotide which has at least 60% sequence identity with SEQ ID NO: 2, SEQ ID NO: 4, or SEQ ID NO: 6;
- the 4CL has at least 60% sequence identity with SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, or SEQ ID NO: 13, or is encoded by a polynucleotide which has at least 60% sequence identity with SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, or SEQ ID NO: 14;
- the BAS has at least 60% sequence identity with SEQ ID NO: 15 or SEQ ID NO: 17, or is encoded by a polynucleotide which has at least 60% sequence identity with SEQ ID NO 16 or SEQ ID NO: 18;
- the BAR has at least 60% sequence identity with SEQ ID NO: 19 or is encoded by a polynucleotide which has at least 60% sequence identity with SEQ ID NO: 20.

[Claim 5]. The cell according to claim 1, wherein the polynucleotide sequence encoding at least one of said enzymes is codon optimized.

[Claim 6]. The cell according to claim 1, wherein the cell is *Corynebacterium glutamicum* ATCC13032.

[Claim 7]. The cell according to claim 1, wherein at least two of the enzymes are encoded by a single recombinant polynucleotide construct.

[Claim 8]. The cell according to claim 1, wherein the cell is *Corynebacterium glutamicum* cell capable of producing at least twice as much L-Tyrosine as compared to *Corynebacterium glutamicum* ATCC13032.

5

[Claim 9]. A method for the production of a cell according to claim 1, comprising

- contacting a *Corynebacterium glutamicum* cell with an expression construct encoding a functional heterologous enzyme with tyrosine ammonium lyase (TAL) activity, and

10 functional 4-coumarate-CoA ligase (4CL) and a functional benzalacetone synthase (BAS), and

- contacting that *Corynebacterium glutamicum* cell with an expression construct encoding a heterologous benzalacetone reductase (BAR).

[Claim 10]. A method for the production of raspberry ketone, comprising:

15 - culturing a cell according to claim 1 under conditions conducive to the production of raspberry ketone and,

- isolating and/or purifying the raspberry ketone from the cell and/or the culture medium.