REVIEW

Functional food additives/ingredients production by engineered *Corynebacterium glutamicum*

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Abstract
Corynebacterium glutamicum is a microbial production host established in the industry 60 years ago. It is mainly used for production of feed and food amino acids. As *C. glutamicum* strain development has been cutting edge since its discovery, it has been engineered for production of a plethora of valuable products. This review will focus on recent developments of *C. glutamicum* strain engineering for biotransformation and fermentation processes towards flavor and fragrance molecules as well as pigments and sweeteners.

Keywords *Corynebacterium glutamicum* · Flavors · Fragrances · Pigments · Sweeteners

Introduction

Fermentation of foods is known since ages. Besides preservation, sensory changes are introduced. Alternatively, sensory molecules such as sweeteners, fragrance and flavor compounds can be added to food to ameliorate their sensory perception. Fermentation processes with *Corynebacterium glutamicum* and *Brevibacterium* species that were reclassified as *Corynebacterium* species, e.g., *B. flavum* to *C. glutamicum* ssp. *flavum* [1] are established in biotechnology for more than 50 years. They primarily targeted the flavor enhancing sodium salt of the amino acid L-glutamate (MSG) and ribonucleoside monophosphates such as inosine monophosphate (IMP) or guanosine monophosphate (GMP) since these contribute to the flavor umami. The reader is referred to excellent reviews on the *C. glutamicum*-based production of amino acids [2] and many other products [3]. By contrast, this review focusses for the first time on the *C. glutamicum*-based production of flavor and fragrance molecules such as indole and patchoulol as well as pigments such as astaxanthin and sweeteners such as xylitol. Successful metabolic engineering strategies are highlighted for

exemplary products and the key performance indicators of the established processes are listed in Table 1.

Flavors

MSG and ribonucleoside monophosphates

C. glutamicum is used at industrial scale to produce monosodium glutamate (MSG), the main component of the umami flavor. Fermentative production of L-glutamate started in late 1950s by Kyowa Hakko Kogyo Co Ltd. [26]. Nowadays, production of 3.2 million tons of L-glutamate/MSG by fermentation is estimated yearly, which accounts for over 40% of the amino acid market [2, 4]. Additionally, production of ribonucleoside monophosphates was demonstrated in *C. glutamicum*. Inosine monophosphate (IMP) and guanosine monophosphate (GMP) act synergistically with MSG to increase umami flavor. Microbial strain improvement for production of ribonucleoside monophosphates in different microorganisms such as *Corynebacterium, Bacillus*, or *E. coli* has recently been reviewed [27]. In *C. glutamicum*, deletion of conversion reactions from IMP to GMP and adenosine monophosphate (AMP) catalyzed by IMP-dehydrogenase (*guaB2*) and adenylosuccinate synthetase (*purA*) resulted in increased intracellular pool of IMP to 22 µmol g^{-1} CDW. Further engineering steps of deleting the glucose 6-phosphate isomerase decreased the IPM titer

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Table 1 Metabolically engineered *C. glutamicum* strains for production of functional food ingredients

Product	Production parameters	Heterologous genes used in metabolic engineering	References
MSG	T: 150 g L^{-1}	–	[4, 5]
IMP	C: 0.0076 mg g^{-1} CDW	–	[6]
Indole	T: 5.7 g L^{-1} (biotransformation from 10 g L^{-1} L-Trp) T: 0.7 g L^{-1}	Overexpression of the codon optimised tryptophanase *tna* from *Providencia rettgeri* Overexpression of the codon harmonised IGP lyase IGL$_{Ta}$ from *Triticum aestivum,* feedback resistant DAHP synthase *aroG*, feedback resistant anthranilate synthase *trpE* and anthranilate phosphoribosyl transferase *trpD* from *E. coli*	[7, 8]
L-theanine	T: 42 g L^{-1}; P: 1.95 g g^{-1} CDW (biotransformation from ethylamine)	Overexpression of codon optimized γ-glutamylmethylamide synthetase *gmaS* from *Methylovorus mays*	[9]
α-farnesene	T: 0.075 g L^{-1}	Overexpression of the codon-optimized farnesene synthase gene from *Malus* x *domestica*	[10]
α-pinene	T: 0.0002 g L^{-1} C: 0.000027 g g^{-1} CDW	Overexpression of the GPP synthase gene from *Abies grandies* and the pinene synthase gene from *Pinus taeda*	[11]
Patchoulol	T: 0.06 g L^{-1} P: 0.0008 g L^{-1} h^{-1}	Overexpression of *ispA* from *E. coli* with a patchoulol synthase gene from *Pogostemon cablin*	[12, 13]
(+)-valencene	T: 0.041 g L^{-1} P: 0.0015 g L^{-1} h^{-1}	Overexpression of *ispA* from *E. coli* and the valencene synthase gene from *Callitropsis nootkatensis*	[14, 15]
Vanillin	T: 0.31 g L^{-1}	Overexpression of feedback-resistant DAHP synthase *aroG* from *E. coli*, a product-resistant chorismate-pyruvate lyase *ubiC* from *E. coli*, carboxylic acid reductase *car* from *Nocardia iowensis,* codon optimised mutant catechol *O*-methyltransferase *comt^m* from *Rattus norvegicus*	[16]
Methyl anthranilate	T: 5.74 g L^{-1}	Overexpression of codon optimised anthranilic acid methyltransferase1 *aamt1* from *Zea mays,* feedback-resistant DAHP synthase *aroG* from *E. coli*	[17]
Astaxanthin	C: 0.003 g g^{-1} CDW T: 0.022 g L^{-1} P: 0.0004 g L^{-1} h^{-1}	Overexpression of lycopene β-cylase *crtY* from *Pantoea ananati*, artificial fusion enzyme CrtZ~W comprising β-carotene hydroxylase and β-carotene ketolase from *Fulvimarina pelagi*	[18, 19]
α-carotene	T: 1 g L^{-1}	Overexpression of mono-functional ε-cyclase and bi-functional β-cyclase gene from *Ostreococcus lucimarinus*	[20]
β-carotene	C: 0.0012 g g^{-1} CDW T: 0.085 g L^{-1} P: 0.0034 g L^{-1} h^{-1}	Overexpression of lycopene β-cylase *crtY* from *Pantoea ananatis*	[18]
Canthaxanthin	C: 0.001 g g^{-1} CDW	Overexpression of lycopene β-cylase *crtY* from *Pantoea ananatis*, β-carotene ketolase from *Fulvimarina pelagi*	[18]
Lycopene	C: 0.002 g g^{-1} CDW	–	[21]
Zeaxanthin	C: 0.0011 g g^{-1} CDW	Overexpression of lycopene β-cylase *crtY* from *Pantoea ananatis*, β-carotene hydroxylase from *Fulvimarina pelagi*	[18]
Allulose	T: 120 g L^{-1} (biotransformation from 400 g L^{-1} D-fructose)	Overexpression of D-allulose 3-epimerase gene from *Clostridium hylemonae*	[22]
Xylitol	T: 31 g L^{-1} (biotransformation from mixture of 10 g L^{-1} glucose, 10 g L^{-1} xylose and 10 g L^{-1} arabinose)	From xylose: gene for xylose reductase from *Rhodotorula mucilaginosa* from arabinose: genes for L-arabinose isomerase, D-psicose 3-epimerase and L-xylulose reductase from *Escherichia coli*, *Agrobacterium tumefaciens* and *Mycobacterium smegmatis*, respectively.	[23]
Squalene	C: 0.011 g g^{-1} CDW T: 0.135 g L^{-1}	Overexpression of genes for squalene synthase from *S. cerevisiae* and FPP synthase IspA from *E. coli*	[10]

Table 1 (continued)

Product	Production parameters	Heterologous genes used in metabolic engineering	References
CoQ10	T: 0.0012 g L^{-1}	Plasmid-borne expression of *ddsA from Rhodobacter sphaeroides*, the *E. coli* ubiquinone biosynthesis genes *ubiA* and as synthetic operons *ubiDIBX* and *ubiGHEF*, chromosomal expression of *ispA* from *E. coli*, replacement of native *ispB* by ddsA from *Pseudomonas denitrificans*; replacement of native genes *actA*, *vdh* and *pcaHG* by *ubiJK*, *aroG*^{D146N} and *ubiC*^{L31A} from *E. coli*, respectively	[24]
Nisin	T: 0.5 g L^{-1}	Expression of a synthetic operon with the genes *nisZ, nisB, nisT* and *nisC* of the nisin Z biosynthesis operon of *L. lactis* B1629	[25]

T Titer in (m)g L^{-1}, *Y* Yield in mg g^{-1}, *P* Volumetric productivity in mg L^{-1} h^{-1}, *C* Content in g g^{-1} CDW

and accumulation of the corresponding nucleobase hypoxanthine was observed [6].

Indole

Recently fermentative production of indole was demonstrated by *C. glutamicum*. Indole is characterized by its specific odor which is described as pungent, floral, with a fecal and animalic musty character. Indole is used as a flavoring of tea and dairy products and as a fragrance in perfumery. Natural indole has limited availability and a high price; therefore, fermentative production offers an attractive alternative. In nature, indole is synthesized from L-tryptophan (L-Trp) in a hydrolytic β-elimination reaction catalyzed by tryptophanases (TNAs) or from indole-3-glycerol phosphate (IGP) in a retroaldol cleavage catalyzed by enzymes with IGP lyase (IGL) activity. Both biosynthetic routes were used in *C. glutamicum* to establish fermentative indole production.

A bioconversion process to produce indole from supplemented L-Trp has been established by the co-expression of the native aromatic amino acid permease gene *aroP* and a bacterial tryptophanase (*tna*) [8]. TNAs hydrolyze L-Trp in a β-elimination reaction yielding indole, pyruvate and ammonia. By mining of bacterial genome databases an array of TNAs were retrieved and the highest production of indole at 0.9 g L^{-1} in *C. glutamicum* chassis strain C1* was achieved based on tryptophanase from *Providencia rettgeri*. However, the product toxicity was observed as a major limiting factor for indole production by *C. glutamicum*. Indole toxicity was studied in *C. glutamicum* ATCC 13032 and the chassis strain C1* and the C1* strain showed higher tolerance to indole compared to its ancestor [28, 29]. Indole toxicity was further circumvented by in situ product recovery (ISPR) by which product is sequestered in the organic layer and maintained at sub-toxic concentrations in the growth medium. Sequestering indole in a food-grade organic phase, dibutyl sebacate, during the fermentation resulted in complete conversion of L-Trp and indole titer of 5.7 g L^{-1}.

De novo production of indole in *C. glutamicum* from glucose was achieved in *C. glutamicum* by indole-3-glycerol phosphate lyases (IGLs) [7, 30] (Fig. 1). IGLs are considered to have evolved from the tryptophan synthase α-subunit (TSA). In tryptophan synthase (TS) two α- and two β-subunits are aligned. Indole is not released from TSA, but is efficiently channeled to tryptophan synthase β-subunit (TSB) for condensation of indole and L-serine to form L-Trp. In contrast, IGLs work as stand-alone enzymes and release free indole. Surprisingly, it was found that *C. glutamicum* TSA (*trpA*) functions as a *bona fide* IGL and can support fermentative indole production, while intrinsically low IGL activity of tryptophan synthase α-subunit (TSA) in the absence of the tryptophan synthase β-subunit was described. Bioprospecting of plant IGLs and bacterial TSAs resulted in identification of additional IGLs that could support fermentative indole production from IGP. Extensive shikimate pathway engineering was performed to increase IGP availability. Efficient conversion of anthranilate to IGP was achieved by deletion of chorismate mutase gene (*csm*) to avoid loss of the intermediate chorismate to the aromatic amino acids L-tyrosine and L-phenylalanine, by deletion of the L-glutamate export gene *yygB* and deletion of *trpL* which alleviates the endogenous trp operon from attenuation control. Using tributyrin as organic layer for in situ product recovery a final titer of 0.7 g L^{-1} indole was achieved by expressing either *C. glutamicum* TNA or IGL from wheat.

L-theanine

L-theanine is the most abundant free amino acid in leaves of tea plants (*Camellia sinensis*) and contributes to the flavor of green tea and shows health benefits. L-theanine is a *N*-ethylated L-glutamate derivative produced in plants by the L-theanine synthetase from L-glutamate and ethylamine. Recently, *C. glutamicum* as well as *E. coli* and *Pseudomonas putida* have been engineered for L-theanine production [9, 31, 32]. In *C. glutamicum* L-theanine
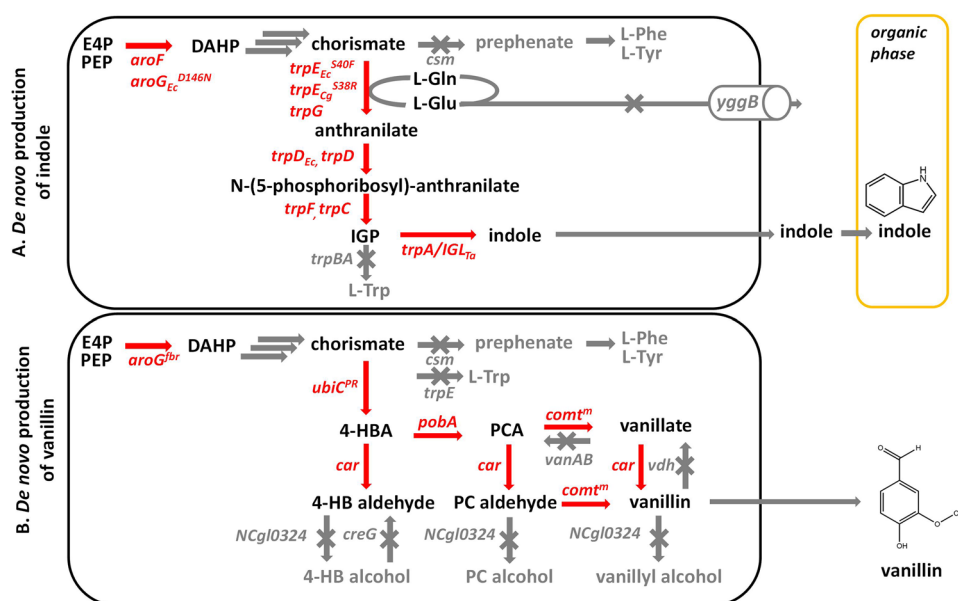
Fig. 1 Indole (**A**) and vanillin (**B**) biosynthesis in metabolically engineered *C. glutamicum*. **A** De novo biosynthetic pathway of indole and **B** de novo biosynthetic pathway of vanillin. Arrows depicted in red indicate gene overexpression, while gene deletions are depicted with grey crosses. The abbreviations are as follows: E4P, erythrose-4-phosphate; PEP, phosphoenolpyruvate; DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate; L-Phe, l-phenylalanine; L-Tyr,l- tyrosine; L-Gln, L-glutamine; L-Glu, L-glutamate; IGP, indole-3-glycerol phosphate; L-Trp, l-tryptophan; 4-HBA, 4-hydroxybenzoate; 4-HB aldehyde, 4-hydroxybenzaldehyde; 4-HB alcohol, 4-hydroxybenzyl alcohol; PCA, protocatechuate (3,4-dihydroxybenzoate); PC aldehyde, protocatechuic aldehyde; PC alcohol, protocatechuic alcohol; *aroF*, DAHP synthase; *aroG*$_{Ec}$^{D146N} and *aroG*^{fbr}, feedback-resistant DAHP synthase from *E. coli*; *csm*, chorismate mutase; *trpE,* anthranilate synthase; *trpE*$_{Ec}$^{S40F}, feedback-resistant *trpE* from *E. coli*; *trpE*$_{Cg}$^{S38R}, feedback-resistant *trpE* from *C. glutamicum*; *trpG*, anthranilate synthase component I; *yggB*, MscS-type mechanosensitive channel; *trpD*, anthranilate phosphoribosyltransferase; *trpD*$_{Ec}$, *trpD* from *E. coli*; *trpFC*, N-(5′-phosphoribosyl)anthranilate isomerase; *trpBA*, tryptophan synthase; IGL$_{Ta}$ IGP lyase from *Triticum aestivum*; *trpA*, tryptophan synthase α-subunit; *ubiC*^{PR}, a product-resistant chorismate-pyruvate lyase from *E. coli*; *pobA*, 4-hydroxybenzoate hydroxylase; *car*, carboxylic acid reductase from *Nocardia iowensis*; *creG*, NAD$^+$-dependent dehydrogenase; *comt*^{m}, a mutant catechol *O*-methyltransferase from *Rattus norvegicus*; *vanAB*, vanillate demethylase; *vdh*, vanillin dehydrogenase; *NCgl0324* aldehyde reductase. Adapted from [7, 16, 30]

production with γ-glutamyl methylamide (GMA) synthetase (GMAS, EC 6.3.4.12) which catalyzes the ATP-dependent ligation of L-glutamate and ethylamine was established in an industrial L-glutamate overproducing strain GDK-9 [9]. Production of ethylamine endogenously by expressing plant-derived L-serine/L-alanine decarboxylases was unsuccessful, therefore a process with exogenous supplementation of ethylamine was established. The highest production at 19 g L^{-1} was established by overexpression of the codon optimized *gmaS* from the methylotrophic bacteria *Methylovorus mays*. The production was further increased by the deletion of the endogenous L-glutamate exporter gene *yggB* (*NCg11221*), resulting in production titer of about 42 g L^{-1} with ethylamine supplementation in 5L bioreactor. In comparison, production of up to 17 g L^{-1} of L-theanine from ethylamine was described in *P. putida* using different carbon sources [31]. In *E. coli* 16 g L^{-1} of L-theanine was produced de novo in strains where a biosynthetic pathway to provide endogenous ethylamine supply via transamination of acetaldehyde was established [32].

Fragrances

Terpenoid fragrances

Corynebacterium glutamicum was also proven as a suitable host organism for the production of volatile terpenoids (Fig. 2). This bacterium naturally produces carotenoids (Fig. 3). Thus, platform strain engineering for terpenoid-derived fragrance compounds relies on the deletion of carotenogenic genes and/or the deletion of prenyltransferases that compete with the heterologous pathways. The isoprenoid precursor molecules isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) are synthesized in the methylerythritol phosphate (MEP) pathway that comprises nine enzymatic steps in *C. glutamicum* [10, 33–35]. Short-chain terpenoids such as α-farnesene, patchoulol, (+)-valencene and α-pinene, derive from farnesyl pyrophosphate (FPP) and geranyl pyrophosphate (GPP) for the latter one. Deletion of *idsA*, the major GGPP synthase gene as well as deletion

Fig. 2 Fragrance compounds being produced with metabolically engineered *C. glutamicum*. Structures of terpenoid- and aromatic-based fragrance molecules are depicted
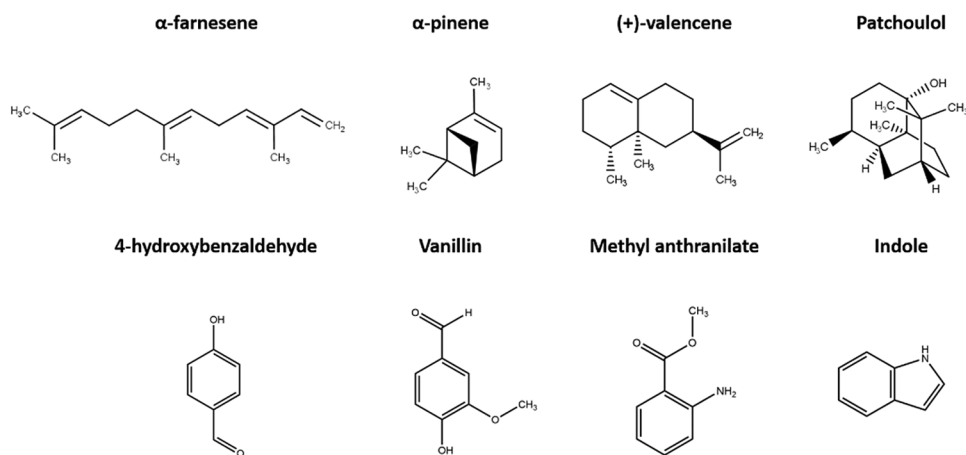
of the carotenogenic operon(s) was shown to be efficient for establishment of short-chain terpenoid production. α-Pinene production could be shown by overexpression of GPP synthase gene from *Abies grandies* and pinene synthase gene from *Pinus taeda* [11]. Production of α-pinene could be improved to 27 μg g^{-1} CDW by an enhanced precursor biosynthesis and isoprenyl pyrophosphate balancing, namely by *dxs* and *idi* overexpression [11]. FPP biosynthesis relied on the FPP synthase gene *ispA* from *E. coli*. α-farnesene production was enabled by codon-optimized overexpression of the farnesene synthase gene from cultivated apple [10]. Interestingly, besides *dxs* and *idi*, the second enzyme Dxr (DXP reductoisomerase) was shown to improve α-farnesene by twofold with maximal titers of 75 mg L^{-1} [10]. The fragrance compound (+)-valencene was the first sesquiterpenoid being produced by *C. glutamicum*. It was shown that overexpression of *ispA* from *E. coli* and valencene synthase gene from *Nootka cypress* resulted in the best (+)-valencene titer with 2.4 mg L^{-1} [15] that could be improved by induction of photo-caged IPTG instead of IPTG to maximal titers of 41 mg L^{-1} [14]. Production of patchoulol was shown by overexpression of *ispA* from *E. coli* with a patchoulol synthase gene from *Pogostemon cablin*. Although the patchoulol synthase gene was optimized for an alga L-production system, patchoulol titers of 0.46 mg L^{-1} were achieved in shake flask experiments from glucose and 0.25 mg L^{-1} from the alternative pentose sugar xylose [12, 13]. In this study maximal patchoulol titers were achieved in two-phase fed-batch fermentation with 50 mg L^{-1} [12, 13].

Vanillin

The aromatic aldehyde vanillin is an important flavoring compound. Several processes for vanillin production have been described starting from ferulic acid, eugenol,

isoeugenol or glucose [36]. The first successful de novo production of vanillin in *C. glutamicum* was recently established [16] (Fig. 1). Production of aldehydes in microbial platforms poses a great challenge due to their rapid reduction to corresponding alcohols via endogenous aldehyde reductase activity. Bioprospecting revealed 27 putative aldehyde reductases in the *C. glutamicum* genome. In this study, a *C. glutamicum* strain producing 4-hydroxybenzyl alcohol was used as the model strain. The deletion of gene *NCgl0324* strongly decreased reduction of 4-hydroxybenzaldehyde to 4-hydroxybenzyl alcohol in vivo. Next, a strain that produces protocatechuate (MA303) was engineered from 4-hydroxybenzoate-synthesizing *C. glutamicum* APS963. Vanillin production was engineered in strain MA303 by overexpressing of the vanillin/vanillyl alcohol biosynthetic pathway consisting of the carboxylic acid reductase from *Nocardia iowensis* (car) and a mutant catechol *O*-methyltransferase from *Rattus norvegicus* (comt^m) genes. Deletion of the aldehyde reductase *NCgl0324* in this strain resulted in the production of 0.31 g L^{-1} vanillin, while deletion of *NCgl0324* in related strains enabled production of 1.18 g L^{-1} protocatechuic aldehyde and 1.36 g L^{-1} 4-hydroxybenzaldehyde. Overcoming the observed product toxicity as e.g., vanillin accumulation lowered the specific growth rate, the final biomass and glucose consumption, holds the potential for further improvements.

Methyl anthranilate

Methyl anthranilate (MANT) is widely used to provide grape scent and flavor in cosmetics and food applications. Fermentative production of MANT was recently described in *E. coli* and *C. glutamicum* [17]. The key to the successful production in both hosts was the optimization of the expression of the anthranilic acid methyltransferase 1 *aamt1* gene from *Zea mays*. Additionally, the anthranilate supply was successfully increased by the expression of the feedback-inhibition
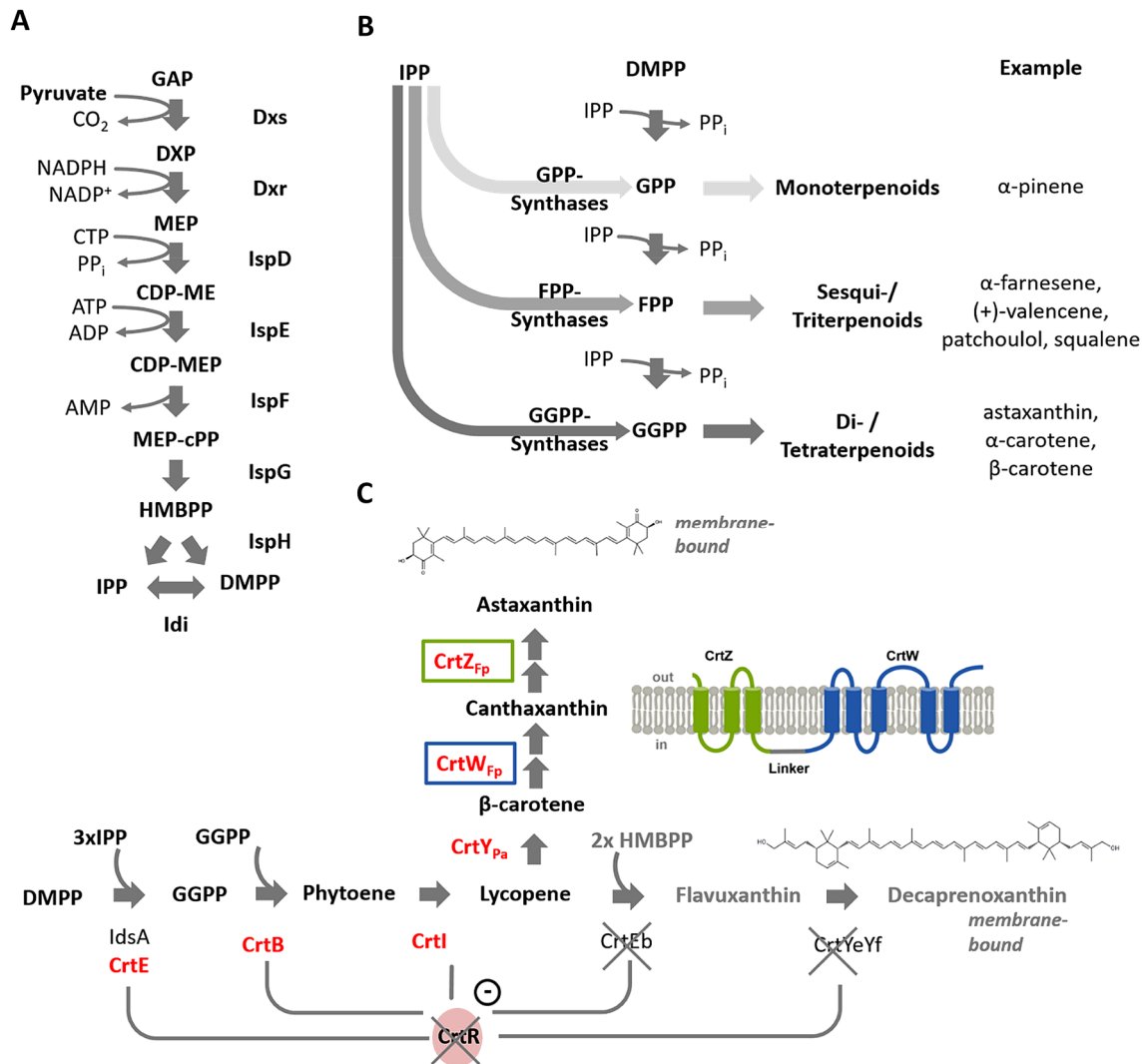
Fig. 3 Terpenoids biosynthesis in metabolically engineered *C. glutamicum*. MEP-pathway (**A**). Enzyme abbreviations are given right to the reactions. Metabolites are shown in bold, cofactors are shown on the left side of the respective reaction. (GAP: Glyceraldehyde 3-phosphate; DXP: 1-deoxy-D-xylulose 5-phosphate; MEP: 2-methylerythritol 4-phosphate; CDP-ME: 4-diphosphocytidyl-2-methylerythritol; CDP-MEP: 4-diphosphocytidyl-2-methylerythritol 2-phosphate; ME-cPP: 2-methylerythritol 2,4-cyclopyrophosphate; HMBPP: 4-hydroxy-3-methyl-2-(E)-butenyl-4-pyrophosphate; IPP: Isopentenyl pyrophosphate; DMPP: Dimethylallyl pyrophosphate). Enzymes: Dxs (1-deoxy-D-xylulose 5-phosphate synthase), Dxr (1-deoxy-D-xylulose 5-phosphate reductoisomerase), IspD (2-C-methylerythritol 4-phosphate cytidylyltransferase), IspE (2-C-methylerythritol 4-diphosphocytidyl kinase), IspF (2-C-methylerythritol 2,4-cyclopyrophosphate synthase), IspG (4-hydroxyl-3-methyl-2-(E)-butenyl pyrophosphate synthase), IspH (4-hydroxyl-3-methyl-2-(E)-butenyl pyrophosphate

reductase). Terpenoid biosynthesis (**B**). Biosynthesis of terpenoid intermediates GPP, FPP and GGPP on the basis of IPP and DMPP by GPP-, FPP- and GGPP synthases is illustrated. Different fragrance and pigment compounds are given as examples. Carotenoid biosynthesis (**C**). The biosynthesis of astaxanthin/decaprenoxanthin starting from the precursors IPP and DMPP are depicted. Red script indicates gene overexpressions, while gene deletions are depicted with grey crosses. Enzymes: IdsA/CrtE (GGPP synthase), CrtB (phytoene synthase), CrtI (phytoene desaturase), CrtEb (lycopene elongase), CrtYeYf (heterodimeric ε-cyclase), CrtY$_{Pa}$ (lycopene β-cyclase from *Pantoea ananatis*), CrtW$_{Fp}$ (β-carotene 4-ketolase from *Fulvimarina pelagi*), CrtZ$_{Fp}$ (β-carotene 3-hydroxylase from *F. pelagi*). The membrane fusion protein CrtZ~W (N-terminal CrtZ with 3 transmembrane helices and C-terminal CrtW with 5 transmembrane helices) is depicted

resistant mutant of 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase from *E. coli* (*aroG*^{S80F}). The availability of the cofactor *S*-adenosyl-L-methionine required by the AAMT1 was optimized by recycling of the SAM reaction product *S*-ribosyl-L-homocysteine (SAH) by activity of

SAH hydrolase *sahH*. Fed-batch production in a bioreactor using two-phase cultivation in which tributyrin was used as organic layer led to the production of 4.47 and 5.74 g L^{-1} MANT in *E. coli* and *C. glutamicum*, respectively. In comparison, in *Saccharomyces cerevisiae* production of 414 mg

L^{-1} methyl anthranilate was achieved [37]. Production of the related compound *N*-methyl anthranilate, a precursor of anticancer acridone alkaloids, in *C. glutamicum* was demonstrated at a final titer of 0.5 g L^{-1} [28, 29].

Pigments

C. glutamicum naturally produces the yellow carotenoid decaprenoxanthin. Decaprenoxanthin is a C50 carotenoid that derives from the isoprenoid precursor molecules isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). In *C. glutamicum*, the methylerythritol phosphate (MEP) pathway synthesizes IPP and DMAPP from the central metabolites pyruvate and glyceraldehyde 3-phosphate (GAP) [33–35]. The genome comprises a major GGPP synthase gene *idsA* (cg2384) [33–35], one major carotenogenic operon (*crtE-cg0722-crtB-crtI-crtYe-crtYf-crtEb*) (cg0723-cg0717) [38, 39] as well as a second functional phytoene synthase gene cg2672 and a glycosyltransferase gene *crtX* (cg0730) [33–35]. Carotenoid biosynthesis is regulated by the MarR-like regulator CrtR [21]. CrtR binds the promoter of the *crt* operon to repress its transcription. GGPP was identified as the major inducer of CrtR (shorter diphosphates interfered less with CrtR binding promoter DNA), thus regulating carotenoid biosynthesis in a metabolite dependent manner [21, 40]. This regulation indicates a feedforward regulatory mechanism of carotenogenesis: first isoprenoid pyrophosphates are synthesized that act as effector molecules on the repressor CrtR and therefore induce transcription of the terminal carotenoid biosynthetic genes.

Decaprenoxanthin represents a rare C50 carotenoid that is not used in industry so far. However, a wide range of C40 carotenoids such as astaxanthin, β-carotene, canthaxanthin, lutein, lycopene and zeaxanthin, are used in the food and feed industries for decades. β-carotene and astaxanthin are dominating the market but the latter one is expected to be the leading product in the future due to diverse applications ranging from coloration of fish feeds to health protection in nutraceutical and cosmetic products due to its excellent antioxidant properties [41]. *C. glutamicum* was engineered for natural production of astaxanthin (Fig. 3). First, the genes *crtEb*, *crtYe* and *crtYf* were deleted in a prophage-cured MB001 strain to prevent biosynthesis of decaprenoxanthin and thus yields accumulation of the central C40 carotenoid lycopene. Moreover, the precursor supply was improved by the chromosomal overexpression of *dxs* under the strong constitutive P*tuf* promoter [33–35]. 1-deoxy-5-xylulose phosphate synthase as the first enzyme in the MEP pathway plays a major role in the regulation of the MEP pathway [42–44]. It is long known that Dxs is feedback regulated by the products IPP and DMPP [43]. Moreover, the coordinated chromosomal overexpression of *crtE-crtB-crtI* as

an artificial operon from the P*tuf* promoter increased lycopene production [18]. Deregulation of the native carotenoid gene expression by deletion of *crtR* resulted in a threefold higher lycopene content (2 mg g^{-1} CDW) [21]. Based on endogenous lycopene production the production of cyclic C40 carotenoids can be established by overexpression of the lycopene β-cyclase CrtY. The lycopene cyclase CrtY from *Pantoea ananatis* is a non-redox flavoprotein that requires NAD(P)H for cyclization of ε-ends of lycopene to γ-carotene (one β-end) and β-carotene (two β-ends) [45]. β-carotene biosynthesis was proven to be very efficient in *C. glutamicum* with contents of up to 12 mg g^{-1} CDW [21]. The heterologous expression was established by a chromosomal integration under the P*tuf* promoter that performed as good as the plasmid-driven overexpression of *crtY* [18]. It is hypothesized that β-carotene in contrast to lycopene could be integrated in a coordinated manner in the plasma membrane and thus can be produced with higher contents.

Based on the β-carotene producing platform strain astaxanthin biosynthesis was shown by three different strategies in *C. glutamicum*. First, it was shown in a combinatorial approach that coexpression of bacterial *crtZ* and *crtW* results in higher astaxanthin contents when both genes were expressed with high translation initiation rates. Second, it could be shown that separate expression of *crtZ* and *crtW* on a two-plasmid system indeed resulted in a higher astaxanthin [18]. Third, the engineering of a membrane-bound fusion protein harboring CrtZ and CrtW yielded the highest astaxanthin content under high glucose concentration (4%) [19]. A robust astaxanthin production under high glucose concentration is necessary for as the process design towards economic relevance is in favor of high cell density fermentations. The fusion protein CrtZ~W comprises the N-terminal CrtZ (3 TMH) and C-terminal CrtW (5 TMH) that are linked by an artificial ten amino acid linker sequence [19]. Although the exact mechanism of the robustness under high glucose concentration is unknown, it was shown very clear that the translational fusion CrtZ~W performed more than twofold better than the transcriptional fusion of CrtZ and CrtW resulting in 22 mg L^{-1} astaxanthin in the mL scale [19]. It was hypothesized that membrane integrity, protein stability or intermediate channeling could explain the superior production properties of the membrane-fusion protein. The astaxanthin derived from *C. glutamicum* represents a natural alternative to algal-based astaxanthin with different product specificities that rely on its free astaxanthin form. Thus, astaxanthin but also other C40 carotenoid production with *C. glutamicum* gain interest from industrial partners to meet the demand for natural pigments.

Sweeteners

Xylitol

Polyols find application as artificial sweeteners with a rising demand due to diabetes and the global epidemic obesity. The polyols contain no calories and they are used e.g., in sugar-free chewing gum as they give rise to a cooling sensation in the mouth [46]. Polyols such as xylitol are poorly absorbed, thus, hardly affecting blood sugar levels, and they do not promote caries as the oral microbiota is unable to digest them. *C. glutamicum* has been engineered for conversion of xylose to xylitol by implementation of xylose reductase from *Rhodotorula mucilaginosa* [23]. Xylitol was also produced from arabinose based on heterologous expression of the genes for L-arabinose isomerase, D-psicose 3-epimerase and L-xylulose reductase from *Escherichia coli, Agrobacterium tumefaciens* and *Mycobacterium smegmatis*, respectively. By combining both approaches with expression of a pentose transport gene from *B. licheniformis*, 27 ± 0.3 g L^{-1} xylitol was produced from pentose rich, acid pre-treated liquor of sorghum stover [23].

D-allulose

The zero-calory sugar D-allulose (or D-psicose) exhibits 70% relative sweetness compared to D-sucrose. A whole-cell bio-transformation using *C. glutamicum* expressing D-allulose 3-epimerase (DAE) from *Clostridium hylemonae* allowed to produce 120 g L^{-1} of D-allulose from 400 g L^{-1} of D-fructose at 55 °C in 1.5 h [47]. The enzyme source appeared critical since DAE from *C. hylemonae* supported higher production as compared to DAE from *Flavonifractor plautii* [48] or a mixture of DAEs from *Paenibacillus senegalensis, Clostridium cellulolyticum*, and *Ruminococcus* sp. [22].

Further additives

Squalene

The triterpene squalene is valued as lipophilic antioxidant. Natural sources (including shark liver) are limited and its use in emulsion-based vaccine adjuvants recently boosted its demand. Upon expression of genes for squalene synthase from *S. cerevisiae*, FPP synthase IspA from *E. coli*, and endogenous Dxs and Idi, squalene production from glucose was enabled. When CRISPRi [49] was used to reduce transcription of the native GGPP synthase gene *idsA*, the highest product titer (0.1053 g L^{-1}) was observed in Biolector cultures with CGXII medium containing 15 g L^{-1} tryptone and

40 g L^{-1} glucose [50]. Additional expression of endogenous *ispDF* increased the titer to about 0.14 g L^{-1} [10].

CoQ10

Coenzyme Q10 (CoQ10) is a lipid-soluble compound with important physiological functions and is sought after in the food and cosmetic industries owing to its antioxidant properties [51]. The menaquinone (MKn) containing *C. glutamicum*, which does not naturally synthesize any CoQ, was engineered for CoQ10 synthesis (Fig. 4). Prior knowledge on overproduction of aromatic compounds and prenyls provided a base strain overproducing both precursors, aromatic 4-hydroxybenzoate via the shikimate pathway and decaprenyl diphosphate via the MEP pathway [52]. Two aspects were important. First, expression of the genes encoding the Ubi complex accessory factors UbiJ and UbiK in addition to the *ubi* genes for the CoQ10 biosynthesis enzymes from *E. coli* improved production of CoQ isoprenologs from CoQ8 to CoQ11. Second, the replacement of the native polyprenyl diphosphate synthase IspB with a decaprenyl diphosphate synthase (DdsA) increased the CoQ10 titer, while the abundance of the unwanted isoprenologs CoQ8, CoQ9, and CoQ11 was reduced significantly [24].

Nisin

The ribosomally synthesized peptide antibiocin nisin shows antimicrobial activity against many Gram-positive bacteria including human pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus* strains. This generally regarded as safe compound, which is approved by FDA and EFSA, is widely used in food preservation [53]. While nisin is typically produced using natural *Lactococcus lactis* strains, *C. glutamicum* has been engineered for synthesis, secretion, and maturation of nisin [25]. To this end, first, a synthetic operon comprising the genes *nisZ, nisB, nisT* and *nisC* of the nisin Z biosynthesis operon of *L. lactis* B1629 was expressed. The resulting pre-nisin required maturation, which was achieved by using a soluble form of the membrane-anchored *L. lactis* B1629 NisZ protease. LC–MS analysis revealed that about 0.5 g L^{-1} nisin was produced [25]. *C. glutamicum* was also engineered for production of other antimicrobial peptides, such as pediocin [54].

Concluding remarks and further directions

Corynebacterium glutamicum processes were successfully scaled to bioreactors with working volumes of several thousand cubic meters for the million-tons per year amino acid production. However, the titers, rates and yields reported here for most of the functional food additives/ingredients
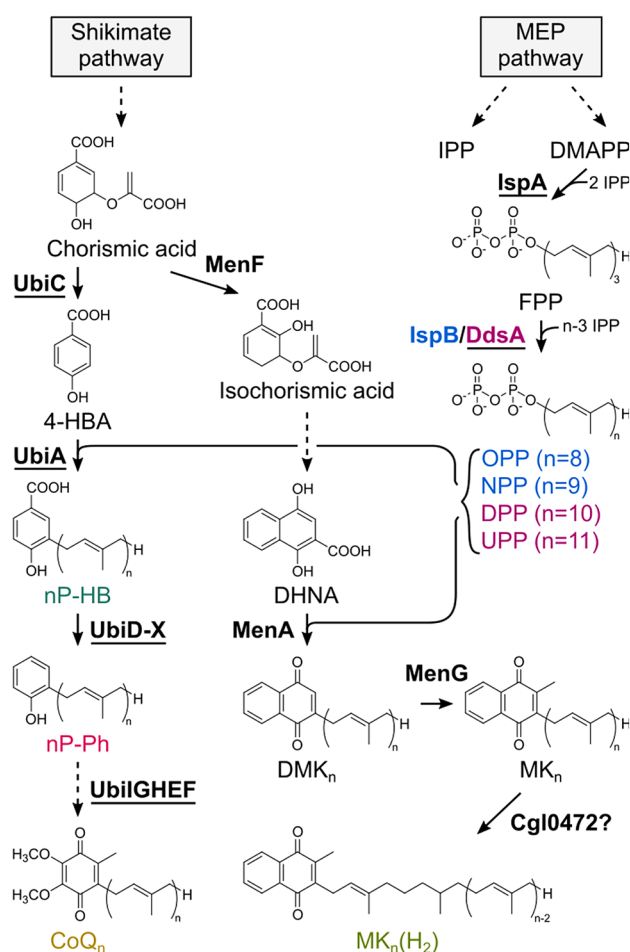
Fig. 4 Metabolic pathway of CoQn and MKn(H2) biosynthesis in engineered *C. glutamicum*. Enzymes are in bold, heterologous enzymes are underlined. As IspB mainly synthesizes NPP and OPP and DdsA mainly synthesizes DPP and UPP, the enzymes and corresponding direct products were marked with matching colors. The question mark indicates that the reaction attributed to Cgl0472 is not experimentally proven. MEP, methylerythritol phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; OPP, octaprenyl diphosphate; NPP, nonaprenyl diphosphate; DPP, decaprenyl diphosphate; UPP, undecaprenyl diphosphate; 4-HBA, 4-hydroxybenzoic acid; nP-HB, 3-n-prenyl-4-hydroxybenzoic acid; nP-Ph, 2-n-prenylphenol; CoQn, coenzyme Qn/ubiquinone-n; DHNA, 1,4-dihydroxy-2-naphthoic acid; DMKn, demethylmenaquinone-n; MKn, menaquinone-n; MKn(H2), dihydromenaquinone-n; IspA, farnesyl diphosphate synthase; IspB, polyprenyl diphosphate synthase; DdsA, decaprenyl diphosphate synthase; UbiC, chorismate-pyruvate lyase; UbiA, 4-hydroxybenzoate octaprenyltransferase; UbiD-X, 3-octaprenyl-4-hydroxybenzoate decarboxylase and flavin prenyltransferase; UbiIGHEF, 2-octaprenylphenol hydroxylase, 2-octaprenyl-6-hydroxyphenol/2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinol methyltransferase, 2-octaprenyl-6-methoxyphenol hydroxylase, ubiquinone/menaquinone biosynthesis methyltransferase, 2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinol hydroxylase; MenF, isochorismate synthase; MenA, 1,4-dihydroxy-2-naphthoate octaprenyltransferase; MenG, demethylmenaquinone methyltransferase; Cgl0472, putative menaquinone oxidoreductase. Reproduced from [24]

have to be improved to achieve and maintain viable economics. This may be achieved on the enzyme, cell or process level. Regarding the enzymes required for optimal pathway operation, new enzymes or variants may be identified by bioprospecting as shown for the IGL and TNA enzymes used for indole production [7, 8, 30]. Enzyme engineering has, for example, been applied to enzymes of aromatic biosynthesis in order to change the substrate scope [55] or alleviating product inhibition as recently shown for 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase [56] and chorismate-pyruvate lyase [57]. Enzyme fusions have been shown to improve, for example, astaxanthin production [19]. These enzyme-based approaches will certainly benefit from combining computational modelling with mechanistic experiments [58] and the rapidly increasing number of protein structures predicted by alphafold [59]. Machine learning has already been applied to enzyme evolution [60] and will be important to come to new-to-nature reactions [61], which eventually may be important to improve production of functional food additives/ingredients. In strain engineering, we see potential in the increasing number and versatility of CRISPR methods [62], including base editors for introduction of SNPs [63]. In this respect, it has to be noted that CRISPR methods can be multiplexed [64]. Genetically encoded biosensors can be used to identify mutants with improved production [65] or enzyme variants [66], or to dynamically control gene expression [7, 30, 67]. The design and application of microbial consortia is an option for division of labor, e.g., access to substrate by one bacterium and conversion to the target product by another [68]. Process intensification makes sure that processes are stable when scaled up and often involve medium optimization. For volatile products, two-layer approaches for in situ product removal (ISPR) offer to reach higher titers by avoiding possible inhibitory effects of the product on the catalyst cell. This concept has been employed, for example, in indole production [8]. The vigorous stirring in stirred tank bioreactors helps partitioning of the product into the organic layer as shown for patchoulol [12, 13]. For cell-bound products, process optimization aims at increasing biomass titers to high-cell density while maintaining high product content within these cells. Sometimes, it is of benefit to co-produce a cell-bond product such as astaxanthin and a secreted product such as lysine [12, 13] when targeting the same application, in this case animal feed additives. For secreted products, partitioning into a second phase is often not possible. One way to improve titers of a growth-inhibitory product is the selection of more tolerant strains as was applied to alleviate indole toxicity [28, 29]. Downstream processing has to comply with two aims: optimal product recovery and compatibility with regulatory issues of uses as food additives or ingredients. *C. glutamicum*, established for decades as excellent producer of food additives such as MSG, has recently been

engineered for production of a wide variety of food additives and ingredients. We anticipate that this development takes up more momentum. First, production scales with *C. glutamicum* belong to the largest known in biotechnology. Second, its products are classified as GRAS and regulatory issues have been taken care of given the established amino acid production processes. Thus, with respect to the production of food additives and ingredients, *C. glutamicum* is competitive with *E. coli* and Baker's yeast. The market and the consumers, thus, have more choices and may rely on better supply reliability.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Informed consent All authors agree with their participation in this paper.

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