Advances in Heterologous Synthesis of Terpenoids in Corynebacterium glutamicum (Postprint)

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Abstract

Terpenoid compounds possess considerable commercial value; however, their production processes are complex and yields are low, which has made microbial heterologous synthesis of terpenoids a research hotspot. Corynebacterium glutamicum harbors pathways for terpenoid pigment synthesis, conferring natural advantages and promising research prospects for the heterologous production of terpenoid compounds. This review represents the first comprehensive overview of terpenoid synthesis in C. glutamicum, providing an introduction to the pathway from three perspectives: terpenoid biosynthetic routes, key enzymes, and global regulatory mechanisms. It summarizes the heterologous synthesis of monoterpenes, sesquiterpenes, and tetraterpenes in C. glutamicum, discusses the challenges that must be addressed for efficient terpenoid production using this organism, and offers recommendations for the high-efficiency synthesis of terpenoid compounds in C. glutamicum.

Full Text

Preamble

Progress of Heterologous Biosynthesis of Terpenoids in Engineered *Corynebacterium glutamicum*

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Abstract

Terpenoids possess considerable commercial value, but their production processes are complex and yields are low. Microbial heterologous synthesis of terpenoids has emerged as a research hotspot. *Corynebacterium glutamicum* contains a native pathway for synthesizing terpenoid pigments, conferring natural advantages and promising research prospects for heterologous terpenoid production. This review provides the first comprehensive examination of terpenoid synthesis in *C. glutamicum*, introducing the pathway from three perspectives: the terpenoid synthesis pathway itself, key enzymes, and global regulatory mechanisms. We summarize advances in heterologous synthesis of monoterpenes, sesquiterpenes, and tetraterpenes in *C. glutamicum*, discuss challenges that must be addressed for efficient synthesis, and offer recommendations for improving terpenoid production in this organism.

Keywords: *Corynebacterium glutamicum*, terpenoids, heterologous biosynthesis

1. Biosynthesis Pathway of Terpenoids in *Corynebacterium glutamicum*

The terpenoid biosynthesis pathway in *C. glutamicum* is illustrated in [Figure 1: see original paper]. This bacterium possesses a complete MEP pathway and all downstream genes required for synthesizing its native C50 carotenoid, decaprenoxanthin. In the MEP pathway, glyceraldehyde-3-phosphate and pyruvate undergo seven enzymatic steps to generate isopentenyl pyrophosphate (IPP), which is isomerized to dimethylallyl pyrophosphate (DMAPP) by the isomerase IDI. The geranylgeranyl pyrophosphate synthase (GGPPS) encoded by *idsA* and *crtE* then catalyzes the condensation of IPP and DMAPP to form geranylgeranyl pyrophosphate (GGPP) [11]. In wild-type *C. glutamicum* ATCC13032, the decaprenoxanthin synthesis genes are organized in two clusters. The first cluster contains *crtE*, *cg0722*, *crtB*, *crtI*, *crtYe*, *crtYf*, and *crtEb*, encoding GGPPS, a putative RND superfamily drug export protein, phytoene synthase, phytoene desaturase, carotenoid C45/C50 -cyclases, and a lycopene elongase, respectively. The second cluster contains *crtB2*, *crtI2-1*, and *crtI2-2*, where *crtB2* encodes a second phytoene synthase, while the remaining two genes lack phytoene desaturase activity. Through the action of enzymes encoded by both gene clusters, IPP and DMAPP are ultimately converted to decaprenoxanthin [12].

1.1 Key Enzymes in the Terpenoid Precursor Synthesis Pathway

Deoxyxylulose-5-phosphate synthase (DXS) is the key rate-limiting enzyme in the upstream MEP pathway. Overexpression of *dxs* using two different approaches both increased intracellular DXS levels and enhanced lycopene accumulation in *C. glutamicum*. When *dxs* was overexpressed using an IPTG-inducible promoter on a plasmid, lycopene content increased from 0.04 ± 0.01 mg/g CDW

to 0.06 ± 0.01 mg/g CDW. Replacement of the native *dxs* promoter with the strong promoter of the elongation factor EF-Tu (*tuf*) in the genome resulted in a two-fold increase, reaching 0.08 ± 0.01 mg/g CDW. Notably, co-overexpression of other MEP pathway genes with *dxs* showed no significant synergistic effects [13].

IspG and IspH are two iron-sulfur proteins in the MEP pathway [14,15]. IspH catalyzes the formation of IPP and DMAPP in unequal amounts [16], and overexpression of *idi* in *C. glutamicum* LYC3-MEP doubled lycopene production compared to the parent strain [13]. In *C. glutamicum*, IdsA serves as the primary GGPPS, while CrtE plays a supplementary role. The optimal catalytic temperatures for IdsA and CrtE are 30–35°C and 25°C, respectively. Both enzymes can condense IPP with DMAPP, geranyl pyrophosphate (GPP), or farnesyl pyrophosphate (FPP) to synthesize GGPP, with IdsA showing highest efficiency using IPP and DMAPP as substrates, and CrtE preferring GPP and IPP. Overexpression of *idsA* leads to lycopene accumulation, while co-overexpression of *idi* to balance intracellular IPP and DMAPP pools can alleviate this accumulation [11].

1.2 Global Regulatory Mechanisms in Terpenoid Biosynthesis

As subunits of RNA polymerase holoenzyme, σ factors are crucial for promoter recognition and transcription initiation. Modulating σ factor expression can enhance product tolerance and yield [17]. Taniguchi et al. [18] overexpressed seven σ factor-encoding genes in *C. glutamicum* and found that *sigA* overexpression increased lycopene production eight-fold. In the wild-type strain, *sigA* overexpression doubled decaprenoxanthin production during stationary phase. In the *sigA*-overexpressing strain, transcription levels of genes involved in thiamine synthesis and aromatic compound degradation were elevated. Thiamine is an essential cofactor for DXS [19], and supplementation with 10 µg/L thiamine or 300 mg/L protocatechuic acid increased decaprenoxanthin production by 10% and 40%, respectively. Deletion of *sigB* increased lycopene production five-fold.

Most multiple antibiotic resistance (MarR) family transcriptional regulators function as repressors. In *Mycobacterium marinum*, *crtR* regulates *crt* operon transcription, and transposon insertion inactivation of *crtR* increased decaprenoxanthin accumulation three-fold [20]. *C. glutamicum* contains a *crtR* ortholog located approximately 200 bp upstream of *crtE*. Henke et al. [21] demonstrated that *crtR* deletion increased *crt* operon transcription 10- to 70-fold and boosted decaprenoxanthin accumulation 10- to 30-fold. Mechanistic studies revealed that CrtR binds to the *crt* operon promoter region between the -10 and -35 elements, located in the intergenic region between *crtR* and *crtE*.

2. Specific Applications of Terpenoid Synthesis Pathways in *C. glutamicum*

The intermediate metabolites IPP and DMAPP in the *C. glutamicum* carotenoid biosynthesis pathway serve as universal precursors for terpenoid synthesis. Geranyl pyrophosphate, farnesyl pyrophosphate, and geranylgeranyl pyrophosphate are direct precursors for monoterpenes, sesquiterpenes, and diterpenes, respectively. Elucidation of this pathway provides the theoretical foundation for heterologous terpenoid synthesis in *C. glutamicum*. Recent years have witnessed significant progress in using *C. glutamicum* as a chassis for terpenoid production, which we discuss below from three perspectives: monoterpenes, sesquiterpenes, and tetraterpenes.

2.1 Monoterpenes

Monoterpenes are abundant in plant essential oils and exhibit antimicrobial, anti-inflammatory, and anticancer activities, making them valuable in food, pharmaceutical, and cosmetic industries [22]. Geranyl pyrophosphate (GPP) is the direct precursor for monoterpenes, synthesized by geranyl pyrophosphate synthase (GPPS). In *C. glutamicum*, both *crtE* and *idsA* encode geranylgeranyl pyrophosphate synthase (GGPPS), though no studies have reported whether these enzymes release the intermediate GPP.

Kang et al. [23] engineered *C. glutamicum* ATCC13032 for heterologous pinene synthesis. Expression of pinene synthase alone failed to produce detectable pinene, but co-expression of pinene synthase from *Pinus taeda* and GPPS from *Abies grandis*, combined with overexpression of *dxs* and *idi* to enhance IPP and DMAPP supply, yielded 27 ± 7 µg/g CDW of pinene. Given the general antimicrobial activity of monoterpenes, the authors investigated pinene toxicity and found that adding 20% n-dodecane as an in situ extraction phase eliminated pinene' s growth inhibition of *C. glutamicum*.

2.2 Sesquiterpenes

Sesquiterpenes are widely distributed in plants and commonly exist as alcohols, ketones, and lactones in essential oils, representing the high-boiling-point fraction with aromatic and biological activities. Farnesyl pyrophosphate (FPP) is their universal precursor, though no evidence indicates that *C. glutamicum* CrtE or IdsA can synthesize or release FPP.

(+)-Valencene is an aromatic compound in citrus fruits used in flavoring and beverages that can be oxidized to nootkatone, a high-value grapefruit-flavored fragrance [24]. Frohwitter et al. [25] successfully constructed a heterologous (+)-valencene synthesis pathway in wild-type *C. glutamicum* ATCC13032. Expression of valencene synthase alone produced no detectable product, suggesting limited FPP precursor supply. However, replacing *crtE* and *idsA* with *ispA* from *E. coli* and *ERG20* from *Saccharomyces cerevisiae* enabled valencene production.

In a strain lacking *crtE* and *idsA* and co-expressing *ispA* and valencene synthase from *Chamaecyparis nootkatensis*, valencene reached 2.41 ± 0.26 mg/L (0.25 ± 0.03 mg/g CDW). Similar to monoterpenes, sesquiterpenes inhibit cell growth. Transcriptomic analysis revealed that n-dodecane extraction minimally affected *C. glutamicum* growth while alleviating valencene toxicity. Comparative transcriptomics under valencene stress suggested that the protein encoded by *mmpl2* may export intracellular valencene, reducing its inhibitory effects.

2.3 Tetraterpenes

Tetraterpenes contain eight isoprene units, with many natural pigments like lycopene, β-carotene, and astaxanthin belonging to this class. Carotenoids have high commercial value in food and feed industries [26]. *C. glutamicum* naturally produces the glycosylated C50 carotenoid decaprenoxanthin through its endogenous terpenoid synthesis pathway [19].

2.3.1 Lycopene Lycopene, a red pigment from tomatoes, exhibits unique biological activity and cardiovascular disease prevention benefits, making it widely applicable in food and pharmaceutical industries [27]. In *C. glutamicum*, lycopene is synthesized from GGPP in two steps. Heider et al. [12] deleted *crtEb* to block lycopene conversion to decaprenoxanthin, achieving 0.03 ± 0.01 mg/g CDW lycopene accumulation. Overexpression of the complete pathway from GGPP (*crtE*, *crtB*, *crtI*) increased production 80-fold to 2.4 ± 0.3 mg/g CDW. Further optimization of IPP precursor supply by overexpressing MEP pathway genes in *C. glutamicum* MB001Δ*crtYeYfEb* raised lycopene to 0.08 ± 0.02 mg/g CDW [13]. Matano et al. [28] engineered *C. glutamicum* MB001Δ*crtYEB* to utilize N-acetylglucosamine, achieving 17.4 ± 0.4 mg/g CDW lycopene. Hadiati et al. [29] constructed strains capable of using hexuronic acids, producing 0.7 ± 0.1 mg/g CDW from galacturonic acid and 0.8 ± 0.3 mg/g CDW from glucuronic acid.

2.3.2 Astaxanthin Astaxanthin is a red C40 carotenoid with exceptional antioxidant activity due to its unique molecular structure, making it valuable in nutraceuticals, cosmetics, and pharmaceuticals [30]. Heider et al. [13] introduced lycopene cyclase gene *crtY*, β-carotene ketolase gene *crtW*, and hydroxylase gene *crtZ* into a lycopene-accumulating *C. glutamicum* strain, producing 1.2 ± 0.5 mg/g CDW astaxanthin. Henke et al. [31] further optimized astaxanthin production to 1.7 ± 0.3 mg/g CDW by balancing *crtW* and *crtZ* expression through combinatorial variation of ribosome binding sites, intergenic distances, and translation start codons—comparable to astaxanthin yields from microalgae.

3. Advances in Genetic Tools and Regulation

3.1 Novel Induction Systems

Inducible expression systems are commonly used for heterologous protein production, but traditional approaches require extensive optimization of induction timing and inducer concentration. Novel light-inducible systems using photocaged IPTG offer advantages including easy automation and reduced contamination risks [32]. Binder et al. [33] employed such a system to control (+)-valencene synthesis in *C. glutamicum*, achieving a six-fold increase in production to 41.0 mg/L.

3.2 Genome Editing and Transcriptional Regulation

Heterologous terpenoid synthesis often requires introducing numerous foreign genes, imposing metabolic burdens on the host. Simple and efficient methods to tune pathway gene expression are essential for maximizing productivity while minimizing burden. CRISPR-based technologies have developed rapidly to address this need. Kim et al. [34] used CRISPR interference for transcriptional regulation of bisabolol and lycopene synthesis in *E. coli*, successfully increasing yields. Yu et al. [35] established a CRISPR-Cpf1 system in *C. glutamicum* and achieved saturated mutagenesis at the G149 site of γ-glutamyl kinase to relieve L-proline inhibition, providing a foundation for applying this technology to transcriptional regulation of terpenoid synthesis.

4. Conclusions and Prospects

The aforementioned advances demonstrate that *C. glutamicum* has achieved considerable success as a microbial chassis for tetraterpene synthesis. However, production of monoterpenes, sesquiterpenes, and triterpenes remains in its infancy, with numerous challenges to overcome.

For instance, although monoterpenes and sesquiterpenes have been successfully synthesized, whether CrtE and IdsA can synthesize or release GPP or FPP requires further investigation. Mutating *ERG20* in *S. cerevisiae* to release free GPP significantly enhanced monoterpene production [36], and similar mutations in *crtE* and *idsA* may yield comparable results. Current precursor supply research has focused solely on the MEP pathway; introducing heterologous MVA pathways represents a promising alternative. Liu et al. increased squalene production in *E. coli* 4.87-fold by introducing the upstream MVA pathway from *Enterococcus faecalis* and downstream MVA pathway from *S. cerevisiae* [37]. Additionally, protein truncation and fusion technologies may further improve simple terpenoid yields, as demonstrated by Jiang et al. [36] who increased geraniol production in *S. cerevisiae* from 43.19 mg/L to 523.96 mg/L by truncating geraniol synthase and fusing it with mutated ERG20.

Furthermore, different heterologous pathways exhibit compatibility issues when introduced into a chassis. For example, geraniol synthases from different sources

show vastly different production levels in *S. cerevisiae* [36], and the same foreign gene expresses differently across chassis [38]. Therefore, exploring more terpenoid synthases and expanding the range of microbial chassis are important for heterologous synthesis.

Finally, biosynthetic pathways for many high-value terpenoids remain uncharacterized, with most research focusing on intermediate synthesis. For instance, casbene is considered an intermediate for complex diterpenoids like phorbol in Euphorbiaceae, yet the detailed pathway from casbene to phorbol remains unclear [39]. Phorbol is a precursor for prostratin, a protein kinase C inhibitor with anti-HIV applications and higher value [40]. Thus, further fundamental research on terpenoid pathway elucidation is essential.

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