Engineering Corynebacterium glutamicum for violacein hyper production

Hongnian Sun, Dongdong Zhao, Bin Xiong, Chunzhi Zhang* and Changhao Bi*

Abstract

Background: Corynebacterium glutamicum was used as a metabolic engineering chassis for production of crude violacein (mixture of violacein and deoxyviolacein) due to Corynebacterium's GRAS status and advantages in tryptophan fermentation. The violacein is a commercially potential compound with various applications derived from l-tryptophan.

Results: Corynebacterium glutamicum ATCC 21850 that could produce 162.98 mg L⁻¹ tryptophan was employed as a novel host for metabolic engineering chassis. Heterologous vio operon from Chromobacterium violaceum was over-expressed in ATCC 21850 strain with constitutive promoter to have obtained 532 mg L⁻¹ violacein. Considering toxicity of violacein, vio operon was expressed with inducible promoter and 629 mg L⁻¹ violacein was obtained in batch culture. Due to the economical coding nature of vio operon, the compressed RBS of vio genes were replaced with complete strong C. glutamicum ones. And extended expression units were assembled to form a synthetic operon. With this strategy, 1116 mg L⁻¹ violacein in batch culture was achieved. Fermentation process was then optimized by studying induction time, induction concentration, culture composition and fermentation temperature. As a result, a titer of 5436 mg L⁻¹ and a productivity of 47 mg L⁻¹ h⁻¹ were achieved in 3 L bioreactor.

Conclusions: With metabolic engineering and fermentation optimization practice, C. glutamicum 21850 (pEC-C-viol1) was able to produce violacein with both titer and productivity at the highest level ever reported. Due to advantages of mature C. glutamicum fermentation industry, this work has built basis for commercial production of violacein.

Keywords: Corynebacterium glutamicum, Metabolic engineering, Violacein, l-Tryptophan

Background

Violacein is a natural indolocarbazole compound formed by condensation of two molecules of tryptophan [1], which was a potential novel pharmaceutical reagent due to its extensive biological properties such as antitumoral [2], antibacterial [3], antiviral [4], and antioxidant [5] activities [6]. Violacein biosynthesis begins with l-tryptophan and is catalyzed by enzymes VioA, B, E, D, and C successively, which were encoded by vioABCDE operon [7]. Several Gram-negative bacteria were reported to produce violacein as their secondary metabolites including Chromobacterium violaceum, Janthinobacterium lividum, Alteromonas luteoviolacea, Pseudoalteromonas luteoviolacea, Duganella sp. B2, etc. [6]. Some of the natural producers were studied to improve their violacein production, such as C. violaceum and Duganella sp. B2 [8, 9]. However, fermentation by natural producers were limited for low productivity, bacterial pathogenicity, and difficulties in large scale culture [10, 11]. Due to these problems, recent research has been focused on metabolic engineering of l-tryptophan over-producing Escherichia coli strain for violacein production [10, 12]. Compared to E. coli, C. glutamicum is generally recognized as safes (GRAS) and exhibits numerous advantages as a microbial cell factory. C. glutamicum strains dominate industrial scale fermentation process to produce amino acids and other compounds for health, cosmetic, food and animal feed [13]. C. glutamicum strain ATCC 21850 is an established industrial l-tryptophan hyperproducer,
which made it an attractive platform since L-tryptophan is direct precursor for the production of crude violacein. In this work, C. glutamicum strains including wild type ATCC 13032 and L-tryptophan producing were engineered as novel chassis for violacein production [14]. Various metabolic engineering and fermentation engineering strategies were applied to C. glutamicum ATCC 21850, and the reported highest violacein titer and productivity were achieved in this work.

Methods

Strains and media

Bacterial strains used in this study are listed in Table 1. pEC-XK99E [15], a C. glutamicum/E. coli shuttle expression vector, was used as plasmid backbone to construct function plasmids. E. coli DH5α was used for plasmid construction and cultured in Luria–Bertani (LB) broth at 37 °C. ATCC 13032 and ATCC 21850 were used as the host strains. C. glutamicum was cultivated at 30 °C in LBHIS medium [16]. Kanamycin at a final concentration of 50 or 25 μg mL⁻¹ was added into the medium for cultivation of E. coli or C. glutamicum when needed.

In fermentative production of violacein with recombinant C. glutamicum, the seeding medium consisted of 50 g L⁻¹ glucose, 10 g L⁻¹ yeast extract, 30 g L⁻¹ corn steep liquor, 4 g L⁻¹ (NH₄)₂SO₄, 0.25 g L⁻¹ MgSO₄·7H₂O, 1 g L⁻¹ KH₂PO₄, 1 g L⁻¹ K₂HPO₄, 0.5 g L⁻¹ Na₂HPO₄, 10 mg L⁻¹ FeSO₄·7H₂O and 10 mg L⁻¹ MnSO₄·H₂O, with pH adjusted to 7.2. The fermentation medium consisted of 60 g L⁻¹ glucose, 50 g L⁻¹ corn steep liquor, 10 g L⁻¹ (NH₄)₂SO₄, 0.5 g L⁻¹ MgSO₄·7H₂O, 1 g L⁻¹ KH₂PO₄, 1 g L⁻¹ K₂HPO₄, 1 g L⁻¹ Na₂HPO₄, 10 mg L⁻¹ FeSO₄·7H₂O, 10 mg L⁻¹ MnSO₄·H₂O, 30 mg L⁻¹ phenylalanine, 30 mg L⁻¹ tyrosine and 10 g L⁻¹ CaCO₃, with pH adjusted to 7.2. Glucose solution was sterilized separately.

Culture conditions for cell growth and violacein production

For violacein production, a single colony from LBHIS plates was inoculated into a 100 mL flask containing 5 mL BHIS media, and cultured at 30 °C and 200 rpm on a rotary shake for 48 h. Then 1 mL of the culture was inoculated into 25 mL of the seed medium in a 250 mL rotary flask and cultivated at 30 °C and 200 rpm for 24 h.

A two-phase process was carried out for violacein production. Firstly, 1 mL of the seed culture was inoculated into 25 mL fermentation medium in a 500 mL rotary flask and cultivated at 30 °C 200 rpm for 12 h, then temperature was dropped from 30 to 20 °C to cultivate for 72 h. Induction was performed with addition of 0.5 mM isopropyl-β-d-thiogalactoside (IPTG) for strains with inducible expression promoters. Fermentation optimization experiments were carried out with various parameters, which might be different from this general condition, and was specified in the text and figure legends.

Fed-batch fermentations were performed in a 3 L fermenter (Bioflow 115, New Brunswick Scientific, USA) with a working volume of 1.5 L, whose fermentation conditions was similar to optimized batch cultures except that temperature of the second phase fermentation was shifted to 20 °C after 18 h. The dissolved oxygen level of the culture was controlled at about 30 % and the pH was maintained at 7.0 by automatic addition of 25 % NH₄OH.

Construction of plasmids

All plasmids were constructed by Golden-gate DNA assembly method [17], and amplified in E. coli DH5α. Then electrotansformed into C. glutamicum ATCC 13032 or ATCC 21850 as described previously [18]. For construction of pEC-vioABCDE, the vio operon was PCR amplified from C. violaceum genomic DNA.

| Table 1 | Strains and plasmids used and constructed in this study |
|---|---|---|
| Strains/plasmids | Relevant characteristics | Sources |
| **Strains** | | |
| E. coli DH5α | F− endA1 thi−1 recA1 relA1 gyrA96 deoR60 dcmΔ(lacZ) M15 Δ(lacZYA-argF)U169 hsdR17(qE, m15) λ− supE44 phoA | Invitrogen |
| ATCC 13032 | C. glutamicum wild-type | ATCC |
| ATCC 21850 | 4- MariT 5- MariT 6-FT 4-APr 4-FPr TyrHxr Phe− Tyr−, tryptophan hyperproducer | ATCC |
| **Plasmids** | | |
| pEC-XK99E | C. glutamicum/E. coli shuttle expression vector, Ptra, lacIq, Kanr | Add gene |
| pEC-vioABCDE | derived from pEC-XK99E, constitutive expression of C. violaceum vio operon | This study |
| pEC-J-vio-1 | pEC-XK99E derivative containing vio operon from J. lividum, expressed under control of inducible promoter Ptra | This study |
| pEC-J-vio-2 | pEC-XK99E derivative containing synthetic J. lividum vio operon with each gene containing complete C. glutamicum RBS sequence | This study |
| pEC-C-vio-1 | pEC-XK99E derivative containing synthetic C. violaceum vio operon with each gene containing complete C. glutamicum RBS sequence | This study |
| pEC-C-vio-2 | pEC-XK99E derivative containing synthetic C. violaceum vio operon with each gene containing complete C. glutamicum RBS sequence, which gene order changed to vioB, vioA, vioE, vioC, vioD | This study |
with primers C-vio-F/C-vio-R. Assembly fragments pEC-ΔlacIq-P1 and pEC-ΔlacIq-P2 were amplified from pEC-XK99E with primers pEC-C1-F/pEC-C1-R and pEC-C2-F/pEC-C2-R respectively.

For construction of plasmid pEC-J-vio1, the vio operon was PCR amplified from the chromosomal DNA of *J. lividum* with primers J-vio-F and J-vio-R. Backbone of pEC-XK99E was divided into two segments to be amplified using primer pairs J-pec1-F/J-pec1-R and J-pec2-F/J-pec2-R respectively. For construction of plasmid pEC-J-vio2, the biosynthetic genes of *vioA, vioB, vioC, vioD, vioE* from *J. lividum* were PCR amplified from the chromosomal DNA with primers J-vioA-F/J-vioA-R, J-vioB-F/J-vioB-R, J-vioC-F/J-vioC-R, J-vioD-F/J-vioD-R, J-vioE-F/J-vioE-R respectively. The backbone was cloned from pEC-J-vio1 with primers J-pec1-F/J-pec2-R.

For construction of pEC-C-vio1, the vio operon was PCR amplified from pKMV-vioA, pKMV-vioB, pKMV-vioC, pKMV-vioD, pKMV-vioE with primers C-vioA-F/C-vioA-R, C-vioB-F/C-vioB-R, C-vioC-F/C-vioC-R, C-vioD-F/C-vioD-R, C-vioE-F/C-vioE-R respectively. The backbone was cloned from pEC-J-vio1 with primers C-vio-F/C-vio-R.

For construction of pEC-C-vio2, *vioB, vioA, vioE, vioC, vioD* were PCR amplified from pKMV-vioB, pKMV-vioA, pKMV-vioE, pKMV-vioC, pKMV-vioD with primers 2C-vioB-F/2C-vioB-R, 2C-vioA-F/2C-vioA-R, 2C-vioE-F/2C-vioE-R, 2C-vioC-F/2C-vioC-R, 2C-vioD-F/2C-vioD-R respectively. The backbone was amplified from pEC-J-vio1 with primers 2C-pec-F/2C-pec-R.

### Analytical method

500 μL samples were obtained from fermentation culture to determine violacein titer as reported previously [9, 12]. The correlation of Absorbance at 570 nm and concentration of crude violacein was determined as illustrated in (Additional file 1: Figure S1A), which was the standard for calculation. Detection of l-tryptophan in fermentation culture was carried by the approach as described recently [9, 12]. The correlation of Absorbance at 600 nm and concentration of l-tryptophan was determined as illustrated in (Additional file 1: Figure S1B), which was the standard for calculation. Glucose was determined using a biosensor analyzer (SBA-40B, Institute of Biology, Shandong Province Academy of Sciences, China). Fermentation broth was centrifuged and diluted 1:100 with 1 N HCl solutions to determine the OD_{600}. Experiments were performed to find that OD_{600} of untreated samples were same as samples after violacein extracted with ethanol. The biomass of one unit OD_{600} was determined to equal around 0.3 g DCW L⁻¹ (Additional file 1: Figure S1C), which was similar to previously reported value [19].

### Results and discussion

*Corynebacterium glutamicum* was engineered to produce violacein

*Corynebacterium glutamicum* was employed as metabolic engineering chassis for production of violacein. Plasmid pEC-XK99E-ΔlacIq was constructed with constitutively expressed vioABCDE operon from and transformed into the model strain *C. glutamicum* 13032. 13032 (pEC-vioABCDE) strain grew to a high cell density but produced no violacein in fermentation medium as illustrated in Fig. 1, although low titer of violacein was obtained in the rich medium LBHIS (Additional file 1: Figure S2). Then the tryptophan producer ATCC 21850 was used as chassis for engineering, which was able to accumulated 163 mg L⁻¹ l-tryptophan at 30 °C in 72 h. The resulting strain 21850 (pEC-vioABCDE) was successfully engineered and produced 532 mg L⁻¹ violacein, higher than its tryptophan titer. This suggested that the violacein producing pathway was successfully expressed and functioned efficiently in ATCC 21850 strain. Probably due to better accumulation and excretion mechanism, violacein synthesis process even pulled more carbon flux to form tryptophan and subsequent violacein. This result proved tryptophan producing *C. glutamicum* a suitable host for production of violacein (Fig. 1). However, since violacein was reported to be an antibacterial reagent, the constitutive production might cause burden on cell, especially in the initial phase of growth. Thus, expression and regulation of *vio* genes should be further optimized for improved cell growth and violacein production.

### Metabolic engineering strategies were applied for improved violacein production

To avoid growth defect in initial growth phase, the *vio* operon vioABCDE, from *J. lividum* was constructed under regulation of Ptrc promoter to form plasmid pEC-J-vio1. When this plasmid was expressed in ATCC 21850,
629 mg L$^{-1}$ violacein was produced when IPTG was added at 12 h after inoculation of seed culture (Fig. 1). This result supported our hypothesis that constitutive production of violacein affect its production.

The vio operon was found to have overlapping genes, which is common in rapidly evolving genomes with high mutation rates such as viruses and bacteria [20]. The main biological function of the overlapped genes was suggested be regulation of gene expression through translational coupling of functionally related polypeptides, and compression of maximum amount of information into shorter DNA sequences (Fig. 2). We hypothesized that such operon structure hampered its heterologous expression, and introduction of complete RBS sequences of the host bacteria to each gene might improve their expression. To test this hypothesis, vio genes from operon of J. lividum were individually cloned. Their RBSs were replaced with strong C. glutamicum RBSs (GAAAGGAGGTTTGGACA) individually [21], and assembled to form a novel synthetic vio operon. This plasmid was designated as pEC-J-vio2 (Fig. 2). Results were shown in (Fig. 1) that both biomass and violacein titer of C. glutamicum ATCC 21850 (pEC-J-vio2) were higher than pEC-J-vio1 (15 vs 20 g DCW L$^{-1}$; 629 vs 815 mg L$^{-1}$, respectively), so the introduction of complete RBS sequences of C. glutamicum to five structural vio genes improved both violacein production and host growth status. Our results proved that the compressed vio operon actually defected its expression in heterologous host, which might be a common problem in genetic and metabolic engineering. Construction of the large plasmid (14049 bps) from six parts was not an easy task, however, the improved production titer suggested that de novo construction of a pathway operon with completed synthetic RBSs is a functional strategy for solve such problems.

To further enhance violacein production, vio genes from another violacein natural producer, C. violaceum, were also individually cloned and assembled to form a similar synthetic vio operon as pEC-C-vio1. With this strategy, violacein production of the strain 21850 (pEC-C-vio1) in batch culture was further improved to reach 1116 mg L$^{-1}$ (Fig. 1).

Inspired by previous success, we aimed to further optimized the modified vio operon. To investigate whether the gene order affects violacein production in C. glutamicum, a novel vio operon was constructed with a different order. The gene order in native violacein producers were all in P$\_\text{vio}$$\cdot$vioA-vioB-vioC-vioD-vioE sequence, which was different from the order of violacein biosynthetic pathway. Tomoko Nishizaki [22] reordered five crt genes of the natural carotenoid cluster in Pantoea ananatis into the order of its metabolic pathway, and enhanced production of zeaxanthin in E. coli. In violacein biosynthesis

![Image](image-url)

Fig. 2 Illustration of engineering strategies with the vio operon. a Violacein synthetic pathway diagram. b Original form of J. lividum vio operon with compressed DNA sequence. c Synthetic J. lividum vio operon with extended complete RBS for each genes. d vio operon with altered gene order.
pathway, the sequence of the reaction catalyzed by the enzymes is ABEDC. In addition, vioB was reported to be rate-limiting step enzyme [1], and VioD is not evolved in formation deoxyviolacein. So that vioD gene was put in the last position of the operson. Thus, vio genes was reordered in a sequence of BAECD (Fig. 2), and plasmid pEC-C-vio2 was constructed. However, results showed that both biomass and violacein titer of 21850 (pEC-C-vio2) strain were lower than pEC-C-vio1 (30 vs 25 g DCW L⁻¹; 1102 vs 629 mg L⁻¹, respectively). This result suggested that the gene order we designed was not optimal than original one. There might be more optimal gene sequences for heterologous expression of vio, however, the enormous work associated with construction of all possible combinations made it impossible to find them. We are planning to study and try to develop some new methods to tackle this problem.

**Excretion of violacein by the high production strain ATCC 21850 (pEC-C-vio1)**

In our study, the culture color of our highest production strain ATCC 21850 (pEC-C-vio1) turned into black. Meanwhile, the lower production strain, for example, ATCC 21850 (pEC-J-vio1) had a purple colored culture, as showed in Fig. 3. It was reported that high violacein producers, either native or heterologous, sometimes excreted violacein into culture [12]. To determine if our high production strain excreted crystalized violacein, microscope was used to observe the culture. The microscopic photos demonstrated that ATCC 21850 (pEC-C-vio1) produced extracellular purple particles similar to the images of previous report [12] which were most possibly violacein crystals, as indicated with arrows in Fig. 3d; while control strain ATCC 21850 (pEC-J-vio1) in the same condition produced no obvious extracellular particles. This microscopic phenomenon might explain the sharply different color between high and low production strains. The extracellular crystal phenotype also implies an economical downstream process for extraction and purification of produced violacein, giving advantages for industrializing this technology.

**Fermentation parameter optimization for violacein production**

Fermentation is a complex process, many parameters needs to be studied and optimized to achieve good production. In our work, basic fermentation optimization was carried out to find optimal culture media, fermentation temperature, IPTG induction concentration and induction time.

Fermentation medium is a key factor of *C. glutamicum* fermentation, in which corn steep liquor is a complex low-cost nitrogen source reported to significantly affect amino acid production [23]. Various concentrations were evaluated in term of violacein production to determine the optimum. To eliminate variations caused by induction concentration, induction time, induction growth status and so on, strain 21850 (pEC-vioABCDE) with constitutive expression of the vio operon was selected as testing strain. As illustrated in Fig. 4a, the highest violacein titer was obtained at 5 % corn steep liquor with addition of 1 % CaCO₃.

Violacein production was reported to benefit from low temperature culturing, and typically the quantity of cell mass is also a major factor for fermentation production. To accumulate large amount of biomass before shifting to low temperature for violacein production, the classic two-phase fermentation process was employed. In this process, cells were cultured at 30 °C and then shifted to another temperature for fermentation product accumulation as previously reported [3, 10, 12, 24]. Here we performed experiments to determine an optimal temperature of the second phase fermentation for 21850 (pEC-C-vio1), selected from 37, 30, 25 and 20 °C (Fig. 4b). A trend of decreasing violacein production with higher temperature was observed, probably due to folding problems of heterologous enzymes at higher temperature [10]. Since the highest biomass was achieved at 30 °C, considering balance between cell growth and violacein production, 20 °C but not lower temperature was selected as the second phase temperature of fermentation.

Due to inducible promoter was employed for vio gene expression, concentrations of the inducer IPTG were studied for optimal fermentation condition of 21850 (pEC-C-vio1), whereas IPTG is toxic to host cell and increases fermentation cost when used at high concentration, lower concentrations might cause inadequate transcription induction [25]. In our work, various concentrations were applied and the optimum was determined to be 0.5 mM as shown in Fig. 4c. IPTG supplementation time is also a key parameter for optimized fermentation process. Theoretically, target enzymes are mainly produced after induction, so appropriate induction time enables balance between biomass accumulation and vio enzymes expression. As shown in Fig. 4d, different induction time was tested for fermentation, and it was determined that induction after 18 h yielded the highest violacein titer at 2344 mg L⁻¹. This was also the highest titer reported in shake flask fermentation without tryptophan addition.

**Fed-batch fermentation of *C. glutamicum* ATCC 21850 (pEC-C-vio1) for violacein production**

Bioreactor fermentation was carried out with the best production strain *C. glutamicum* ATCC 21850 (pEC-C-vio1) in a 3 L fermenter with the determined optimal
parameters. As illustrated in Fig. 5, at 18 h after inoculation before induction, glucose consumption rate was about 1 g L\(^{-1}\) h\(^{-1}\), growth rate was 0.822 g DCW L\(^{-1}\) h\(^{-1}\) with no obvious lag phase. There was no violacein accumulation at this point. After induction, between 18 and 75 h, glucose consumption rate was about 0.6 g L\(^{-1}\) h\(^{-1}\) and growth rate decreased to 0.264 g DCW L\(^{-1}\) h\(^{-1}\) probably owing to change of temperature from 30 to 20 °C. within less than 6 h after induction, fermentation culture gradually became purple indicating initiation of violacein production. And violacein productivity from 24 to 75 h was measured to be 88 mg L\(^{-1}\) h\(^{-1}\). When glucose decreased to 6 g L\(^{-1}\), 51 g L\(^{-1}\) was fed to the reactor, which was consumed subsequently at a consumption rate of 1.1 g L\(^{-1}\) h\(^{-1}\). However, violacein productivity decreased at this stage probably due to product inhibition. Biomass reached 46.5 g DCW L\(^{-1}\) at 100 h, and began to decrease probably due to similar reason.

During the fermentation process, 101 g glucose was consumed and 5436 mg L\(^{-1}\) crude violacein was obtained with an overall productivity of 47 mg L\(^{-1}\) h\(^{-1}\). The highest titer reported was 1.75 g L\(^{-1}\) with a productivity of 36 mg L\(^{-1}\) h\(^{-1}\), which was achieved in Xing’s lab in 2015 [12]. Thus, Both production titer and productivity in this work are currently the highest from both native or heterologous hosts, but the yield at 0.054 g-vioalcein/g-glucose is lower than 0.116 reported previously [12].

**Conclusion**

*Corynebacterium* was used as a metabolic engineering chassis for production of violacein, due to its GRAS status and advantages in tryptophan fermentation. With extensive metabolic engineering and fermentation optimization practice, *C. glutamicum* 21850 (pEC-C-vio1) was able to produce violacein with both titer and productivity at the highest level ever reported. In the engineering
Fig. 4  Fermentation parameter optimization.  

**a** Violacein production and biomass of 21850 (pEC-vioABCDE) with 1, 3, 5 and 7 % corn steep liquor supplementation in fermentation. 1 % CaCO₃ was added to 5 % steep liquor culture for further improvement.  

**b** Temperature optimization for 21850 (pEC-J-vio1) of the second-phase of fermentation. violacein and biomass at 20, 25, 30, 37 °C was illustrated.  

**c** IPTG concentration was optimized for 21850 (pEC-J-vio1) fermentation at 0.25, 0.5, 1.0 mM for 21850 (pEC-J-vio1). Corresponding violacein production and biomass was illustrated.  

**d** Optimization of induction time of 21850 (pEC-C-vio1) for violacein production. After inoculation, 0.5 mM IPTG was added at 6, 12, 18, 24, 30 h and then fermentation was performed at 20 °C for 72 h with addition of IPTG. Experiments were carried out in triplicate.

---

Fig. 5  Fed-batch fermentation of violacein with *C. glutamicum* ATCC 21850 (pEC-C-vio1) in 3 L NBS BioFlo 115 fermenter
process, multiple engineering strategy was applied to
the vio operon. To be specific, different promoters were
applied; compressed genes in vio operon were artifi-
cially extended and rearranged in various sequences;
and vio operons from different violacein producers were
expressed and compared. In fermentation optimization
part, four important parameters were studied, including
culture media, temperature of two phase fermentation,
induction concentration and induction time. With these
effort, crude violacein production of C. glutamicum
was increased from zero to a titer of 5436 mg L\(^{-1}\) as illus-
trated in Fig. 6. The titer and productivity we achieved
in this work represent highest level to date. Future work
may be focused on systematically metabolic engineering
of the complete synthetic pathway and related metabolic
network to further improve production, productivity and
yield.

### Additional files

Additional file 1: Table S1. Strains, plasmids and oligonucleotides used
in this study. Figure S1. The linear relationship between Absorbance at
570 nm and concentration of crude violacein. Figure S2. Batch cultiva-
tions of C. glutamicum in LBHIS broth.

Additional file 2. Plasmid profiles and gene sequences.

---

| Violacein titer | Increase fold |
|-----------------|--------------|
| 0 mg/L          | 0            |
| 532 mg/L        | 1            |
| 629 mg/L        | 1.18         |
| 815 mg/L        | 1.53         |
| 1116 mg/L       | 2.10         |
| 2344 mg/L       | 4.41         |
| 5436 mg/L       | 10.22        |

Fig. 6 Summarization of metabolic engineering and fermentation optimization practice for successful violacein microbial cell factory construction.
References

1. Balibar CJ, Walsh CT. In vitro biosynthesis of violacin from L-tryptophan by the enzymes ViOa-E from Chromobacterium violaceum. Biochemistry. 2006;45:15444–57.

2. Platt D, Amao S, Mehta T, Vercutseyee K, Myles EL, Johnson T, Triveedihi V. Violacin inhibits matrix metalloproteinase mediated CXCR4 expression: potential anti-tumor effect in cancer invasion and metastasis. Biochem Biophys Res Commun. 2014;455:107–12.

3. Choi SY, Kim S, Lyuck S, Kim SB, Mitchell RJ. High-level production of violacin by the newly isolated Duganella violaeinigra str. NI28 and its impact on Staphylococcus aureus. Sci Rep. 2015;5:15598.

4. Andriighetti-Fröhner C, Antonio R, Creczynski-Pasa T, Barardi C, Simões C. Cytotoxicity and potential antiviral evaluation of violacin produced by Chromobacterium violaceum. Memórias do Instituto Oswaldo Cruz. 2003;98:843–8.

5. Konzein M, De Marco D, Cordova CA, Vieira TO, Antônio RV, Creczynski-Pasa TB. Antioxidant properties of violacin: possible relation to its biological function. Bioorgan Med Chem. 2006;14:8307–13.

6. Durán M, Ponezi AN, Faljoni-Alario A, Teixeira MFS, Justo GZ, Durán N. Potential applications of violacin: a microbial pigment. Med Chem Res. 2011;21:1524–32.

7. Hoshino T. Violacin and related tryptophan metabolites produced by Chromobacterium violaceum: biosynthetic mechanism and pathway for construction of violacin core. Appl Microbiol Biotechnol. 2011;91:1463–75.

8. Mendes A, de Carvalho J, Duarte MT, Durán N, Bruns R. Factorial design and response surface optimization of crude violacin for Chromobacterium violaceum production. Biotechnol Lett. 2001;23:1963–9.

9. Wang H, Jiang P, Lu Y, Ruan Z, Jiang R, Xing X-H, Lou K, Wei D. Optimization of culture conditions for violacin production by a new strain of Duganella sp. B2. Biochem Eng J. 2009;44:119–24.

10. Rodrigues AL, Trachtenmann N, Becker J, Lohanatha AF, Blotenberg J, Bolten DJ, Korneli C, de Souza Lima AO, Porto LM, Sprenger GA, Wittmann C, Systems metabolic engineering of Escherichia coli for production of the anti-tumor drugs violacin and deoxyviolacin. Metab Eng. 2013;20:29–41.

11. Yang C, Jiang P, Xiao S, Zhang C, Lou K, Xing X-H. Fed-batch fermentation of recombinant Chromobacter freundii with expression of a violacin-synthesizing gene cluster for efficient violacin production from glycerol. Biochem Eng J. 2011;57:55–62.

12. Fang MY, Zhang C, Yang S, Cui JY, Jiang PX, Lou K, Wachi M, Xing XH. High crude violacin production from glucose by Escherichia coli engineered with interactive control of tryptophan pathway and violacin biosynthetic pathway. Microb Cell Fact. 2015;14:8.

13. Ikeda M, Takeno S. Amino acid production by Corynebacterium glutamicum. In: Yukawa H, Inui M, editors. Corynebacterium glutamicum. Berlin: Springer; 2013. p. 107–47.

14. Herry DM, Duncan LK. Cloning of the trp gene cluster from a tryptophan-hyperproducing strain of Corynebacterium glutamicum: identification of a mutation in the trp leader sequence. Appl Environ Microbiol. 1993;59:791–9.

15. Jakoby M, Kramer R, Burkovski A. Nitrogen regulation in Corynebacterium glutamicum: isolation of genes involved and biochemical characterization of corresponding proteins. FEMS Microbiol Lett. 1999;173:303–10.

16. Eggeling L, Bitt M. Handbook of Corynebacterium glutamicum. Boca Raton: CRC Press; 2005.

17. Hillson NJ, Rosengarten RD, Keasling JD. JS DNA assembly design automation software. ACS Synth Biol. 2012;1:14–21.

18. Tauch A, Kirchner O, Löfler B, Göttker S, Pühler A, Kalinowski J. Efficient electrottransformation of Corynebacterium diphtheriae with a mini-repli-con derived from the Corynebacterium glutamicum plasmid pGA1. Curr Microbiol. 2002;45:362–7.

19. Peters-Wendisch PG, Wendisch VF, Paul S, Eikmanns BJ, Sahl H. Pyruvate carboxylase as an anaplerotic enzyme in Corynebacterium glutamicum. Microbiology. 1997;143:1095–103.

20. Krakauer DC. Stability and evolution of overlapping genes. Evolution. 2000;54:731–9.

21. Kang MS, Han SS, Kim MY, Kim BY, Huh JP, Kim HS, Lee JH. High-level expression of Corynebacterium glutamicum of nitrile hydratase from Rhodococcus rhodochrous for acrylamide production. Appl Microbiol Biotechnol. 2014;98:4379–87.

22. Nishizaki T, Tsuge K, Itoya M, Doi N, Yanagawa H. Metabolic engineering of carotenoid biosynthesis in Escherichia coli by ordered gene assembly in Bacillus subtilis. Appl Environ Microbiol. 2007;73:1355–61.

23. Lee PC, Lee WG, Lee SY, Chang HN, Chang YK. Fermentative production of succinic acid from glucose and corn steep liquor by Anaerolobacter succiniciproducens. Biotechnol Bioprocess Eng. 2000;5:379–81.

24. PX Jiang, HS Wang, Zhang C, Lou K, Xing XH. Reconstruction of the violacin core of Duganella sp. B2 in different heterologous hosts. Appl Microbiol Biotechnol. 2010;86:1077–88.

25. Hannig G, Makrides SC. Strategies for optimizing heterologous protein expression in Escherichia coli. Trends Biotechnol. 1998;16:54–60.