ORIGINAL ARTICLE

Reconstruction of the Violacein Biosynthetic Pathway From *Chromobacterium violaceum* in *Escherichia coli*

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ABSTRACT

Introduction: Violacein, a bis-indole produced by various bacteria is known to display extensive pharmaceutical properties. Unfortunately, low productivities in natural violacein producers have led to inconsistent violacein supply, limiting its application as future therapeutics. Heterologous expression systems such as *Escherichia coli* and *Pichia pastoris* offers an alternative means of producing these high-valued secondary metabolites. This work describes the development of a genetic system for violacein heterologous production in *E. coli*. **Methods:** A violacein producer, *C. violaceum*, MTH01, was isolated from the limnological grounds of Universiti Sains Malaysia, Penang. Using gene specific primers, the entire 7.3 kb violacein gene cluster was successfully amplified from *C. violaceum* MTH01 DNA, cloned into pUC19 vector (pVio19) and then subcloned into pET-3a and pET-11b, yielding recombinant vectors, pVio3a and pVio11b, respectively. For heterologous expression, parameters such as carbon sources, temperature, inducer (IPTG) and L-tryptophan were optimised. Violacein extracted from purple-pigmented *E. coli* transformants were analysed using TLC and FTIR. **Results:** After a few days, *E. coli* transformants containing either pVio3a or pVio11b developed purple colonies, indicating that violacein was successfully expressed in *E. coli*. TLC analysis of the pigment extract revealed Rf values comparable to deoxyviolacein, an intermediary metabolite in violacein biosynthesis, while FTIR spectra revealed the presence of amine and carbonyl groups, both of which are characteristics of an indole. **Conclusion:** The *E. coli* heterologous system described here could utilize either glucose or glycerol as carbon source. Addition of L-tryptophan to growth medium was necessary for successful expression of the violacein pathway.

Keywords: Bis-indole; Violacein; *Chromobacterium violaceum*; Heterologous expression

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INTRODUCTION

Violacein, a secondary metabolite produced naturally by various bacteria has been shown to possess extensive pharmaceutical potential including as anticancer, antibacterial [1], antifungal [2], antiparasitic [3] and antiviral properties [4]. This bis-indole ((3E)-3-[5-(5-hydroxy-1H-indol-3yl)-2-oxo-1H-pyrol-3-ylidene]-1H-indol-2-one) is generated by oxidation and coupling of two modified tryptophan molecules [5]. Violacein biosynthesis is catalysed by a series of five enzymes, namely, VioABCDE which is encoded by a 7.3 kilo-bases gene cluster consisting of genes, vioA, vioB, vioC, vioD, and vioE [6-7].

The numerous interesting biological potential with therapeutic relevance of violacein has piqued the scientific community's interest into developing this metabolite as novel medications for the future. Unfortunately, despite its vast potential as future therapeutics, large or industrial scale production of violacein using natural-violacein producing bacteria is hampered by several factors. These factors include low yields in natural violacein-producing bacteria [8], unstable violacein-producing bacteria variant strains [9] and the potential for these natural violacein producers to become opportunistic pathogens [10-11]. One method for overcoming these limitations is by producing this valuable metabolite using established heterologous expression like *E. coli* and yeast.

This work describes the reconstruction of the violacein biosynthetic gene cluster and development of a genetic system for heterologous production of violacein in *E. coli*. The entire 7.3 kilo base violacein biosynthetic gene cluster was cloned into T7 expression vectors, pET3a and pET11b. Parameters such as carbon source, culture medium, supplementation of inducer and precursor to growth medium, that could influence heterologous expression of foreign genes in *E. coli* cells were also evaluated.

MATERIALS AND METHODS

Isolation and Identification of Purple-pigmented Bacteria

Soil samples were collected from the limnological areas of Tasik Harapan (5o21′13.5′N 100o 17′59.3″E) at Universiti Sains Malaysia, Pulau Pinang. About 1 g of the soil sample was resuspended in 10 mL sterile distilled water. Subsequently 1 mL of the soil suspension was transferred into 9 mL sterile distilled water. This mixture was serially diluted before being inoculated on Nutrient agar and incubated at 30 °C for 24 h. A purple-pigmented soil isolate, subsequently designated as MTH01, was chosen for further characterisation.

Identification of MTH01 was determined by 16S ribosomal DNA (rDNA) sequencing. Isolation of MTH01 genomic DNA was carried out using G-spin Total Genomic DNA Extraction (iNtRON Biotechnology, Korea). The 16S rDNA gene of MTH01 was amplified using primers 16SF (5′-CAGGCCTAACACATGCAAGTC-3′) and 16SR (5′-GGGCGGWGTGTACAAGGC-3′) and carried out according to standard PCR protocol. The amplified PCR fragment was sent for commercial sequencing. Analysis and alignment of DNA sequences was carried out using the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu) [12].

Amplification of the Violacein Gene Cluster

The Hotstart PCR method was used to amplify the violacein biosynthetic gene cluster consisting of genes vioABCDE from MTH01 genomic DNA. Due to the size of the violacein operon, amplification of this gene cluster was carried out in two segments, i.e. Fragment A containing vioAB and Fragment B containing vioCDE. Primers for this purpose were designed based on the nucleotide sequence of *C. violaceum* ATCC 12472 violacein biosynthetic gene cluster (GenBank Accession No. NC_005085.1). The primers for Fragment A were ABF(5′-GGGACCAAGCTTAATCCATATGAAGCATCTTCCGATATCTGCATTGTCG-3′) and ABR (5′-CATTTCAGGCCTCTCTAGATAGTTTCCACAAG-3′). HindIII (AAGCCT), NdeI (CATATG) and XbaI (TCTAGA) restriction sites were incorporated into these primers. Primers for Fragment B were CEF (5′-GCTTGTGGAAAGCTTTCTAGAGAGGCCTG-3′) and CER ATCCTAGCCGCTTGGCGGCGAAGACGGCGTCG-3′), with restriction sites for XbaI (TCTAGA) and BamHI (GGATCT) incorporated. Amplification of Fragment A was done using Q5® High Fidelity 2X Master Mix (New England Biolabs, USA) while Fragment B was amplified using Taq98TM Hot Start 2X Master Mix (Lucigen, USA). PCR was performed using Veriti® 96-well Fast Thermal Cycler (Applied Biosystems, USA) according to this thermal profile; an initial denaturation at 98 °C for 30 s (Q5® High Fidelity 2X Master Mix) or 2 min (Taq98TM Hot Start 2X Master Mix), followed by 30 cycles of template denaturation at 98 °C for 10 s, primer annealing at 60 °C for 15 s, and DNA extension for 4.5 min (Q5® High Fidelity 2X Master Mix) or 3 min (Taq98TM Hot Start 2X Master Mix). The PCR mixtures were then subjected to a final extension at 72 °C for 7 min. The PCR products were purified using MEGAquick-spinTM Total Fragment DNA Purification kit (iNtRON Biotechnology, Korea) before being used for cloning purposes.

Construction of the Violacein Biosynthetic Gene Cluster in Expression Vectors

The construction and assembly of the entire violacein gene cluster into cloning vector pUC19 (Takara Bio, Japan) was carried out in two stages. Initially, Fragment B was cloned into the XbaI-BamHI site of pUC19 to generate a recombinant clone, pMM2. Subsequently, Fragment A was inserted into the HindIII-XbaI site of pMM2 to generate pVio19. The size of the final DNA insert carried by pVio19 was validated by conducting a HindIII -BamHI double digestion and visualised using agarose gel electrophoresis. The DNA insert harboured by pVio19 was also sent for sequencing. Analyses of the DNA sequences were done using BLAST software at http:www.ncbi.nim.nih.gov/BLAST/ [13].

For heterologous gene expression studies, the DNA insert carried by pVio19 was subcloned into the NdeI-BamHi sites of expression vectors, pET-3a (Novagen, USA) and pET-11b (Novagen, USA). Recombinant pET-3a and pET-11b carrying the entire violacein biosynthetic gene cluster were designated as pVio3a and pVio11b respectively.

Heterologous Expression of the Violacein in *E. coli* BL21 (DE3) and Extraction of Pigment from Induced Cells

Escherichia coli BL21 (DE3) {F- ompT hsdSB(rß-mß-) gal dcm λ(DE3 [lacI lacUV5-T7 gene1, ind1, sam7 nin5]} (Promega, USA) was used for heterologous gene expression studies. Preparation and transformation of competent *E. coli* BL21 (DE3) cells with either pVio3a or pVio11b was carried out according to [14] and plated on LB agar (100 µg/mL ampicillin). *E. coli* transformants carrying either pVio3a or pVio11b were designated as *E. coli* Vio3a and *E. coli* Vio11b, respectively. A single colony of either *E. coli* Vio3a or *E. coli* Vio11b was inoculated into 5 mL LB (100 µg/mL ampicillin) and incubated at 37 °C with 180 rpm agitation, overnight. A volume of 0.2 mL of the overnight culture was inoculated into 200 mL of M9-YE medium [15] supplemented with 100 µg/mL ampicillin, selected carbon source (glucose or glycerol) and 0.7 g/L of L-tryptophan as precursor. The cultures were incubated at 37 °C with 180 rpm agitation for at least two to three hours to an OD_{600} value of 0.5. To induce violacein expression, 0.4 mM of 1 M IPTG was added to *E. coli* Vio3a inoculum and 1.0 mM of 1 M IPTG added to *E. coli* Vio11b inoculum respectively.

The cell cultures were further incubated at 25 °C for three days to enhance the production of violacein. Prior to extraction of pigments from the induced E coli cells, the bacterial culture was centrifuged at 4000 x g for 20 min. The pelleted cells were washed three times with distilled water, followed by extraction with absolute methanol. The methanolic extract was air-dried in the fume hood.

Characterisation of Extracted Pigment

The crude pigment extracted from recombinant *E. coli* Vio3a and *E. coli* Vio11b were analysed using UV-Visible (UV-Vis) Spectroscopy, Thin Layer Chromatography (TLC) and Fourier Transform Infrared (FTIR) spectroscopy. The maximum wavelength λ_{max} of the methanolic extract was determined using UV-Vis spectrophotometer between wavelengths 400 to 800 nm. Characterisation by TLC was carried out using 0.20 mm pre-coated silica aluminium sheet and developed using methanol/ethyl acetate (8:12). Spots developed were visualised under UV and white light. The FTIR spectrum of the pigment extracts was detected using Thermo Nicolet Nexus (Thermo Scientific, USA), while data spectral collection and analysis was conducted using OMNIC™ software. A drop of methanolic extract of the crude pigment was placed and pressed between a pair of sodium chloride (NaCl) plates followed by analysis using FTIR between 4000 and 400 cm^{-1}.Violacein pigment extracted from the natural producer strain, *C. violaceum* MTH01 was used as the control in the UV-VIS, TLC and FTIR studies.

RESULTS

Isolation and Identification of Purple-pigmented Bacteria

A purple coloured isolate, MTH01, was successfully isolated from the soil inoculum which was sourced from the limnological areas of Tasik Harapan, USM. The intense purple hues displayed by MTH01 strongly suggests that this isolate is a natural producer of the bis-indole, violacein, and was chosen for further characterisation. Species identification via 16S rDNA sequencing showed that the 16S rDNA sequence of MTH01 (Genbank Accession: OP703624) displayed at least 99% identity to published *C. violaceum* databases.

Isolation, Cloning and Characterisation of MTH01 Violacein Biosynthetic Gene Cluster

Published sequences of the 7.23 kb biosynthetic gene cluster indicated that this region of the *C. violaceum* genome has very high GC content, around 64.83% [16]. Therefore, due to the high GC content and relatively large size of the target PCR fragment, the strategy was to amplify the violacein biosynthetic gene cluster separately in two segments i.e. Fragment A (vioAB) and Fragment B (vioCDE). Using violacein specific primer pairs ABF/ABR and CEF/CER, two PCR fragments of size 4.3 kb (Fragment A) and 3 kb (Fragment B), respectively, was successfully amplified from the genome of *C. violaceum* MTH01 (Fig. 1). Fragment B (vioCDE) was successfully cloned into the XbaI-BamHI site of pUC19 to generate recombinant pUC19 clone of 5.6 kb in size and this clone was designated as pMM2. Subsequently, insertion of Fragment A (vioAB) into the HindIII-XbaI site of pMM2 gave rise to a recombinant clone, pVio19, of 9.9 kb in size. Double digestion conducted on pVio19 using HindIII and BamHI gave two band sizes of approximately 2.6 kb and 7.3 kb, which corresponds to the predicted sizes of the backbone vector, pUC19 and DNA insert (Fig. 2).

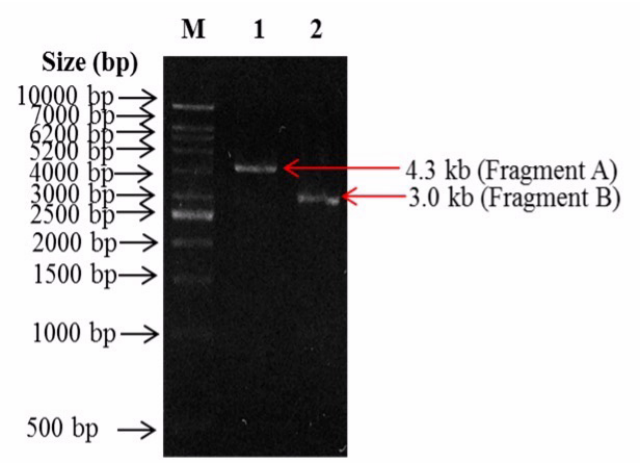

Figure 1 : Agarose gel analysis of amplified PCR fragments, Fragment A (vioAB) and Fragment B (vioCDE). Lane M: VC 1 kb DNA ladder, Lane 1: Fragment A (vioAB, 4.3 kb), Lane 2: Fragment B (vioCDE, 3 kb).

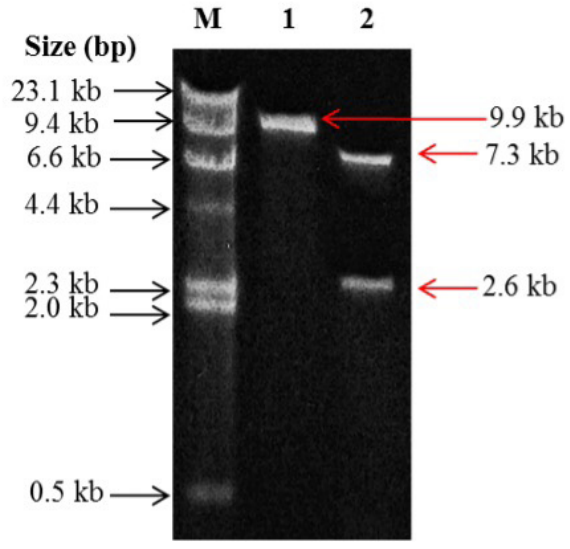

Figure 2 : Agarose gel analysis of recombinant pUC19 clone, pVio19. Restriction double digestions on pVIo19 using enzymes HindIII and BamHI. Lane M: Lambda HindIII DNA marker, Lane 1: Linearised pVio19 (9.9 kb), Lane 2: pVio digested with HindIII and BamHI. gave two fragments of size 2.6 kb (pUC19 backbone vector) and 7.3 kb (vioABCDE).

DNA sequence analyses of the cloned insert harboured by pVio19 revealed that the overall organization of the violacein operon (7334 base pairs in size) isolated from strain MTH01showed a 99% identity with published violacein biosynthetic gene cluster of Chromobacterium sp. Subsequent analysis of the translated DNA using BLAST indicated that the cloned insert contained all five genes of the violacein of the violacein operon, i.e. vioA, vioB, vioC, vioD and vioE.

Cloning and Heterologous Expression of the Violacein Biosynthetic Operon into Expression Vectors, pET-3a and pET-11b

For heterologous gene expression studies, the MTH01 violacein operon harboured by pVio19 was subcloned expression vectors, pET-3a and pET-11b, yielding recombinant vectors, pVio3a and pVio11b, respectively. Expression of the violacein operon carried by pVio3a and pVio11b was successfully achieved in *E. coli* BL21(DE3) cells. Appearance of purple colonies could be seen among the transformants carrying either pVio3a (*E. coli* Vio3a clone) or pVIO11b (*E. coli* Vio11b clone) between three to five days of incubation on LB plates (100 µg ampicillin/mL). When grown on LB agar plates, it is worth mentioning that the transformants developed purple colonies regardless whether the inducer, IPTG was present or absent. However, these *E. coli* clones displayed a more fastidious nature when cultivated in M9-YE liquid broth. The cells could utilize either glucose or glycerol as the carbon source, and for the purple pigment to be developed, L-tryptophan must be added to M9-YE. Shifting the incubation temperature from 37 °C to 25 °C also helped promote the development purple pigment. When grown in liquid medium such as M9-YE, the purple pigment precipitates either as discrete particles or present as cell clumps.

Characterisation of Pigment Extracted from *E. coli* Vio3a and *E. coli* Vio11b cells

The violacein extract (in methanol) from *C. violaceum* MTH01 displayed a maximum peak with strong absorption at 560 nm (data not shown). This λ_{max} value is within the UV-Vis spectrum of violacein, as reported by others [17]. There was no distinct single peak for the pigment extracted from *E. coli* Vio3a while the λ_{max} for pigment from *E. coli* Vio11b cells displayed a maximum absorption at 480 nm (data not shown). Several absorbance peaks at different wavelengths were detected, indicating that the samples were a mixture of compounds. As a result, the UV-Vis spectral data obtained was inconclusive in terms of determining the identity of the functional groups present in both extracts from *E. coli* Vio3a and *E. coli* Vio11b cells.

Fortunately, the TLC profile provided a clearer picture of the chemical species abundance for both extracts from *E. coli* Vio3a and *E. coli* Vio11b cells, as seen in Fig. 3. Under white light, the TLC profile for

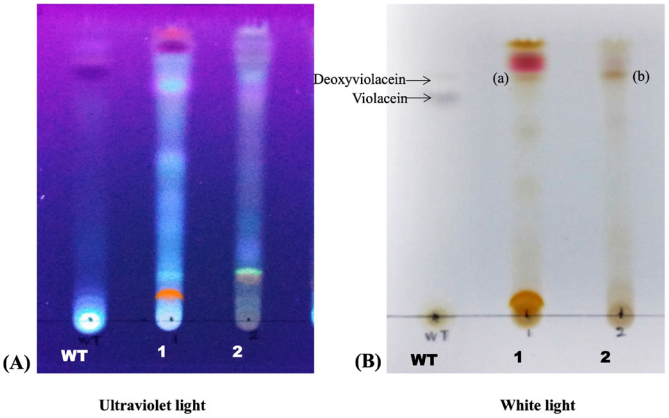

Figure 3 : Separation of the crude extract from *C. violaceum* strain MTH01, E. coli Vio3a and E. coli Vio11b using Thin layer chromatography (mobile phase: ethyl acetate/methanol: 12:8). Lane WT: violacein pigment crude extract from *C. violaceum* strain MTH01 with Rf 0.72 (indicative of violacein) and Rf 0.78 (indicative of deoxyviolacein), Lane 1: crude pigment extract from E. coli Vio3a with Rf 0.78(a), Lane 2: crude pigment from Vio11b with Rf 0.78 (b).

the violacein standard from MTH01 showed two pigments; i.e. deoxyviolacein (pink) and violacein (purple) with Rf values 0.78 and 0.72 respectively. For crude pigment extract from *E. coli* Vio3a and *E. coli* Vio11b cells, there was a distinct band co-migrating at an Rf value of 0.78, suggesting that deoxyviolacein was the dominant chemical species present in both extracts.

Further insights into the functional groups of these compounds was obtained using FTIR. Overall, crude pigment from *E. coli* Vio3a and *E. coli* Vio11b FTIR spectral pattern is almost similar with violacein extract from *C. violaceum* MTH01 (Fig. 4). The FTIR spectrum for pigment extract from both *E. coli* Vio3a and *E. coli* Vio11b demonstrated the presence of amine (C-N) and carbonyl (C=O) of groups, which are characteristics of an indole. However, for the FTIR spectral of *E. coli* Vio3a extract, low transmittance (%) was also observed in the range of 600 cm-1 to 1600 cm-1. This low transmittance (high absorbance) is an indication that apart from indole, the *E. coli* Vio3a extract could possibly be comprised of mixed compounds with alkenes and alkanes as the functional groups.

DISCUSSION

The current work describes the attempts to reconstruct the violacein biosynthetic operon from *C. violaceum* strain MTH01 into T7 expression vectors and achieve heterologous expression of violacein in *E. coli* cells, BL21 (DE). The successful expression of genes and the recombinant production of foreign proteins or metabolites using a heterologous system is a challenging and intricate task. Among the issues that must be addressed are leaky expression, protein

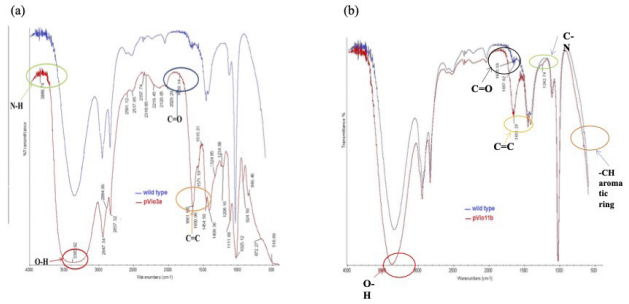

Figure 4 : Comparison of FTIR spectra for violacein pigment crude extracts from *C. violaceum* strain MTH01 cells, E. coli Vio3a cells and E. coli Vio11b cells. The FTIR spectrum for crude pigment from E. coli Vio3a (a) demonstrated the presence of a carbonyl of an amide (C=O) group (1661.94 cm-1), alcohol (OH) group (3383.93 cm^{-1}), amine (C-N) group (1025.12 cm^{-1}), carbonyl (C=O) group (1650.96 cm^{-1}), out of plane =C-H (849.46 cm^{-1}) and alkene (C=C) group (in the range of 1640 cm^{-1} to 1680 cm^{-1}). The FTIR spectrum for crude pigment from E. coli Vio11b (b) showed the presence of alcohol (OH) group (3356.06 cm^{-1}) and amine (C-N) group (1242.74 cm^{-1}). The presence of carbonyl (C=O) and alkene (C=C) group was observed at 1651.23 cm^{-1}. The out of plane band for C=H was observed at 788.96 cm^{-1}.

instability, insoluble and toxic protein products. As a result, the selection of expression vectors and host strains is critical for protein expression, solubility, and yield. Apart from this, optimisation of parameters that are known to influence microbial pigment production also needs to be addressed.

To generate an *E. coli* clone capable of producing violacein as a secondary metabolite, the cell must be able to express all five enzymes, VioABCDE, involved in the catalytic conversion of L-tryptophan into the bisindole violacein. Hence, to achieve violacein production the *E. coli* cells, the entire violacein biosynthetic cluster, comprising of genes vioABCDE, had to be cloned into expression vectors and introduced into the cells. Overall, the genome of *C. violaceum* has a very high GC (64.83%) [16] content. The targeted violacein biosynthetic gene cluster is not only high in GC content, but also very long, measuring 7.3 kilo base pairs in length. It is a well-established fact that amplification of high GC content genes by PCR is a major challenge during the creation of recombinant GC-rich DNA constructs [17]. Therefore, to minimise problems associated with high GC content, PCR amplification and cloning of this long violacein biosynthetic operon was carried out by splitting this targeted operon into two separate fragments, i.e. Fragment A and Fragment B. The amplified Fragment A (vioAB) and Fragment (vioCDE) were then reassembled into pUC19, yielding the complete violacein biosynthetic gene cluster with all genes arranged contiguously and transcribed as an operon.

For gene expression studies, this violacein biosynthetic operon was subcloned into T7 expression vectors, pET3a (T7 promoter) and pET11b (T7 promoter with lac operator and lacI repressor). These pET expression vectors, which were derived from pBR322 plasmid, and comes under the control of the T7 phage promoter, have been shown to achieve a tighter control over basal expression in comparison to other *E. coli* promoter-based systems such as trc and lacUV5 (18). These vectors are also touted to be extremely effective, allowing for high levels of target protein expression, up to 50% of total cellular protein production [19]. Expression of the violacein biosynthetic operon cloned into both pET3a and pET11b expression vectors was successfully achieved in *E. coli* BL21 (DE3) cells. *Escherichia coli* BL21 (DE3) strain was chosen as host because it possesses several traits that could enhance the yield of recombinant protein produced. This strain is deficient in Lon and OmpT proteases, enzymes that mediate the degradation of abnormal or foreign proteins [18,20]. This is an important feature because recombinant protein degradation could be reduced, thereby increasing product stability and yield. Apart from this, *E. coli* BL21 (DE3) is equipped with the lacI and the lacUV5-driven T7 RNA polymerase cassette [18]. Ideally, in this kind of scenario, derepression of the lacUV5 promoter would only happen upon induction by IPTG, allowing over-expression of T7 RNA polymerase and thus expression of the T7-controlled target gene from pET expression vectors. However, it was observed that when cultured on Luria agar plates, *E. coli* Vio3a and *E. coli* Vio11b cells developed purple colonies even in the absence of IPTG. Despite the strong selectivity of the T7 promoter, residual "leaky" expression of proteins from pET-3 constructs have been acknowledged [21]. It was, however, perplexing to note that the pET-11b construct, which is equipped with extra transcriptional control element such as the lac operator, also failed to demonstrate more stringent regulation of the target genes.

In this study, transformed *E. coli* cells carrying pVio3a (*E. coli* Vio3a) or pVio11b (*E. coli* Vio11b) developed purple colonies after a few days of incubation, indicating the successful expression of the violacein biosynthetic gene cluster. Nevertheless, the development of the purplish hues by *E. coli* Vio3a and *E. coli* Vio11b when plated on plate agar was less intense when compared to the natural producer strain *C. violaceum* MTH01. Although the pigment/product yield extracted from *E. coli* Vio3a and *E. coli* Vio11b was not particularly impressive, the fact that the violacein biosynthetic gene cluster was successfully expressed in the *E. coli* cells is encouraging. The FTIR analysis confirmed that the pigments expressed by *E. coli* Vio3a and *E. coli* Vio11b contained amine (C-N) and carbonyl (C=O) of groups, which are

characteristics of an indole. Incidentally, the TLC profile also indicated that instead of violacein, deoxyviolacein, was the dominant chemical species present in both extracts.

Parameters such as carbon source, temperature, culture medium, aeration rate, and addition of the precursor L-tryptophan, to growth medium were tested to see if they influenced the heterologous expression of violacein in E.coli cells. The heterologous expression system developed here could use either glucose or glycerol as carbon source, good aeration was necessary and pigment production appears to be most favourable at 25°C. However, based on the product yield and quality of pigment obtained, there appears to be no discernible advantage to culturing the recombinant strains in rich growth media such as Luria Bertani or defined M9-YE medium. L-tryptophan plays a major role for successful violacein biosynthesis [22]. Hence, although *E. coli* can produce a reasonable amount of tryptophan via its amino acid biosynthesis pathway, an adequate amount of L-tryptophan was added to the growth medium. This is a pre-emptive measure to ensure that L-tryptophan would not be the rate-limiting determinant in the violacein biosynthesis.

Various factors known to influence the successful expression of recombinant proteins/products were taken into consideration in the design and development of an expression system for heterologous production of violacein in *E. coli* cells. Despite this, it is important to note that the dynamics of violacein biosynthesis are also dependent on the intricate balancing of transcripts and proteins/enzymes, as well as the action of promoter and ribosome binding sites [20, 23-24]. In violacein biosynthesis, VioA catalyses the condensation of two L-tryptophan molecules to 2-imino-3(Indole-3-yl) propionate. This indole is then converted to protodeoxy violaceinate by enzymes VioB and VioE, and subsequently converted to violacein by the catalytic action of VioD and VioC [5]. In the absence of VioD, the violacein biosynthetic pathway may produce deoxyviolacein as the dominant metabolite [22, 25]. Therefore, in this heterologous expression system, VioD could be the limiting factor or impoverished, resulting in deoxyviolacein as the main metabolite produced.

CONCLUSION

Achieving successful gene expression using a heterologous system is a very intricate matter and all aspects from gene expression, bioprocess optimization and optimisation of parameters that are known to influence microbial pigment production needs to be taken into consideration.

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