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Full Length Research Paper

Efficient expression of TdiA, a single-module nonribosomal peptide synthetase in *Escherichia coli* Rosetta (DE3) for enzymatic synthesis of bis-indolylquinone

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TdiA, a nonribosomal peptide synthetase, catalyzes the carbon-carbon bond formation in biosynthesis of bis-indolylquinone natural products. The TdiA gene (*tdiA*) containing several codons rarely used by *Escherichia coli*, was cloned from the genome of *Aspergillus nidulans* and optimized in two strains of *E. coli*: BL21 (DE3) and Rosetta (DE3), which is a rare codon optimizer strain. The effects of initial isopropyl-β-D-thiogalactopyranoside (IPTG) concentration and induction time on the enzyme expression level were investigated in two strains. The results indicated that the amount of TdiA expressed in Rosetta (DE3) was about 6-fold higher than that in BL21 (DE3). The activity of TdiA expressed by Rosetta (DE3) in enzymatic synthesis of bis-indolylquinone using indole-3-pyruvic acid (IPA) as substrate was generally the same as that expressed in BL21 (DE3). Based on the optimal culture system using Rosetta (DE3), the yield of TdiA achieved 10.32 mg/L under the appropriate conditions. This efficient expression of TdiA would be in favour of advancing the totally enzymatic preparation of bis-indolylquinone natural products.

Key words: Nonribosomal peptide synthetase, *Aspergillus nidulans*, *Escherichia coli* Rosetta (DE3), rare codons, bis-indolylquinone.

INTRODUCTION

TdiA, a nonribosomal peptide synthetase (971 amino acids) from *Aspergillus nidulans*, was organized in three distinct domains including (1) an adenylation domain, (2) a thiolation domain, and (3) a thioesterase/cyclase (Bouhired et al., 2007). These domains resemble nonribosomal peptide synthetases and the TdiA catalyzes carbon-carbon bond formation, which is a key mechanism responsible for the biosynthesis of the class of bis-indolylquinone natural products (Schneider et al., 2007). The bis-indolylquinone compounds have received close attention for its antiretroviral activity, anti-HIV activity, antidiabetes activity and cytotoxic effects in the submicromolar range (Fredenhagen et al., 1997; Kaji et al., 1998; Zhang et al., 1999).

Recently, the *tdiA-E* gene cluster for bis-indolylquinone metabolic product Terrequinone A, was discovered in the genome of the filamentous fungus *A. nidulans* applying genomic and chemical analyses along with microarray techniques (Bok et al., 2006). It is observed that *Escherichia coli* has become first choice organism for recombinant protein expression due to convenient handling and its long history of use in biotechnology. However, (Balibar et al. 2007) have overproduced *tdiA* gene in the bacterial host *E. coli* and the TdiA expression in *E. coli* BL21 (DE3) with isopropyl-β-D-thiogalactoside (IPTG) induction yielded only 1.6 mg L^{-1} of tagged

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enzyme. The protein production is still not a trivial task and optimal cultivation conditions are hard to set for large sample sets. High frequency or clusters of codons rarely used in the expression host appears sometimes to cause translational frame shifting, premature translational termination and misincorporation of amino acids, which would reduce the quality of the product or prevent a high expression yield (Tegel et al., 2010).

Each kind of microbes has their own favourable codons and the protein expression is highly affected by significant differences in codon usage between organisms (Huang et al., 2012). In particular, Arg codons AGA, AGG, CGG, CGA, Ile codon AUA, Leu codon CUA, Gly codon GGA, and Pro codon CCC are commonly used by eukaryotes but rarely used by *E. coli* (Fu et al., 2007). By comparing codons in the *tdiA* gene cloned from *A. nidulans* with those used by *E. coli*, it is clear that several codons are underrepresented. A complete compilation of codon usage of the sequences placed in the GenBank database can be found at http://www.kazusa.or.jp/codon/ (Nakamura et al., 2000), and *E. coli* displays frequencies of about 1.14% rare Arg codons and 0.6% of rare codon CCC. As there are 32 rare Arg and 16 rare codons CCC in the *tdiA* gene from *A. nidulans* (about 3.2 and 1.65% in 971 codons, respectively), excessive rare codon usage in the target gene has been implicated as a cause for low level protein expression (Sorensen et al., 1989; Zhang et al., 1991). However, the strain *E. coli* Rosetta (DE3), a derivative of BL21 (DE3), contains a plasmid of pRARE (CmR) encoding rare tRNAs in *E. coli*. This would enhance the expression of eukaryotic proteins that contain codons rarely used in *E. coli* such as AUA, AGG, AGA, CGG, CUA, CCC and GGA. The aim of this work is to improve expression of the *tdiA* gene from *A. nidulans* and increase TdiA productivity via the adaptation of a rare codon optimizer strain and the optimization of cultivation conditions of the new recombinant *E. coli*.

MATERIALS AND METHODS

Bacterial strains, plasmids and media

The donor strain anamorph *A. nidulans* A4 (ATCC 38163) was purchased from ATCC. The host strain *E. coli* DH5α, BL21 (DE3) [F^-omp T hsd S_B ($r_B^-m_B^-$) gal dcm lacY1 (DE3)] and Rosetta (DE3) [F^-omp T hsd S_B ($r_B^-m_B^-$) gal dcm lacY1 (DE3) pRARE (argU, argW, ileX, glyT, leuW, proL) (Cm^r)] applied in this work were purchased from Beijing TransGen Biotech (Beijing, China). The expression vector pET24a was available in our laboratories and the cloning vector pEASY-Blunt simple cloning vector was purchased from Beijing TransGen Biotech (Beijing, China). The cloning and transformation were carried out as those described by (Sambrook and Russell, 2001).

Amplification and cloning of the *tdiA* gene

The genome of anamorph *A. nidulans* A4 served as the DNA template was based on the method introduced by Balibar et al. (2007). Primers were designed based on the published *tdiA* gene sequence (GenBank: EF550581.1), and an NdeI (CATATG) restriction site at the beginning of the amplified fragment and a *Sal*I (GTCGAC) restriction site at the end of the amplified fragment were involved. The forward and reverse primers were 5′-CAAGTTACATATGGCACCAAGCAAGACCGAGATC-3′ and 5′-CAAGTCGACCAGGCCCCGCTCCCTCAGTACATT-3′ (NdeI sites and Salt sites are underlined), respectively. Primers were synthesized by the Shanghai Sangon Biotech Company (Shanghai, China). The *tdiA* gene was amplified using polymerase chain reaction (PCR), where KOD Plus was used as DNA polymerase (Toyobo, Osaka, Japan). The amplified product was purified using PCR purification kit (Promega, USA).

Generation of recombinant *Escherichia coli* Rosetta (DE3) harboring TdiA, a nonribosomal peptide synthetase cDNA

The PCR product was first cloned into the pEASY-Blunt simple cloning vector (TransGen, Beijing, China) by blunt-end ligation and sequenced by the Shanghai Sangon Biotech Company (Shanghai, China). Next, it was digested with restriction enzymes (*Nde*I and *Sal*I). The gene was purified and ligated with pET24a(+) open by the same enzymes at 16°C overnight, yielding an expression vector *tdiA*-pET24a(+). The ligation system contained purified gene fragment, linearized pET24a(+), ddH_2O, and Ligation Solution I (TakaRa Dalian, China). The final expression plasmids *tdiA*-pET24a(+) were screened and amplified in *DH5a* and then was transformed into *E. coli* Rosetta(DE3) and *E. coli* BL21 (DE3) to obtain *tdiA*-pET24a(+)/*E. coli* Rosetta(DE3) and *tdiA*-pET24a(+)/*E. coli* BL21 (DE3), respectively.

Expression and purification of TdiA, a nonribosomal peptide synthetase in *Escherichia coli* Rosetta (DE3)

The *E. coli* BL21 (DE3) and Rosetta (DE3) containing *tdiA*-pET24a(+) cultured overnight was inoculated at 5% ratio into 300 ml LB medium (Kan 30 μg mL^{-1}) in a 1000 ml conical flask and cultured at 30°C in a shaker set at 200 rpm until the culture reached a logarithmic phase (OD600 = 0.60 - 0.80). Isopropyl-β-D-thiogalactoside (IPTG, Sigma) was added to a final concentration of 40 μmol L^{-1} and the temperature was adjusted down to 15°C. The induction period for *E. coli* BL21 (DE3) and Rosetta (DE3) was 48 h. The cell suspension was centrifuged at 5,000×g for 15 min and resuspended in 25 mM Tris-HCl (pH 8.0) buffer containing 500 mM NaCl. The cells were disrupted by three cycles of sonication (5 min, 60 W, 3 s power, and 3 s pulse) on ice using a high-intensity sonicator (Misonix Sonicator 4000, Qsonica, LLC, Newtown, Connecticut, USA). The cell debris was removed by centrifuged at 11,000×g for 30 min.

The supernatant containing the soluble protein fraction was purified by Ni-NTA gravity flow column (GE Healthcare). The column was washed with buffer (50 mM NaH_2PO_4, 300 mM NaCl, 25 mM imidazole at PH 8.0) when the soluble protein fraction was loaded onto the column. Non-specific proteins bound to the column were eluted thrice using buffer containing 50, 70 and 90 mM of imidazole, respectively. Finally, TdiA was eluted with buffer containing 250 mM of imidazole. The eluted protein fractions were desalted by dialysis three times against 25 mM HEPES (pH 7.5), 150 mM NaCl and 10% glycerol. The protein concentration and proportion were assayed by Bradford method (Bradford, 1976).

Fractions were collected and analyzed using sodium dodecyl sulfate-polyarylamide gel electrophoresis (SDS-PAGE). 20 μL of the purified protein was loaded onto a 12% (w/v) separating gel and a 5% stacking gel SDS–PAGE, the gel was run at 80 and 180 V constant voltage for protein concentration and separation gel, respectively. The gels were stained with Coomassie Blue.

TdiA enzymatic assay

Reactions to measure product formation by TdiA were carried out as the method described by (Balibar et al. 2007). 5 mM TdiA was incubated with the mixture containing 50 mM Tris (pH 8.0), 10 mM $MgCl_2$, 200 µM CoA and 3 µM Sfp. After 45 min at 25°C, the reaction was initiated by addition of 5 mM ATP and 200 mM indole-3-pyruvic acid (IPA), 100 µl of the reaction mixture were quenched using 200 µl MeOH after 4 h and then centrifuged at 16,000 g to pellet protein. The supernatant containing the product was analyzed by high performance liquid chromatography (HPLC). The HPLC analysis was performed on a Shimadzu LC-20A (Shimadzu Corporation, Kyoto, Japan) with a Phenomenex 250 × 4.6 mm C_{18} 5 µm Luna column using a gradient of 5 to 100% acetonitrile over 50 min starting in H_2O; absorbance at 280 nm was monitored. The molecular mass of the synthesized didemethylasterriquinone D was confirmed by a Shimadzu LCMS 2010EV liquid chromatography (LC) mass spectrometry (MS) (Shimadzu Corporation, Kyoto, Japan) analysis using negative spray ionization. One unit of enzymatic activity (U) was defined as the amount of TdiA required to catalyze the transformation of 1 µmoL of IPA to didemethylasterriquinone D per minute.

RESULTS

Cloning and sequence analysis of the full-length cDNA encoding TdiA

The *tdiA* gene was cloned and amplified by PCR from *A. nidulans* A4, using the cDNA as the template and specific primers and sequenced. The forward primer contained an *Nde*I restriction site preceding the ATG start codon, while the reverse primer contained a *Sal*I site. The amplified fragment were digested with *Nde*I and *Sal*I and inserted into the *Nde*I and *Sal*I sites of pET expression vector, followed by ligation. The recombined plasmids pET24a(+)-TdiA were transferred into *E. coli* DH5α and were then introduced into *E. coli* BL21 (DE3) and Rosetta (DE3), respectively.

Rare codons analysis of the *tdiA* gene

Each kind of microbes has their own favourable codons and protein expression is highly affected by significant differences in codon usage between organisms. The protein expression is highly affected by significant differences in codon usage between organisms. *E. coli* was used as the most popular host for the expression of heterologous proteins and the expression of heterologous proteins in it is strongly affected by codon bias. Recent studies suggested that clusters of AGG/AGA, CUA, AUA, CGA or CCC codons can reduce both the quantity and quality of the synthesized protein. In addition, it is likely that an excess of any of these codons, even without clusters, could create translational problems (Kane, 1995). In particular, Arg codons AGA, AGG, CGG, CGA, Ile codon AUA, Leu codon CUA, Gly codon GGA, and Pro codon CCC are commonly used by eukaryotes but rarely used by *E. coli* (Zahn, 1996). This phenomenon occurs when the codon usage of the mRNA coding for the foreign protein differs from that of the bacterium. The ribosome pauses upon encountering a rare codon and may detach from the mRNA, thereby the yield of protein expression is reduced.

Some strategies have emerged for solving codon usage bias such as codon optimization of the foreign coding sequence by silent mutagenesis or increasing the availability of underrepresented tRNAs by host modification (Huang et al., 2012; Rosano and Ceccarelli, 2009; Sorensen and Mortensen, 2005). While codon optimization is a cumbersome and expensive process, modifying host availability of rare tRNAs is an easier approach. Several bacterial strains have been engineered to overcome this effect. *E. coli* Rosetta (DE3) contains a plasmid of pRARE (CmR) encoding rare tRNAs and enhances the expression of eukaryotic proteins that contain codons rarely used in *E. coli*: AUA, AGG, AGA, CGG, CUA, CCC and GGA.

By comparing the codons in the *tdiA* gene cloned from *A. nidulans* with those used by *E. coli*, it is clear that several codons are underrepresented (Figure 1). Since there are 32 Arg rare codons (AGG, AGA, CGA), 16 Pro rare codons (CCC) and 8 Ile rare codons (ATA) in the *tdiA* gene (971 codons) from *A. nidulans* (about 3.2, 1.65 and 8.2% in 971 codons, respectively), *E. coli* displays frequencies of about 1.14% rare Arg codons, 0.6% of Pro rare codons (CCC) and 0.5% Ile rare codons (ATA). A complete compilation of codon usage of the sequences placed in the GenBank database can be found at http://www.kazusa.or.jp/codon/. The number of rare *E. coli* codons in a DNA sequence can be determined at http://nihserver.mbi.ucla.edu/RACC/.

Expression and purification of the recombinant TdiA

The positive construct was chosen and confirmed by PCR coupled with sequencing analysis and then expressed under control of the T7 promoter. The recombinant pET24a(+)-*tdiA* was over-expressed in *E. coli* BL21 (DE3) and Rosetta (DE3) following induction with IPTG. The purification steps of *E. coli* Rosetta (DE3)/pET24a(+)-TdiA are shown in Table 1. The final specific activity of the purified TdiA expressed by Rosetta (DE3) was showed an increase of over 2.3-fold with respect to crude activity. The specific activity of *E. coli* Rosetta (DE3)/pET24a(+)-TdiA was 2.30 U/mg (Table 1). The protein migrated as a single band with an apparent molecular mass of 105.5 kDa. The SDS-PAGE result via Glyko BandScan showed that the TdiA expressed in Rosetta (DE3) was approximately 24.2% of the total soluble protein, which was about 6-fold higher than that in BL21 (DE3) (Figure 2). The activity of TdiA expressed by Rosetta (DE3) on biosynthesis was generally the same as BL21 (DE3) (Figure 5).

The effect of IPTG concentration and induction time on

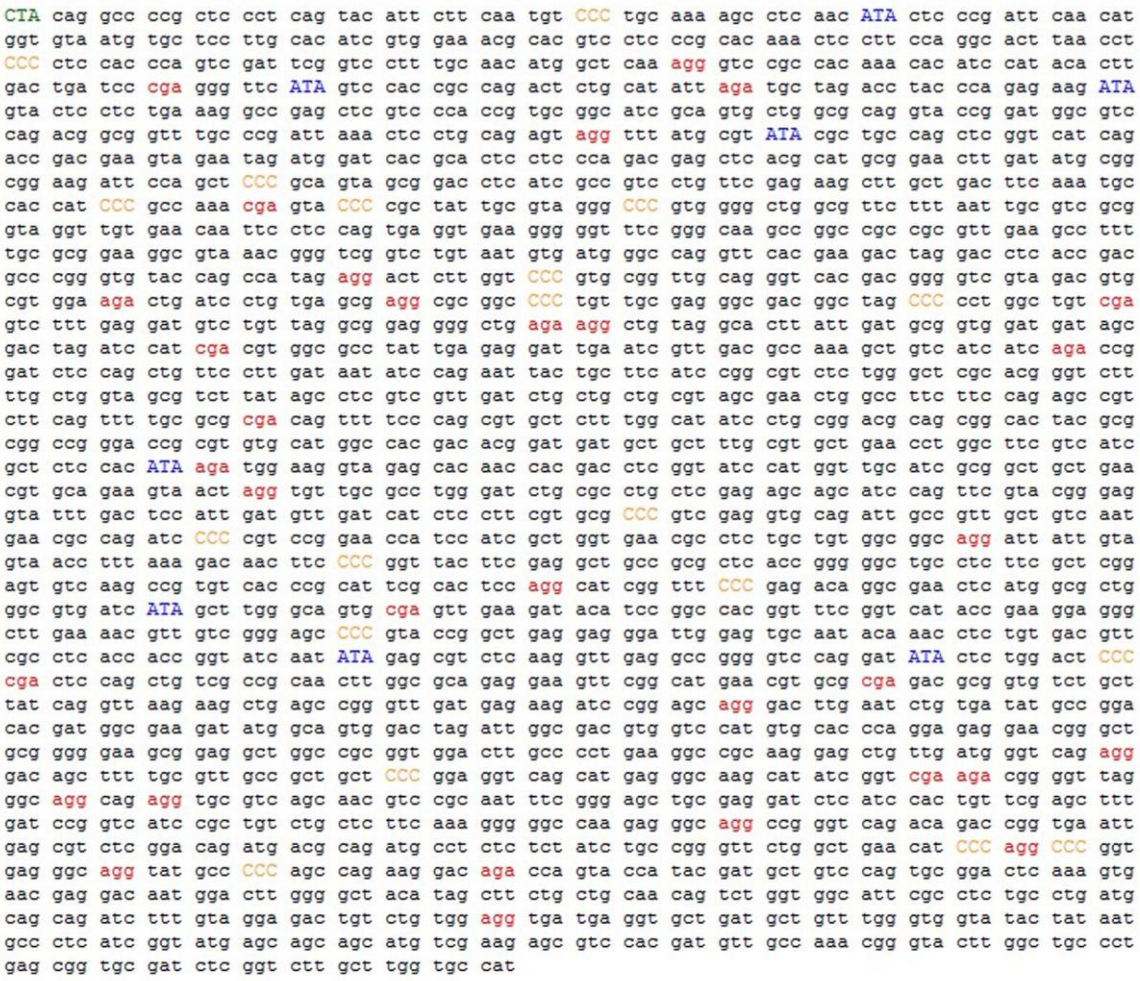

Figure 1. The nucleotide sequences of *tdiA* gene. (Red, rare Arg codons AGG, AGA, CGA; Green, rare Leu codon CTA; Blue, rare Ile codon ATA; Orange, rare Pro codon CCC).

Table 1. TdiA production expressed in *E. coli* Rosetta (DE3)/pET24a-TdiA.

Step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification (-fold)	Yield (%)
Crude extract	80	99.45	98.7	0.99	-	100
Ni^{2+}-affinity column	10	10.18	23.4	2.30	2.3	23.7

the growth of *E. coli* Rosetta (DE3)/pET24a(+)-TdiA was evaluated and the results are shown in Figures 3 and 4. IPTG added after batch cultures were carried out for OD_{600} was approximately 0.6 to 0.8, and the cultivation temperature was lowered from 30 to 15°C after induction. The cells were harvested after cultivation for 48 h. The results indicated that at low IPTG concentration, *E. coli* Rosetta (DE3) have a good cell growth, and the growth of *E. coli* Rosetta (DE3)/pET24a(+)-TdiA reach the plateau after induced 24 h. The TdiA produced by *E. coli* Rosetta (DE3) was 10.18 mg L^{-1} (Table 1) and about 6.5-fold higher than that in BL21 (DE3) (Balibar et al., 2007).

TdiA biosynthesis of bis-indolylquinone natural products

TdiA resembles nonribosomal peptide synthetases using an assembly line logic comprising multiple modules; these enzymes use a thiotemplated mechanism to activate, tether and modify amino acid monomers, sequentially elongating the peptide chain and finally

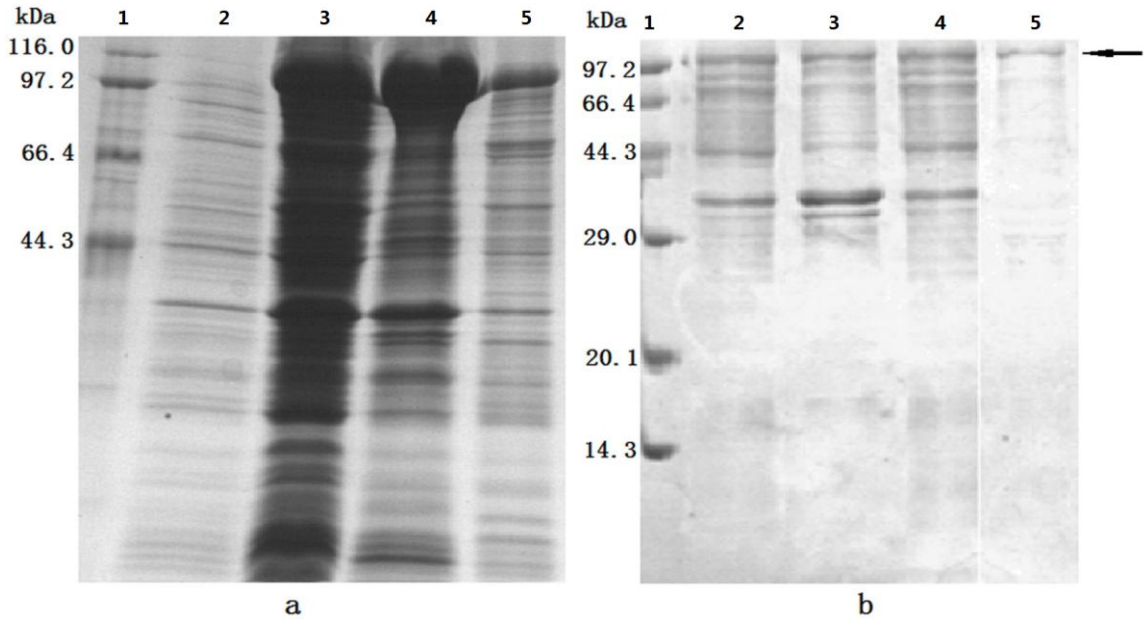

Figure 2. Expression of foreign TdiA in the recombinant *E. coli* Rosetta (DE3)/pET24a(+)-TdiA and *E. coli* BL21 (DE3) /pET24a(+)-TdiA. (a) Lane 1: the high-molecular weight protein-size marker, from top to bottom: 116.0, 97.2, 66.4, and 44.3 kDa. Lane 2: whole cells of *E. coli* Rosetta (DE3)/pET24a(+)-TdiA without induction. Lane 3: whole cells of *E. coli* Rosetta (DE3)/pET24a(+)-TdiA which was induced under the optimal conditions. Lane 4: soluble proteins of *E. coli* Rosetta (DE3)/pET24a(+)-TdiA. Lane5: purified *E. coli* Rosetta (DE3)/pET24a(+)-TdiA after NI-NTA affinity chromatography. (b) Lane 1: the low-molecular weight protein-size marker, from top to bottom: 97.2, 66.4, 44.3, 29.0, 20.1 and 14.3 kDa. Lane 2: whole cells of *E. coli* BL21 (DE3) /pET24a(+)-TdiA which was induced under the optimal conditions. Lane 3: insoluble proteins of induced *E. coli* BL21 (DE3)/pET24a(+)-TdiA. Lane4: soluble proteins of *E. coli* BL21 (DE3)/pET24a(+)-TdiA. Lane 5: purified *E. coli* BL21 (DE3)/pET24a(+)-TdiA after NI-NTA affinity chromatography.

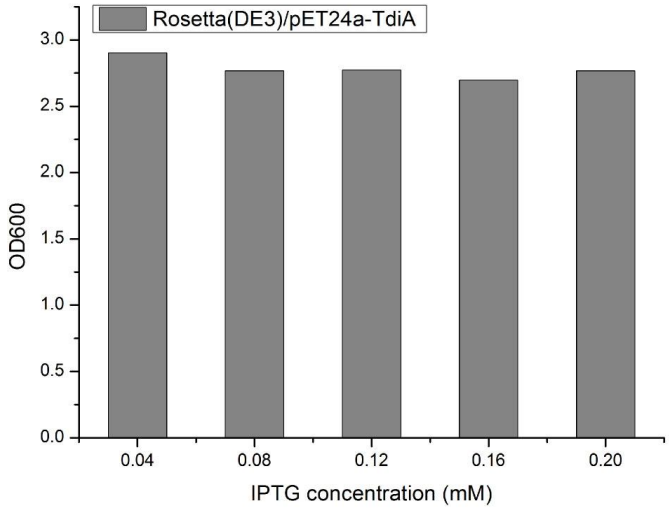

Figure 3. Effect of IPTG concentration on growth of *E. coli* Rosetta (DE3)/pET24a(+)-TdiA.

releasing the complete peptide (Balibar et al., 2007; Stein et al., 1996). Magarvey et al., 2006 performed an ATP-PPi exchange assay on IPA to address the substrate specificity of TdiA. In the present work, we assay the transformation of IPA to didemethylasterriquinone D biocatalyzed by TdiA and HPLC, and determine the enzymes activity and the ability to form bis-indolylquinone. The methods for biosynthesis of bis-

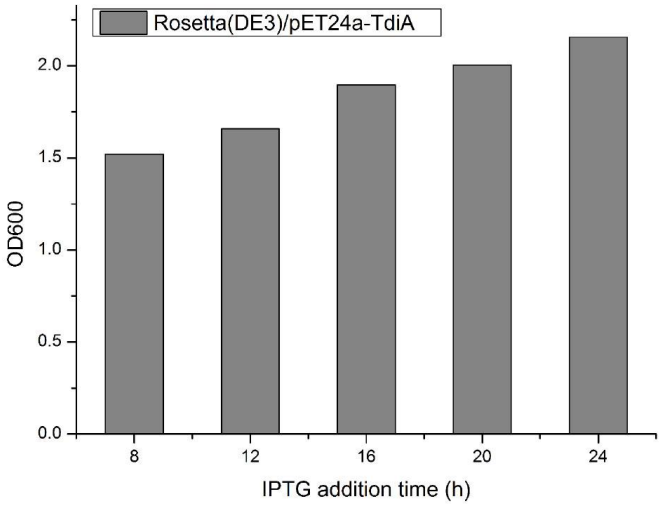

Figure 4. Effect of IPTG induction time on growth of *E. coli* Rosetta (DE3)/pET24a(+)-TdiA.

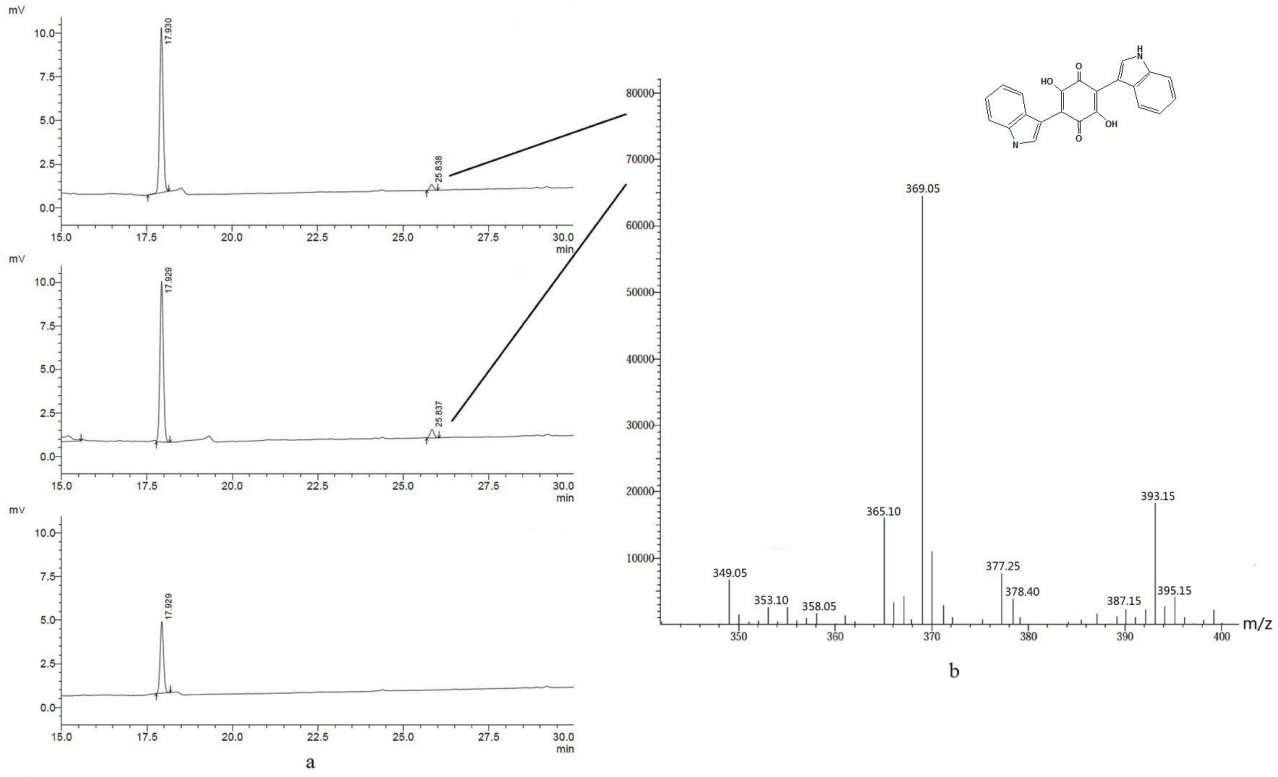

Figure 5. The HPLC chromatogram of the reaction mixture quenched with methanol (a) and mass spectrum (MS) of didemethylasterriquinone D (b) ($M\text{-}H^+$ = 369.05). The HPLC showed the formation of the substrate IPA and product of transformation of didemethylasterriquinone D (R_t =17.929 min, indole-3-pyruvic acid; R_t = 25.837min, didemethylasterriquinone D). The MS also showed the formation of didemethylasterriquinone D ($M\text{-}H^+$ = 369.05).

indolylquinone by TdiA were described by (Balibar et al. ,2007). Figure 5 shows the HPLC chromatogram of TdiA biotransform IPA to Di-demethylasterriquinone D. We can infer that there was no significant difference in TdiA activity for *E. coli* Rosetta (DE3) and BL21 (DE3) (Figure 5a). The mass spectrum confirmed the molecular mass of didemethylasterriquinone D ($M\text{-}H^+$ = 369.05) (Figure 5b) catalyzed by TdiA.

DISCUSSION

E. coli host as the first choice for overexpressing heterologous proteins has numerous advantages, including inexpensive culture conditions, very well known genetic background, easy manipulation and amenability to high density fermentation procedures (Sahdev et al., 2008). However, the expression of heterologous proteins in *E. coli* is strongly affected by codon bias. In the present work, Rosetta (DE3) strains were used to enhance the expression of eucaryotic proteins which contain codons rarely used in *E. coli*. The existence of rare codons in foreign gene can cause insufficient tRNA pools, which will lead to translational stalling, premature translation termination, translation frame shifting, and amino acid mis-incorporation (Fu et al., 2008). Consequently, one of the effective ways to get high expression levels of proteins was to improve the abundance of tRNAs for rare codons in the host cells (Fu et al., 2007). Several groups have shown that the yield of protein whose genes contain rare codons can be improved when the cognate tRNA is increased within the host (Brinkmann et al., 1989; Rosenberg et al., 1993).

The *tidA* gene cloned from a filamentous fungi *A. nidulans* (Galagan et al., 2005) to construct recombinant *E. coli* for bis-indolylquinone compounds production. The bis-indolylquinones exhibit a range of biological activities against cancer and diabetes (Zhang et al., 1999). The *tidA* gene was expressed in *E. coli* BL21 (DE3) and yielded only 1.6 mg L^{-1}(Balibar et al., 2007). As there are 32 rare Arg and 16 rare codons CCC in the *tdiA* gene from *A. nidulans* (about 3.2 and 1.65% in 971 codons, respectively), excessive rare codon usage in the target gene has been implicated as a cause for low level protein expression.

In this work, a rare codon optimizer, *E. coli* Rosetta (DE3), was applied to express the *tdiA* gene from *A. nidulans*. The results of this study showed that the activity of TdiA expressed by *E. coli* Rosetta (DE3) was the same as the ordinary *E. coli* BL21 (DE3), while the yield of TdiA production by *E. coli* Rosetta (DE3) was about 10.32 mg L^{-1}, and about 6.5-fold higher than that produced by BL21 (DE3), which explained that the codon favour ability of the host strain was an important factor for enzyme expressed by a foreign gene, and the higher TdiA product was able to promote the di-demethylasterriquinone D biosynthesis. Further study of TdiA catalyzes carbon-carbon-bond formation in biosynthesis and the class of bis-indolylquinone natural products is in progress.

ACKNOWLDGEMENTS

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