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# Strategies for increasing heterologous expression of a thermostable esterase from *Archaeoglobus fulgidus* in *Escherichia coli*

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ABSTRACT

Heterologous proteins expressed in bacteria are used for numerous biotechnological applications. *Escherichia coli* is the most commonly used host for heterologous protein expression because of its many advantages. Researchers have been studying proteins from extremophiles heterologously expressed in *E. coli* because the proteins of extremophiles are strongly resistant to extreme conditions. In a previous study, a thermostable esterase Est-AF was isolated from *Archaeoglobus fulgidus* and expressed in *E. coli*. However, further studies of Est-AF were difficult owing to its low expression levels in *E. coli*. In this study, we used various strategies, such as changing the expression vector and host strain, codon optimization, and optimization of induction conditions, to increase the expression of Est-AF. Through codon optimization and by changing the vector and host strain, Est-AF expression was increased from 31.50 ± 0.35 mg/L to 61.75 ± 0.28 mg/L. The optimized expression system consisted of a codon-optimized *Est-AF* gene in a pET28a(+)-based expression plasmid in *E. coli* Rosetta cells. The expression level was further increased by optimizing the induction conditions. The optimized conditions were induction with 0.4 mM isopropyl-b-D-1-thiogalactoside (IPTG) at 37 °C for 5 h. Under these conditions, the expression level of Est-AF was increased from 31.5 ± 0.35 mg/L to 119.52 ± 0.34 mg/L.



## 1. Introduction

Heterologous protein expression is an important biotechnological tool that is used to produce proteins for research and industry [1,2]. The most commonly used host for high-level heterologous protein expression is *Escherichia coli*, because of its numerous advantages [3,4]. First, *E. coli* grows fast, with a doubling time of approximately 20 min under optimal culture conditions. Next, *E. coli* cultures can easily reach a high density; in liquid culture, the density limit of *E. coli* is approximately 200 g dry cell weight. In addition, *E. coli* can be cultured in medium made with inexpensive components. Lastly, exogenous DNA can be introduced into *E. coli* using a simple transformation method.

Extremophiles can grow under extreme conditions, such as at high and low temperatures, high pressure, high salinity, in the presence of radiation, and at high and low pH, and most extremophiles are Archaea [5,6]. Generally, the proteins of a given organism show optimum function under conditions similar to its optimal growth conditions [7,8]. Therefore, proteins from extremophiles are also resistant to extreme conditions. Many researchers use *E. coli* to produce high levels of the target protein for studies and biological applications [9–14].

However, heterologous protein expression can be limited by numerous factors [3], including little or no production due to toxicity of the expressed protein, codon bias, and other limiting factors in batch cultivation. In addition, the heterologous protein can form insoluble inclusion bodies due to disulfide bonding, interaction with chaperones, and a slow production rate. Lastly, the heterologous protein may not be produced in an active form.

Protein structure elucidation methods, such as X-ray crystallography, NMR spectroscopy, and cryo-electron microscopy, require high concentrations of protein [13,15], as do industrial applications of biocatalysis [16,17].

In a previous study, we identified an esterase in *Archaeoglobus fulgidus* (Est-AF) and expressed it in *E. coli* [18]. The Est-AF showed high thermostability and enzyme activity toward (*R,S*)-ketoprofen ethyl ester, but low enantioselectivity toward (*S*)-ketoprofen ethyl ester. Therefore, we improved the enantioselectivity of Est-AF toward (*S*)-ketoprofen ethyl ester by error-prone polymerase chain reaction (PCR) and site-saturated mutagenesis [19]. However, we

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had difficulty doing further studies, such as crystallization and structural analysis of Est-AF, because the protein was expressed at low levels in *E. coli*.

In this study, we increased the expression levels of Est-AF in *E. coli* by changing the expression vector and host strain and optimizing the codons of the *Est-AF* gene for *E. coli*. Then, we elucidated the reason for the increased expression of Est-AF by measuring *Est-AF* mRNA levels using RT-PCR. Finally, the expression of Est-AF was further increased by optimizing the induction conditions, including the temperature, isopropyl-b-D-1-thiogalactoside (IPTG) concentration, and time.

## 2. Materials and methods

### 2.1. Strains, plasmids and growth conditions

The original Est-AF construct, containing the wild-type *Est-AF* gene cloned into pQE30 [pQE30-AF(WT)], was described previously [18]. The pET28a(+) vector was used to express Est-AF in the following host strains: *E. coli* BL21(DE3), HMS174(DE3), and Rosetta™(DE3). The pET28a(+) vector and host strains were purchased from Novagen (USA). Host strains were cultured in Luria-Bertani (LB) medium (MB Cell, USA) at 37 °C with shaking at 180 rpm. Competent cells were prepared by the conventional $CaCl_2$ method [20].

### 2.2. Construction of expression systems

The wild-type *Est-AF* gene was amplified by PCR with primers Forward (*Bam*HI) 5′-CGGGATCCATGGAAAGAA-TAACTCTCGAAATCGAT-3′ and Reverse (*Hind*III) 5′- CCCAAGCTTT-TACAGCTTCTCAATAAAATTTTTTAGGG-3′ (restriction sites are underlined). The primers were synthesized by Macrogen (Korea). The 50-μL PCR reaction mixture contained 1 × reaction buffer, 200 μM deoxynucleotide triphosphate mix, 25 pM each primer, 10 ng of template [pQE30-AF(WT)], and 5 units of *Pfu* DNA polymerase (BEAMS Biotechnology, Korea) in ultra-pure water. The PCR cycling conditions were as follows: 95 °C for 45 s, 52 °C for 30 s, and 72 °C for 1 min and 30 s for 25 cycles.

The codons of *Est-AF* were optimized using the OPTIMIZER server (http://genomes.urv.es/OPTIMIZER/) [21]. The optimized *Est-AF* gene was synthesized by Integrated DNA Technologies (USA).

The wild-type or codon-optimized *Est-AF* genes were subcloned into pQE30 or pET28a(+) at the *Bam*HI and *Hind*III sites. The restriction enzymes *Bam*HI and *Hind*III were purchased from New England Biolabs (USA). The recombinant plasmids were transformed into *E. coli* XL1-blue, BL21(DE3), HMS174(DE3), or Rosetta™(DE3) cells for expression.

### 2.3. Expression and purification

The transformed *E. coli* cells were grown in LB broth containing ampicillin (100 mg/mL) or kanamycin (50 mg/mL) to an optical density at 600 nm ($OD_{600}$) of 0.4–0.5, at which point, esterase expression was induced by the addition of 1 mM IPTG to the culture medium. After 3 h of induction at 37 °C, the cells were harvested by centrifugation at 2560 × *g* for 20 min, and then stored at −20 °C overnight. The cells were resuspended in 50 mM sodium phosphate buffer (pH 8.0) containing 10 mM imidazole and were lysed by sonication (Sonics, USA). Cell-free extracts were prepared by incubation at 75 °C for 30 min to denature all proteins other than Est-AF and centrifugation at 2560 × *g* for 20 min. The His-tagged Est-AF protein was purified from the supernatant by affinity chromatography with nickel-nitrilotriacetic acid resin (QIAGEN, Germany) according to the manufacturer's instructions. The eluted protein was dialyzed twice against 50 mM Tris–HCl buffer (pH 8.0) to remove the imidazole.

### 2.4. Optimization of induction conditions

The induction conditions were optimized as determined by the final concentration of the purified Est-AF. To determine the optimum induction temperature, expression was induced at various temperatures (23 °C–44 °C) using 1 mM IPTG for 3 h. To determine the optimum IPTG concentration, expression was induced at 37 °C with various concentrations of IPTG (0.2 mM–1.6 mM) for 3 h. To determine the optimum induction time, expression was induced at 37 °C with 0.4 mM IPTG for various periods (1 h–8 h). Est-AF was purified using the method described above. Cell growth under the tested conditions was assessed by measuring the $OD_{600}$.

### 2.5. Analytical procedures

The concentration of the purified Est-AF was determined by the Bradford method using PRO-MEASURE protein measurement solution (Intron Biotechnology, KOREA) with BSA as the standard [22].

The purity and molecular weight of Est-AF were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) as described previously [23]. The SDS-PAGE system was purchased from Bio-Rad Laboratories (USA).

The quantity of *Est-AF* mRNA was determined by real-time reverse transcriptase PCR (RT-PCR). The quenching of *Est-AF* mRNA was performed by RNAprotect™ Bacteria Reagent (QIAGEN, Germany). RNA was extracted with the RNeasy® Mini kit (QIAGEN, Germany). The cDNA was synthesized from the extracted RNA using cDNA EcoDry™ Premix (Clontech, USA). The primers were designed using Primer Express® Software v3.0.1 and were synthesized by Macrogen (Korea). The 16S ribosomal RNA of *E. coli* (GenBank accession number J01859.1) was included as an internal control. The RT-PCR was performed using Power SYBR® Green PCR Master MIX (Thermo Fisher Scientific, USA) in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA). All experiments were performed according to the manufacturer's instructions.

The enzyme activity of purified Est-AF was determined by high-performance liquid chromatography (HPLC) using (*R,S*)-ketoprofen ethyl ester as described previously [18]. The HPLC system and Chirex Phase 3005 column were purchased from Waters (USA) and Phenomenex (USA), respectively.

## 3. Results

### 3.1. Codon optimization of the Est-AF gene and construction of expression systems

The wild-type *Est-AF* gene, which is composed of 744 bases (248 codons), was optimized using the OPTIMIZER server (Table 2). After codon optimization, 164 bases were substituted and 148 codons were modified. The optimized *Est-AF* gene shared 78% nucleotide identity with the wild-type gene. The codon usage of the wild-type and codon-optimized *Est-AF* genes are compared in Table 2. The codon adaptation index (CAI), effective number of codons (ENc), and GC content of the codon-optimized *Est-AF* gene were 1, 21, and 50.4%, respectively (see Table 3).

The expression systems were composed of combinations of *Est-AF* gene, expression vectors, and host strains (Table 1). The wild-type or codon-optimized *Est-AF* gene was subcloned into an expression vector [pQE30 or pET28a(+)]: pQE30-AF(WT), pQE30-AF(OP), pET28a(+)-AF, and pET28a(+)-AF(OP). Expression from the pQE30-AF(WT) and pQE30-AF(OP) constructs was controlled by the T5 promoter, and expression from pET28a(+)-AF(WT) and