



Enhanced Expression of an *Acinetobacter baumannii* specific recombinant endolysin in *Escherichia coli*

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Article Info	Abstract
<p>Document Type: Research Paper</p> <p>Received 26/04/2024 Received in revised form 15/06/2024 Primary Accepted 22/07/2024 Accepted 01/08/2024</p> <p>Published 12/08/2024</p> <p>Keywords: Prophage Lysin, Recombinant Enzyme, expression improvement, PlyF307</p>	<p>Prophage endolysin PlyF307, a peptidoglycan-destroying enzyme previously identified through the screening of the <i>Acinetobacter baumannii</i> genome, has shown the ability to kill numerous clinical isolates of <i>A. baumannii</i> in its recombinant form. <i>A. baumannii</i> is an extremely antibiotic-resistant Gram-negative hospital pathogen that is distributed worldwide. In this study, we used <i>Escherichia coli</i> BL21(DE3) and BL21(DE3) pLysS as a recombinant protein expression host to produce His-tagged PlyF307. Expression was done in Luria-Bertani (LB), Terrific Broth (TB), and auto-inducing medium, and different concentrations of β-d-1-thiogalactopyranoside (IPTG) were used for inducing. Induction was performed several times during the growth logarithmic phase. Bacterial cells were harvested at different post-induction times. Extraction and purification of the recombinant endolysin were performed using different lysis buffers and sonication programs. According to the experimental results, expression inducing was done with 0.1 mM IPTG at OD₆₀₀ = 0.9. The incubation temperature was 37 °C before and after induction time. Finally, 520-570 mg of recombinant his-tagged PlyF307 (19.7 kD) was purified in different batches using 250 mM imidazole from 8- h post-induction harvested <i>E.coli</i> BL21(DE3) pLysS- PlyF307 cultured in 1- l Luria- Bertani broth (LB) medium in baffled flasks. The purified recombinant protein was verified using the western blotting technique. In conclusion, the strong positive net charge and bacteriolytic activity of the PlyF307 make it a suitable candidate for use in therapeutics and other biotechnology applications. Enhancement of the recombinant endolysin production yield was considerable in this study and absolutely will be helpful to achieve this purpose, and this improved expression can be a significant step toward the scaling-up of the enzyme production in <i>E. coli</i>.</p>

1. Introduction

Endolysins, or Lysins, are peptidoglycan hydrolyzing enzymes (PGH) used by phages to cleave the cell wall of bacterial hosts during the final stage of the lytic cycle (Yuan et al., 2021). When endolysins access peptidoglycan, they can degrade it, disturbing the bacterial cell's osmotic stability, destroying it, and leading to cell death

(Murray et al., 2021). This specified killing mechanism of endolysins can be exploited by applying it as recombinant proteins from the outside to target intended bacteria (Schmelcher & Loessner, 2021). The use of endolysins to trigger cell death can be used as an alternative to antibiotic treatment. People have used antibiotics to treat infectious diseases for more than half a century.

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Over-utilization and misappropriation of antibiotics have caused an increase in the number of antibiotic-resistant strains. These resistant strains are different from multidrug-resistant (MDR), extensively drug-resistant (XDR), and pan-drug-resistant (PDR) (Rahman et al., 2021). Therefore, endolysins could be developed as a practical choice for eradicating this type of infectious bacteria (Murray et al., 2021). Therefore, the exogenous application of endolysins as antimicrobial treatments has been receiving much interest in finding a solution to antibiotic resistance concerns (Tassell et al., 2016).

Typically, there are two types of endolysins: a modular assembly of at least two independent functional domains related through a short linker and a globular structure with only an enzymatically active domain (EAD). The EAD can cleave different bonds in the peptidoglycan structure of the bacterial cell wall, while the one-cell wall binding domain (CBD) identifies the bacterial cell wall specifically and binds it so the catalytic domain can begin its function (Chang, 2020). Endolysins are usually categorized into five groups depending on the bond they break in the PG structure. Enzymes such as *N*-acetylmuramidases (lysozymes), transglycosylases, and *N*-acetylglucosaminidases have catalytic activity on the sugar backbone of peptidoglycan, while endopeptidases, such as *N*-acetylmuramoyl-L-alanine amidases, strike the peptide part and cleave the amide bond between L-alanine in pentapeptide moiety and *N*-acetylmuramic acid in the sugar backbone (Miroshnikov et al., 2006).

The cell wall structures in Gram-positive and Gram-negative bacteria also differ; a thick PG layer that is attached covalently to polysaccharides, teichoic acids, and peptidoglycolipid can be observed in Gram-positive bacteria. Gram-negative bacteria have a thinner PG layer located between the inner and outer membranes and Lipopolysaccharides (LPS). Some porins can be observed across the outer membrane in irregular distribution (Lai, Chen, Ho, Xia, & Leung, 2020). Therefore, excellent PG lysis occurs exogenously in Gram-positive

bacteria because they are not covered by an excessive **outer membrane** (Schmelcher & Loessner, 2021). In contrast, Gram-negative bacteria are partly resistant to treatment with endolysin because they have a second outer membrane that inhibits directed contact between the endolysin and peptidoglycan part of cell walls (Lim et al., 2014). Using the outer membrane permeabilizers, e.g., chelators, is the preferred approach for improving the efficiency of endolysins against Gram-negative bacteria as biocontrol promoters. Chelator agents such as ethylenediaminetetraacetic acid (EDTA) have primarily been used in this approach (Briers & Lavigne, 2015).

Appropriate biocontrol effect of recombinant endolysins can occur by applying enough of the protein. Therefore, adequate concentration accessibility of these proteins could be the first step to using the unique eliminator of resistant bacterial strains, particularly for surface disinfection in hospitals and pathogen detection (Loessner, 2005; Rahman et al., 2021). However, decreasing the process cost is an important factor to be considered in the production of recombinant proteins on a large scale. This includes the complete production process, from selecting an appropriate expression host and culture medium to downstream processing stages such as cell harvesting, lysis, and purifying of the protein (Schmelcher & Loessner, 2021).

In this research, we examined some important factors in recombinant protein expression, including host strain, expression temperature, inducing time, and IPTG concentration, to improve the expression of recombinant his-tagged PlyF307 in *E.coli*.

2. Materials and methods

2.1 Expression of recombinant lysin in different *E. coli* strains

Recombinant plasmid pET28a-PlyF307 was transformed into *E. coli* BL21(DE3) and *E. coli* BL21(DE3) pLysS as expression hosts. Induction

was done by 1mM IPTG (Thermo Fisher Scientific, USA) at OD₆₀₀= 0.7 to 0.9, and incubation was continued for different times (4, 8, and 16 h) at 37 °C with a 180 rpm shaking rate (IKA; KS4000I control, Germany)(Khurana, Pratibha, & Kaur, 2017).

2.2 Expression in different culture media

Various culture mediums were used in several conditions to obtain the best amount of recombinant endolysin expression. The Luria-Bertani broth (LB) (BioBasic, Canada), Terrific Broth (TB) (BioBasic-Asia Pacific Pte Ltd, Singapore), and auto-induction mediums (AIM) were employed at 37 °C for expression of the protein in *E. coli* BL21(DE3) pLysS as the host (Modi et al., 2023). The auto-inducing medium was prepared following the protocol described by Fox and Blommel (2009).

2.3 Expression by different concentrations of inducer at different times of bacterial host growth

The final concentration of 0.1, 0.5- and 1-mM IPTG was examined at OD₆₀₀ of 0.7 to 0.9 for inducing recombinant endolysin expression (Epoch Microplate Spectrophotometer, USA). After induction, all samples were run on SDS-PAGE gel, and the increase in protein expression was evaluated by normalized quantity (Alikhani et al., 2017).

2.4 Recombinant endolysin extraction

For recombinant protein extraction from the host, buffer A (PBS 0.1 M, Triton X100 1%, PMSF 1%, 2-mercaptoethanol 17mM and Imidazole 10 mM, pH=8) and buffer B (NaH₂PO₄ 50 mM, NaCl 300 mM, 2-mercaptoethanol 17 mM, Glycerol 10% and Imidazole 10 mM, pH=8) were used for bacterial lysis and recombinant protein extraction (Berg et al., 2001; Tran et al., 2018). Different cycles (1, 2, and 3 cycles per minute; at pulses of 30 and 15; 10" on and 30, 15, and 10" off, respectively) of sonication were applied with 50 and 75 % of the full power of the sonication device for different volume of bacterial suspensions (Fapan Ultrasonication, Iran).

Materials, including PBS, Triton X100, Glycerol, PMSF, 2-mercaptoethanol, and Imidazole, were purchased from Bio Basic (Bio Basic Inc., Canada), and the other chemical materials were from Merck & Co., Inc (Germany).

2.5 Recombinant endolysin purification

A Ni-NTA column (Qiagen, Germany) was used to purify the his-tagged recombinant PlyF307. The wash and elution buffers, including 20- and 250-mM imidazole, respectively, were the same as buffer A. Purification fractions were detected using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and validated by western blotting using anti-His HRP conjugated antibody (Sigma-Aldrich, Germany). According to the ProtParam tool, the estimated molecular weight of PlyF307 recombinant protein was approximately 19.7 kDa. The purified enzyme was dialyzed for 4 h in the PBS buffer (pH=8) to remove residual imidazole. Freeze-drying was done after buffer exchanging (Alikhani et al., 2017). A PBS buffer containing 1M sucrose and 0.4 M mannitol, pH=8, was added to the dialyzed enzyme at a ratio of 1:10 to protect recombinant endolysin during freeze-drying (Johnson et al., 2002). Finally, the concentration of the obtained recombinant PlyF307 was measured at OD₂₈₀ with ϵ % of 9.37 (Epoch Microplate Spectrophotometer, USA).

3. Results and Discussion

3.1 Expression in *E. coli* BL21(DE3) pLysS was higher than *E. coli* BL21(DE3)

There are many genetically engineered *E. coli* for recombinant protein production that vary in certain specifications, making them suitable for precise purposes (Bakhtiari & Vaez., 2024). In this study, we used a very characterized strain of *E. coli* BL21(DE3) that is employed from the lab to the industrial scale of recombinant protein production (Studier., 2005). In addition, we used a modified BL21(DE3) (that expresses lysozyme) and a given protein in parallel to prevent recombinant protein toxicity in the host. Many reports have used this strain for toxic proteins (Li et al., 2024). According to the SDS-PAGE results,

E. coli BL21(DE3) pLysS shows a higher amount of expressed PlyF307 (Fig 1), so this strain was applied as the expression host, and further tests were applied to this strain.

3.2 Expression of recombinant endolysin was similar in TB and LB mediums

An important factor affecting recombinant protein expression is the medium used for the host culture. For example, Rezai and Zarkesh-Esfahani produced a higher amount of human growth hormone in LB compared to a 4YT (yeast extract, 5 g/l; Tryptone, 8 g/l; NaCl, 5 g/l, pH = 7.4)

medium (2012). Another study produced 1805.50 mg/l of nattokinase via culture condition optimization (Modi et al., 2023). Khurana reported that changing the culture media led to a 1.3-fold increase in recombinant lipase expression in *E. coli* (2017). In this study, the recombinant protein PlyF307 concentration expressed in different mediums showed that *E. coli* BL21(DE3) pLysS-pET28a-PlyF 307 had a higher expression amount in the Terrific Broth (TB) and Luria- Bertani (LB) than the auto-inducing medium (Fig 2). The incubation temperature was 37 °C and the best time for harvesting of bacteria was 8 h after adding the inducer.

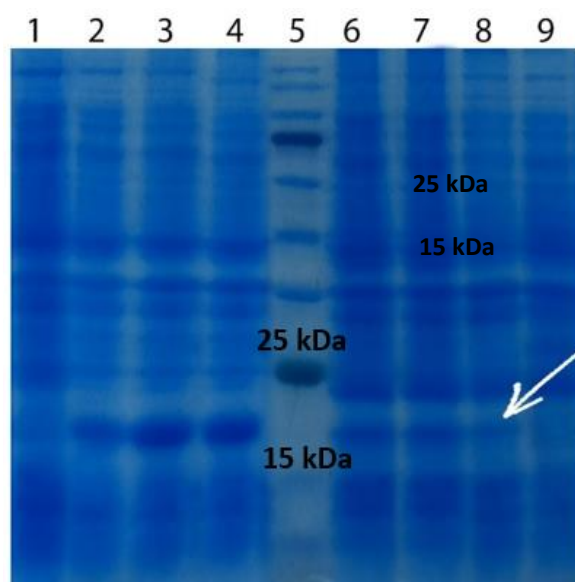


Figure 1: Expression Pattern of PlyF307 in *E. coli* BL21(DE3) and *E. coli* BL21(DE3) pLysS at Different Times after Induction

Note. 1- 4; expression in *E. coli* BL21(DE3) pLysS: 1. Before induction, 2. 4 h after induction, 3. 8 h after induction, 4. 16 h after induction, 5. Protein ladder (pre-stained Protein Ladder, Sinaclon), 6 -9; expression of recombinant PlyF307 in *E. coli* BL21(DE3): 6. 16 h after induction, 7. 8 h after induction, 8. 4 h after induction, 9. Before induction. The PlyF307 (19.7 kDa) band is shown with a white arrow.

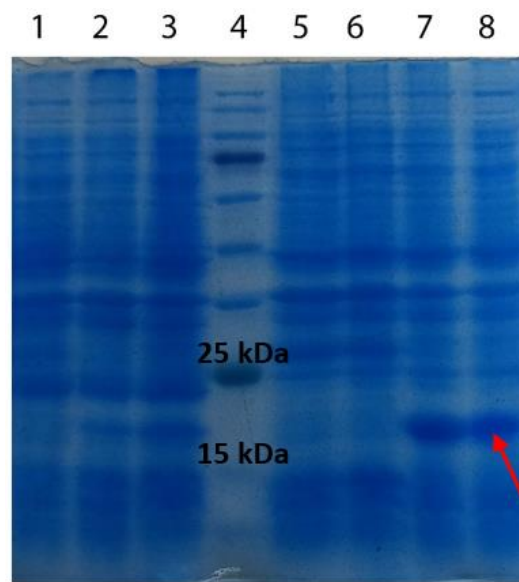


Figure 2: SDS-PAGE of Recombinant PlyF307 in *E. coli* BL21(DE3) pLysS in Different Mediums

Note. 1. Early-stage sample of *E. coli* BL21(DE3) pLysS-PlyF307 in Auto-inducing Medium 2. Overnight sample of *E. coli* BL21(DE3) pLysS-PlyF307 in auto-inducing medium 3. Overnight sample of *E. coli* BL21(DE3) pLysS-PlyF307 in auto-inducing medium (more sample loading) 4. Protein ladder (pre-stained Protein Ladder, Sinaclon) 5. Terrific Broth medium (before induction) 6. Luria-Bertani broth medium (before induction) 7. Terrific Broth medium (after induction) 8. Luria-Bertani broth medium (after induction). The plyF307 (19.7 kDa) band is shown with a red arrow.

3.3 Expression of recombinant endolysin was identical in different concentrations of inducer

Inducer concentration and induction time are important agents for recombinant protein production in *E.coli* (Mühlmann et al., 2017).

Fazaeli's study investigated parameters such as temperature, IPTG concentration, incubation time and media components (2018). Their experiments showed that the expression of recombinant cholesterol oxidase was significantly enhanced from 3.2 to 158 U/l bacterial culture (Fazaeli et al., 2018). In this study, different concentrations of IPTG were used to induce induction in some optical density of the host; however, protein expression yields were almost identical in different IPTG amounts. Therefore, a final IPTG concentration of 0.1 mM at $OD_{600} \approx 0.9$ of *E. coli* BL21(DE3)-pLysS was shown to be the most appropriate and cost-effective for the expression process (Fig 3).

3.4 Recombinant endolysin extraction from the bacterial host in different conditions

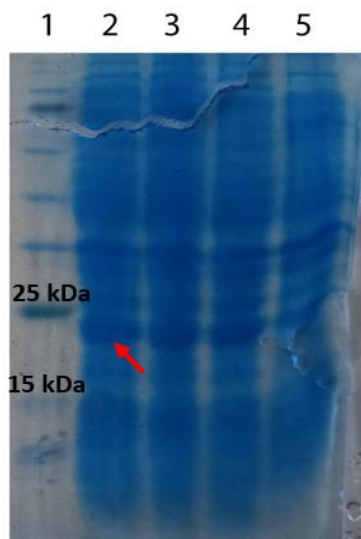


Figure 3: SDS-PAGE of Recombinant Protein His-tagged *PlyF307* Expression in Different Concentrations of IPTG as Inducer

Note. 1. Protein ladder (pre-stained Protein Ladder, Sinaclon); 2. 1mM IPTG; 3. 0.5mM IPTG; 4. 0.1mM IPTG; 5. Without IPTG. The *PlyF307* (19.7 kDa) band is shown with a red arrow

3-5 Purification of recombinant endolysin

The recombinant *PlyF307* was purified successfully from cell lysate using nickel-NTA affinity chromatography with buffer A containing 250 mM imidazole as the elution buffer. The purified enzyme concentration was measured at OD_{280} with $\epsilon_{\%}$ of 9.37. Finally, 520-570 mg of

E. coli BL21(DE3) pLysS as an expression host was harvested after 8 hours and extraction was done using buffer A (PBS 0.1 M, Triton X100 1%, PMSF 1%, 2-mercaptoethanol 17mM and Imidazole 10 mM, pH=8) and B (NaH_2PO_4 50 mM, NaCl 300 mM, 2-mercaptoethanol 17 mM, Glycerol 10% and Imidazole 10 mM, pH=8) as a lysis buffer after isolating bacterial mass from the culture medium. Bacterial cells were incubated in the lysis buffers and sonicated at 50 and 70 % power in different cycles. Similar amounts of soluble protein in different sonication conditions were observed in buffer A. However, the sonication program was set into 10 "on and 10 "off for one minute per mL of bacterial lysate and 50% of full sonication power (Fig 4).

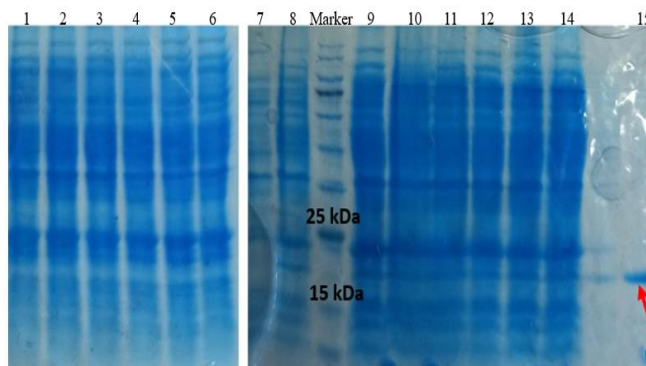


Figure 4: SDS-PAGE of Extracted Bacterial Soluble Proteins and Recombinant *PlyF307*

Note. 1. Lysis buffer B, 30" on 30" off for 1 min, power 70%; 2. Lysis buffer B, 15" on 15" off for 1min, power 70%; 3. Lysis buffer B, 10" on 10" off for 1 min, power 70%; 4. Lysis buffer B, 30" on 30" off, power 50%; 5. Lysis buffer B, 15" on 15" off for 1 min, power 50%; 6. Lysis buffer B, 10" on 10" off for 1 min, power 50%; 7. Bacterial sample before induction; 8. Bacterial sample after induction, Marker: (Pre-stained Protein Ladder, Sinaclon); 9. Lysis buffer A 10" on 10" off for 1min, power 50%; 10. Lysis buffer A, 15" on 15" off for 1min, power 50%; 11. Lysis buffer A, 30" on 30" off, power 50%; 12. Lysis buffer A, 10" on 10" off for 1min, power 70%; 13. Lysis buffer A, 15" on 15" off for 1min, power 70%; 14. Lysis buffer A, 30" on 30" off, power 70%; 15. The purified *PlyF307* (19.7 kDa) band is shown with a red arrow.

purified protein was obtained per 1 liter of LB medium in different batches (Fig 5A). Western blotting was used to ensure the correct protein had been purified; the result shows the correct location of the recombinant enzyme as a 19.7 kDa protein (Fig 5B).

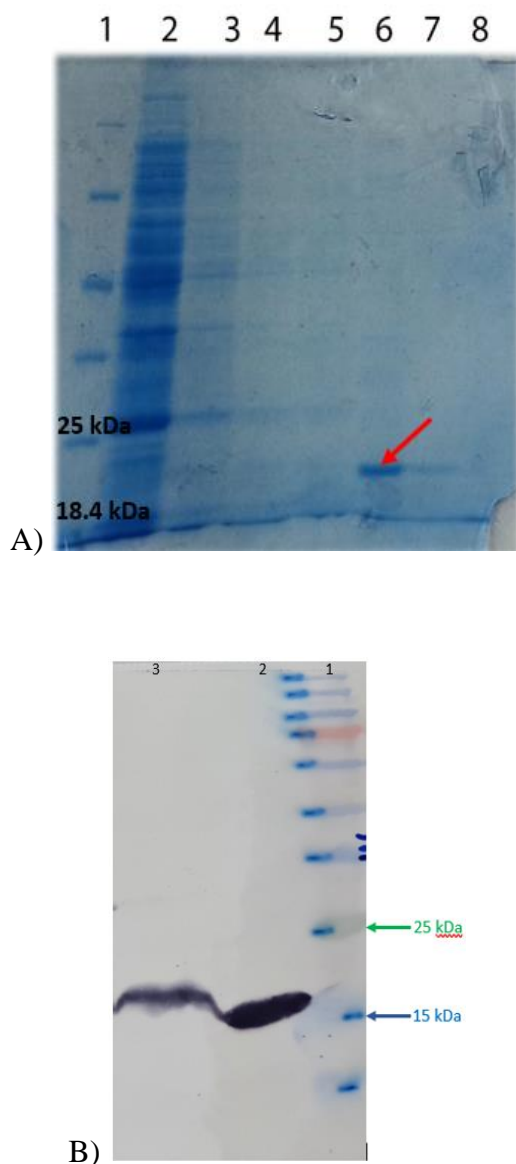


Figure 5: A SDS-PAGE of Purification Fractions of Recombinant *PlyF307*

Note. 1. Un-stained protein ladder (Sinaclone, Iran); 2. Flow through; 3. Wash 1; 4. Wash 2; 5. Elution 1; 6. Elution 2; 7. Elution 3; 8. Elution 4. The purified *PlyF307* (19.7 kDa) band is shown with a red arrow. B Western Blot of recombinant *PlyF307*; 1. Pre-stained protein ladder (Sinaclone, Iran); 2 & 3. Recombinant *PlyF307*.

4. Conclusion

In this study, we successfully developed an improved protocol for efficient production of recombinant *PlyF307* endolysin using *E. coli* BL21(DE3) pLysS as the expression host. The established purification protocol using Ni-NTA affinity chromatography yielded 520-570 mg of pure protein per liter of LB medium. The identity

and purity of the recombinant enzyme were confirmed through SDS-PAGE and western blot analysis, revealing the expected molecular weight of 19.7 kDa. This improved production protocol represents a significant step toward the industrial-scale manufacturing of *PlyF307*, a promising antimicrobial agent against antibiotic-resistant *A. baumannii*.

Conflict of interest

The authors declare that they have no competing interests.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Conflict of Interest

The authors declare no conflict of interest.

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