Microbiology, Metabolites and Biotechnology
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Enhanced Expression of an *Acinetobacter baumannii* specific recombinant endolysin in *Escherichia coli*

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Article Info

Document Type: Research Paper

Received 21/12/2024
Received in revised form 17/01/2025
Accepted 18/02/2025

Published 7/05/2025

Keywords:
Prophage Lysin, Recombinant Enzyme, expression improvement, PlyF307

Abstract

Prophage endolysin PlyF307, a peptidoglycan-destroying enzyme previously identified through the screening of the Acinetobacter baumannii genome, has shown the ability to kill numerous clinical isolates of *A. baumannii* in its recombinant form. *A. baumannii* is an extremely antibiotic-resistant Gram-negative hospital pathogen that is distributed worldwide. In this study, we used *Escherichia coli* 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), 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 induction was done with 0.1 mM IPTG at OD_{600} = 0.9. The incubation temperature was 37 °C before and after the 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 *E.coli* 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 will be helpful in achieving this purpose. This improved expression can be a significant step toward the scaling-up of the enzyme production in *E. coli*.

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, and resulting in cell destruction and death (Murray et al., 2021). This specified killing mechanism of endolysins can be exploited by applying them as recombinant proteins from the outside to target intended bacteria (Schmelcher &

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DOI: 10.22104/mmb.2025.7277.1159 Please cite this article as: F. Khosravi Node, Microbiology, Metabolites and Biotechnology (MMB), https://armmt.irost.ir/article_1509.html

Loessner, 2021). The use of endolysins to trigger cell death can be used as an alternative to antibiotic treatment. People have been using antibiotics for more than half a century to treat infectious diseases. 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, further development of endolysins could be a practical choice for eradicating this infectious bacteria (Murray et al., 2021) with the exogenous application of endolysins as antimicrobial treatments 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. The enzymatically active domain (EAD) can cleave different bonds in the peptidoglycan structure of the bacterial cell wall, while the one-cell wall binding domain (CBD) identifies specifically the bacterial cell wall and binds with it allowing the catalytic domain to start functioning (Chang, 2020).

Endolysins are usually categorized into five groups based on the type of 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, including N-acetylmuramoyl-L-alanine amidases, strike the peptide part cleaving the amide bond between L-alanine in the pentapeptide moiety and N-acetylmuramic acid in the sugar backbone (Miroshnikov et al., 2006).

However, cell wall structures in Gram-positive and Gram-negative bacteria differ. A thick PG layer can be observed in Gram-positive bacteria attached covalently to polysaccharides, teichoic acids, and peptidoglycolipids. Gram-negative bacteria have a thinner PG layer located between the inner and outer membranes and Lipopolysaccharides (LPS), and some irregularly distributed porins can be observed across the outer membrane (Lai et al., 2020). Therefore, PG lysis happens exogenously well in Gram-positive bacteria, and 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 the peptidoglycan part of the cell walls (Lim et al., 2014).

The use of outer membrane permeabilizers, e.g., chelators, is the most popular approach for improving the efficiency of endolysins against Gram-negative bacteria as biocontrol promoters, with chelator agents such as ethylenediaminetetraacetic acid (EDTA) being the most common (Briers & Lavigne, 2015). An appropriate biocontrol effect of recombinant endolysins can be achieved by applying an adequate amount of the protein. Therefore, having access to an adequate concentration of these proteins will be the first step to using this 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 consider when producing 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

The recombinant plasmid pET28a-PlyF307 was transformed into *E. coli* BL21(DE3) and *E. coli* BL21(DE3) pLysS as the expression hosts. Induction was done by 1mM IPTG (Thermo Fisher Scientific, USA) at OD_{600}= 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

To obtain the best amount of recombinant endolysin expression, Various culture media were used in several conditions. 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 (NaH_2PO_4 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 volumes 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 OD_{280} with $\varepsilon_{\%}$ of 9.37 (Epoch Microplate Spectrophotometer, USA).

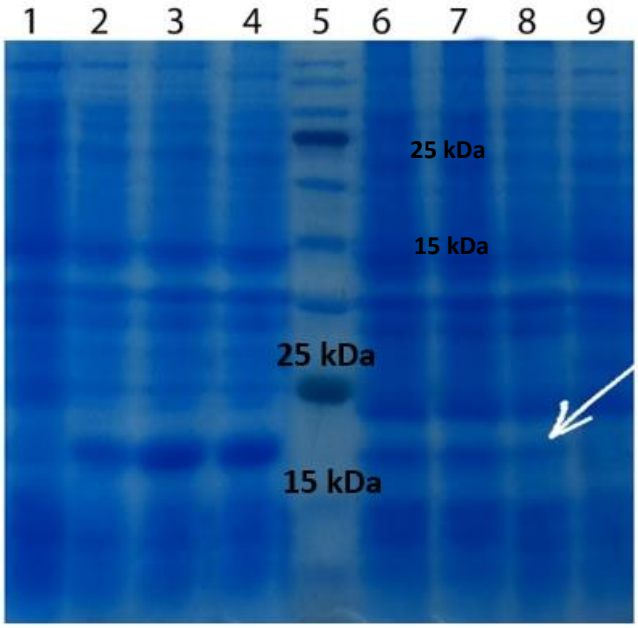
3. Results and Discussion

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 the very characterized strain of *E.*

coli BL21(DE3) which is employed in both the lab and the industrial scale of recombinant protein production (Studier., 2005). In addition, we used a modified BL21(DE3) that expresses lysozyme and 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 showed 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.

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

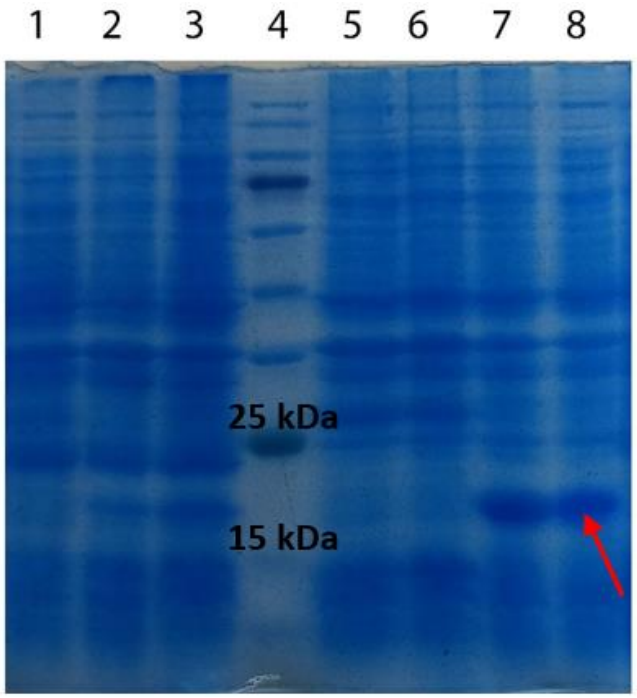


Note. 1- 4; Expression in *E. coli* BL21(DE3) pLysS: 1. Before induction, 2. 4 h after induction, 3. 8 h after induction, 4. 16 h after induction, 5. Protein ladder (pre-stained Protein Ladder, Sinaclon), 6 –9; expression of recombinant PlyF307 in *E. coli* BL21(DE3): 6. 16 h after induction, 7. 8 h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

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

An important factor that can affect 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 media 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 2: *SDS-PAGE of Recombinant PlyF307 in E. coli BL21(DE3) pLysS in Different Media*

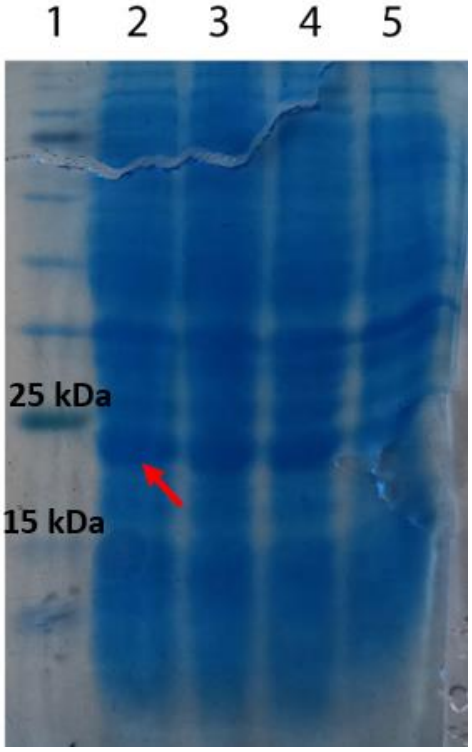


Note. 1. Early-stage sample of *E. coli* BL21(DE3) pLysS-PlyF307 in Auto-inducing Medium 2. Overnight sample of *E. coli* BL21(DE3) pLysS-PlyF307 in auto-inducing medium 3. Overnight sample of *E. coli* BL21(DE3) pLysS-PlyF307 in auto-inducing medium (more sample loading) 4. Protein ladder (pre-stained 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 $OD_{600} \simeq 0.9$ of *E.coli* BL21(DE3)-pLysS was shown to be the most appropriate and cost-effective for the expression process (Fig 3).

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

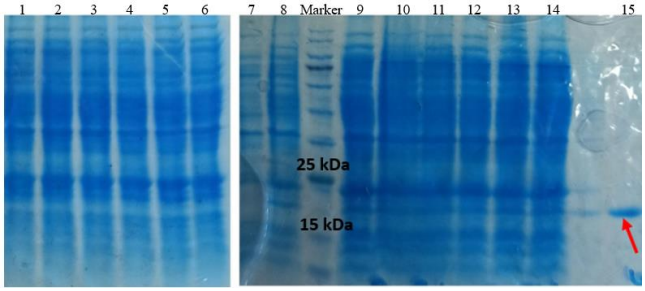


Note. 1. Protein ladder (pre-stained Protein Ladder, Sinaclon); 2. 1mM IPTG; 3. 0.5mM IPTG; 4. 0.1mM IPTG; 5. Without IPTG. The PlyF307 (19.7 kDa) band is shown with a red arrow.

3.4 Recombinant endolysin extraction from the bacterial host in different conditions

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*



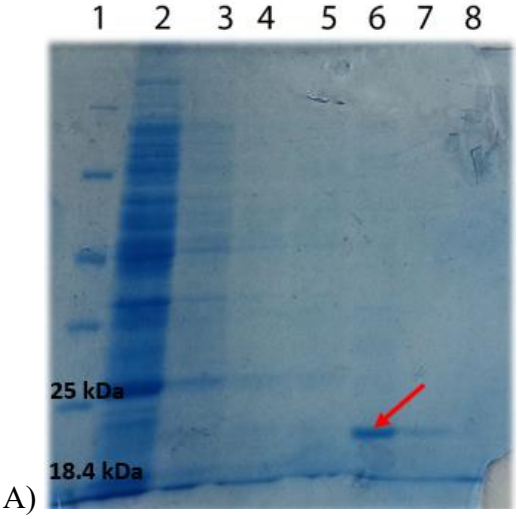
Note. 1. Lysis buffer B, 30″ on 30″ off for 1 min, power 70%; 2. Lysis buffer B, 15″ on 15″ off for 1min, power 70%; 3. Lysis buffer B, 10″ on 10″ off for 1 min, power 70%; 4. Lysis buffer B, 30″ on 30″ off, power 50%; 5. Lysis buffer B, 15″ on 15″ off for 1 min, power 50%; 6. Lysis buffer B, 10″ on 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.

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 $\varepsilon_{\%}$ of 9.37. Finally, 520-570 mg of 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 (Sinaclon, 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.

Acknowledgment

The authors kindly appreciate the assistance of Dr. Pilar Garcia Suarez regarding the research progress.

Ethical approval

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

Conflict of Interest

The authors declare no conflict of interest.

Authors' Contributions

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