Optimization of engineered-modular endolysins expression conditions in *Escherichia coli* NiCo21(DE3) for the control of Streptococcal infection

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Abstract. Streptococci are Gram-positive bacteria which cause diverse animal and human diseases and systemic infections. Antibiotic therapy for this pathogen is often unsuccessful and contributes to the development of antibiotic resistance. Bacteriophage modular endolysins, as well as their chimeric constructs, represent promising antimicrobials with high specificity against these bacteria and unlikely to evoke antimicrobial resistance. Our previous work has developed the recombination and fusion engineered-modular endolysins design (namely E₇C₉ and E₇C₉E₉, respectively) from a particular modular endolysin E₇C₉ to obtain novel antimicrobial with enhanced activity. In this study, we investigated further the optimization of the engineered-modular endolysin expression conditions in *Escherichia coli* NiCo21(DE3). Accordingly, clone selection, final inducer concentration, and incubation temperature were optimized. To obtain a recombinant clone which exhibits optimum protein expression level, expressions of the recombinant protein were conducted in shake flask. Subsequently, the soluble and insoluble protein crude extracts were collected by lysis and denaturation treatments, respectively, and verified by SDS-PAGE and Western blot analysis. The result showed that the selected clone for optimum expression of E₇C₉, E₇C₉, and E₇C₉E₉ was clone 1, 3, and 5, respectively. Moreover, the optimum final inducer concentration for both E₇C₉ and E₇C₉ was 2000 μM, whereas for E₇C₉E₉ was 500 μM. Besides, the optimum incubation temperature for protein expression for E₇C₉E₉ was incubation at 37°C and followed by induction at 25°C, and for both E₇C₉ and E₇C₉ were both incubation and incubation at 25°C. Furthermore, these three proteins were found to be expressed as insoluble proteins. Collectively, these results could contribute to the development of ‘tailor-made’ antimicrobials by modular endolysin engineering, which can be used for the control of Streptococcal infection.

1. Introduction
Streptococci (such as *Streptococcus agalactiae* and *S. dysgalactiae*) are Gram-positive bacteria which cause diverse animal and human diseases. This pathogen can cause bovine mastitis, which is the most costly disease in dairy cattle farming worldwide, including Indonesia, and significantly reduces milk
quality, quantity, and safety [1] [2] [3]. This contagious agent mainly transmitted from cow to cow through the milking process, and to human through exposure to farm animals. Moreover, this pathogen can cause fatal infection of neonatal sepsis and meningitis in baby [4] [5], skin, soft tissues or joints, as well as bacteremia in human [6]. Thus, this pathogen can cause systemic infections which could turn into severe zoonotic or foodborne illness, death and even outbreaks in animal and human, and carries a high economic burden [3] [7] [8].

Antibiotic therapy for this pathogen is often unsuccessful and contributes to the development of antibiotic resistance. Thus, this pathogen is categorized as a serious threat with regards to antibiotic-resistance with the number of antibiotic-resistant infections per year continues to increase [9]. Often, for every new antibiotic introduced, it was usually followed by the identification of antibiotic-resistant bacteria, even recently, no major new types of antibiotics are introduced [9] [10]. Therefore, an appropriate strategy for this infection control, which should be more pathogen-specific and refractory to resistant strain formation, need to be improved. This strategy includes the development of engineered-modular endolysins since modular endolysin has several characteristics which make it suitable for its application as antimicrobial.

Bacteriophage endolysin represent promising antimicrobial against these bacteria in animal and human which is more highly pathogen-specific and unlikely to evoke antimicrobial resistance, for the bacteriophage and host have co-evolved, their highly conserved target peptidoglycan (PG) bond sites, and externally applied agent acting on the cell envelope avoids intracellular resistance mechanisms [3] [7] [11] [12] [13] [14]. Endolysins from a Gram-positive phage-host background are generally characterized by a modular architecture, featuring separated distinct enzymatically active domain (EAD) for catalytic activity for cell wall disruption, and cell wall-binding domain (CBD) for substrate recognition [15] making them have the ability to inhibit growth or kill bacteria [5] [11]. Moreover, their modular design renders them amenable to engineering for ‘tailor-made’ antimicrobials [15]. Previous studies demonstrated that Streptococcal phage modular endolysins and their combination uses and chimeric constructs exhibited strong activity, and enhanced-synergistic effect and maintained-parental specificities, respectively [2] [7].

Given the previous success of modular endolysins as antimicrobial agents and the potential of its modular design, as well as since there have been no reports describing the utilization of bacteriophage endolysins for antimicrobial in Indonesia, it is worthwhile to study further the antimicrobial potential of these proteins. It includes the development of engineered-modular endolysins with novel enhanced-activity, such as through recombination and fusion technologies [16], and more cost-effective in their application. Moreover, this development might also lower the dose of antimicrobials, yielding increased treatment efficacy, further reducing the chance of resistance development, and in some instances, may also solve solubility problems ensuring efficient production and purification of otherwise insoluble lytic proteins [15]. Therefore, this research evaluated the potential of engineered-modular endolysins for the treatment of Streptococcal infection.

Our previous work has developed the recombination and fusion engineered-modular endolysins design (namely E_{1}C_{9} and E_{2}C_{9}E_{n}, respectively) from a particular modular endolysin E_{9}C_{9} by utilizing its modular architecture featuring separated distinct EAD and CBD to obtain novel antimicrobials with enhanced activity (patent registration on the process). The E_{2}C_{9} was designed as recombination of an EAD and a CBD, whereas the E_{2}C_{9}E_{n} was designed as a fusion of two EADs and a CBD, from two different modular endolysins. This research, therefore, subsequently investigated the optimization of the engineered-modular endolysins expression conditions in *Escherichia coli* Nico21(DE3) using a rhamnose-based tightly regulated expression system. Accordingly, clone selection, final inducer concentration, and incubation temperature were optimized.
2. Materials and Methods

2.1. Recombinant Bacterial Strains
The recombinant bacterial strains of *E. coli* Nico21(DE3) harbouring the verified-recombinant rhamnose-based tightly regulated pDSR684 expression vector containing the gene encoding engineered-modular endolysin or modular endolysin were used to evaluate the optimization of expression conditions of the recombination and fusion engineered-modular endolysins in this research. These engineered-modular endolysins were derived from *Streptococcus suis* bacteriophage and *Streptococcus agalactiae* prophage modular endolysins.

2.2. Optimization of Engineered-Modular Endolysins Expression Conditions
Optimization of engineered-modular endolysins expression conditions was performed to obtain optimized selected clone, final inducer concentration, and incubation temperature. The recombinant strain was precultured in Luria Bertani (LB) medium containing 100 μg/mL ampicillin at 37°C, and incubated while shaking at 150 rpm for 16 h [17] [18]. The preculture was then subcultured in LB medium containing 0.2% glucose and 100 μg/mL ampicillin at incubation temperature variations of 18, 25, and 37°C with shaking at 150 rpm and grown to an OD600 nm of 0.3-0.5 [5] [12] [17] [18]. Subsequently, the culture was induced with L-rhamnose to final concentration variations of 0, 10, 100, 500, 1000, 1500, and 2000 μM, respectively [17] [18]. The growth was then continued to an OD600 nm of 3.0 at induction temperature variations of 18 and 25°C for the subculture incubation temperature of 18 and 25°C, respectively, and 25 and 37°C for the subculture incubation temperature of 37°C [17] [18] [19] [20] [21].

Cells were collected by centrifugation at 4000×g and 4°C for 10 min [18], the supernatant was separated, and the cell pellet was subsequently subjected to denaturation treatment. The cell pellet was resuspended in denaturation buffer (100 mM NaH2PO4, 6 M urea, 10 mM Tris-HCl, and 10 mM of imidazole; pH 8) [21], and phenylmethanesulfonyl fluoride (PMSF) was added afterward to a final concentration of 1 mM. Cells suspension was then incubated at room temperature with shaking at 250 rpm for 1 h. Total protein crude extract was collected after centrifugation at 10,000×g for 30 min at 4°C.

The protein expression was further verified in optimized conditions (selected clone, final inducer concentration, and incubation temperature) in induced and noninduced conditions. Moreover, soluble and insoluble protein crude extracts were collected from verified optimized protein expression conditions by lysis and denaturation treatments, respectively. After that, cells were resuspended in lysis buffer (50 mM NaH2PO4, 300 mM NaCl, and 10 mM of imidazole; pH 7.4), and PMSF was subsequently added to a final concentration of 1 mM [18] [19] [20] [21]. Soluble protein crude extract was then isolated by sonication with 5 times pulses for 1 min alternated with 30 s pauses, and the supernatant was collected after centrifugation at 20,000×g for 15 min at 4°C [18] [21]. The cell pellet was resuspended again in denaturation buffer, and PMSF was added afterward to a final concentration of 1 mM. Cells suspension was then incubated at room temperature with shaking at 250 rpm for 1 h. Insoluble protein crude extract was collected after centrifugation at 10,000×g for 30 min at 4°C.

2.3. Characterization of the Recombinant Protein
The recombinant protein was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The crude protein sample was diluted (1:1) with Laemmli sample buffer (Biorad) containing β-mercaptoethanol (Sigma Aldrich). The solution was then heated in boiling water for 15 min to denature the proteins. Samples and molecular weight markers were loaded onto SDS-PAGE, having a 4% stacking gel and 10% separating gel [22]. The electrophoresis was run at a constant voltage of 90 V. Protein bands were stained with Blue Light Staining (Vivantis) and destained with water according to the manufacturer’s protocol. The recombinant protein expressed in optimized conditions was verified by Western blot using HisDetector™ Ni-HRP Conjugate Western Blot Kit according to the manufacturer’s protocol.
3. Results and Discussions
Optimization of engineered-modular endolysins expression conditions in E. coli Nico21(DE3) using a rhamnose-based tightly regulated expression system has been investigated. E. coli Nico21(DE3) was chosen as the preferred expression host due to its potential for obtaining recombinant His-tagged target proteins with reduced levels of host protein contamination [23]. Moreover, a rhamnose-based tightly regulated expression system was chosen as an expression vector since it appears to be a viable alternative of E. coli expression system for stable cloning and production of functional fully intact full-length recombinant proteins to overcome difficulties found in the commonly used prokaryotic expression systems [17]. This vector, which is tightly regulated by L-rhamnose inducer and D-glucose represser under the control of the rhaB promoter and corresponding regulatory genes [24], could prevent leaky expression if 0.2% of glucose is added into the medium [17].

The results of SDS-PAGE analysis of total protein from the optimization of clone selection among 7 clones showed that the selected clone for optimum expression of EφCφ, EλCφ and EλCφEφ were clone 1, 3, and 5, respectively. These results were verified with the protein band showing molecular weights of approximately 28.6, 30.6, and 48.2 kDa, respectively, which is similar to the predicted molecular weight based on its amino acid sequences (Fig 1). The difference in the expression level of the recombinant protein of each clone might associate with the difference of the plasmid copy number within each clone.

![Figure 1](image1.png)  
**Figure 1.** The results of the SDS-PAGE analysis of total protein from the optimization of clone selection (clone 1-7). M: protein standard marker, WT: wild type, C: clone.

Moreover, the optimum final concentration of L-rhamnose inducer for both EλCφ and EφCφ was 2000 μM, whereas for EλCφEφ was 500 μM (Fig 2). These results showed that the addition of EADλ into the EφCφ to form fusion engineered-modular endolysin designed could minimize the inducer requirement for protein expression compare to that recombination engineered-modular endolysin and template modular endolysin used in this research. Previously, research had been shown that the optimum final concentration of L-rhamnose inducer for nontoxic TphoA, mildly toxic GFP, and toxic MalE-NTR proteins expression were 1000 μM [17]. A similar result was also reported for Int282 protein expression in E. coli under the control of a rhamnose-based regulated promoter [18]. These results suggest that variability in the amino acid sequence in protein related to the tertiary structure of the protein [19], thus it might affect the optimum inducer requirement for the protein expression in the expression host.
Figure 2. The results of the SDS-PAGE analysis of total protein from the optimization of the final concentration of L-rhamnose inducer optimization (0, 10, 100, 500, 1000, 1500, and 2000 μM). M: protein standard marker, WT: wild type.

The optimum incubation temperature for protein expression for E\(_{\lambda}\)C\(_{\phi}\)E\(_{\phi}\) was incubation at 37ºC and followed by induction at 25ºC, and for both E\(_{\lambda}\)C\(_{\phi}\) and E\(_{\phi}\)C\(_{\phi}\) were both incubation and induction at 25ºC (Fig 3). A previous study also indicated that the optimum expression incubation temperature for Int282 protein expression in E. coli under the control of a rhamnose-based regulated promoter was room temperature [18]. This relatively low optimum expression incubation temperature could facilitate correct protein folding, thus enabling efficient expression and localization of the target protein [17].

Figure 3. The results of the SDS-PAGE analysis of total protein from the optimization of the incubation temperature for protein expression with incubation and followed by induction temperature variations, i.e. 18, 25, 37/25, and 37ºC. M: protein standard marker, WT: wild type.

Furthermore, the induced condition of the optimized expression conditions of E\(_{\lambda}\)C\(_{\phi}\), E\(_{\phi}\)C\(_{\sigma}\), and E\(_{\lambda}\)C\(_{\varphi}\)E\(_{\psi}\) was verified to give the optimum protein expression compared to the noninduced one (Fig 4). The results showed that the three proteins attained their optimum protein expression in induced condition, whereas there was relatively no protein expression in noninduced one. A previous study also revealed that there was only a slight or no leaky expression of noninduced Int282 protein expression in E. coli under the control of a rhamnose-based regulated promoter [18]. Therefore, these results support that the rhamnose-based expression system used works in a tightly regulated fashion, as previously described [17] [24].
Figure 4. The results of the SDS-PAGE analysis of the total protein of verified noninduced and induced optimized expression conditions. M: protein standard marker, WT: wild type, ni: noninduced total protein, and i: induced total protein.

Moreover, these three proteins were found to be expressed as insoluble proteins. SDS-PAGE and Western blot analysis (Fig 5) observed the presence of insoluble proteins (inclusion-body) showing the predicted molecular weights of each $E_\lambda C_\phi$, $E_\phi C_\phi$, and $E_\lambda C_\phi E_\phi$ in insoluble protein crude extract. The formation of this insoluble protein might be due to the expression in less oxidative cytoplasm of $E. coli$, which could not efficiently maintain the functionality of the protein than the periplasm. Besides, the use of the plasmid which has a strong ribosome binding site (promoter system) and belongs to the high copy number type of plasmid could generate high protein expression level which may lead to the inclusion body formation of the protein [25]. Previous works showed similar experience that several endolysins suffer from a low solubility during or after expression, and the recombinant endolysins solubility could be improved with recombination of EAD and CBD of different endolysins or expression and purification protocols modification [16]. Moreover, the $E_\lambda C_\phi E_\phi$ and $E_\phi C_\phi$ were observed to have the highest and higher level of protein expression, respectively, compared to the $E_\phi C_\phi$. These results showed that the addition of EAD$_\lambda$ into the $E_\phi C_\phi$ to form fusion and recombination engineered-modular endolysins could facilitate the improvement of protein expression level, as well as the downstream process (purification as well as characterization of the protein). These results also suggest that different amino acid sequence in a protein determines the conformation of the protein structure, which could limit or improve the expression level of the protein [19] [20].

Figure 5. The results of the SDS-PAGE and Western blot analysis of crude extracts of soluble and insoluble proteins. M: protein standard marker, WT: wild type, s: soluble-protein crude extract, and i: insoluble-protein crude extract.

4. Conclusions
The engineered-modular endolysins ($E_\gamma C_\phi$ and $E_\delta C_\phi E_\phi$) and modular endolysin ($E_\phi C_\phi$) have been successfully expressed in $E. coli$ NiCo21(DE3) using a rhamnose-based tightly regulated expression system as insoluble proteins with molecular weights of approximately 30.6, 48.2, and 28.6 kDa, respectively. The optimized-expression conditions for these proteins, i.e., the selected clone for optimum expression of $E_\phi C_\phi$, $E_\delta C_\phi$, and $E_\lambda C_\phi E_\phi$ were clone 1, 3, and 5, respectively. Moreover, the optimum
inducer final concentration for both E₃Cₘ and E₄Cₘ was 2000 μM, whereas for E₃CₘEₖ was 500 μM. Furthermore, the optimum incubation temperature for protein expression for E₃CₘEₖ was incubation at 37°C and followed by induction at 25°C, and for both E₃Cₘ and E₄Cₘ were both incubation and induction at 25°C. Collectively, these results provided the possibility of engineered-modal endolysin production as an antimicrobial candidate, which can be employed in future experiments in the treatment of Streptococcal infection.

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6. References
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