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Optimizing Expression of Recombinant HPV16 E6 Mutant (*RecmE6*) for Cervical Cancer Immunotherapy Development

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ABSTRACT
Cervical cancer ranks as the fourth leading cause of cancer-related death among women worldwide. The disease is primarily associated with *Human Papillomavirus* (HPV), particularly HPV type 16, which is frequently detected in cervical cancer cases. Immunotherapy has emerged as a promising therapeutic approach that can potentially reduce mortality by harnessing the immune system to target cancer cells. This study aimed to optimize the expression of a recombinant mutant E6 protein from HPV16 (RecmE6) to produce sufficient and high-quality antigen suitable for immunotherapeutic applications. Expression conditions were optimized by varying temperature, isopropyl-β-D-thiogalactopyranoside (IPTG) concentration, and incubation time. The highest yield of RecmE6 was obtained using 1 mM IPTG at 37°C for 8 hours. SDS-PAGE analysis confirmed successful expression, showing a prominent protein band at approximately 16 kDa. These findings demonstrate that the optimized parameters effectively enhance the expression of RecmE6, supporting its potential use in further development of cervical cancer immunotherapy.

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1. INTRODUCTION

Cervical cancer is one of the deadliest diseases in the world, and it is predicted to increase by 77% over the next 25 years (IAFRC, 2024). According to 2024 data from the Ministry of Health of the Republic of Indonesia, there were 408,661 new cases and 242,988 deaths from cervical cancer in the country. Persistent infection by the human papillomavirus (HPV) is recognized as the primary cause of cervical cancer (Serrano., et al, 2018).

Over 150 types of *Human Papillomavirus* (HPV) have been identified and classified into three groups based on their role in the progression of cervical cancer (Stark and Živković, 2018). The most common high-risk HPV type found in cervical cancer patients is HPV-16, which has a prevalence of 55,4% (Marlina., et al, 2016). HPV-16 produces oncoproteins E6 and E7 that inactivate tumor suppressor proteins such as p53 and pRb. These proteins are essentials for regulating the cell cycle and apoptosis. As a result, cervical epithelial cells undergo changes that lead to a condition known as cervical intraepithelial neoplasia (CIN), which can progress to cervical cancer. Consequently, HPV-16 is recognized as the dominant subtype is the development of cervical cancer, accounting for over 50% of cases worldwide (Chaberek., et al, 2022).

Various effort have been made to address cervical cancer, including the World Health Organization's (WHO) launch of a global strategy aimed at eliminating the diseases by 2024. This strategy includes the HPV vaccination for 90% of girls and young women, screening for infections in 70% of women of reproductive age, and early intervention of pre-cancerous conditions in 90% of new cervical cancer cases (WHO, 2024). Several treatment options are available for cervical cancer patients, including surgical to remove cancerous tissue, radiation and chemotherapy (Johnson., et al, 2019). However, these methods often do not yield optimal results, particularly for metastatic cancer, and can lead to significant side effect for patients.

One promising therapeutic approach is immunotherapy (Castle., et al, 2021). Compared to chemotherapy, immunotherapy offers greater target specificity. Immunotherapy helps immune system to identify and eliminate cancer cells. Immunotherapy is also considered safer than chemotherapy or radiotherapy (Wagner., et al, 2021). Several studies have demonstrated the potential of HPV-specific immunotherapy, which modified the HPV

antigen to effectively stimulate the T-cell immune response (Stevanović., et al, 2019). Therefore, developing an immunotherapy strategy targeting the E6 protein could be a promising option for treating cervical cancer.

The mutant E6 protein (RecmE protein) is designed to reduce the oncogenic potential of the original protein while maintaining its ability to stimulate an immune response (Sobhani., et al, 2020). Mutations are introduced into the functional regions of the oncoprotein while preserving the main immunogenic epitopes. As a result, this protein is expected to be harmless to host cells yet still recognized by the immune system as an effective antigen that triggers a specific immune response.

Optimization in protein expression method is a crucial step for producing sufficient quantities of high-quality antigens to support further immunotherapy research. Additionally, the selection of appropriate plasmid vector will enhance protein expression in host cells, such as *Escherichia coli* (Casali and Preston, 2023). Key parameters, including temperature of cell culture, induction with Isopropyl-β-D thiogalactopyroside (IPTG) and the length of incubation period, significantly influence the level of protein expression (Kaur., et al, 2018).

This study aims to determine the optimal conditions for expressing the recombinant E6-mutant protein (RecmE6), which is intended for the use in future immunotherapy for cervical cancer. The result of this study are expected to contribute in the development of more specific and targeted cancer immunotherapy strategies, and serve as an important step in reducing future cervical cancer mortality rates.

2. RESEARCH METHOD

a. Research Sample

This study is an experimental study based on the visualization result from SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis). The sample used in this research consisted of recombinant E6 mutant gene fragment (RecmE6) that had mutations at the position of F47R, L50A, R102A, R131A and 4C/4S as studied by (Zanier., et al, 2013). The mutant E6 gene fragment was modified to include the Kozak sequence (GCCACC) at the 5' end of BamHI restriction site, and codon optimization was applied at various points to enhance the translation efficiency. The mutant E6 DNA fragment then inserted into the pcDNA3.1(+)-N-6His plasmid vector, resulting in an expected molecular weight of 16 kDa for the E6 protein.

b. Transformation of recombinant plasmid

The recombinant plasmid containing the mutant E6 gene fragment was then inserted into *E.coli*-competent DH5α cells using the heat shock method. The result of the transformation were then spread on Luria-Bertani (LB) agar medium containing ampicillin at a final concentration of 100 μg/ml for the selection of transformant colonies. This was done overnight at 37℃. The appearance of colonies on the selective medium indicated successful transformation. These colonies were subsequently propagated overnight in LB broth medium using a shaker incubator set to 37℃ at 200 rpm.

c. Optimization of protein expression

Before conducting the optimization step, the transformant culture must be refreshed by preparing a new culture in a shaker incubator until the OD 600 reaches 0.4-0.6. Once this is achieved, the optimization step is performed. This involves the induction of protein expression with Isopropyl-β-D-thiogalactopyranoside (IPTG) at concentration of 0.1 mM, 0.5 mM, and 1 mM. The culture was incubated at 37℃ and 200 rpm for 3-4 hours. The protein was then harvested according to the protocol outlined by Sambrook and Russel (2006) with Tris-HCl buffer and 1% SDS. The supernatant was then mixed with Laemmli Buffer in 1:1 ratio and analyzed with SDS-PAGE using Coomasie Blue staining.

After determining the optimal IPTG concentration, a subsequent optimization step was conducted to examine the incubation temperature. The incubation temperature for culture was set at 37℃, 30℃, and 25℃ in a shaker incubator with 200 rpm for 24 hours. The result was then analyzed with SDS-PAGE. The final step was for optimal incubation time, varying between 4, 6, 8, and 24 hours. The test was conducted with the optimal IPTG concentration and temperature from previous step. Again, the result was then analyzed with SDS-PAGE.

3. RESULT AND DISCUSSION

RESULT

a. Detection of *E.coli* DH5α transformant in LB agar

The method used to identify successfully transformed bacteria is antibiotic resistance-based selection. The pcDNA3.1(+)-N-6His plasmid contains an Ampicillin resistance gene (AmpR), which means that only E.coli DH5α cells that have been successfully transformed will express the antibiotic resistance gen and grow on the LB agar medium.

Figure 1[I] clearly demonstrates that *Escherichia coli DH5α* without plasmid cannot grow on media with ampicillin. Figure 1[II], as the the control medium without *E.coli*, also shows there is no colony growth. Meanwhile, Figure 1[III] shows that *Escherichia coli DH5α* without plasmid can grow on media without

ampicillin with colonies spread across the entire agar surface. These three controls are necessary to validate that the colony growth on the agar media is due to the successful transformation.

The results of transformant cell growth are displayed in Figure 1A, 1B and 1C. White colonies are present on all three plates, indicating that the RecmE6 recombinant plasmid was successfully transformed into *E.coli* DH5α cells, which expresses the ampicillin resistance gene (AmpR). Plate A shows a smaller number of colonies compared to plate B and C.

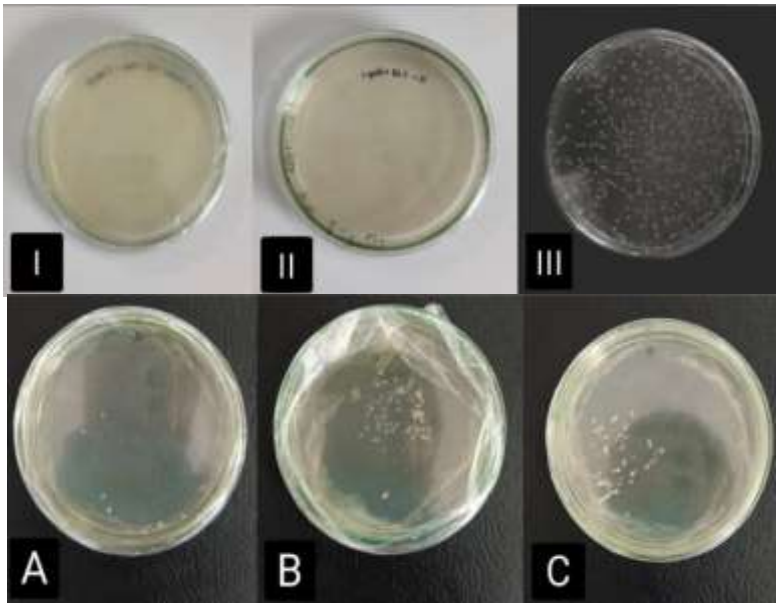

Figure 1. Transformant detection result
[I] *E. coli* without plasmid (LB + Amp)
[II] No cell control (LB + Amp)
[III] *E. coli* without plasmid (LB without Amp)
[A] *E. coli* Transformant A (Heatshock 1)
[B] *E. coli* Transformant B (Heatshock 2 - Repetition)
[C] *E. coli* Transformant C (Heatshock 3 - Repetition)

b. SDS-PAGE Analysis for Optimal IPTG Concentration

Figure 2 illustrates the results of optimizing IPTG concentration. When compared to the protein ladder, target protein bands were observed around 16 kDa for sample groups B and C, whereas sample group A did not display any protein bands. This indicates a difference in expression capabilities among sample A, B and C.

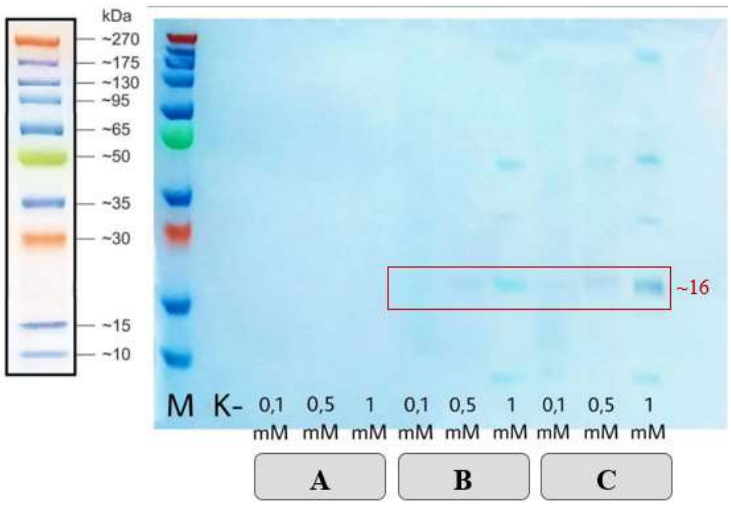

Figure 2. SDS PAGE analysis for optimization of IPTG concentration
[M] Protein Ladder, [K-] Negative control (without IPTG), [A] *E. coli* Transformant from plate A
[B] *E. coli* Transformant from plate B, [C] *E. coli* Transformant from plate C

The protein expression result for both sample groups, B and C, show a consistent pattern: as the concentration of IPTG increases, protein expression also increases. The thickest protein band, indicating the highest level of protein expression, is observed at an IPTG concentration of 1 mM.

c. SDS-PAGE Analysis for Optimal Incubation Temperature

Testing was conducted using the optimal IPTG concentration of 1 mM to determine the optimal incubation temperature. Figure 3 shows the results of SDS-PAGE with varying incubation temperatures.

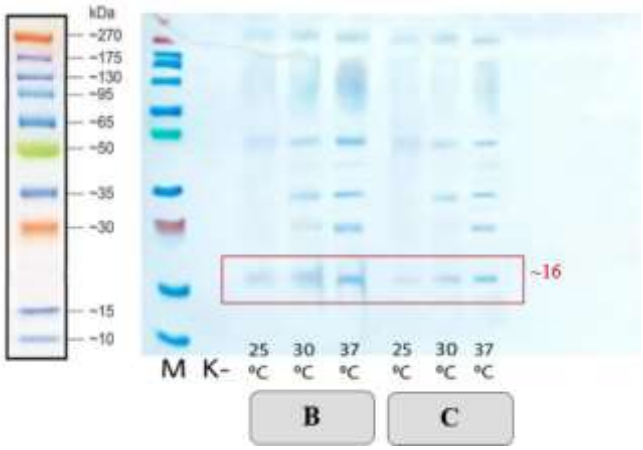

Figure 3. SDS-PAGE analysis for incubation temperature optimization
[M] Protein Ladder
[K-] Negative control (without IPTG)
[B] *E. coli* Transformant from plate B
[C] *E. coli* Transformant from plate C

Based on previous analyses, sample A did not produce any protein bands, indicating that it was unsuitable for protein expression. As a result, samples B and C will be used for further testing.

The SDS-PAGE results showed that, at varying incubation temperature, the RecmE6 target band at 16 kDa produced a thicker protein band at 37℃, compared to 25℃ and 30℃. In addition, there is no protein band target protein in the negative control lane (K-).

d. SDS-PAGE analysis for Optimal Incubation Time

The optimal incubation time was tested using 1 mM IPTG at 37℃. The results are shown in Figure 4. The result clearly demonstrate that the thickest protein bands are produced by samples incubated for 8 and 24 hours. In comparison, the intensity of protein expression is very low at 4 and 6 hours.

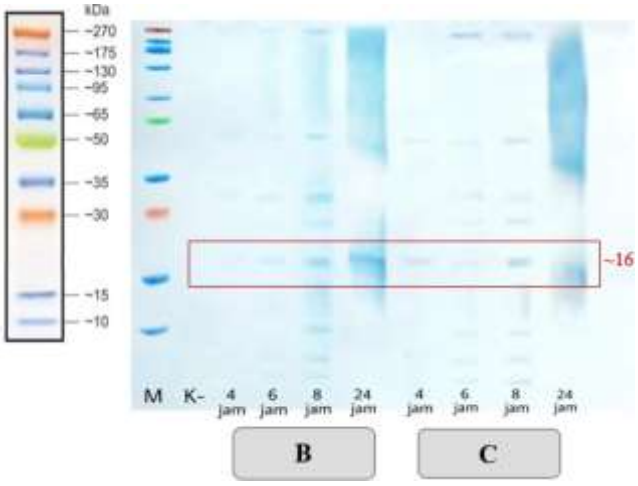

Figure 4. SDS-PAGE analysis for optimal incubation time.
[M] Protein Ladder, [K-] Negative control (without IPTG), [B] *E. coli* Transformant from plate B
[C] *E. coli* Transformant from plate C
**Jam = hours*

Thus, the optimal incubation time for protein expression are 8 or 24 hours. However, it is important to note that there are clear smears appears at 24 hours incubation. This may indicate a protein degradation at this period. This factor is considered when determining the optimum incubation time. The protein expression was dependent on IPTG induction, as evidenced in the figure that there is no protein target band on K- lane.

DISCUSSION

a. Detection of *E.coli* DH5α transformant in LB agar

This study aims to determine the optimal conditions for expressing recombinant E6 mutant protein using the *E.coli* DH5α expression system. The recombinant plasmid containing the mutant E6 gene was transformed into *E.coli* using the heat shock method. The results of the transformation were validated through antibiotic selection. Three types of controls were used to verify the transformation results (Figure 1 [I], [II], and [III]), and the selection results for the transformed cells are presented in Figure 2 [A], [B], and [C].

The result in Figure 1[I] showed no colony growth, indicating that *E.coli* DH5α cells did not grow. This lack of growth can be attributed to the absence of plasmid containing an antibiotic resistance gene. The purpose of using this control was to ensure that there was no contamination during the transformation process. Therefore, the results of this control indicate that the transformation process was free from contamination by other bacteria.

Figure 1 [II] shows no visible colony growth, indicating that the medium and ampicillin are not contaminated. In contrast, Figure 1 (III) displays abundant colony growth, suggesting that Escherichia coli DH5α cells are healthy and capable of rapid growth. This growth also demonstrates that the LB agar medium provides adequate nutrients for bacterial proliferation. The results from the control samples in Figure 1 [I], [II], and [III] were used to validate the success of the transformation process and to ensure that there were no contaminations or technical errors during the procedure.

The result of the recombinant plasmid transformation are shown in figure 1A, 1B, and 1C. The white colonies in Figure 1A demonstrated limited growth. This could due to uneven heat distribution during the transformation process, which could have caused many cells to die from overheating. Such conditions could lead to physiological stress and damage to cells, affecting the protein expression level.

b. SDS-PAGE Analysis for Optimal IPTG Concentration

The result indicate that the Negative control (K^-) does not produce any protein bands in SDS-PAGE. This result suggests that without IPTG induction, the protein expression does not occur efficiently. The IPTG (Isopropyl-β-D-1-thiogalactopyranoside) is commonly used as an inducer of gene expression in lac promoter-based systems, particularly in *Escherichia coli* (Larentis., et al, 2014). The LacI protein can repress the lac promoter, which prevents the transcriptions of the target gene and consequently blocks translation and protein synthesis. The IPTG works by binding to the LacI protein, thereby releasing the inhibition of the lac promoter and allowing gene transcription to occur (Larentis., et al, 2014). An additional advantage of the IPTG is that it is not degraded by *E.coli*, leading to stable protein production that is not affected by the metabolic regulation of the bacteria cells (Faust., et al, 2015).

Escherichia coli can express proteins without IPTG treatment because it has a natural inducer, lactose, but this process is slower and less stable (Faust., et al, 2015). *E.coli* can metabolize lactose and causes fluctuations in lactose concentrations within the cell, resulting in inconsistent protein expression levels. Therefore, using IPTG as an inducer is crucial to ensuring optimal, stable, and efficient protein expression.

During the IPTG concentration optimization, differences in the thickness of protein bands were observed in the SDS-PAGE results. Band thickness reflects the amount of protein expressed, with thicker bands indicating higher levels of protein expression. The results indicate that variations in IPTG concentration affect the expression level of *RecmE6* protein. The thickest band was observed in the 1 mM of IPTG, suggesting that this concentration resulted in the highest expression of *RecmE6*. In contrast, thinner protein bands were seen at concentration of 0.1 mM and 0.5 mM, indicating that these concentrations were not optimal for *RecmE6* protein expression.

c. SDS-PAGE Analysis for Optimal Incubation Temperature

The three temperature variations used, 25°C, 30°C, and 37°C, affect protein expression levels and stability differently. At 25°C, the target protein band (16 kDa) appears very faint and nearly invisible. This low temperature for protein expression in an Escherichia coli-based system leads to slower translation processes. Consequently, while the protein produced is more likely to fold correctly, the overall quantity is very low. Additionally, a distinct smear can be observed at 25°C. This may happen because cellular proteases remain active at this temperature, leading to the degradation of proteins before they can be properly detected in SDS-PAGE.

At 30°C, the expression of the target protein showed an increase compared to 25°C, which was evident from the thicker and clearer protein bands observed around 16 kDa. This suggest that 30°C is an optimal temperature for protein expression, as the rate of translation increases while still allowing for proper protein folding. Furthermore, the smear band is significantly reduced at this temperature, indicating that protein degradation is less frequent compared to lower temperature.

At 37°C, the expression of the RecmE6 protein reached its peak, as evidenced by the clear and prominent 16 kDa band. This indicate that IPTG induction at this temperature significantly enhances protein production. *Escherichia coli* typically grows at 37°C which is considered as its optimal growth temperature in laboratory settings (Eramus., et al, 2024). This preference is due to its natural habitat in the intestines of mammals, including humans, where body temperature averages around 37°C (Basavaraju and Gunashree, 2023). Consequently, the entire biological system of *Escherichia coli* operates most efficiently at this temperature, from metabolism to gene expression, allowing bacteria to proliferate and survive within the host.

The enzyme involved in *Escherichia coli* metabolism also exhibit optimal activity at 37°C (Eramus., et al, 2024). The lower temperatures reduce the enzyme activity, leading to slower bacterial growth. On the other hand, higher temperature can cause denaturation of the enzyme structures, disrupting their biological function and inhibiting cell survival. Additionally, optimal growth of *Escherichia coli* at 37°C is further supported by the stability of its membrane (Eramus., et al, 2024). At the lower temperature, the cell membrane becomes stiffer, which hampers nutrient transport and substance exchange. Conversely, if the temperature is excessively high, the membrane become too fluid, compromising its integrity and risking cell leakage or death. Therefore, maintaining temperature stability is essential for the metabolic efficiency of *Escherichia coli.*

d. SDS-PAGE Analysis for Optimal Incubation Time

A protein band with a molecular weight of approximately 16 kDa began to appear after 4 hours of incubation, although it was still very faint. This suggests that the protein expression process had started, but it was not yet optimal. At 6-hour incubation, the protein band was more clearly visible compared to the 4-hour incubation, indicating increased protein production. However, the band at 6 hours remained thin and less distinct, suggesting that protein synthesis was still ongoing and had not yet reached its maximum expression levels.

Eight hours after induction, the 16 kDa protein band appeared thicker and more defined, suggesting an increase in protein expression, although it was still not optimal. In comparison to the 24-hour incubation results, the protein expression after 8 hours remain less than optimal. While the protein band was noticeably thicker after 24 hours, the presence of a smear indicated potential degradation or overexpression of the protein.

There are two possible causes of smearing on protein lanes. First, excessive protein expression could result in overly thick band that appear diffuse or unclear (Sambrook and Russell, 2006). This is likely due to the considerable time difference between the 8-hour and 24-hour incubation, leading to significant protein accumulation. Second, smearing may also indicate protein degradation caused by prolonged incubation. Degrades proteins may break into smaller fragments, which migrate further down the gel, resulting in diffuse bands rather than sharp ones.

Furthermore, the 8-hour incubation period corresponds to the transition between the late logarithmic phase and the early stationary phase of cell growth. At this stage, cells remain in a relatively optimal condition for protein synthesis before viability begins to decline in the death phase. During the logarithmic (exponential) phase, cells undergo rapid growth and primarily focus on DNA replication and the synthesis of other cellular components, which may limit the optimal expression of the protein (Nurjayadi., et al, 2018). Consequently, harvesting the bacterial cells too early during this phase may result in lower protein yield due to insufficient accumulation of the target protein.

As the cell enter the early stationary phase, their growth rate begins to decline due to nutrient depletion or the accumulation of metabolic byproducts. However, during this phase, the expression system often reaches its optimal capacity for recombinant protein production. Cellular metabolism remains sufficiently active to sustain protein expression without significant degradation (Mutmainnah and Rijal, 2020). Moreover, the physiological state of the cells is generally more stable during the logarithmic phase, allowing for optimal accumulation of the target protein before environmental stressors begin to adversely affect cell viability. Therefore, harvesting recombinant protein during the early stationary phase is strongly recommended.

Optimization of incubation time revealed that protein expression peaked at 8 hours and 24 hours. However, the 24-hour yielded a smearing band, indicated protein degradation. At this point, 8-hour incubation is the optimal incubation time to obtain highest quantity of the intact protein.

4. CONCLUSION

This study aimed to determine the optimal condition for RecmE6 protein expression. The findings demonstrated that the optimal expression of the RecmE6 protein was achieved with 1 mM IPTG, at a temperature of 37°C, and after 8-hour incubation. This was indicated by the most intense and distinct protein band observed at approximately 16 kDa. Protein expression increased proportionally with IPTG concentration, reaching its peak at 1 mM, while lower concentration (0.1 and 0.5 mM) resulted in fainter bands. Among all tested conditions, the combination of 37°C and an 8-hour incubation produced the highest level of protein expression.

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