Effects of Growth Medium on Extracellular Secretion of Human Epidermal Growth Factor in *Escherichia coli* by Co-expression with *Bacillus cereus* Phospholipase C

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Abstract. Human Epidermal Growth Factor (hEGF) is a small, mitotic growth polypeptide that promotes the proliferation of various cells and is widely applied in clinical practices, especially in therapeutic uses of wound healing. Since it has a lot of benefits, production of recombinant hEGF (rhEGF) in a large scale is needed. Some methods have been used in this protein production, one of them was the production of rhEGF using extracellular secretion in *Escherichia coli*. Previous research have been done using co-expression method with phospholipase C from *Bacillus cereus* to increase the amount of rhEGF. Phospholipase C *B. cereus* have been used in several protein expression and was proved that it could increase the secretion of recombinant protein through hydrolytic mechanism of cell membrane. In addition, growth condition is one of some major factors which can affect the yields of produced protein. Different compositions of bacterial growth medium often lead to different result. This paper studies how rich-nutrient Terrific Broth (TB) medium and Luria Bertani (LB) medium produced different rhEGF results when it was co-expressed with phospholipase C *B. cereus*. rhEGF was characterized using SDS-PAGE and confirmed by western blot using anti-mouse EGF, and its concentration was measured using ELISA. rhEGF was successfully characterized after co-expression in TB medium and the concentration was 503.48 μg/mL. rhEGF was better produced in TB medium rather than in LB medium since TB medium has richer composition.

1. Introduction
Diabetes mellitus is a metabolic disorder disease characterized by chronic hyperglycemia due to defect in insulin secretion, insulin action, or both [1]. One of the most devastating and significant complications of diabetes and is associated with neuropathy and/or peripheral of the lower limb in patients suffering diabetes mellitus is diabetic foot ulcer (DM). The prevalence of diabetic foot ulcer in the diabetic population is around 4–10% [2]. Generally, diabetic foot ulcer is hard to be cured, therefore latest treatments have been done to cure this problem by using Human Epidermal Growth Factor protein to accelerate wound healing [3]. A previous study reported that recombinant hEGF can be considered as therapeutic agents in wound healing of diabetic foot ulcer and it was proven that the recovery of ulcer can be done in only about 8 weeks [4]. A number of recombinant proteins have been...
reported that they can be used as therapeutic agents. In addition, the market sales of recombinant pharmaceutical proteins has gradually increased. Increasing the needs of recombinant proteins as therapeutic agents suggests the needs of large amounts production of recombinant proteins with low production costs using recombinant DNA technology [5]. Extracellular expression in *Escherichia coli* host are the most commonly used methods [6]. Since it has limited space of the periplasmic membrane, this type of secretions in *E. coli* has several disadvantages due to the low secretion products [7]. In another case, extracellular expression in *E. coli* can be enhanced through hydrolytic membrane mechanism by co-expression with *Bacillus cereus* Phospholipase C, especially for protein with low molecular weight [8]. Since hEGF is only 6.2 kDa, which is one of the low molecular proteins in size, therefore in this study we enhanced the secretion of recombinant hEGF by co-expression methods with *B. cereus* Phospholipase C.

Like many other expression processes, the amount of produced protein depends on the survival and the ability of the host cell in producing the protein during cultivation. In addition, the survival of the cells is much affected by culture conditions. One of the crucial conditions affecting the amount of cell density and its survival is the composition of growth medium. Recombinant *E. coli* can be grown at high density in general media such as Luria Bertani broth (LB), the synthetic M9 minimal salt medium, Super broth (SB), and Terrific broth (TB) [9] in 37 °C, and pH between 6.4 and 7.2 [10]. The most common used media are TB and LB since they are economically effective, and their compositions are easy to be found. A study used those two types of media in expressing a fleunter growth hormone (fGH) and reported that the use of TB medium produced higher amount of fGH since TB has richer composition than LB [11]. Therefore, in this research we optimized the growth medium using LB and TB in producing higher amount of hEGF during co-expression process by *B. cereus* phospholipase C.

2. Materials and Methods

2.1. Bacterial strain, vector construction and materials

This research used *E. coli* BL21 (DE3) strain that was taken from our laboratory stock. Other reagents including medium compositions, antibiotic, inducer, and SDS-PAGE reagents were obtained from Sigma Aldrich (Singapore). Western blot reagents and analysis were supported by Research Center of Biotechnology, Indonesian Institute of Science (LIPI, Bogor, Indonesia). The construction of plasmid pETDuet-1 which harbor genes encoding *B. cereus* phospholipase C and hEGF was designed by our team and purchased from Genescript, Novagen (Madison, WI, USA).

The gene encoding *B. cereus* phospholipase C was gained from protein data bank (accession code: 1AH7) and it was designed to be inserted to multiple cloning site 1 (MCS1) in the plasmid. Besides, genes encoding PelB and hEGF were elaborated from our previous research [12] and were designed to be inserted to multiple cloning site 2 (MCS2). hEGF gene was fused with gene encoding PelB signal peptide in C terminal in order to help the migration of hEGF through Sec-dependent secretion mechanism.

2.2. Cultivation conditions

The plasmid described before was used to change chemically competent *E. coli* BL21(DE3) cells. These engineered *E. coli* strains were cultured in shake flasks to produce test proteins. A 5 mL of Luria Bertani medium (1% [w/v] tryptone; 0.5% [w/v] yeast extract; 1% [w/v] NaCl) was added with 100 μg/mL ampicillin. The medium was then inoculated with frozen glycerol stock from engineered *E. coli* strain (100 μL), and then incubated in a rotary shaker (200 rpm) at 37 °C for up to 8 h. Aliquots from this seed culture (5% [v/v]) were used to inoculate 50 mL of Luria Bertani and Terrific Broth medium (0.5% [w/v] glycerol; 1.2% [w/v] tryptone; 2.4% [w/v] yeast extract; 0.23% [w/v] KH2PO4; and 1.64% [w/v] K2HPO4) supplemented with 100 μg/mL ampicillin, which was then shaken in a rotary shaker (200 rpm) at 37 °C. Then isopropyl β-d-thiogalactopyranoside (IPTG) inducer was added to a final concentration of 0.1 mM when the optical density of culture at 600 nm (OD600) increased to
1.5, and incubation was continued at 25 °C [8]. The each of culture samples were then collected every 20 h in cultivation medium, periplasm, and cytoplasm.

2.3. Protein characterization and analysis
The collected culture was then centrifuged in 10,000 rpm in 4 °C for 30 minutes and the supernatant was gained as medium fraction. Some of the pellets was dissolved with TSE buffer (5% [v/v] Tris-Cl pH 8.0, 1 M; 20% [w/v] sucrose; 10% [v/v] EDTA 0.5 M) to extract the periplasm and then incubated in the ice bath for 20 minutes. Periplasmic fraction was gained as the supernatant after its being centrifuged in 20,000 rpm, 4 °C, for 30 minutes. Some other pellets from the first centrifugation was dissolved in lysis buffer (1% [v/v] Tris-Cl pH 7.5, 1M; 0.2% [v/v] 0.5 M EDTA) and the suspension was then disrupted using ultrasonic disintegration to obtain proteins inside cytoplasm. Then it was centrifuged in 10,000 rpm in 4 °C for 30 minutes and the supernatant was collected. Protein characterization was performed by Tricine SDS-PAGE (gel concentration: 15%) following the newly developed protocol [13]. The sample was then confirmed by western blot using Ig anti-mouse EGF (as the primary antibody) and IgG anti-mouse EGF (as the secondary antibody) in a nitrocellulose membrane. The proteins in the membrane were then visualized by alkaline phosphatase substrate. The concentration of rhEGF in cultivation medium was then measured by ELISA which was purchased from Qiayee (Bio-Technne, USA).

3. Results and Discussions
The genes encoding PLC and PelB-hEGF were inserted to pETDuet-1 plasmid and this recombinant plasmid was used to transform E. coli BL21(DE3). The modified E. coli strain was then cultivated in shake flasks using two different mediums with different compositions. After 61 h of expression in those different mediums, hEGF was produced in both medium but with different concentration. In expression using LB medium, rhEGF was produced in low level. This data was supported by SDS-PAGE result which was not showing band around 6.2 kDa (below 10 kDa) in medium fraction. This result also shows that the localization process of rhEGF from cytoplasm to periplasm and culture medium was not successfully happened (Figure 1).

Figure 1 shows us that rhEGF was still localized in the periplasm even after 61 hours post induction. The probability of hEGF which might present in the medium fraction was low since there was no band produced in the medium fraction in 20, 40, and 61 hours post induction. This result also shows that PLC was not well characterized around 28 kDa both in medium and periplasm. Looking up to the cytoplasm, SDS-PAGE result shows the present of PLC and rhEGF band (Figure 2). This result tells us that both PLC and rhEGF were actually produced even since 20 h post induction but they were not migrated successfully to periplasm and culture medium.

Different result was obtained from expression in TB medium. Figure 3 shows that hEGF band below 10 kDa was produced in higher intensity than the band produced in figure 1. This means that the co-expression process in TB medium produced rhEGF in higher concentration than in LB medium. Besides, figure 3 tells us about the migration of rhEGF from periplasm to the culture medium. In 20 h after induction, rhEGF was present in periplasm but not in culture medium. After 40 h, it was present both in periplasm and culture medium, indicating the migration of the protein. Looking up to 61 h after induction, all of rhEGF in periplasm has been moved to the culture medium since there was no rhEGF band in periplasm. In conclusion, co-expression in TB medium produced rhEGF in high amount and provided a good migration of the protein to the culture medium.
The rhEGF production was better using TB medium than LB medium. This might be happened because of richer composition which exist in TB. TB medium contains higher concentration of tryptone and yeast extract (having roles as carbon source as amino acids and vitamins) than LB medium (TB: 1.2% tryptone, 2.4% yeast extract, while LB: 1% tryptone, 0.5% yeast extract). Besides, TB has phosphate buffering and glycerol as carbon source. Higher content of carbon sources can be used to support the growth of biomass yields up to five times higher than LB. Regarding to the results of volumetric recombinant protein, TB is superior when compared to LB as a result of higher cell density [14] and/or increased specific productivity per biomass. On the other hand, amino acids form a primary carbon source in LB medium which then be constituted by catabolizable amino acids, while the fermented sugar concentration is equivalent to 0.5% yeast extract which is estimated to be less than 100 μM. As a result, growth in the LB medium stops when the culture’s optical density at 600nm (OD600) reached 7 when the amino acids catabolism has been depleted. Catabolism of amino acids lead to alkalinization of the medium up to pH 9 due to excessive ammonium excretion [14-15]. The optimal growth temperature for E. coli is 37 °C, and pH between 6.4 and 7.2 [10]. Thus, significant pH
fluctuation of the medium will affect the survival of the cell and lead to cell death. As a result, cells cannot express the protein and the production of protein will be stopped, resulting the low yield of protein. In TB medium, the presence of phosphate buffering will attenuate pH drifts during cultivation, resulting the prevention of cell death caused by pH fluctuation.

![Figure 3](image1.png)

**Figure 3.** The SDS-PAGE analysis of the culture supernatant and periplasmic fraction in expression with TB medium. P0: periplasmic fraction before induction, M: protein marker, M0: medium fraction before induction, P20: periplasmic fraction 20 h after induction, M20: medium fraction 20 h after induction, P40: periplasmic fraction 40 h after induction, M40: medium fraction 40 h after induction, P61: periplasmic fraction 61 h after induction. M61: medium fraction 61 h after induction.

Another characterization was performed by western blot. This method was done to confirm the presence of rhEGF through binding activity with its antibody. Figure 4 shows the result of western blot and a band was shown up, confirming that the expressed protein was right. The concentration of rhEGF in TB medium culture media was measured using ELISA, with the concentration of 503.48 μg/mL.

![Figure 4](image2.png)

**Figure 4.** Western blot analysis result. Std: hEGF standard, M: protein marker, M61: culture medium 61 h after induction after co-expression in TB medium.

### 4. Conclusion
In this study showed the optimized culture medium for production of rhEGF protein. TB medium was found to be the best medium since it can support better growth and produce more recombinant protein than LB medium. This medium may also be suitable to produce other high-yield recombinant proteins. This result may also be a consideration in continuing the research by scaling up the volume fermentation so that we can produce rhEGF in high amount. Then, the large number of active recombinants rhEGF produced by this method can be utilized as a therapeutic candidates of diabetic foot ulcer.
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