Expression of recombinant human granulocyte-colony stimulating factor in Escherichia coli using various induction methods

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Abstract. Granulocyte-colony stimulating factor (G-CSF) is a glycoprotein that has several therapeutic applications. It consists of 174 amino acids and manufactured by recombinant DNA technology. Until now, the Escherichia coli expression system is still become the first choice for producing recombinant proteins. It is because of this organism is simple to culture in low-cost medium and easy to scale up. In the course to find the most efficient way to produce a high yield of recombinant human G-CSF, we compared several types of medium with different induction methods. In this experiment, recombinant E. coli NiCo21(DE3) harbouring gene encoding rh-GCSF proteins were cultured in various media including auto-induction, non-induction, and IPTG-induction. To determine the protein expression profile, culture sampling was done every 12 h (up to 60 h). Then, the optical density at λ 600 nm was measured using UV spectrophotometer and rh-GCSF protein expression were characterized using SDS-PAGE and western blot analyses. ImageJ software was used to calculate the amount of rh-GCSF protein yield using Bovine Serum Albumin (BSA) with known concentration as a standard. Result of this experiment concluded that simple auto-induction medium from Imperial College could produce good amount of rh-GCSF proteins (117 µg/mL) with relatively low production cost and short incubation time.

1. Introduction

Recombinant human granulocyte-colony stimulating factor (rh-G-CSF) protein is an effective biopharmaceutical in stimulating neutrophil production and reducing the duration of neutropenia-induced chemotherapy [1]. In a human body, hG-CSF is a hematopoietic growth factor that regulates the proliferation and differentiation of hematopoietic precursors of neutrophil granulocytes [2]. Naturally occurring h-G-CSF is a glycosylated protein containing 174 amino acids residues (MW = 18.8 kDa) and relatively hydrophobic. The O-linked carbohydrate chain is attached to thr 133 and has a little impact on the three-dimensional structure of the h-G-CSF molecule [3]. It also contains a free cysteine at position 17 and two intramolecular disulfide bonds at positions 36/42 and 64/74 [4, 5].

Recombinant DNA technology provides several simple techniques to transfer and express the desired gene within a particular host cell. Selecting the right production host is important because it affects the quality and quantity of the final product. Escherichia coli (E. coli) bacteria offering several...
advantages than other host systems including high growth rates on relatively simple and inexpensive medium, easily transformed with exogenous DNA, and high protein yields\cite{6,7}. Moreover, molecular genetics and cellular physiology of \textit{E. coli} cells are well-known. Thus, it is possible to produce recombinant proteins in simple and inexpensive ways.

IPTG-induced medium is currently the most efficient method for expressing recombinant proteins in \textit{E. coli}. However, because of its high cost and toxicity, IPTG is not suitable for large-scale production. Auto-induction method is known has several advantages including less expensive inducer (lactose) and not required of culture monitoring\cite{8,9}. On the other side, some studies reported that growth medium without IPTG induction has been able to produce a significant amount of proteins. This can be occurred because of the presence of lactose in the growth medium or leakage at the \textit{lac} promoter, resulting in the expression of the proteins.

In the production of recombinant proteins, the main goal is to get a high yield protein products along with the good biological activity. Besides, easy and inexpensive growth medium is also highly considered to get low production cost. In order to find the most efficient medium for producing the recombinant G-CSF proteins in \textit{E. coli} NiCo21(DE3), we compare its expression using five variant of culture medium including auto-induction, non-induction, and IPTG-induced medium.

2. Material and Methods

2.1. Microorganism

The \textit{E. coli} NiCo21(DE3) transformant harbouring gene encoding rh-GCSF in pJExpress414 expression vector was used in the present work. This microorganism was obtained from Protein Engineering and Drug Delivery System Laboratory (Research Centre for Biotechnology, Indonesian Institute of Sciences).

2.2. Medium for growth and expression

Medium that used in this study were Terrific Broth (TB) (1.2% Tryptone, 2.4% Yeast Extract, 0.015% MgSO$_4$, 0.65% KH$_2$PO$_4$, 0.33% (NH$_4$)$_2$SO$_4$, 0.71% Na$_2$HPO$_4$, 0.05% glucose and 0.2% lactose), auto-induction medium from Imperial College London (IC) (2% Tryptone, 0.5% Yeast Extract, 0.5% NaCl, 0.3% KH$_2$PO$_4$, 0.6% NaH$_2$PO$_4$, 0.6% glycerol, 0.05% glucose and 0.2% lactose), 2xYT (1.6% Tryptone, 1% Yeast Extract, 0.5% NaCl) and Lysogeny Broth (1% Tryptone, 0.5% Yeast Extract, and 1% NaCl)\cite{10,11}. All medium were sterilized using autoclave except for glucose and lactose which sterilized by membrane filtration. The medium used for growth and expression was the same, except of lactose removal and addition of 0.1% glucose in the pre-culture preparation.

2.3. Selection of high-level expressing colony

A bacterial glycerol stock of \textit{E. coli} NiCo21(DE3) transformant was diluted to $10^6$ dilution with sterile water and spread 30 µL on LB agar plate containing 100 µg/mL Ampicillin (LB-Amp). The plate was then incubated overnight at room temperature (23-25°C). Nine single colonies were picked and each inoculated into LB-Amp medium, followed by overnight incubation at 37°C with continuous shaking at 150 rpm. This culture was diluted 1:50 with LB-amp medium and grown until the optical density (OD$_{600}$) 0.8 was reached. After that, IPTG with a final concentration of 1 mM was added and growth was continued overnight at room temperature. The colony that produces a largest amount of rh-GCSF proteins was replicated on new LB-Amp agar plate and used for further expression analysis.

2.4. Growth condition for rh-GCSF proteins expression

Pre-cultures were prepared by growing selected \textit{E. coli} NiCo21(DE3) transformants in various media including TB, IC, 2xYT, and LB which already supplemented with 100 µg/mL Ampicillin. The cultures were grown overnight at room temperature with continuous shaking at 150 rpm. It was then diluted 1:50 with fresh medium containing 100 µg/mL Ampicillin and growth was continued for 60 h. Culture sampling was carried out in every 12 h for protein expression characterization and OD$_{600}$ measurements. For IPTG-induced expression, the cultured was prepared in LB-Amp medium and
added with IPTG to a final concentration of 1 mM when OD$_{600}$ 0.8 was reached, then incubation was continued for 60 h.

2.5 Protein characterization

The samples for protein expression characterization were prepared by mixing the pellet cells from 1 mL culture with 50 µL Denaturing Lysis Buffer (8 M Urea, 10 mM Tris-Cl, 100 mM Na$_2$HPO$_4$) and 50 µL Reducing Sample Buffer, subsequently heated at 100°C for 10 min. SDS-PAGE analysis was performed using 15% acrylamide gel. To each lane of the gel, 5 µL samples was loaded and the electrophoresis performed in SDS-PAGE buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS) using Bio-Rad Mini-PROTEAN® at 90 volt for 110 min. To calculate the yield of rh-GCSF protein expression, the standard Bovine Serum Albumin (BSA) with known concentration was loaded in the same gel. After that, the gel was immersed in Coomassie blue staining for 1 h and destained using hot water. If the protein band was already cleared, then the gel was subjected to ImageJ software [12].

Western blot analysis was done after the proteins had been separated by SDS-PAGE. Briefly, the gel, nitrocellulose membrane, and Whatman paper were immersed in the transfer buffer (25 mM Tris base, 192 mM Glycine, and 20% methanol) and then placed in western blot apparatus (Trans-Blot® Cell, Bio-Rad) at 90 V for 2 h. The membrane was incubated in blocking buffer [10% skim milk in Tris-buffered saline pH 7.6 (50 mM Tris-Cl and 150 mM NaCl)] for 1 h at room temperature, subsequently washed 3 x 10 min with TBS-T (0.1% Tween-20 in TBS). The membrane was then incubated with anti-G-CSF mouse monoclonal IgG antibody (1:2000 dilution, Santa Cruz Biotechnology Inc., USA) in blocking buffer for 1 h. After that, the membrane was washed 3 x 10 min with TBS-T and incubated with AP-conjugated goat anti-mouse IgG antibody (1:3500 dilution, Santa Cruz Biotechnology, Inc., USA) for 1 h. Finally, the rh-GCSF proteins band was visualized using 1 mL NBT-BCIP (Thermo Scientific, USA) after the membrane had been washed with TBS-T.

3. Result and Discussion

3.1 Selection of high-level expression colony

In order to get a high-level expressing colony, thus the expression level of *E. coli* NiCo21(DE3) transformant harbouring pJExpress414-GCSF was screened. The single colony was obtained from diluting the glycerol stock and spread it on LB-Amp agar plate. After that, the protein expression level from selected colonies was checked by inducing the culture with IPTG, followed by SDS-PAGE characterization. Figure 1 showed the band of rh-GCSF proteins that have an approximate size of 18 kDa which corresponds to its theoretical size [3]. From this analysis, we found that colony number 9 produced a higher amount of rh-GCSF proteins in comparison to other colonies. Therefore, it was chosen for further protein expression analysis.

![Figure 1](image-url)

**Figure 1.** Colony selection of *E. coli* NiCo21(DE3) transformant harbouring pJExpress414-GCSF. Lane 1-9, total proteins from colony number 1-9 (respectively).

Colony selection was observed as one of many important factors for high-level recombinant protein production in *E. coli*. It is important because colonies harbouring the same expression vector might result in different expression level. Sivashanmugam et al. reported that low yield of protein expression
was often found if we used glycerol stock for pre-culture preparation, even though this glycerol stock previously produced a high yield protein [13]. This situation occurred quite often when we produced human proteins that tend to toxic to bacterial cells. Therefore, screening of E. coli transformant was important to select a high-level expressing colony.

3.2 Expression of rh-GCSF proteins in various induction methods

Medium selection and expression induction method were other important factors in producing high-yield recombinant proteins. The medium composition such as the type of carbon sources, salt, mineral, and buffering system also important to be considered before we performed the protein production [14]. In order to optimize the protein production at low production cost, we compared the expression of rh-GCSF proteins in auto-induction (TB and IC medium), non-induction (LB and 2xYT medium) and IPTG-induced in LB medium. We also monitored the expression protein profiles and cell densities by culture sampling per 12 hours in order to determine the optimum incubation time for producing the highest amount of recombinant proteins.

Pre-cultures were prepared by inoculating colony number 9 into various media including TB, IC, 2xYT, and LB. To prevent the protein expression in pre-culture, we have removed the lactose from TB and IC medium. The room temperature was chosen for incubation because too saturated pre-cultures might result in plasmid instability. Besides, the basal leakage of the T7 expression system usually resulted in poor yield of proteins [13]. To maintain the basal expression prior to induction, 0.1% glucose was added into all pre-cultures medium. By the next morning, the pre-culture was diluted 1/50 with a fresh medium containing 100 µg/mL Ampicillin and sampling was began after 12 h of incubation. For IPTG-induced culture, the incubation time was started after the addition of IPTG. After all samples have been collected, then the characterization was done using SDS-PAGE and the protein was quantified using imageJ software analysis. Figure 2 showed SDS-PAGE analysis of rh-GCSF proteins in various media.

![SDS-PAGE analysis of rh-GCSF proteins in various media.](image-url)

Figure 2. Electrophoregram of SDS-PAGE analysis. (A) TB auto-induction; (B) IC auto-induction; (C) IPTG-induction; (D) 2xYT non-induction; (E) LB non-induction medium.
Table 1 showed the total protein yields for the time course of rh-GCSF expression in various media. TB and IC were used as auto-induction medium which contains lactose as an inducer for protein expression. A bit different from auto-induction medium described by Studier (2005), there was no metal mix addition in both TB and IC which expected to reduce the production cost. Moreover, the addition of metal mix was generally carried out for expressing the recombinant protein which required a metal cofactor for its activity.

| Medium            | Time of incubation |
|-------------------|--------------------|
|                   | 12 h   | 24 h   | 36 h   | 48 h   | 60 h   |
| TB                | -      | 54.3   | 96.4   | 101.5  | 162.47 |
| IC                | -      | 58.7   | 117.2  | 102    | 91     |
| LB                | 3.6    | 4.17   | 8.8    | 12.7   | 14.2   |
| 2xYT              | -      | -      | -      | -      | -      |
| IPTG-induced (LB) | 60.7   | 63.9   | 79.8   | 74.4   | 70.7   |

Table 2. The optical density (OD_{600}) of E. coli NiCo21(DE3) transformant in various media

| Medium            | Time of incubation |
|-------------------|--------------------|
|                   | 12 h   | 24 h   | 36 h   | 48 h   | 60 h   |
| TB                | 1.8    | 3      | 3.8    | 3.9    | 4.4    |
| IC                | 2.1    | 3.1    | 3.7    | 3.5    | 3.2    |
| LB                | 3.3    | 3.5    | 3.5    | 3.8    | 3.7    |
| 2xYT              | 2      | 2.8    | 2.8    | 2.8    | 2.8    |
| IPTG-induced (LB) | 3.5    | 3.5    | 3.6    | 3.5    | 3.5    |

As seen in Table 1, there was no protein observed in both TB and IC medium at 12 h of incubation. This phenomenon was common if we used the auto-induction system in high-density shaking cultures. In this system, the cells would grow until quite high density without expressing target proteins. Many factors including glucose and amino acids would inhibit the expression until they were depleted and then allowed the lactose to induce the expression of target proteins [10]. Until 36 h of incubation, the IC medium produces the highest protein yield. However, the yield started to decrease at 48 h which corresponded with its optical density (Table 2). This situation might occur because of the depletion of carbon source, amino acids, and other components which important for growth and expression.

Different from IC medium, the growth of recombinant E. coli and also the production of rh-GCSF proteins were increased gradually until 60 h using TB auto-induction medium. TB medium contains a higher amount of yeast extract than IC medium, causing the culture to continue growing and producing the proteins. However, until 48 h of incubation, the target protein produced by IC auto-induction medium was still higher than TB. It might result from the addition of glycerol which served as a good carbon and energy source.

LB and 2xYT were common media used for expression using traditionally IPTG-induction. We used this medium in this experiment because sometimes the basal expression of T7 promoter could produce a significant amount of target proteins which expected to provide a simple, cost-effective and competitive methods for producing rh-GCSF proteins. However, our experiment showed that no significant expression was found using LB and 2xYT medium. This result was in contrast with research conducted by Zhang et al. which successfully produced membrane proteins in E. coli BL21(DE3) cytoplasmic space by omitting IPTG as an inducer in LB medium [15]. Moreover, no expression of rh-GCSF proteins can be observed in 2xYT medium using SDS-PAGE analysis.

Nair et al. were also reported that the good expression level of rh-GCSF can be achieved using a common medium without any induction [16]. However, in their experiment, only in the addition of more than 1% (w/v) yeast extract could produce the recombinant proteins. As said by Fu et al., the
higher amount of yeast extract increases the expression of human parathyroid hormone fusion proteins without the addition of inducer [17].

Grossman et al. concluded that cyclic AMP is required for unintended induction. Their findings showed that un-induced expression of lac-controlled genes occurs when cell cultures reached the stationary phase and glucose has disappeared. In the presence of glucose, the levels of cyclic AMP were low, thus the transcription from the lac promoter was low [18]. This study could explain why a little bit expression of rh-GCSF was found in LB medium.

In this experiment, traditional IPTG-induced expression was performed as a control. Table 1 showed that higher production of rh-GCSF proteins was found at 36 h of incubation which corresponds to its optical density at the same hour (Table 2). However, the amount of total proteins using IPTG-induction was lower than TB and IC auto-induction medium. This result indicated that the auto-induction medium has more advantage than using IPTG-induction or non-induction medium.

To prove that the 18 kDa proteins found on SDS-PAGE analysis were rh-GCSF protein, then western blot analysis was carried out using anti-GCSF mouse monoclonal IgG antibody. For negative control, a total proteins of E. coli NiCo21(DE3) non-transformant was included in this analysis. Figure 3B showed 18 kDa bands were detected in TB, IC, LB, and IPTG-induced medium. A very pale band was detected in 2xYT media proving that there was a very low expression has occurred yet cannot be detected using SDS-PAGE analysis. No band was detected in negative control.

![Image of SDS-PAGE and western blot analyses of rh-GCSF expression](image)

**Figure 3.** Electrophoregram of (A) SDS-PAGE and (B) western blot analyses of rh-GCSF expression at 60 hours of incubation. Lane 1, IPTG-induction; lane 2, LB non-induction; lane 3, 2xYT non-induction; lane 4, IC auto-induction; lane 5, TB auto-induction; lane 6, E. coli NiCo21(DE3) non-transformant.

We reported that auto-induction medium is more convenient than both of non-inducing and IPTG-induction medium. In this method, the expressing colony is simply inoculated into auto-induction medium and no need to monitor the culture growth nor adding the inducer at the proper optical density to get a high amount of recombinant proteins. Furthermore, the yield of target proteins is higher than traditional IPTG-induced expression.

4. **Conclusion**
After all the analyses have been conducted, we conclude that IC auto-induction medium has a pretty good ability to produce a high-yield of rh-GCSF proteins with a relatively low cost and short production time (117 µg/mL in 36 h of incubation).

5. **Acknowledgment**
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6. References

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