

Optimization Expression and Stability Test of Recombinant Human Interferon Alfa 2a Fusion Protein in *Escherichia coli* BL21 (DE3)

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Abstract

The rhIFN α 2a is expressed as a fusion protein containing thioredoxine and polyhistidine sites at its N terminal. Our previous research has obtained recombinant human IFN α 2a (rhIFN α 2a) protein that expressed predominantly as a soluble form in *E. coli* BL21 (DE3). Through systematic approach of various culture conditions, the aim of current this research is to acquire the best condition and its stability of recombinant rhIFN α 2a fusion protein in a culture under study. Expression optimization performed by using three parameters, *i.e.*: temperature, induction time and inducer concentration. Various IPTG concentrations are 0.25, 0.5, 0.75, and 1.0 mM. The incubation time of bacterial cell culture carried out in 3, 4, and 5 hours at temperature 28, 30, and 37 °C. The best condition was used to analyze the stability of rhIFN α 2a protein expression up to ten generation. The expressed protein was analyzed using SDS PAGE and CBB staining. The optimal culture condition was found to be 37 °C temperature with 4 hours time of induction and 1 mM IPTG concentration. Stability analysis revealed that the rhIFN α 2a protein expression remained stable until the tenth generation with molecular weight, approximately, 36 kDa.

Key words: rhIFN α 2a fusion protein, *E. coli* BL21 (DE3), expression, stability test

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Introduction

Interferon α 2a is a cytokine that comprises of 165 amino acids with two disulfide bonds (Cys1 to Cys29; Cys98 to Cys138) with 19 kDa in size (Rae *et al.*, 1995). Interferon α 2a has been used for the therapy of non-hodgkin's lymphoma, hairy cell leukemia, chronic myelogenous leukemia, AIDS-related kaposi's sarcoma, and chronic hepatitis C (Jonasch & Haluska, 2001; Huckans *et al.*, 2007). Currently the recombinant of IFN α 2a is produced by using recombinant DNA techniques and available commercially.

Almost all of commercial recombinant IFN α is produced and purified in *Escherichia coli*. Interferon α which is expressed in *E. coli* mostly forms insoluble aggregates in cytoplasm that is called inclusion bodies (Swaminathan & Khanna, 1999; Beldarraín *et al.*, 2001; Srivastava *et al.*, 2005), it is generally a misfolding protein form and biologically inactive (Villaverde & Carrio, 2003). Various strategies to improve the solubility of recombinant protein produced in

E. coli can be performed by limiting protein aggregation in vivo with cultivation at lower temperatures, decreasing of the induction agent concentration, and the inducible promoter expression system (Baneyx & Mujacic, 2004). The expression of recombinant protein as a fusion protein containing thioredoxine tag enable high-level expression of soluble protein (Kapust & Waugh, 1999), by reducing the tendency of the formation of inclusion bodies (Lilie *et al.*, 1998).

Using pET-32b(+) expression vector, our previous research has successfully expressed a soluble rhIFN α 2a protein in *E. coli* BL21(DE3). The protein was produced as a fusion protein with 36 kDa in size containing N terminal thioredoxine and polyhistidine sites (Kusumawati *et al.*, 2013). Expression of recombinant protein in the soluble form has some has enabled rapid and relatively easy purification step (Fathallah *et al.*, 2009). With respect to achieve the best culture condition for soluble rhIFN α 2a protein expressed in *E. coli* BL21(DE3), in this current research, we performed a systematic approach study in

finding the exact optimal temperature, IPTG concentration, and incubation time. To obtain comprehensive information of how stable the expression of the protein under study, using the best condition found, test of stability was performed up to ten generation.

Materials and Methods

Media, reagents and bacterial strain. *Escherichia coli* BL21 (DE3) (Novagen) was used as the host for expression of fusion protein. Transformant clone number 3 *E. coli* BL21(DE3) that has recombinant vector pET-32b(+)-IFN α 2a from the previous research was used for expression of recombinant protein. Bacto tryptone (Becton Dickinson), bacto yeast extract (Becton Dickinson) and sodium chloride (Merck) was used for bacterial growth medium. IPTG (Roche) was used for induction expression of fusion protein. Coomassie brilliant blue R-250 staining solution was used for stained the result of SDS PAGE.

Expression optimization of rhIFN α 2a fusion protein. Transformant clones number 3 *E. coli* BL21(DE3) was used for optimization expression of rhIFN α 2a fusion protein. Twenty microliter of glycerol stock culture was grown in 3 mL of liquid LB medium with ampicillin and incubation at temperature 37°C for overnight with shaking 200 rpm. Five hundred microliter of stock culture was grown in 10 mL liquid LB medium with 50 μ g/ml ampicillin and incubation at temperature 37°C for 2 hour (OD₆₀₀: 0.5) with shaking 250 rpm. The induction of bacterial cell culture was carried out by addition IPTG with various concentrations are 0.25, 0.5, 0.75, and 1.0 mM and incubation at temperature 28, 30, and 37 °C with shaking 250 rpm. Harvesting of 1 mL cultures performed at 3, 4, and 5 hours after induction. Cultures was harvested by centrifugation 8,000 rpm for 10 minutes to separated cultivation medium (supernatant) and bacterial cells (pellet) and then stored at -20 °C for overnight. The pellet was resuspended in PBS buffer.

Stability test of expression rhIFN α 2a fusion protein. The stability test of expression rhIFN α 2a fusion protein was performed until ten generation. Twenty microlitre of glycerol stock

culture was grown in 3 mL of liquid LB medium with ampicillin and incubation at temperature 37 °C for overnight with shaking 200 rpm. One hundred fifty microlitre of stock culture was grown in 3 mL liquid LB medium with ampicillin and incubation at temperature 37 °C for 2 hours with shaking 250 rpm. Induction was carried out by addition of IPTG to a final concentration of 1 mM and incubation at temperature 37 °C with shaking 250 rpm. Harvesting of cell culture was performed at 4 hours after induction. Bacterial cell culture was divided into 1 mL and harvested by centrifugation 8,000 rpm for 10 minutes. The pellet stored at -20 °C for overnight and then resuspension with 200 μ l 1×PBS buffer.

Protein characterization. The rhIFN α 2a fusion proteins are characterized by SDS-PAGE method followed CBB stain. Ten microlitre of the pellet resuspension add equal volume of 2×sample loading buffer was boiled at 95 °C for 4 minutes and subjected to SDS-PAGE analysis using 12 % acrylamide gel. The protein was visualized with coomassie brilliant blue stain. The result was analyzed by densitometry method with automatic digitizing system using UN-SCAN-IT gel version 6.1. To avoid extensive variability of images among Figures 1, 2, 3, and 4, the calculation of data analysis was performed using average background of the images as the basis.

Results

To find the optimum condition of hIFN alpha 2a expression, the *E. coli* BL21 (DE3) cell harboring recombinant plasmid was cultured and grown in different temperatures (28, 30, and 37 °C), IPTG concentration (0.25, 0.5, 0.75, and 1.0 mM) and incubation time (3, 4, and 5 hours). Following incubation with several conditions, the media were collected by centrifugation and sonicated. The obtained media was then characterized using SDS-PAGE and CBB staining analysis.

Incubation at 28 °C (Figure 1) showed that IPTG concentration and time of incubation have impact on protein expression. However, it is shown that the time of incubation has more effect than that of IPTG concentration. At the same IPTG concentration, protein expression increased with the increase of time incubation.

On the other hand, at the same incubation time, protein expression was also increase with the increase of IPTG concentration. However, the increase of protein expression was more dependent on incubation time with the highest result was at 5 hours and 0.75 mM IPTG.

To evaluate the effect of temperature increase in protein expression, the cell was incubated at 30 °C at various incubation time and IPTG concentration (Figure 2). The results showed that expression at 30 °C of incubation

appears similar to that of 28 °C where IFN protein expression increased with the increase of incubation time; and the highest expression occurs at 5 hours of incubation time. Meanwhile, the increase of IPTG concentration also increases IFN expression. Interesting to note that expression at 0.5 mM of IPTG was lower than that of 0.25 and 0.75 mM. Overall, the expression at 30 °C was higher than that of 28 °C.

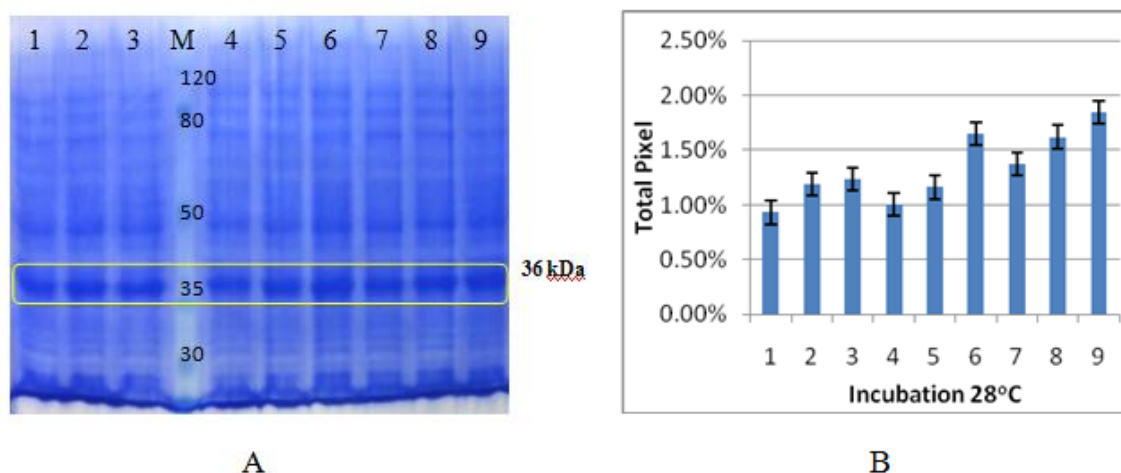


Figure 1. Profile CBB staining of rhIFN α 2a protein expression at 28°C bacterial incubation. (A) Lane (1) 3 hours (0.25 mM IPTG); (2) 4 hours (0.25 mM IPTG); (3) 5 hours (0.25 mM IPTG); (M) Marker; (4) 3 hours (0.5 mM IPTG); (5) 4 hours (0.5 mM IPTG); (6) 5 hours (0.5 mM IPTG); (7) 3 hours (0.75 mM IPTG); (8) 4 hours (0.75 mM IPTG); (9) 3 hours (0.75 mM IPTG). (B) Expression level analysis by UN-SCAN-IT.

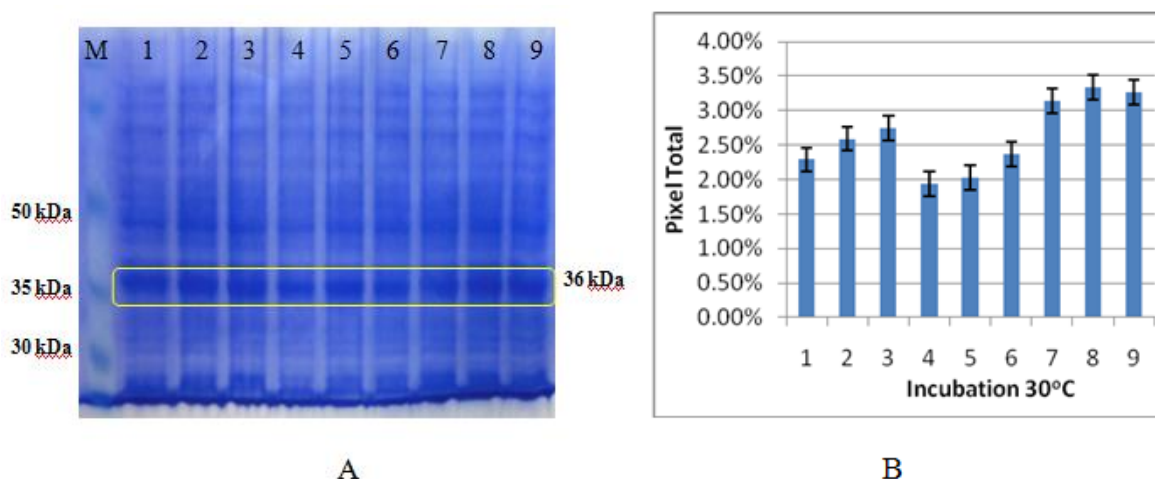


Figure 2. Profile of CBB staining of rhIFN α 2a protein expression at 30°C bacterial incubation. (A) Lane (M) Marker; (1) 3 hours (0.25 mM IPTG); (2) 4 hours (0.25 mM IPTG); (3) 5 hours (0.25 mM IPTG); (4) 3 hours (0.5 mM IPTG); (5) 4 hours (0.5 mM IPTG); (6) 5 hours (0.5 mM IPTG); (7) 3 hours (0.75 mM IPTG); (8) 4 hours (0.75 mM IPTG); (9) 3 hours (0.75 mM IPTG). (B) Expression level analysis by UN-SCAN-IT.

To have better evaluation of the effect of higher temperature on the expression of IFN, the cell was incubated at 37 °C (Figure 3). The data showed that the expression of IFN protein

level at 37 °C, on the average, was much higher than that of 28 and 30 °C. The influence of time of incubation and IPTG concentration

on expression was not seen clearly at 37 °C incubation.

To study more about the effect of temperature and IPTG, the cell was grown at different temperatures (28, 30 and 37 °C) and various incubation times (3, 4 dan 5 hours). The results clearly showed that in the presence of 1 mM IPTG the IFN protein expression increased sharply with the increase of time incubation and temperature (Figure 4).

The stability test of expression rhIFN α 2a fusion protein was performed until ten generation. Since the highest protein expression occurred at 37 °C, the stability test was performed at this temperature (Figure 5). The results showed that, overall, in this stability test the expression increased and peaked at generation seven and followed by slow decline.

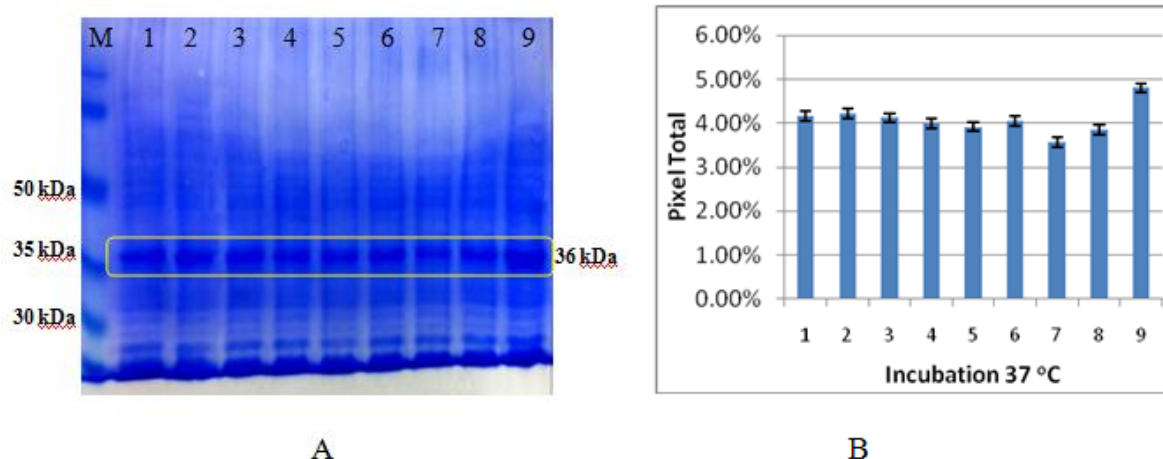


Figure 3. Profile of CBB staining of rhIFN α 2a protein expression at 37°C bacterial incubation. (A) Lane (M) Marker; (1) 3 hours (0.25 mM IPTG); (2) 4 hours (0.25 mM IPTG); (3) 5 hours (0.25 mM IPTG); (4) 3 hours (0.5 mM IPTG); (5) 4 hours (0.5 mM IPTG); (6) 5 hours (0.5 mM IPTG); (7) 3 hours (0.75 mM IPTG); (8) 4 hours (0.75 mM IPTG); (9) 3 hours (0.75 mM IPTG). (B) Expression level analysis by UN-SCAN-IT.

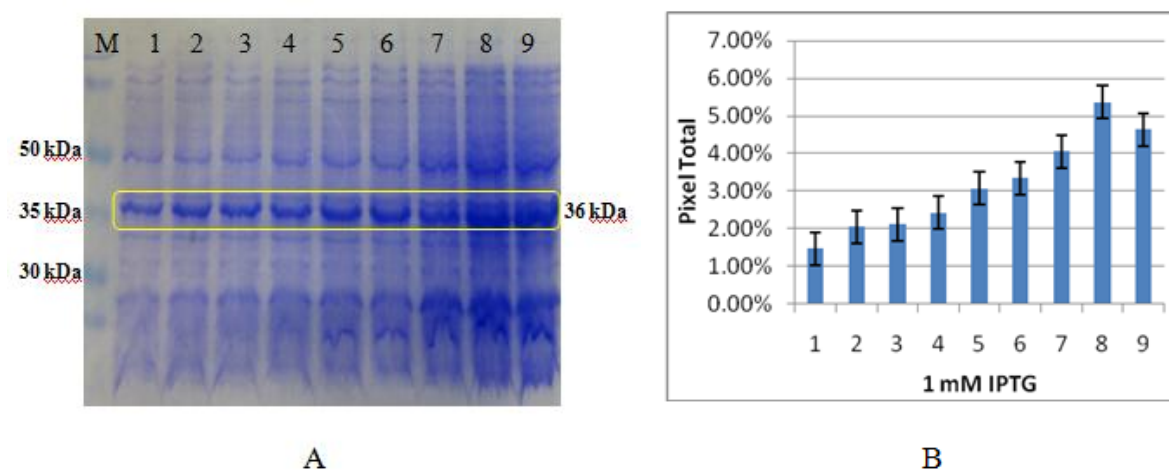


Figure 4. Profile of CBB staining of rhIFN α 2a protein expression at 28, 30 and 37 °C bacterial incubation with 1 mM IPTG concentration. (A) Lane (M) Marker, (1) Pellet 3 hours (28 °C), (2) Pellet 4 hours (28 °C), (3) Pellet 5 hours (28 °C), (4) Pellet 3 hours (30 °C), (5) pellets 4 hours (30 °C), (6) pellet 5 hours (30 °C), (7) pellet 3 hours (37 °C), (8) pellet 4 hours (37 °C), (9) pellet 5 hours (37 °C). (B) Expression level analysis by UN-SCAN-IT.

Discussion

The advance of recombinant DNA technology and its application in the pharmaceutical industry has brought a growing

market within the human medical biotechnology industry. With this, the availability of approved biopharmaceuticals has increased tremendously in the last several years. Currently, one of the most successful

recombinant biopharmaceutical ingredients used for the production of human medicines are produced bacteria. Bacteria grow quickly and can generate high quantities of recombinant protein. To obtain maximum usage of expression system including bacteria, however, several variables have to be taken into consideration. Various strategies for optimizing the production of a recombinant protein is through the choice of culture medium, mode of cultivation, control the

expression system (Kleman & Strohl, 1994), and genetic engineering of the target protein (Makrides, 1996; Sørensen & Mortensen, 2005). To achieve maximum results of *E. coli* BL21 (DE3) for the expression of rhIFN α 2a fusion protein, a systematic approach study in finding the exact optimal temperature, IPTG concentration, and incubation time for the best culture to grow was the main purpose of this study.

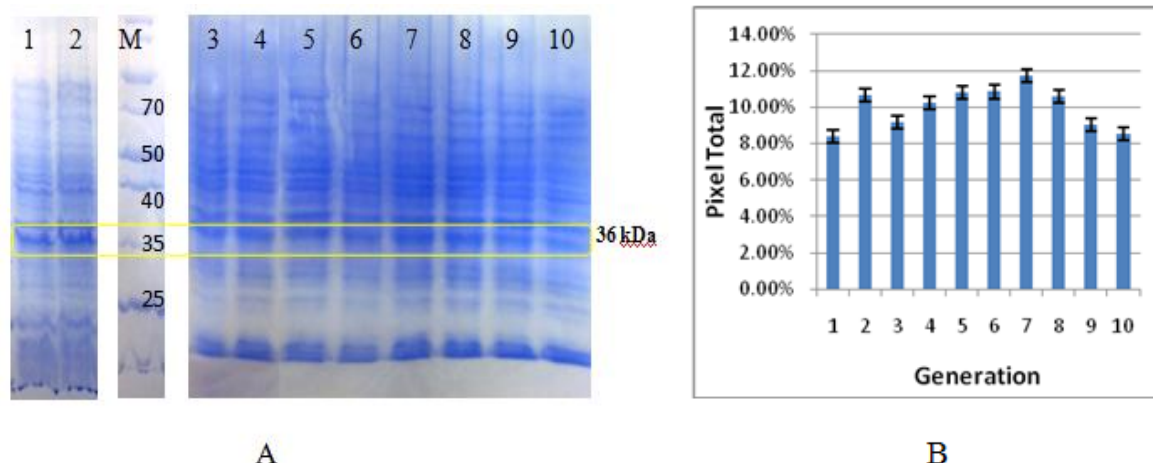


Figure 5. Profile of CBB staining of rhFN α 2a protein expression. (A) Lane (1) first generation; (2) second generation; (M) Marker; (3) third generation; (4) fourth generation; (5) fifth generation; (6) sixth generation; (7) seventh generation; (8) eighth generation; (9) ninth generation; (10) tenth generation. (B) Expression level analysis by UN-SCAN-IT.

Analyses of SDS-PAGE and CBB stains as shown in Figures 1-4, overall, showed that the expression level of protein under study increased at various levels as the result of various temperatures, IPTG concentrations and incubation times. Interesting to note that, at 28 °C incubation temperature (Figure 2), looks like time of incubation has more effect than that of IPTG concentration. Protein expression level keeps increasing significantly with the increase of time incubations even though IPTG concentration remains the same. On the other hand, protein expression level was also increase at lower magnitude at increasing of IPTG concentration when the incubation time did not change. Thus, in this case, the protein expression level was more dependent on incubation time with the highest result was at 5 hours and 0.75 mM IPTG.

The effect of temperature on protein expression was further evaluated at 30 and 37 °C with the same IPTG concentration (Figure 2 and 3). The results showed that expression at 30 °C of incubation appears similar to that of

28 °C with the highest occurred at 5 hrs of incubation time. Interesting and significant finding was obtained when the incubation was at 37 °C where the data obtained from this temperature explained that time of incubation and IPTG concentration did not have much influence on protein expression. In addition, over all, the expression of IFN protein level at 37 °C was much higher than that of 28 and 30 °C. Our data concluded that the best growth temperature of *E. coli* is 37 °C and this data was also shared by previous work (Arthuso *et al.*, 2014). However, with respect to fact that 37 °C is the best temperature for growth, lower temperature is still often used as lower temperature can reduce protein aggregation (Baneyx & Mujacic, 2004). Prolonged induction at low temperatures and decreased amounts of IPTG can increase solubility of recombinant protein (Gopal & Kumar, 2013).

Taken together, the obtained data demonstrated that, at 0.25-0.75mM IPTG concentration, temperature played more dominant role; and the time of incubation

played significant role only when the incubation temperatures was at 28 and 30 °C. To further evaluate consequences of inducer for protein expression, the IPTG concentration was increased to 1 mM (Figure 4). The data clearly showed that the expression level at 28 and 30 °C keep increasing with the increase of incubation time. However, at 37 °C, even though there is an increase of protein expression (from 3 to 4 hours incubation time), this increase was immediately followed by a decrease at 5 hours of incubation time proving that increase of incubation time was more essential at 28 and 30 °C than that of 37 °C.

Having important and essential data for optimal temperature, IPTG concentrations, and incubation time for the sake of expression protein, further evaluation was performed on the stability of the culture itself. Since stability of expression of recombinant protein is essential, we feel that this evaluation was important to perform. In conjunction with Figure 5, the stability test was carried out at 37 °C, 1 mM IPTG and 4 hours incubation time for ten generations. With the exception of slight decrease in generation 9 and 10, data of overall expression study (generation 1 to 10) shows that the expression of rhFN α 2a fusion protein with the molecular weight at 36 kDa in all lanes remains stable until the tenth generation and successfully maintained in the recombinant plasmid in *E. coli* BL21 (DE3). The stability of plasmid is influenced by the plasmid properties (Thiry & Cingolani, 2002), medium composition, host inheritable background (Summers, 1991; Zhao *et al.*, 2001) and also by the process parameters such as culture conditions (Craynest *et al.*, 1996), culture temperature, and expression protein toxicity (Corchero & Villaverde, 1998). Since the protein nicely expressed until ten generation and the band clearly visible, we assume that all the culture conditions were acceptable for the cell to grow and express the protein of interest. In addition of toxic protein, (Hägg *et al.*, 2004) reported that the instability of protein expression may be caused by instability of plasmids in bacterial cell growth cultures which is mainly due to uneven distribution of plasmids to daughter cells during cell division. Plasmid instability often overcame with the use of antibiotic pressure (Peteranderl *et al.*, 1990; Hägg *et al.*, 2004; Jana & Deb, 2005).

Conclusion

The optimum condition of rhIFN α 2a protein expression has been performed and established. The best result was obtained at 37 °C incubation temperature with harvesting time 4 hours incubation time and 1 mM IPTG concentration. The time of incubation has significant effects when the incubation temperatures were at 28 and 30 °C than that of 37 °C. With the exception of slight decrease in generation 9 and 10, analysis of protein expression stability revealed that, up to 10 generation, the level of expression relatively stable.

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