

Full Length Research Paper

Screening for the optimal induction parameters for periplasmic producing interferon- α 2b in *Escherichia coli*

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Accepted 5 August, 2010

Screening for optimum induction parameters to improve the production of periplasmic interferon- α 2b (PrIFN- α 2b) by recombinant *Escherichia coli* was conducted using shake flask culture. Recombinant *E. coli* Rosetta-gami 2(DE3) harboring the plasmid pET26b containing IFN- α 2b gene under the control of the T7lac promoter was used, where the induction was accomplished by isopropyl β -D-1-thiogalactopyranoside (IPTG). The induction parameters (inducer concentration, point of induction, induction temperature and the length of induction) were analyzed to find the suitable range to be used for further optimization process. From the analysis, narrow range of induction temperature from 16 to 30°C and IPTG lower than 2 mM were found suitable for induction of PrIFN- α 2b. On the other hand, early log phase was the preferred time to initiate the induction and the length of induction was dependent on the combination of other induction parameters used.

Key words: Interferon- α 2b (IFN- α 2b), induction parameter, *Escherichia coli*, periplasm, shake flask culture.

INTRODUCTION

Human interferon- α 2b (IFN- α 2b) is a member of the cytokine family with the ability to induce antiproliferative, antiviral and immunomodulating activities. It has been approved for the treatments of gastrointestinal tract diseases, various cancers including hairy cell leukemia, Acquired Immunodeficiency Syndrome (AIDS)-related Kaposi's sarcoma, chronic myelogenous leukemia, chronic hepatitis B and cirrhosis (Pestka et al., 2004; Billiau, 2006; Chelbi-Alix and Wietzerbin, 2007). For a massive therapeutic use of IFN- α 2b, large quantities of this cytokine are currently produced in recombinant strains of *Escherichia coli* and other genetically modified hosts

(Pimienta et al., 2002; Srivastava et al., 2005; Ghosalkar et al., 2008).

The *E. coli* recombinant protein expression system has been, and is still the system of choice for the production of IFN- α 2b. Indeed, IFN- α gene do not have introns (Baron and Narula, 1990). Targeting the recombinant protein into periplasmic space of *E. coli* offer several advantages. For example, N-terminal amino acid residue of the secreted product can be identical to that of the naturally secreted gene product, and protease activity is considered to be much lower in the periplasmic space than in the cytoplasm. Thus, production in periplasmic space may avoid problem related to protein degradation. Since the periplasm contains far fewer native host proteins, purification of target protein could be simplified (Schumann and Ferreira, 2004; Choi et al., 2006; Terpe, 2006).

Generally, foreign protein expression causes a metabolic burden on the cell which may reduce the growth

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Abbreviations: PrIFN- α 2b, Periplasmic interferon- α 2b; IPTG, isopropyl β -D-1-thiogalactopyranoside.

rate, cell yield, product expression and plasmid stability. In addition, environmental factor such as temperature also significantly affect the cell metabolism (Bronikowski et al., 2001; Han and Lee, 2006). Furthermore, environmental factors also have a pronounced effect on protein folding and stability during protein expression, and frequently studied to improve solubility of the proteins (Donovan et al., 1996; Weickert et al., 1996). The level of isopropyl β -D-1-thiogalactopyranoside (IPTG) used to induce protein expression can be varied to adjust the extent of metabolic burden imposed on the cell (Donovan et al., 1996; Hansen et al., 1998). Since IPTG is not metabolized, it permits the separation of the physiological function of lactose as a carbon source from its function in the regulation of lac gene expression. These properties make IPTG ideal in industrial applications for the large scale production of expressed proteins. However, strong induction may cause the formation of inclusion bodies, which are inactive (Kopetzki et al., 1989).

Strong induction may also increase the death rate of host cells (Donovan et al., 1996). The maximum yield of foreign protein from the fermentation process is depended on the point in the growth cycle at which expression is induced and also the length of induction (Donovan et al., 1996). Some recombinant strains are highly influenced by the induction; where growth and viability is drastically reduced following the induction, especially periplasmic expression (Balagurunathan and Jayaraman, 2008). Longer induction time may also cause exhaustion to nutrients available in the culture (Chen and Morgan, 2006). For this reason, it is important to balance the induction capacity as well as protein production based on the environmental condition employed during the expression of target protein.

Therefore, the objective of this study is to analyze the effect of different environmental factors during induction such as temperature, IPTG concentration, point of induction and length of induction on growth of recombinant *E. coli* and the expression of periplasmic interferon- α 2b (PrIFN- α 2b). The information gathered may be used for optimization of the fermentation process, which aims at improving the production of recombinant protein in the periplasmic space.

MATERIALS AND METHODS

Microorganism

E. coli strain Rosetta-gami 2(DE3) (Cat. No. 71351, Novagen, USA) harboring pET-26b-IFN was used for the production of recombinant PrIFN- α 2b. The details of the preparation of this strain are described in Ramanan et al. (2010a). The stock cultures were kept at -20 and -80 °C in 10% (v/v) glycerol, for immediate and long-term use.

Medium and fermentation

The culture medium consisted of 47.6 g/l terrific broth (TB) (Cat. No.

71754-3, Merck, USA), 4 g/l glycerol (Cat. No. 356352, Merck, USA), 30 mg/l kanamycin (Cat. No. K4378, Sigma-Aldrich, USA) and 34 mg/l chloramphenicol (Cat. No. 220551, Merck, USA) which were used in this study.

The inoculum was prepared by inoculating 1% (v/v) of stock culture into 50 ml Falcon tube containing 10 ml medium. The tube was incubated at 37 °C in incubator shaker, agitated at 250 rpm for 24 h and this culture was used as standard inoculum for all fermentations. The flasks were seeded with 2% (v/v) inoculum and then incubated at 37 °C on a rotary shaker (Certomat, B. Braun, Germany) agitated at 250 rpm. All the fermentations were conducted in 250 ml Erlenmeyer flask containing 50 ml medium. During the fermentation, different concentrations of IPTG were added to the culture at different fermentation times according to the need of each experiment.

Experimental design

The screening for suitable induction strategy was carried out by analyzing the suitable range of individual effect on the production of recombinant PrIFN- α 2b in periplasmic *E. coli*. The strength of induction was examined using different IPTG concentrations, followed by investigating the suitable induction point and induction temperature. The combinations of lowest and highest factor of each variable were used to analyze the suitable length of induction time for the maximum production of PrIFN- α 2b.

In order to investigate the effect of IPTG concentration, the cultures were induced after 2 h of fermentation with different concentrations of IPTG (ranging from 0.1 to 3 mM) for 20 h of induction time. The effect of induction point was studied by inducing the fermentation cultures at two points, which represents early and late-log phase that corresponds to cell density of ~0.6 and 9 measured at A_{600} , respectively. IPTG with a concentration 0.1 mM was added to the cultures and the fermentation was continued until 12 h of induction time at 37 °C.

To investigate the effect of different induction temperatures on periplasmic production, the recombinant *E. coli* was first cultured at 37 °C. After 2 h, the cultures were induced with 0.1 mM IPTG and then incubated at different induction temperatures (16, 30 and 37 °C) up to 22 h of cultivation. To screen the suitable length of induction, 8 sets of interactions of all induction strategies (IPTG, point of induction and induction temperature) were performed in several shake flask cultures. All fermentations were carried out in triplicates. During the fermentation, samples were withdrawn at time intervals and stored at -20 °C prior to analysis.

Analytical procedure

The harvested culture samples were centrifuged at 10,000 \times g (Micro 22R, Hettich Zentrifugen, Germany) for 20 min to obtain the cell pellets for protein extraction. The protein fraction from periplasmic space was extracted using osmotic shock method as described by Ramanan et al. (2009). Briefly, the cell pellets were resuspended in ice cold water and incubated for 5 - 15 min and then centrifuged for 20 min at 10,000 \times g, at 4 °C. The cell pellets were then resuspended in 1 ml osmotic shock solution, containing 20% (w/v) sucrose, 33 mM Tris-HCl (pH 8.0) and 0.5 mM EDTA. The cell suspension was vortexed and then centrifuged at 10,000 \times g, at 4 °C. The harvested shrunk cells were re-suspended in ice-cold water and incubated at room temperature for 5 - 15 min with shaking. The periplasmic protein was recovered after separating the cells from the solution by centrifuging at 10,000 \times g for 20 min.

The samples were analyzed for total protein content using Bradford method (Bradford, 1976) according to Bio-Rad protein assay kit manual (Bio-Rad, USA). Bovine serum albumin (BSA) was used as a standard in a linear range (0.1 to 0.5 mg/ml). The amount

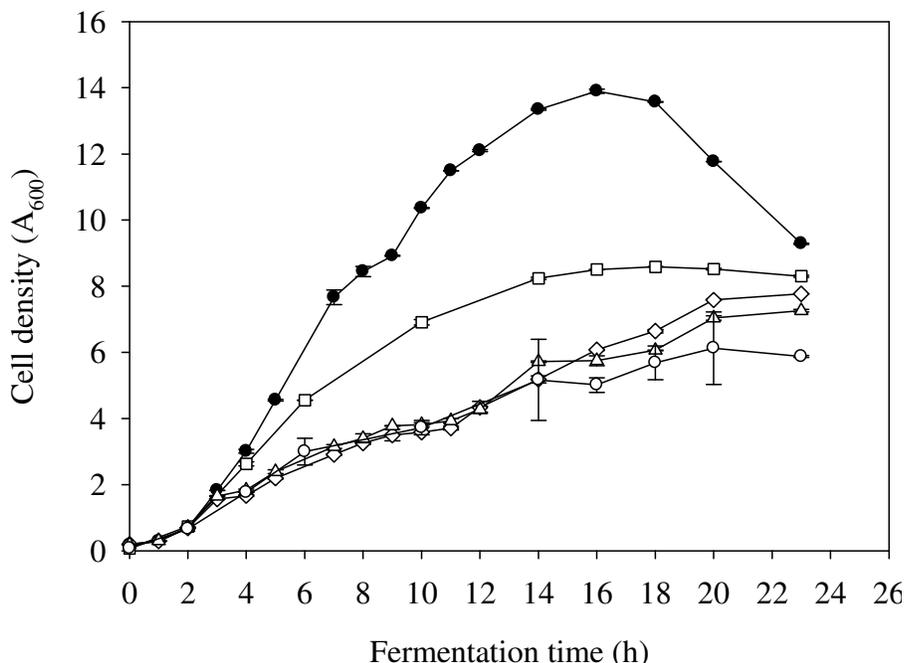


Figure 1. Growth profiles of recombinant *E. coli* when induced with different IPTG concentration at A_{600} 0.6 at 37°C. [●] not induced; [□] 0.1 mM IPTG; [◇] 1 mM IPTG; [△] 2 mM IPTG; and [○] 3 mM IPTG. The error bar represents the standard deviation.

of PrIFN- α 2b was measured using biosensor chip in Biacore3000 as described in our previous study. The biosensor chip was prepared as described elsewhere (Ramanan et al., 2010b, Ramanan et al., 2009).

RESULTS

Effect of IPTG concentration

The effect of different IPTG concentrations on growth of recombinant *E. coli* is shown in Figure 1. After the induction, growth rate of recombinant *E. coli* was reduced and severe reduction was observed when higher IPTG concentration was used. The effect of different IPTG concentrations on the production of periplasmic protein, PrIFN- α 2b and specific product yield are shown in Table 1. At 8 h of induction time, 0.1 mM IPTG yielded the highest PrIFN- α 2b production as well as the secretion of periplasmic protein. When the concentration of IPTG was increased from 0.1 to 3 mM, production of both periplasmic protein and PrIFN- α 2b was greatly reduced. On the other hand, the highest specific product yield was obtained at 4 h of induction using 1 mM IPTG. The reduction of periplasmic protein, PrIFN- α 2b and specific product yield were observed after 4 h of induction time at 1 and 3 mM of IPTG. On the other hand, similar reductions of all the responses were observed after 8 h of induction time at 0.1 mM of IPTG. From the results of this study, it can be concluded that the production of PrIFN- α 2b was inhibited at higher IPTG concentrations.

Effect of induction point

Induction point is the point at which the target protein starts to express. Several aspects such as the ability of host cells to produce protein and the availability of protein expression tools need to be considered in the initiation of protein expression. Effect of induction point, early- and late- log phase, on growth of recombinant *E. coli* and the production of PrIFN- α 2b is shown Figure 2. At early-log phase induction, the profile of growth rate, periplasmic protein, PrIFN- α 2b and specific product yield were in the increasing trend until the end of fermentation. Furthermore, the level of periplasmic protein, PrIFN- α 2b and specific product yield were higher at early-log phase induction than at late-log phase induction.

At late-log phase induction, periplasmic protein PrIFN- α 2b and specific product yield were drastically dropped towards the end of fermentation. However, the final growth obtained during late-log phase induction was higher than at the early-log phase induction.

Effect of induction temperature

The influence of induction temperature (from 16 to 37°C) on growth of recombinant *E. coli* and PrIFN- α 2b production is shown in Figure 3. Growth of recombinant *E. coli* was reduced when the cultivation temperature was reduced from 37 to 16°C (Figure 3A). Growth was greatly inhibited at low temperature (16°C). The highest PrIFN-

Table 1. Production of PrIFN- α 2b and periplasmic protein when induced with different IPTG concentrations at $A_{600} \sim 0.6$ at 37°C.

Parameter	IPTG concentration (mM)	Post induction time (h)		
		4	8	12
PrIFN- α 2b (ng/ml)	0.1	18.28 (0.40)	49.04 (1.30)	23.75 (1.17)
	1	22.58 (1.31)	20.32 (0.49)	4.87 (1.32)
	2	21.66 (1.92)	10.36 (0.76)	6.54 (0.81)
	3	3.24 (0.37)	2.66 (0.29)	3.43 (0.16)
Periplasmic Protein (mg/ml)	0.1	0.07 (0.01)	0.30 (0.05)	0.168 (0.03)
	1	0.06 (0.02)	0.09 (0.02)	0.120 (0.03)
	2	0.07 (0.03)	0.09 (0.01)	0.101 (0.01)
	3	0.03 (0.01)	0.05 (0.01)	0.209 (0.01)
Specific product yield (μ g/g)	0.1	7.46	12.62	5.05
	1	32.93	8.68	1.20
	2	19.21	5.19	2.07
	3	2.16	1.37	1.19

The samples were taken at different time intervals (4, 8 and 12 h after induction) from each culture induced with different IPTG concentrations. The value in bracket is the standard deviation. Specific product yield were calculated with the average values.

α 2b was produced at 30°C, while production of PrIFN- α 2b was very low at 16 and 37°C (Figure 3B). Although the production of PrIFN- α 2b and protein was maintained at high level for cultivation at 30°C, towards the end of fermentation (at 12 h after induction), the yield at 16°C was increased rapidly and yielded higher specific productivity than at 30°C. This is because at lower temperature, the expressed protein tends to be in soluble form and also tend to transfer across the membrane at a higher level. To improve the specific product yield for fermentation with induction temperature at 16°C, the induction time was extended.

Length of induction time

Eight sets of fermentation condition involving the interaction of each of the induction strategy was analyzed to obtain the suitable length of induction time for maximum production of PrIFN- α 2b. The growth profiles of each culture during the fermentation along with the total periplasmic protein and PrIFN- α 2b production for early- and late-log phase induction are shown in Figures 4 and 5, respectively. Different combinations of induction condition gave different results. For induction at early-log phase, production of PrIFN- α 2b was increased until the end of cultivation irrespective of the difference in temperature and induction strength. However, for induction at late-log phase, the production of PrIFN- α 2b was decreased at certain point after induction and it depended on the temperature. For instance, the drop in the production of PrIFN- α 2b was noticed after 8 h of induction at low temperature (16°C) (Figures 5a and b) and after 4 h at high temperature (37°C) (Figures 5b and d). The induction

strength was attributed to the level of expression in all conditions. As described above, the total periplasmic protein profile followed similar trend of growth profile in all experiments indicating that the secretion is associated with cell growth.

DISCUSSION

In order to screen the suitable induction parameters for enhancement of recombinant protein production, several aspects need to be considered. One of the most important factors is induction strength. The induction strength was not necessarily high as it could cause metabolic burden to the growing cells, and hence would reduced the yield. The normal range of induction strength used for protein expression ranged from 0.005 to 5 mM IPTG. However, 1 mM IPTG was widely used (Donovan et al., 1996). For example, to induce IFN- α 2b production as inclusion bodies in *E. coli* 1 mM IPTG has been used (Srivastava et al., 2005; Valente et al., 2006). On the other hand, 2 mM of IPTG was used to induce IFN- α 2b production in peri-plasmic *E. coli* (Barbero et al., 1986). In our case, the use of IPTG at concentration higher than 1 mM greatly influenced the specific product yield and the total amount of product. Although 1 mM IPTG gave the highest specific product yield, the cell growth was inhibited which in turn, reduced the final amount of the targeted protein.

Another induction factor that influenced the production of recombinant protein in periplasmic *E. coli* was the point at which the induction was made. Commonly, the optimum point for induction was at mid-log phase (Peng et al., 2004; Vásquez-Bahena et al., 2006). In some instance,

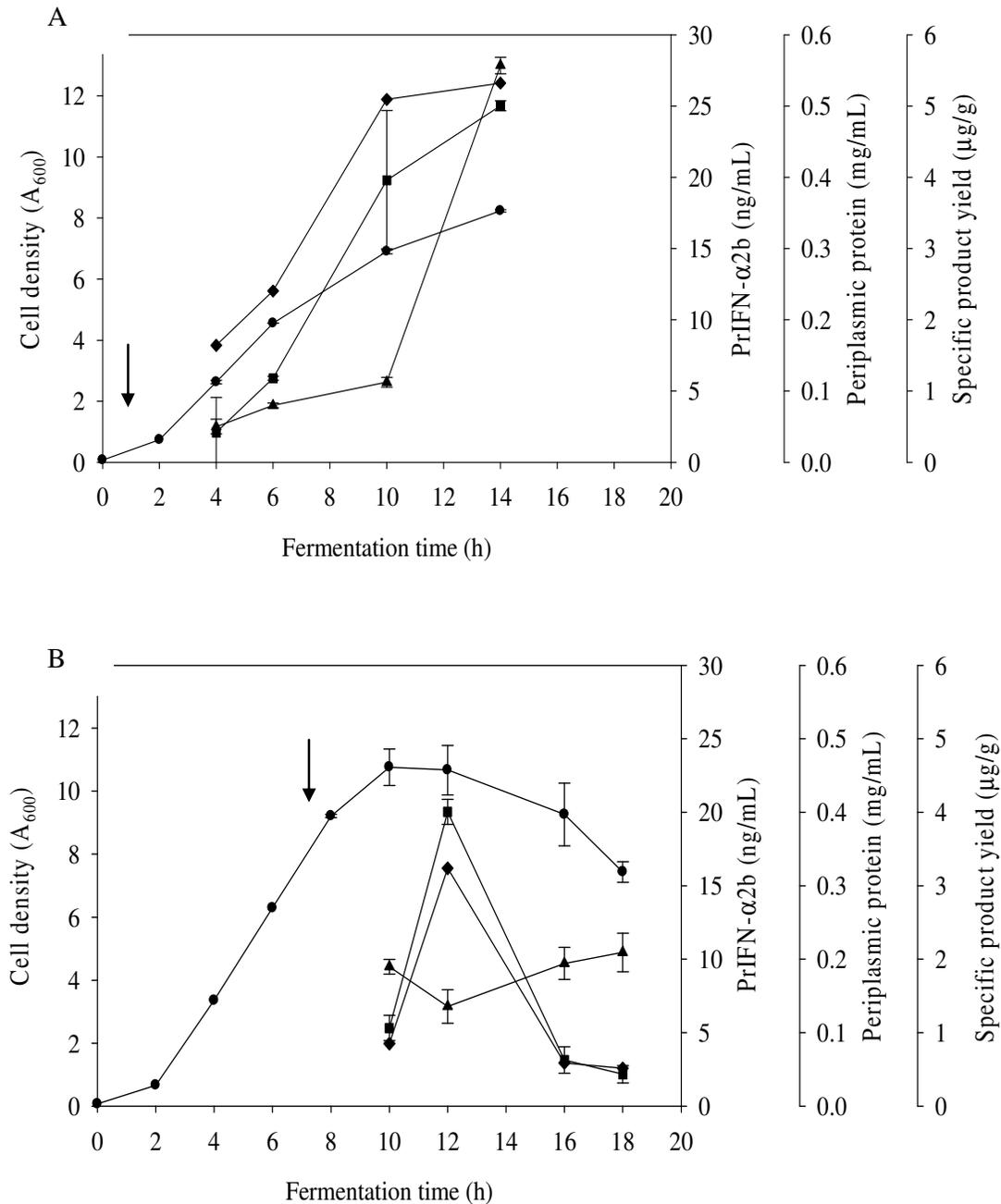


Figure 2. Production of PrIFN- α 2b and periplasmic protein when induced at different induction point. A. Early log phase ($A_{600} \sim 0.6$); B. late log phase ($A_{600} \sim 9$) with 0.1 mM IPTG at 37°C; [●] cell density; [■] PrIFN- α 2b; [▲] periplasmic protein; [◆] specific product yield. The error bar represents the standard deviation and the arrow represents induction starting point.

induction was also performed at a stationary phase (Yildir et al., 1998). Indeed, the induction point would depend on the response of strains during the induction. Strains whose growth and/or viability are drastically reduced after induction, late-log or stationary phase induction would provide high cell densities, which in turn, increased the production of the required protein. In this case, the addition of IPTG at early-log phase might utilized the

cellular machinery for the foreign protein expression which reduced the production of protein required for cell proliferation (Donovan et al., 1996).

Sandén et al. (2003) analyzed the limiting factors in *E. coli* when induced at different induction points with respect to specific growth rate. They found that, the ribosome (represented by rRNA) was degraded upon induction at high specific growth rate due to high production level. At

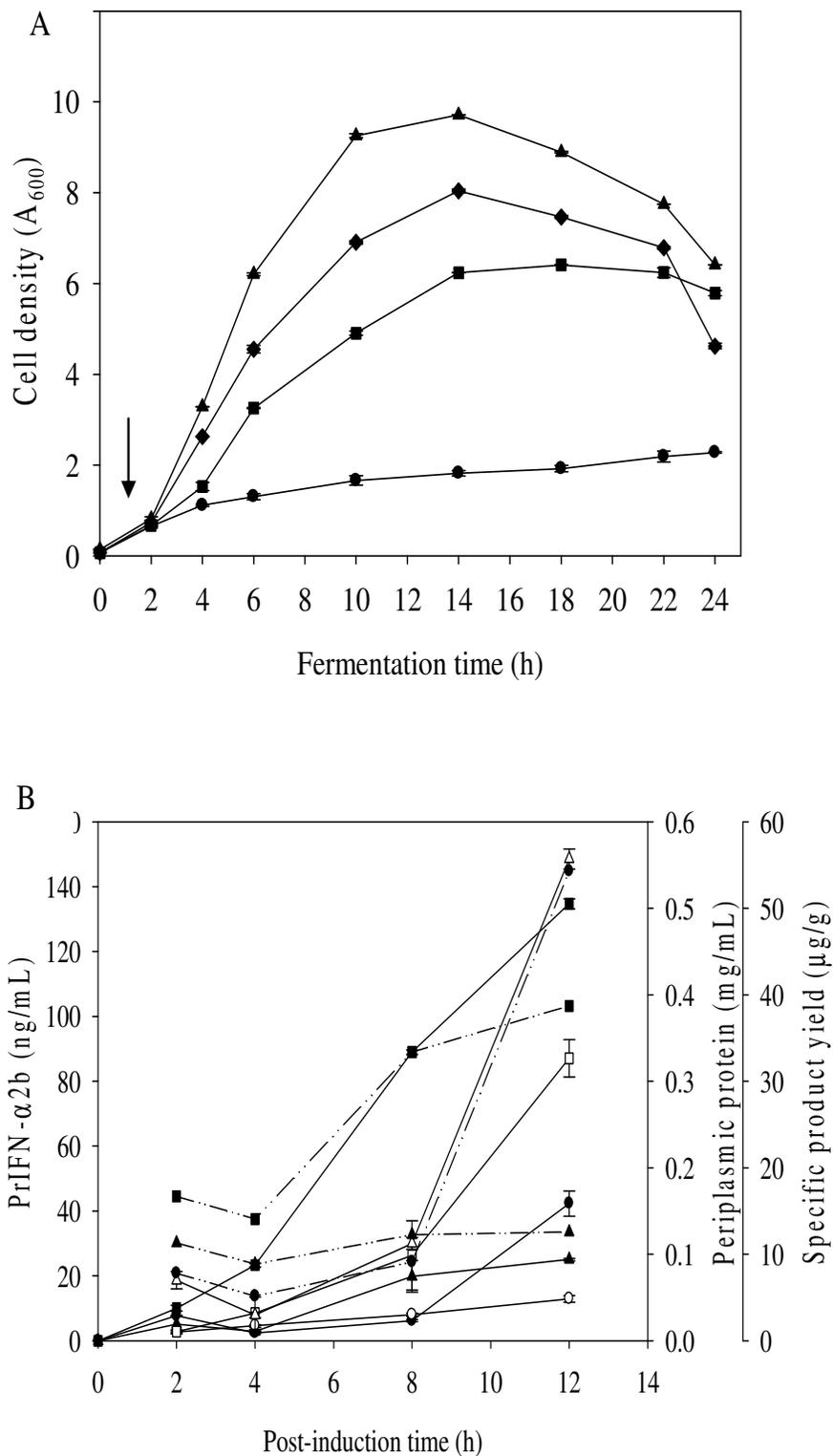


Figure 3. Effect of temperature on A. Growth of recombinant *E. coli* RG 2 (DE3); B. PrIFN- α 2b production for culture, initially cultivated at 37°C, induced with 0.1 mM IPTG after 2 h of cultivation. Symbols for A: [●] induced at 16°C; [■] induced at 30°C; [◆] induced at 37°C; [▲] normal growth without induction; B.: [●] PrIFN- α 2b at 16°C; [■] PrIFN- α 2b at 30°C; [▲] PrIFN- α 2b at 37°C; [○] protein at 16°C; [□] protein at 30°C; [△] protein at 37°C. The line of dash-dot-dot represent specific product yield, with symbols: [●] PrIFN- α 2b at 16°C; [■] PrIFN- α 2b at 30°C; [▲] PrIFN- α 2b at 37°C. The error bar represents the standard deviation and the arrow indicates the induction starting point.

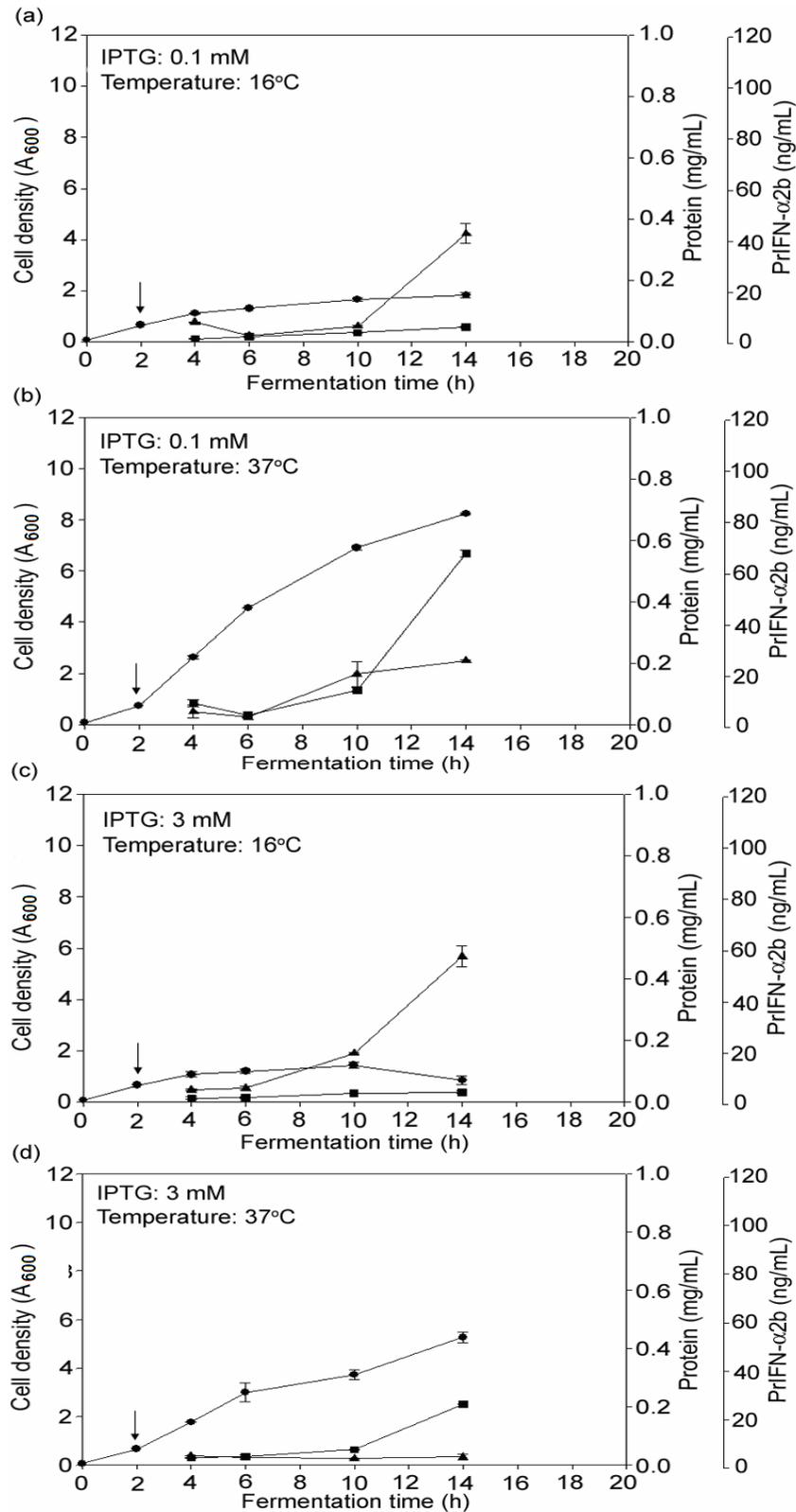


Figure 4. Growth profile of *E. coli* and production of PrIFN- α 2b and periplasmic protein during fermentation when induced at early log phase with different IPTG concentrations and induction temperatures. [●] Cell density; [■] protein; [▲] PrIFN- α 2b. The error bar represents the standard deviation and the arrow represents induction starting point.

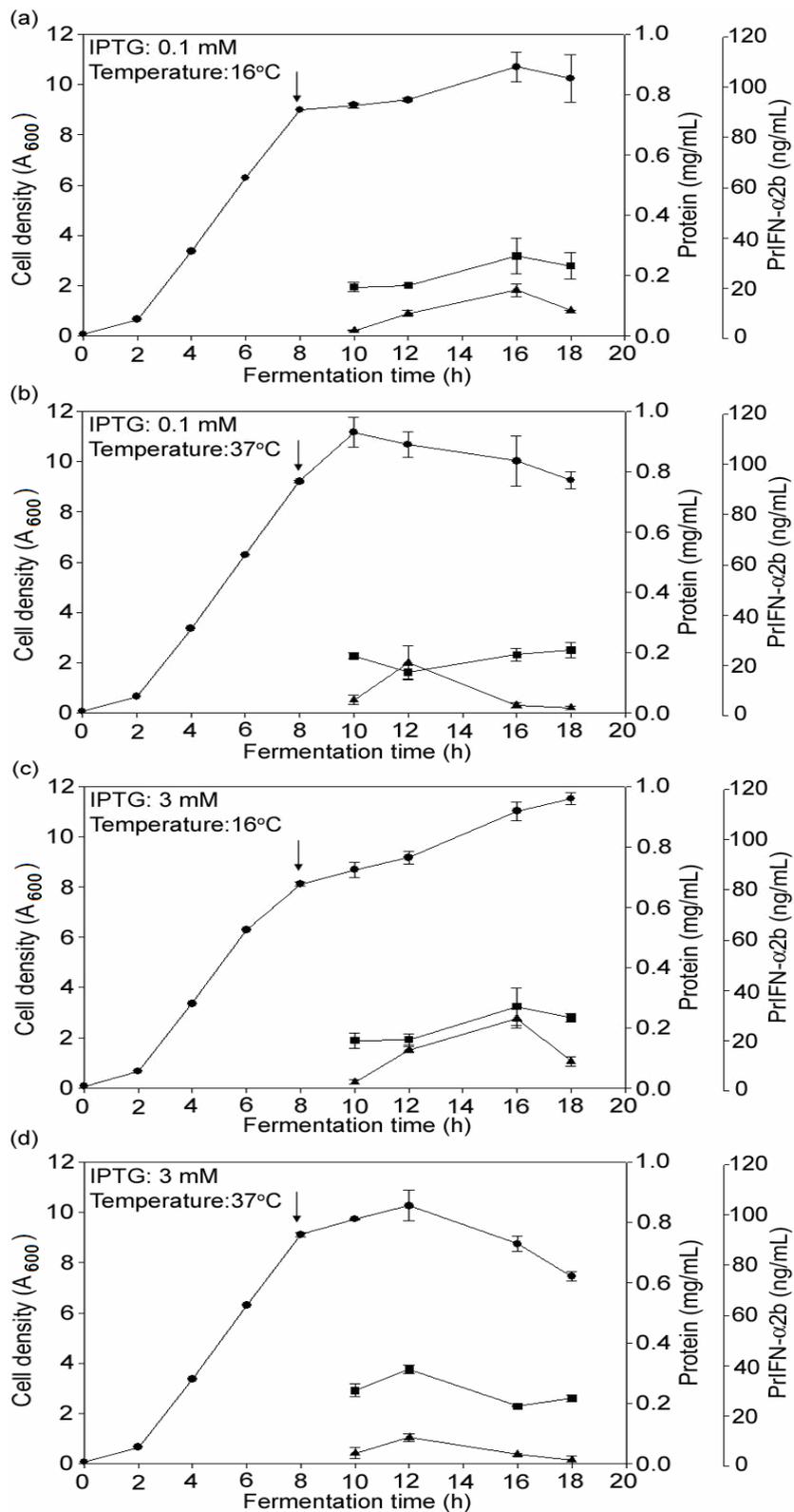


Figure 5. Growth profile of *E. coli* and production of PrIFN- α 2b and periplasmic protein during fermentation when induced at late log phase with different IPTG concentrations and induction temperatures. [●] Cell density; [■] protein; [▲] PrIFN- α 2b. The error bar represents the standard deviation and the arrow represents induction starting point.

accumulation of acetic acid. In addition, induction at a lower specific growth rate would make carbon (energy) to be a limiting factor. Similar findings were also obtained in this study. Additionally, high energy is required for the translocation process of proteins into periplasm. Therefore, induction range between early-log phase and middle-log are preferred for improvement of PrIFN- α 2b production.

The temperature during induction also plays a significant role in determining the maximum product yield in periplasmic *E. coli*. It is also a major factor that influences the activity of enzymes inside the cells and the solubility of protein produced. In fact, maximal activity of the *lac* and *tac* promoters occurs at temperatures between 37 and 39 °C (Donovan et al., 1996; Bronikowski et al., 2001). It is not surprising that at this cultivation temperature, *E. coli* produced higher recombinant protein production as well as protein itself. However, the effect is different for periplasmic expression (Mergulhao et al., 2004; Mergulhao et al., 2005; Mergulhao and Monteiro, 2007). When more proteins were produced, the transport machinery of *E. coli* seems to be limited. Hence, the rate of transfer of premature protein was lowered (Mergulhao et al., 2004; Mergulhao and Monteiro, 2007; Balagurunathan and Jayaraman, 2008). In this case, high expression rate which was driven by high induction strength was not favorable for periplasmic expression. Hence, lower temperature that facilitates soluble protein production was favorable to enhance the rate of protein transfer across the membrane (Gasser et al., 2008).

At lower temperature (e.g. 16 °C) the metabolism of cells is reduced, followed by inhibition of enzyme activity responsible for protein expression. In addition, the biological function of the membrane would be lost as the temperature decreases. Membranes are essentially colloidal solutions of phospholipids and protein in a fluid phase and it is only in this fluid phase that they are biologically functional. When the temperature decreases, membranes become increasingly viscous with decreasing membrane fluidity. Below certain temperature, these membranes change to a gel (solid) phase when biological function is lost (Nedwell, 1999). As a consequence, the synthesis rates of enzymes belonging to the biosynthetic machinery (e.g., RNA polymerase enzyme) and translational factors decreased more strongly in the culture synthesizing the recombinant protein (Hoffmann et al., 2004).

High level expression using strong promoter like *tac* and T7 systems require a short induction period (Sandén et al., 2003). Longer induction period are applied when the level of expression is low and the availability of nutrients is high. In some cases, the production might be reduced to zero and at worst, the protein product is degraded. Furthermore, reduction of growth could also be seen when the nutrients became exhausted (Chen and Morgan, 2006). Increase in the time for induction process usually has relative effect on the protein leakage from periplasmic space into the culture medium (Bäcklund et al., 2008) and this leakage is partly associated with auto-

cell lysis (Somerville et al., 1994). In our case, the length of induction was based on the fermentation condition employed. Longer induction time showed higher PrIFN- α 2b production when low level of induction strength was introduced. On the other hand, lower PrIFN- α 2b production was observed when high expression strength was introduced. Besides, the time of induction (induction point) also played a significant role in determining the duration of induction time. Hence the length of induction could be determined once the other conditions were chosen for higher expression.

Conclusion

Results from this study have demonstrated that the induction strategy greatly influenced the *E. coli* fermentation for enhancement of PrIFN- α 2b production. Lower strength of induction, narrow range near the middle level of induction point and temperature was preferred to maximize the PrIFN- α 2b production by the recombinant *E. coli*. Length of induction could be determined based on the future optimized condition. Besides, further optimization of these factors using response surface methodology to enhance the PrIFN- α 2b production in recombinant *E. coli* RG2 (DE3) is being carried out in our laboratory.

ACKNOWLEDGEMENT

We gratefully acknowledge the financial grant received from the Ministry of Science, Technology and Innovation (MOSTI), Malaysia (Vote No. 54953-01).

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