Production of recombinant human interferon gamma by batch fermentation in *E. coli* system

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ABSTRACT

To improve the expression of recombinant human interferon gamma (rhIFN-γ) protein in recombinant E. coli Rosetta DE3 bacterial cells, the effect of different factors for the over-production of rhIFN-γ, including type of culture media, pH and type of inducer, has been evaluated in experimental shaking flasks. A simple batch fermentation process was carried out and the growth rate of recombinant E. coli Rosetta DE3 has been monitored using optical density measurements and wet cell weight. The LB medium was used and the dissolved oxygen level was maintained at 30-40% of air saturation, by control of the inlet air and agitation rate. The pH of media was sustained at pH 7. The inducer was lactose at 2mM final concentration, added after 3 h fermentation, and the stationary phase of growth started at 9 h of batch process. The final cell weight of the batch fermentation was 7g/l media after 12 h of starting process. The immunogenicity of rhIFN-γ protein has been tested against the rabbit polyclonal human interferon antibodies by western blot analysis. Based on these small-scale experiments, optimum operating conditions were chosen to scale up a better pilot process production of rhIFN-γ protein.

***Key words**: Recombinant human interferon gamma – Batch fermentation- Lactose inducer - E. coli Rosetta DE3.*

INTRODUCTION

In recent years, considerable effort and attention have been directed toward the use of prokaryotic systems, particularly *Escherichia coli* (*E. coli*) for the expression and production of human genes for both basic research and commercial applications**.** Human IFN-γ is a glycosylated protein with a total molecular weight of 25 kDa and is composed of 143 amino acid residues. The recombinant hIFN-γ expressed in *E. coli* is not glycosylated and has a molecular weight of 17 kDa, but it is still physiologically active (Banerjee *et al.,* 2009 and Hernandez *et al.,* 2008). *Escherichia coli*, requires specific formulations of growth media for use in cloning, plasmid DNA preparation, and protein expression (Sezonov *et al.,* 2007). In order to optimize the production of recombinant proteins four strategies have been used, namely choice of culture media, effect of pH, type and concentration of inducer (Babaeipour *et al.,* 2009).

The production of therapeutic proteins in industry is often dependent on microbial fermentation. The batch fermentation is a simple process in which the cells increase in the size and not in their number. It is a closed system which contains limited amount of nutrient medium (Stanbury *et al.,* 1995). Therefore, the present work focused on

production of rhIFN-γ more enhanced by tuning recombinant protein expression in batch culture of *E. coli* using lactose as an inducer.

MATERIALS AND METHODS

Microorganism and vector system

The gene coding for hIFN-γ was isolated from *in vitro* stimulated human peripheral blood mononuclear cells (Boyum, 1968). The isolated hIFN-γ cDNA was cloned into the expression vector pET-15b (Novagen, Wiscosin, USA) and transformed into the host competent bacterial cells, Rosetta (DE3) (Novagen, Wiscosin, USA) for expression of rhIFN-γ protein (Okasha *et al.,* 2011).

Factors affecting optimization in shaking flask

Culture media

LB agar medium containing 100µg/l ampicillin and 35 µg/l chloramphenicol was used routinely to grow *E. coli* Rosetta DE3 pET-15b/hIFN-γ. Two types of media were tested for optimization of rhIFN-γ expression in shaker flasks. Lauria Broth (LB) medium is consisting from 10g/l tryptone, 10g/l NaCl, 5gm/l yeast extract and Terrific Broth (TB) media consisting 10.2g/l tryptone, 20.4g/l yeast extract, 0.4% glycerol, 0.72M K_2HPO_4, 0.17M KH_2PO_4

Overnight cultures were inoculated with a single colony of the recombinant strain harboring pET-15b/hIFN-γ into 10 ml of LB or TB medium in a shake flask at 250 rpm and incubated overnight at 37°C. The overnight culture (10 ml) was used to inoculate 100 ml of each of the two media (LB and TB). The cultures were incubated at 37°C with shaking at 250 rpm until the OD_{600} reached 0.6. Isopropylthio-D-galactoside (IPTG) was added as inducer of recombinant protein expression at final concentrations 2mM and the incubation continued for 3 h. The cells were harvested by centrifugation at 5000rpm for 5 min, 4°C and suspended in lysis buffer (50mM tris HCl pH 7.5, 5mM EDTA pH8, 100mM NaCl, 1mM PMSF). The bacterial pellets were separated and subjected to 15% SDS-PAGE for protein analysis according to Sambrook *et al.* (1989).

pH of the media

The optimal pH for growth of *E. coli* is near neutral. *E. coli* cells can grow reasonably well over a range of three pH units from pH 5.5 to 8.5 (Russell and Diez-Gonzalez, 1997). The growth curve of the recombinant *E. coli* clone was followed by measuring the OD_{600} of the LB medium at an hour intervals for 3 h culture at 37°C and 250 rpm.

Shaking rate

Growth of *E. coli* in shake flasks is dependent on supplies of oxygen by shaking at relatively high speeds. The optimum flask shaking range is between 200 and 350rpm (McDaneil and Bailey, 1968). In our study three different shaking speeds have been tested 150rpm, 250rpm and 350rpm and growth of recombinant *E. coli* has been monitored by measuring the OD_{600} of the LB medium at 3h intervals for 9 h cultures at 37°C.

Inducers

It was possible to assess the effects and interactions of the induction variables on the expression of rhIFN-γ using different inducers: IPTG and lactose, with different concentrations, with a view to improving process productivity and reducing the production costs (Larentis *et al.,* 2014). For induction of rhIFN- γ protein in bacterial cells, two different inducers have been evaluated. After 3 h of growth of bacteria either IPTG or lactose at different concentrations (0.5-2mM) were added to the medium. At the end of the culture, the bacterial pellets were separated

and subjected to 15% SDS-PAGE for protein analysis.

Expression of rhIFN-γ in Batch fermentation system

Large-scale production of recombinant proteins in *E. coli* requires growth of cells in fermentor. Simple batch fermentations involve growth of fermenting organisms in a fixed amount of medium, without additional nutrients were applied (Coligan *et al.,* 2004). Batch culture was inoculated by adding 500 ml of an overnight incubated LB medium, supplemented with antibiotics: ampicillin and chloramphenicol (biotech-Biobasic Co., Ontario, Canada) into the bioreactor (model BioFlo 310 New Brunswick Scientific, New York, USA) containing 5000 ml of LB medium. Cultivation temperature was controlled at 37°C. The pH was controlled at pH 7 by the addition of 25% (w/v) NH_4OH or 3 M H_3PO_4. Dissolved oxygen was controlled at 30–40% of air saturation by controlling both the inlet air and agitation rate at 350rpm. Foam was controlled by adding non-silicon antifoaming reagent (Sigma Aldrich Co., Munich, Germany). The growth was monitored by measuring OD_{600} of samples taken at 3 h intervals using spectrophotometer (model 634, Varian Co., Ontario, Canada). After 3 h, 2mM lactose final concentration was used as inducer of protein expression. After 12 h of batch fermentation process; the cells were pelleted at 6000 rpm and 4ºC for 10 min. In order to analyze the rhIFN-γ protein expression by SDS-PAGE analysis, 1ml at each time interval was centrifuged in at 6000 rpm and 4ºC for 10 min. Finally, the bacterial pellet of 5000 ml medium obtained from batch fermentation was freezed at −20°C.

Evaluation of rhIFN-γ immunogenicity by immunoblotting analysis

Recombinant hIFN-γ protein samples from batch fermentation (1.5µl of each) and standard rhIFN-γ (1µg) (Invitrogen Co., New York, USA) were loaded onto 15% denaturing acrylamide gels (Sambrook *et al.,* 1989). SDS-PAGE and Western blotting were done essentially as described by Burnette (1981). Immunodetection was done by the use of rabbit poly anti human interferon gamma (Komabiotech, Biosource, Montrial, Canada) as a primary antibody, antirabbit IgG alkaline phosphatase conjugate (Sigma Aldrich Co., Munich, Germany) as a secondary antibody and NBT/BCIP ready to use solution substrate (Sigma Aldrich Co., Munich, Germany).

RESULTS

Factors affecting optimization in shaking flask

Culture media

The growth curve of the recombinant *E. coli* clone was followed by measuring the OD_{600} of the culture indicated that growth in LB medium is higher than in TB medium (Fig. 1). The OD_{600} of LB medium was 3.5 after 6 h however; the OD_{600} of TB medium was 2.7. Also, the SDS-PAGE in (Fig. 2) showed that the expected band of rhIFN-γ protein expressed in bacterial lysate of LB medium is higher than the same band appeared in bacterial lysate of TB medium. Therefore, the LB is the optimum medium for batch fermentation.

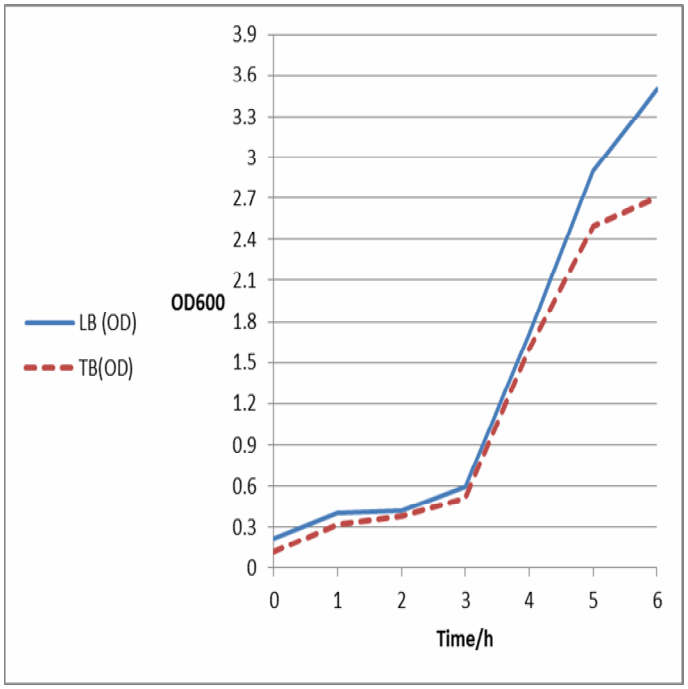


Fig. (1): Growth curve of recombinant E. coli clone in TB and LB culture media as represented by measuring of the OD_{600}

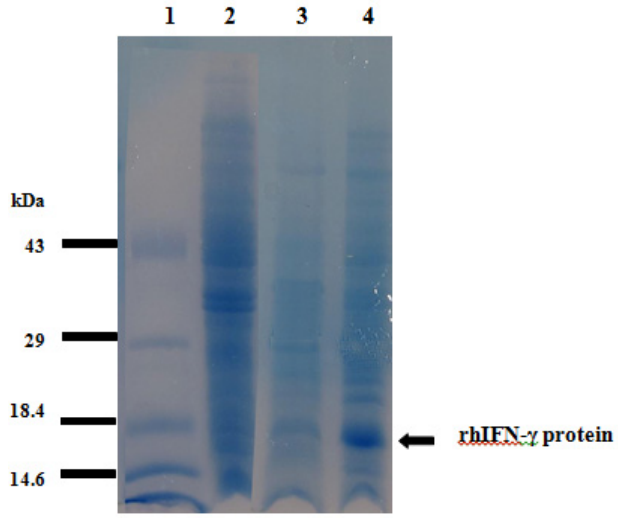


Fig. (2): SDS-PAGE of induced bacteria using 2mM lactose. Lane 1: pre-stained Mwt protein standard (GIBCO, BRL). Lane 2: bacterial pellets of non-induced LB sample. Lane3: bacterial pellets of induced recombinant IFN-γ TB media using 2mM IPTG. Lane4: bacterial pellets of induced recombinant IFN-γ LB media using 2mM lactose.

pH of the media

The growth curve of the recombinant *E. coli* clone in Fig. (3) concluded that *E. coli* bacteria gave best growth at pH 7 due to the OD_{600} reached 3.3 after 6 h of incubation, whereas it was only 2.2 and 2.5 at pH6 and 8 respectively.

Shaking rate

The OD_{600} of recombinant *E. coli* were monitored for 9 h, at 350rpm shaking speed the OD_{600} reached 3, at 250rpm the OD_{600} reached 1.5, and at 150 rpm the OD_{600} was the lowest to be 1 as shown in (Fig. 4), indicating that the 350 rpm shaking speed gave higher growth rate.

Type of Inducer

The effect of inducer type IPTG or lactose; and concentration on the growth of bacteria was evaluated by analyzing the wet cell weight (WCW) of withdrawn samples. The WCW using lactose and IPTG is shown in (Fig. 5). By increasing the concentration of the inducers (Lactose and IPTG), the WCW increased. The diagram in (Fig. 5) showed that 2mM lactose gave the highest WCW. As represented in SDS-PAGE in (Fig. 6), the rhIFN-γ was expressed successfully at definite Mwt; the expected bands of rhIFN-γ protein expressed in bacterial lysate of 1.5mM and 2mM lactose were higher than the same bands appeared in bacterial lysate of 0.5mM and 1mM lactose.

Large-scale production of rhIFN-γ by Batch fermentation

Using the optimized conditions of experimental shaking flasks in the 7.5 L fermentor by batch process increased the growth of bacteria containing the expressed protein. The growth curve was followed by measuring density at 600nm (OD_{600}) and by WCW (Fig.7). This indicated that the exponential phase of recombinant *E. coli* cells was initiated at 3 h up to 9 h and from 9 h the stationary growth phase started.The SDS-PAGE of batch fermentation samples (Fig. 8), revealed that the expression of rhIFN-γ protein has been expressed successfully and the Mwt of expressed recombinant protein has the same Mwt compared to standard rhIFN-γ and prestained standard protein.

Evaluation of rhIFN-γ immunogenicity by immunoblotting analysis (Western Blot)

Western blot analysis using polyclonal anti-human interferon gamma antibodies showed a specific protein band which has Mwt of 17 KDa, recognized both; the recombinant protein and the standard hIFN-γ (Fig.9). Induction of fermentation by lactose increased the expression of the rhIFN-γ protein over the noninduced cells.

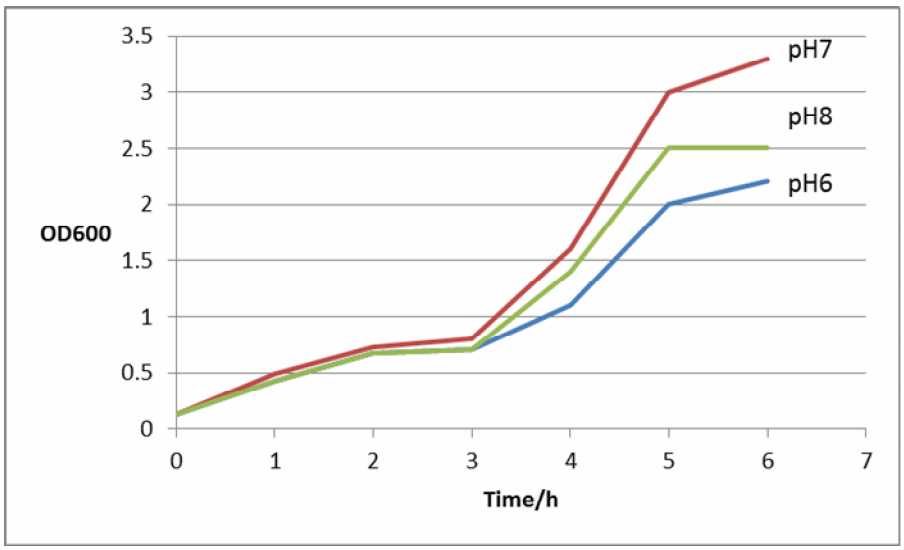


Fig. (3): Growth curve of recombinant E. coli in LB culture media at pH 6, 7 and 8.

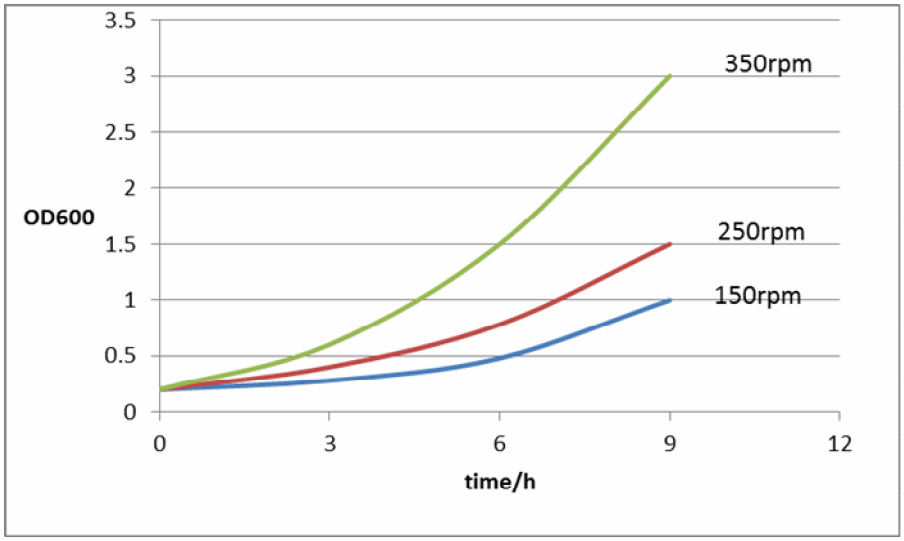


Fig. (4): Growth curve of recombinant E. coli in LB media at shaking speeds 150rpm, 250rpm, 350rpm.

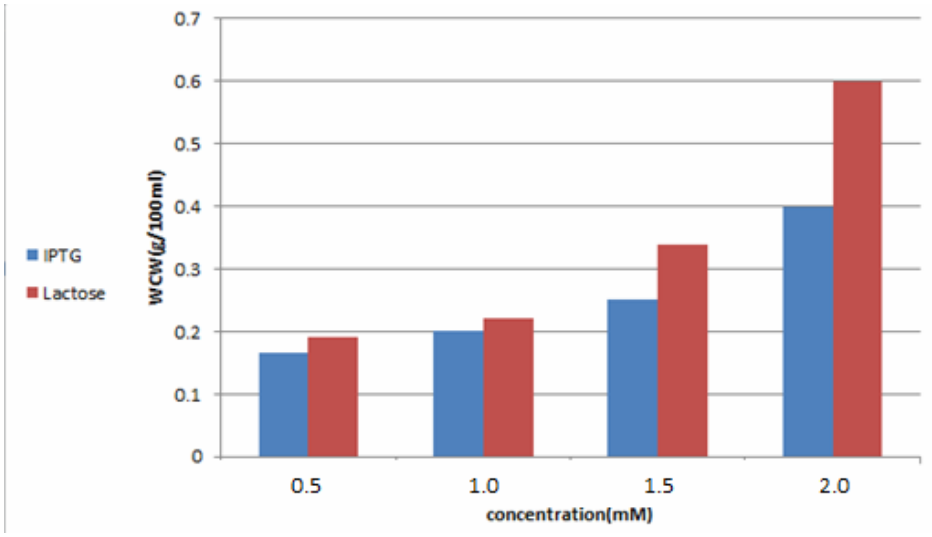


Fig. (5): WCW of bacterial cells induced with IPTG or lactose at concentrations 0.5, 1, 1.5 and 2mM.

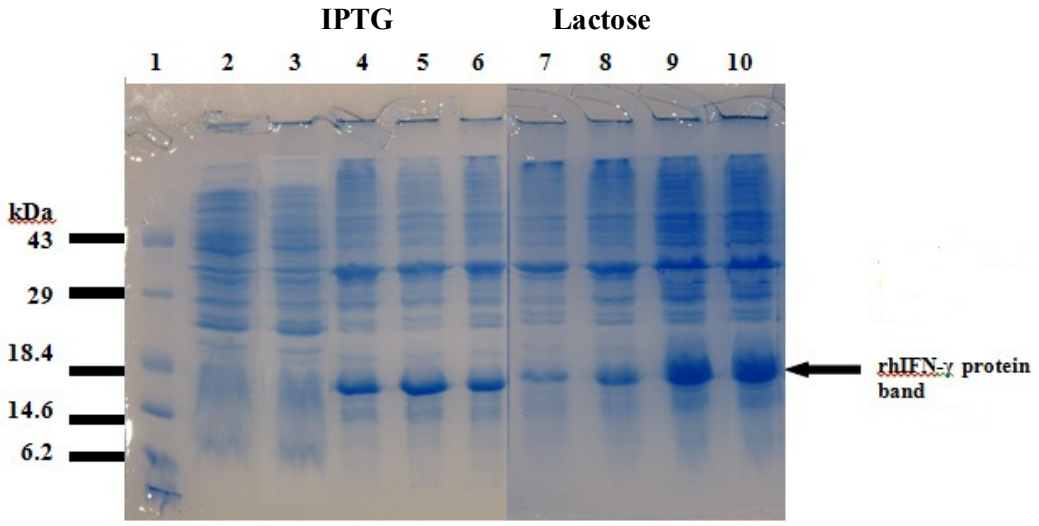
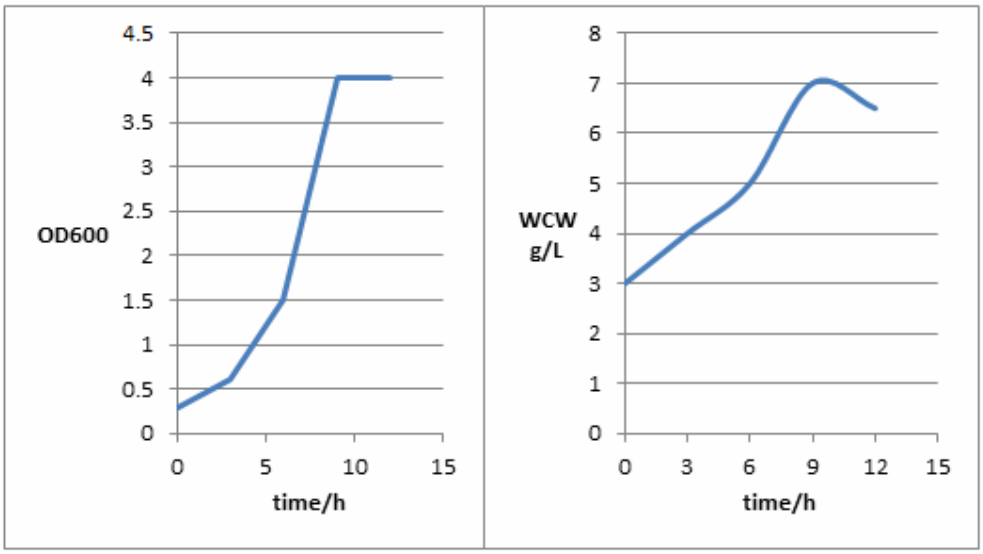


Fig. (6): SDS-PAGE of induced bacteria using IPTG or lactose. Lane 1: low Mwt standard prestained protein (GIBCO, BRL). Lane 2: non-induced sample. Lanes 3-6: represent induction with IPTG at final concentration 0.5, 1, 1.5, 2mM. Lanes 7-10: represent induction with lactose at final concentration 0.5, 1, 1.5, 2mM respectively.

DISCUSSION

The goal of this study was the establishment of protocol for highly production of rhIFN-γ protein in *E. coli* cells. In order to improve rhIFN-γ protein expression, different factors have been tested in experimental shaking flasks to optimize production on large-scale fermentation. Factors affecting expression and production of rhIFN-γ protein such as type of media, type of inducer, shaking rate and effect of pH were evaluated. The optimum condition of each factor was applied to batch fermentation for highly protein production. The rhIFN-γ protein was expressed successfully in TB and LB. To realize the optimum media for rhIFN-γ protein expression, OD_{600} value was detected. The growth curve and SDS-PAGE revealed that LB medium provided a high bacterial growth and protein expression. In addition to growth medium, pH is also important for *E. coli* growth rate and cell density. Rosetta host strains are BL21 derivatives that commonly studied for its abilities to evolve to three different pH environments pH6, 7 and 8 to detect the optimum acidity for high growth. The growth curve of recombinant *E. coli* showed that neutral pH7 is the ideal one.



(a) **(b)**

Fig. (7): The batch fermentation. (a) The growth curve of recombinant E. coli clone (OD_{600}). (b) Represents biomass concentration by detecting WCW.

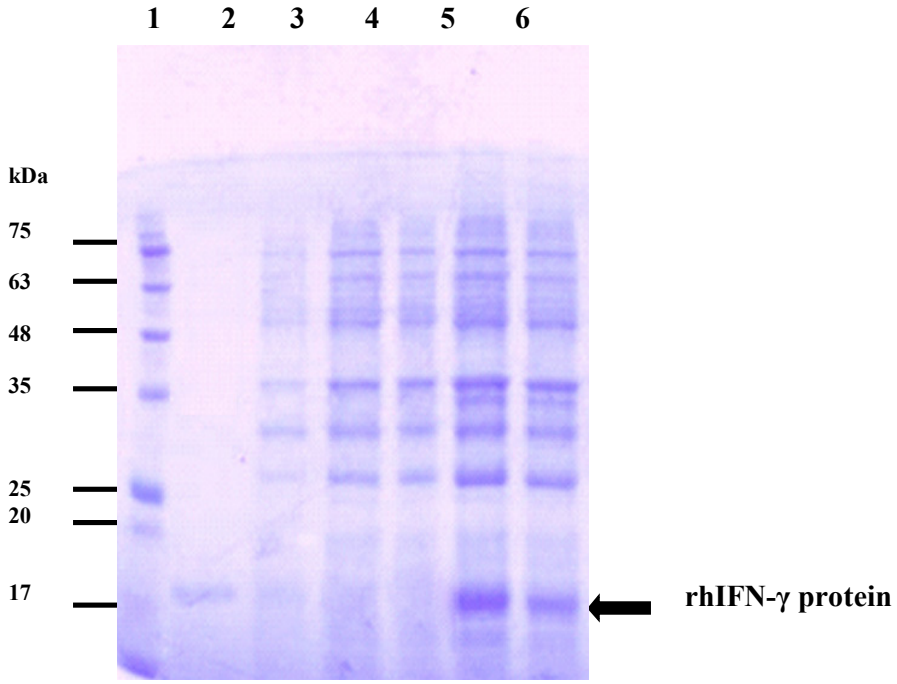


Fig. (8): SDS-PAGE of samples taken at 3 h intervals. Lane1: prestained Mwt protein marker (Genedirex). Lane2: rhIFN-γ protein standard with concentration 1mg/ml. Lanes3-7: represent the samples from fermentation culture media at 0, 3, 6, 9, and 12 h intervals.

The pET15b expression vector harboring hIFN-γ gene contains T7 promoter that only matches phage T7 RNA polymerase that transcribes mRNA to make all the necessary proteins for *E. coli* and rhIFN-γ protein under the transcriptional control of a lacUV5 promoter. Thus, the system can be induced by lactose or its non-hydrolysable analog IPTG (Rosano and Ceccarelli, 2014). Four different concentrations of each inducer have been used in this study; the results were evaluated by measurement of WCW and rhIFN-γ protein electrophoresis. It has been concluded that the optimum inducer for rhIFN-γ protein expression was lactose at 2mM concentration. The recombinant *E. coli* lag phase according to bacterial growth curve was noticed at the first 3 h of batch process. On the other hand, the exponential phase was taken about 3 h. Stationary phase, where the rate of cell growth matches the rate of cell death, was started at 9 h of batch process. The addition of lactose after 3 h into the fermentor induced efficiently expression of rhIFN-γ. As shown in Fig. (8), the maximum amount of rhIFN-γ was achieved after 3 h of post induction. However, Khalilzadeh *et al.,* (2003) recorded that maximum rhIFN-γ protein expression in fed batch process was achieved after 5 h of post induction using 3mM IPTG as inducer.

Total wet cell weight of batch fermentation for rhIFN-γ protein was reached to 7g/l after 12 h giving a total of 35g in one batch fermentation. In conclusion, we were able to optimize a protocol that enhances production of recombinant human interferon gamma expressed in *E. coli* system. This will help in production of large amount of this

therapeutic protein by scaling up this technology, and optimization of rhIFN-γ protein purification.

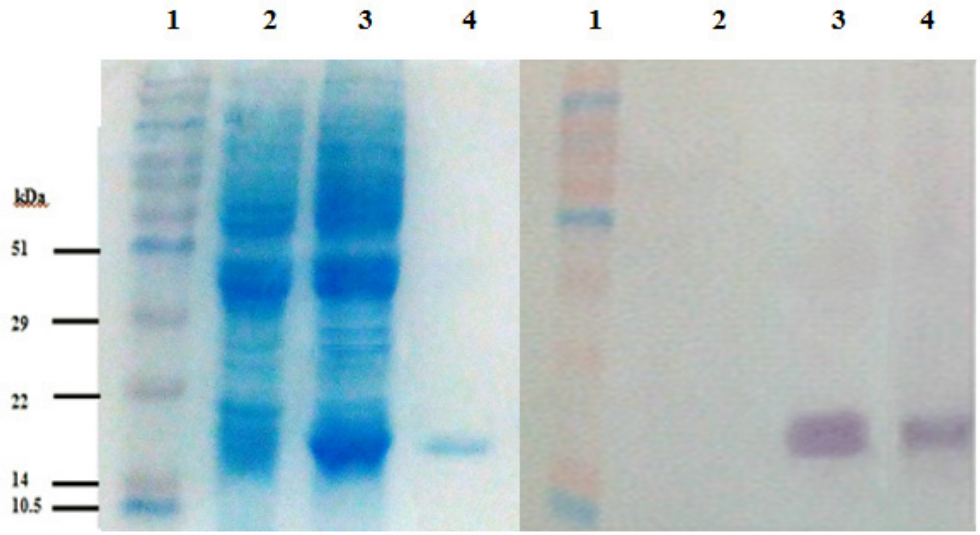


Fig. (9): Western blot analysis of bacterial lysate. Lane (1): prestained Mwt protein standard. Lane (2): Noninduced bacteria cells. Lane (3): Induced bacterial cells. Lane (4): recombinant standard hIFN-γ.

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REFERENCES

Babaeipour, V.; Shojaosadati S. A.;Khalilzadeh R.; Maghsoudi N. and Farnoud A. M. (2009). Enhancement of human γ-interferon production in recombinant *E. coli* using batch cultivation. Appl. Biochem. Biotechnol., 160: 2366-2376.

Banerjee, S.; Salunkhe, S. S.; Deshpande, A. A., Mandi, N. S.; Mandal, G.; *et al.* (2009). Over-expression of proteins using a modified pBAD24 vector in *E. coli* expression system. J. Biotech. Lett., 31: 1031-1036.

Boyum, A. (1968). Isolation of mononuclear cells and granulocytes from human blood. J. Scandin. Clin. Lab. Invest., 97: 77-89.

Burnette, W. N. (1981). Western blotting, electrophoretic transfer of proteins from sodium dodecylesulfate-polyacrylamide gels tounmodified nitrocellulose and radiographic detection with antibodies and radioiodinated protein A. J. Anal. Biochem., 112: 195-203.

Coligan, J. E.; Dunn, B. M.; Speicher, D. W.; Wingfield, P. T. and Ploegh, H. L. (2004). Current Protocols in Protein Science. John Wiley & Sons Inc., Washington, USA. Chapter 5. pp: 541-548.

Hernandez, V. B.; Maldonado, L. T.; Rivero, E. M.; Rosa, B.; Acevedo, L. O. and Rodreguiz A. L. (2008). Optimization of human interferon gamma production in

Escherichia coli by response surface methodology. Biotechnol. and Bioproc. Engin., 13: 7-13.

Khalilzadeh, S.A.; Shojaosadati, A. and Bahrami, N. M. (2003). Over-expression of recombinant human interferon-gamma in high cell density fermentation of *Escherichia coli.* Biotech. Lett., 25: 1989-1992.

Larentis, A. L.; Nicolau, J. F.; Gdos, E. S.; Vareschini, DT; Almeida, F. V.; Reis M. G.; Galler, R. and Medeiros M. A. (2014). Evaluation of pre-induction temperature, cell growth at induction and IPTG concentration on the expression of a leptospiral protein in *E. coli* using shaking flasks and micro bioreactor. BMC Research Notes, **7**: 671-671.

McDaniel, L. E. and Bailey E. G. (1968). Effect of shaking speed and type of closure on shake flask cultures. App. Microbiol., 17:286-290.

Okasha, H.; Saber, M. A.; Abdullah, N. M. and Abd El-Azeem, E. M. (2011). Application of biotechnology for production of recombinant protein (human interferon gamma). MSc. Thesis, Faculty of Science, Ain Shams University,Egypt.

Rosano, G. L. and Ceccarelli E. A. (2014). Recombinant protein expression in *Escherichia coli:* advances and challenges. Front in Micorbiol., 5: 172-172.

Russell, J. B. and Diez-Gonzalez F. (1997). The Effects of Fermentation Acids on Bacterial Growth. Advances in Microbial Physiology. 39: 205-234.

Sambrook, J.; Fritsch, E. F. and Maniatis, T. (1989). Molecular Cloning: A laboratory manual. 2 nd edition, Cold spring Harbor Laboratory Press, NY, USA. Vol. III. pp: B18.

Sezonov, G.; Joseleau-Petit D. and D'Ari R. (2007): *Escherichia coli* physiology in Luria-Bertani broth. Bacteriol. J., 189: 8746-8755.

Stanbury, P. F.; Whitaker, A. and Hall S. G. (1995). Principle of fermentation technology. 2nd edition, Elsevier Ltd, Oxford, UK. Chapter2. pp: 21-31.

الملخص العربي

انتاج انترفيرون جاما الآدمي المدمج عن طريق التخمر الدفعي لبكتيريا الإيشيريشيا كولاي

محمد علي صابر [١]، هند عكاشة أحمد [١] ، مجدي محمود محمد [٢]، محمود حسن رميح [١]
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أجريت دراسات من أجل تحسين انتاج انترفيرون جاما الآدمي (rhIFN-γ) المدمج في بكتيريا الايشيريشيا كولاي سلالة (Rosetta DE3) وقد تم تقييم ظروف الإنتاج المثلى والتي تضمنت نوع الوسط ودرجة الأس الهيدروجيني المناسبة ونوع المحفز لانتاج البروتين في قوارير. وقد وجد أن اللاكتوز هو المحفز الأمثل في التركيز النهائي (2mM) ومن ثم تطبيق ذلك المحفز في عملية التخمر الدفعي للخلايا وتم رصد معدل نمو سلالة الايشيريشيا كولاي المؤتلفة (Rosetta DE3) باستخدام قياسات الكثافة الضوئية والوزن الرطب للخلايا. تم الحفاظ على مستوى الأكسجين الذائب بنسبة ٣٠-٤٠٪ من تشبع الهواء عن طريق السيطرة على معدل مدخل الهواء والاهتزاز .استمر ثبات الأس الهيدروجيني للوسط البكتيري عند ٧. في النهاية كانت كثافة الخلايا للتخمر الدفعي تعادل ٧ جرام لكل لتر بعد ١٢ ساعة من عملية التخمر الدفعي. وقد لوحظ ثبات معدل النمو بعد ٩ ساعات من عملية التخمر الدفعي، وقد تم اختبار القدرة المناعية لبروتين انترفيرون جاما الآدمي المدمج (rhIFN-γ) ضد الأجسام المضادة الخاصة بالبروتين بإستخدام ويسترن.. اعتمادا على تلك الدراسات يمكن استخدام ظروف الانتاج المثلى لرفع مستوى انتاج انترفيرون جاما الآدمي المدمج (rhIFN-γ) بتكلفة مناسبة.