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The Expression of Human Granulocyte Macrophage Colony Stimulating Factor by Heat-Induction in *Escherichia coli*

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Abstract

A self-regulated high-copy number plasmid containing chloramphenicol resistant gene, for the production of recombinant proteins under the regulation of bacteriophage λpL promoter, was constructed. The designed 5024 base pair expression plasmid contained a heat sensitive repressor cI857 coding gene to regulate the function of λpL promoter under heat shock induction. Using the constructed vector, for the first time, periplasmic production of human granulocyte macrophage colony stimulating factor (hGM-CSF) under heat induction was demonstrated. The developed hGM-CSF expressing system in this study has potential for the secretion of nearly 100% of expressed proteins into the *Escherichia coli* periplasmic space upon temperature up-shift.

Keywords: Recombinant human granulocyte macrophage colony stimulating factor (rhGM-CSF); lambda (λ) PL; Heat shock-induction

1. Introduction

Human granulocyte macrophage colony stimulating factor (hGM-CSF) has been used for treatment of myelodysplastic syndrome, neutropenia and chemo-therapy induced myelosuppression [2,19,35]. There are also several investigations in progress to examine its potential for further clinical use [1]. Production of recombinant hGM-CSF (rhGM-CSF) in several heterologous expression systems such as mammalian [17,36], yeast [6,24] and bacterial [3,4,15] cells have been reported so far. The produced rhGM-CSF in bacteria, unlike those expressed in yeast or mammalian cells lacks carbohydrate, but exhibits higher biological activity *in vitro* than the natural one. Several reports on secretion of hGM-CSF in *E. coli* have been published so far [4,7,13,15,20,26]. We also reported the periplasmic expression of hGM-CSF in two IPTG-inducible *E. coli* expression systems equipped with either T7 or lac promoters [4,9]. Such systems have provided powerful tools in basic researches, whereas they are not desirable in large-scale production of therapeutic proteins,

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because of the toxicity and cost of the non-degradable chemical inducers [12,22].

In order to overcome the limitation of our previously reported IPTG-inducible systems for the production of rhGM-CSF a practical approach was the application of a non-chemically inducible expression system such as heat-inducing expression systems, equipped with either pL or pR promoters of *coliphage*-λ, in combination with their temperature sensitive repressor coding gene cI857 [23, 28]. These promoters are recognizable by bacterial RNA-polymerase and therefore have the advantage of less restricted choice of host strain [5,21]. The cI857 gene product is active at 28°C and inactive at 42°C and represses the transcription from λ promoters [23]. However, the appropriate conditions for optimum induction of λpL promoter in such expression systems have not been defined clearly [12]. The λpL-equipped regulatory compound was also applied for the production of T7 RNA polymerase in T7 expression system by Tabor and Richardson [34], and Gupta *et al*. [12] in which two plasmids were used, one for the expression of T7 RNA polymerase under a heat induction mediated λpL-promoter, and the other for the expression of the gene of interest under T7-promoter regulation. The situation of carrying two types of plasmids in a single host may lead to a decrease in both, plasmid stability and the host growth rate. Therefore, it is assumed that the problem of plasmid-instability might be dramatically decreased by the application of a single plasmid system, carrying both the λpL promoter regulated gene in combination with the λ-cI857 repressor coding gene. Schmidt *et al*. [30] reported the potential of such a single plasmid heat inducible system for the production of 4.5 g of recombinant human insulin B-chain fusion protein per liter. In present study, the primary steps for the construction of first generation of a heat inducible hGM-CSF expressing plasmid, controlled by λpL promoter and its λ-cI857 repressor gene on a single high copy-number plasmid is reported. Using this system, we examined the periplasmic expression of hGM-CSF in *E. coli* after temperature up-shift under various inducing conditions. The possible use of some genetic factors for the improvement of the plasmid is discussed.

2. Materials and Methods

2.1. Bacterial Strains, Plasmids, and Primers

The TG1 strain of *Escherichia coli* (*Strategene*) was used as cloning and expression host. Plasmids pBS-sk and pBC-sk (*Strategen*) were used for the cloning of the synthetic DNA fragment and the construction of the expression vector. Plasmids pGP1-2 [34] and pET26-GM-CSF [4] were used as sources for the repressor cI857 coding gene and pelB::hGM-CSF coding cassette respectively. A double-stranded fragment of λpL promoter carrying *Xba*I and *Sac*I restriction sites on its 3' and 5' regions respectively, was constructed by annealing of five oligonucleotides namely, PL1, PL2, PL3, PL4 and PL5 (Table 1).

2.2. Media, Enzymes and Chemicals

Luria-Bertani (LB) (containing 10 g/l Bacto-tryptone, 5 g/l Bacto yeast extract, and 10 g/l NaCl, pH 7.0, all were purchased from *Merk*-Germany), was used as culture medium, and ampicillin (100 mg/ml), kanamycin (60 mg/ml) or chloramphenicol (50 mg/ml) were added when required to maintain selection pressure. X-gal (40 µg/ml) and IPTG (1 mM) were used for blue-white screening of the desired recombinant colonies. Enzymes *Sac*I, *EcoR*I, *Bam*HI, Taq DNA polymerase and T4 DNA ligase were purchased from the *Roche*-Germany. Polyclonal rabbit-antiserum raised against hGM-CSF was prepared in *NRCGEB* (Iran). Immuno-reactive materials were detected using horse-radish peroxidase-conjugated goat anti-rabbit antibody (*Tebsan*-Iran). Standard hGM-CSF was purchased from Novartis (*Switserland*).

Table 1. The sequences of the oligonucleotides used for the construction of λpL promoter

Name	Sequence
λpL	CAACCATCTGCGGTGATAAATTATCTCTGGCGGTGTTGACATAAATACCACTGGCGGTGATACTGAGCACT
PL1	5'-CAACCATCTGCGGTGATAAATTATCTCTGGCGGTGT-3'
PL2	5'-TGACATAAATACCACTGGCGGTGATACTGAGCACT-3'
PL3	5'-CTAGAGTGCTCAGTATCACCGCCAGT-3'
PL4	5'-GGTATTTATGTCAACACCGCCAGAGAT-3'
PL5	5'-AATTTATCACCGCAGATGGTTGAGCT-3'

2.3. DNA Manipulations

2.3.1. General Techniques

Plasmid DNA isolation, restriction digestions as well as sub-clonings were performed according to standard methods [29]. Commercially prepared columns (*Roche*-Germany) were applied for the purification of DNA from agarose gel and direct PCR products. Colony-screenings were taken place either based on α-complementation test in selective X-gal/IPTG containing LB plates, according to standard methods [29] or by performing direct PCR on colonies; briefly, after suspension in ddH2O, the bacteria were heated for 5 minutes at boiling temperature and used directly as template in PCR. After selecting the recombinant clones based on PCR tests, restriction analysis was employed to confirm the recombinant plasmids. The sequences of the recombinant clones were determined using ABI 373A automated sequencer (*MWG*-Germany) using specific primers.

2.3.2. Reconstruction of λpL Promoter

A 75 bp DNA fragment containing a major section of λpL promoter was constructed by annealing of equal amounts of 5 complementary kinased oligoenucleotides (Table 1) designed based on a double stranded λpL promoter sequence from the λ-phage complete sequence (GENBANK/M17233). An equi-molar mixture of the oligonucleotides was heated at 94°C for 5 min. Annealing was completed by cooling down the mixture to room temperature, followed by ligation of the oligonucleotides, using T4 DNA ligase.

2.4. Expression Analysis

2.4.1. Growth and Inducing Conditions

Bacterial inocula were prepared by growing isolated colonies, obtained from frozen stock, growing on selective medium. The recombinant bacteria were grown at 28°C until OD_{600}= 0.5-0.8, followed by a temperature-shift from 28°C to either 37°C, 40°C or 42°C in a shaking incubator. The cells were harvested and then total, cytoplasmic and periplasmic protein patterns of the bacteria taken from different induction times were examined by SDS-Poly acrylamide gel electrophoresis (SDS-PAGE) and western blotting method (see section *2.4.3*).

2.4.2. Preparation of Cytoplasmic and Periplasmic Proteins

Periplasmic osmotic-shock fluid from hGM-CSF producing bacteria was obtained by a modified method of Libby *et al.,* [20]. Briefly, on the micro-scale, 1.5 ml fermentation broth with OD_{600} =1 was centrifuged at 15,000 g for 5 min and the pellet was collected. All the subsequent steps were carried out at 4°C. The pellet was resuspended in 15 µl of ice-cold TES buffer (0.2 M Tris-HCl, 0.5 M EDTA, 0.5 mM sucrose, pH 8.0) for 20 min, shaking every second minutes. 22.5 µl of ice cold double-distilled water was added and the incubation was continued for 30 min on ice. The cells were centrifuged at 16,000g for 20 min and the pellet was saved as cytoplasmic fraction. Tri-chloro acetic acid was added to the supernatant up to 12% of the final volume. The mixture was centrifuged at 16,000g for 20 min. The pellet was dissolved in 1X sample buffer and boiled for 5 min and saved as periplasmic fraction for further protein analysis.

2.4.3. SDS-PAGE and Western Blotting

SDS-PAGE was performed by a modified method described by Lammli *et al.* [16] and gels were stained with commassie brilliant blue. Electeroblotting of proteins onto nitrocellulose membrane (Amersham-Pharmacia Biotech) was carried out in a transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) at 86 mA, overnight. For the Western analysis, the electeroblotted membrane was probed with a polyclonal antiserum prepared against hGM-CSF (NRCGEB-Iran). The hGM-CSF-antibody complex was then treated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin and visualized using a solution of 4-chloronaphtol with hydrogen peroxidase as enzyme substrate.

2.4.4. rhGM-CSF Expression Assay

The protein pattern of the recombinant bacteria, visualized on commassie brilliant blue stained SDS-PAGE gel, was scanned by a beckmann mode R-112 densitometric gel scanner for the estimation of rhGM-CSF ratio related to the total bacterial proteins.

3. Results

3.1. Construction of Recombinant Plasmid for the Expression of hGM-CSF

Construction of the 5024 bp recombinant plasmid (pZGY3) was carried out in four cloning steps (Fig. 1). In the first step, a 1100 bp *EcoR*I/*Pst*I fragment, containing cI857 gene, was isolated from pGP1-2 plasmid [34] and ligated into an *EcoR*I/*Pst*I digested pBS(sk) plasmid (Fig. 1A) and the new construction was named pZGY1. In parallel, the constructed 75bp λ promoter (see Materials and Methods) was inserted into

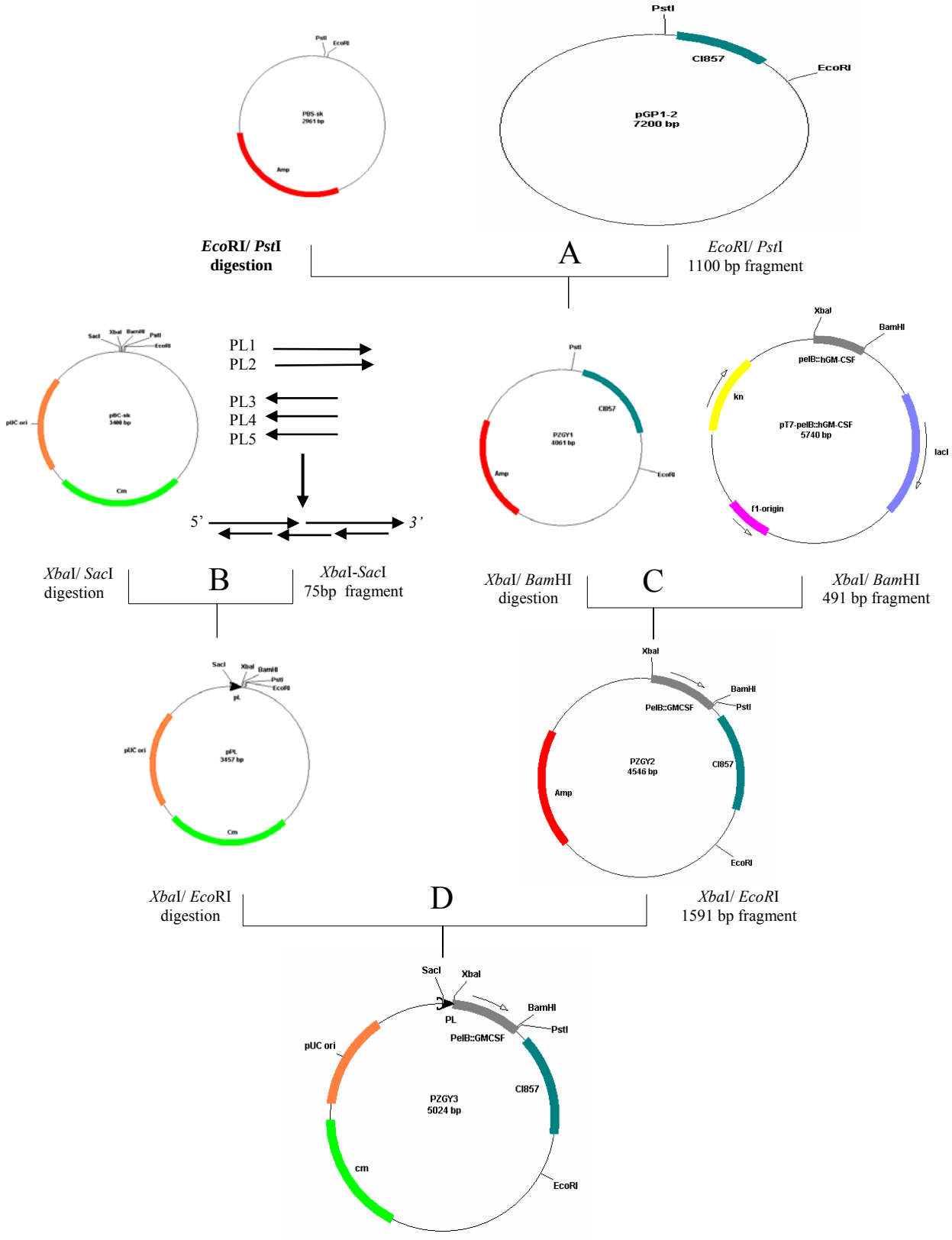

Figure 1. Schematic view of the stepwise progress of the construction of pZGY3 plasmid.

the *Xba*I/*Sac*I digested pBC(sk) plasmid and the resulted plasmid was named pPL (Fig. 1B). The insertion of a 491 bp *Xba*I/*BamH*I fragment containing ribosome binding site and pelB::GM-CSF coding cassette [3], into the pZGY1 construction was performed in the third step (Fig. 1C) and the resulted plasmid was named pZGY2. In the final step, a 1591 bp *Xba*I/*Eco*RI fragment, including rbs, pelB signal sequence, hGM-CSF cDNA and the cI857 gene, isolated from the pZGY2 plasmid was ligated into the *Xba*I/*Eco*RI digested pPL vector (Fig. 1D), thereby the pelB::hGM-CSF coding fragment was located downstream of the λpL promoter. All the cloning steps were confirmed by restriction analysis, followed by the nucleotide sequence determination of the cloned fragments.

3.2. Expression Analysis

3.2.1. Analysis of the Periplasmic Expression of hGM-CSF

The total and periplasmic protein patterns of the recombinant bacteria, obtained 4 h after incubation at 42°C, showed the expression of a 14.4 kDa protein, corresponding to the molecular weight of hGM-CSF (Fig. 2A). The expressed protein was also recognizable by rabbit serum directed against hGM-CSF (Fig. 2B). The protein bands with similar size to mature hGM-CSF appeared in the protein patterns of negative control, in SDS-PAGE were not detectable in the western analysis (Fig. 2 lanes 2). As no protein corresponding to the precursor of hGM-CSF (pelB::hGM-CSF) was detected in the immuno-blots obtained from the total and cytoplasmic proteins of the induced recombinant bacteria, it was concluded that the secretion process was efficient enough to direct a complete processing of the pelB::hGM-CSF followed by releasing of the rhGM-CSF into the periplasm, although the expression level was low.

3.3. Optimization of Induction Conditions

It is well established that when a recombinant bacterium containing strong promoter is induced, prolonged induction tends to disrupt the cellular machinery, leading to poor specific growth rate [18,31]. Therefore, for any expression system, an optimized induction condition has to be defined. In the present system, first it was decided to determine the optimum duration of the heat shock. After growing the cells at 28°C until an appropriate cell density, thereby the λpL promoter is repressed, a temperature up-shift (28°C→42°C) was carried out and sample collection was performed at different time points. A basal-level of hGM-CSF expression occurs by the recombinant bacteria in un-induced condition (Fig. 3B lane 2), that is not comparable with the expression level after induction. The electrophoresed pattern of the rhGM-CSF in the bacterial periplasm after temperature up-shift (at 42°C) and its relative amount are presented in Figure 3. These data show that a maximum expression level occurs when the heat shock is continued for 6 hours (Fig. 3C). In order to set a temperature profile for induction, the relative amounts of the mature hGM-CSF in the periplasm after 6 hours of induction at 37°C, 40°C or 42°C were compared. Although the differences were not significant, but the expression level were relatively higher in the cases of inductions at 37°C and 40°C (data not shown).

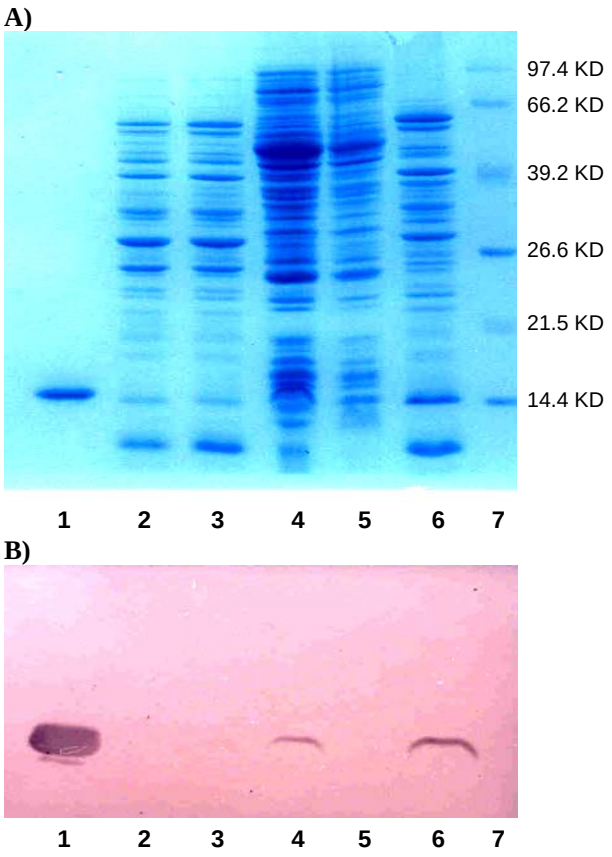

Figure 2. Protein patterns obtained from total, cytoplasm and periplasm of the recombinant bacteria: (A) SDS-PAGE , (B) Western blot. Lane 1, standard hGM-CSF; Lane 2, negative control (periplasmic proteins of *E. coli* TG1 strain containing pZGY2); Lane 3, periplasmic proteins of un-induced cells; Lanes 4, 5 and 6; the total, cytoplasmic and periplasmic proteins of the recombinant bacteria, respectively; Lane 7, protein markers.

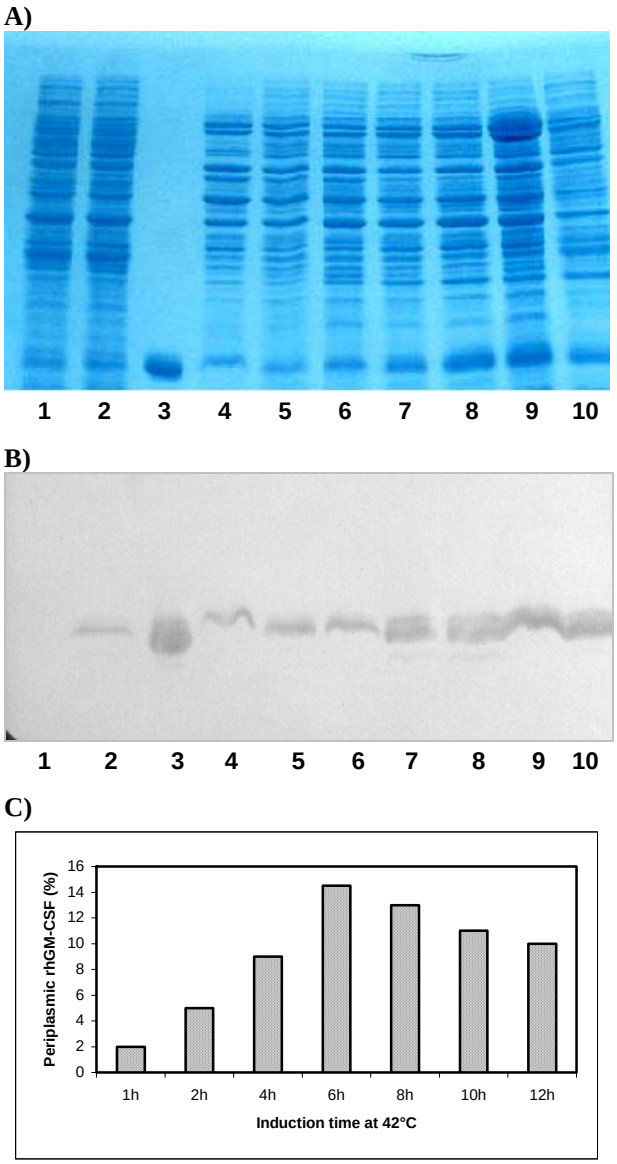

Figure 3. Analysis of periplasmic GM-CSF synthesis at different induction times at 42°C. (A) SDS-PAGE. (B) Western blot. (C) Estimation of periplasmic hGM-CSF. Lanes 1, negative control (periplasmic proteins of *E. coli* TG1 strain containing pZGY2); Lanes 2, periplasmic proteins of un-induced cells; Lanes 3, standard hGM-CSF; Lanes 4-10: periplasmic proteins of the recombinant bacteria after 1 h, 2 h, 4 h, 6 h, 8 h, 10 h and 12 h of heat induction, respectively.

4. Discussion

The constructed λpL heat-inducible expression vector in this study has shown potential for the expression of heterologous proteins in *E. coli* under heat induction. By inserting a pelB::hGM-CSF coding cassette next to a ribosome binding site originated from a pET expression vector (*Novagen*), in a chloramphenicol resistant plasmid, periplasmic production of hGM-CSF under heat induction in *E. coli* was demonstrated. The results showed that the recombinant protein is processed and transferred to periplasm completely. Comparing to the previous works on heat inducible expression systems with two plasmids [12,33,34], this system has the advantage of carrying a single self-regulated plasmid in addition to higher chance to remain stable in a growing host.

There are various suggestions for the optimum inducing condition of λpL promoter in a heat inducible system [8,12] including; a temperature up-shift from 30°C to 42°C for 15 min, followed by reducing temperature to 37°C to induce transcription [14], or a temperature increase to 42°C after a suitable cell-density has been obtained [27,28,32]. There are also suggestions on optimum heat-shock duration, at specific temperatures [25]. In the system presented in this study, after a suitable cell-density has been obtained at 28°C, a duration of 6 hours temperature up-shift led to the highest production level of hGM-CSF. The expression plasmid described in this report would eliminate the need for chemical inducers particularly in large-scale cultivation of recombinant bacteria in which the application of chemical non-degradable inducers such as IPTG are not desirable. In contrast to the systems where an exogenous and toxic RNA polymerase such as T7-bacteriophage RNA polymerase is used for the expression of target protein, this system carries regulatory elements, which are recognized by *E. coli* transcription machinery. Therefore it should function properly in a wide range of *E. coli* strains. The presented results were obtained from the production of rhGM-CSF in shake-flask cultures, using LB medium. However, for the over-production of the recombinant protein in high-cell density culture, cells are required to grow in defined medium to allow the application of a specific feeding-protocol.

It has been suggested that placement of a strong terminator at the 3′ end of a gene, enhances the expression level of the corresponding protein [10]. Moreover, considering the possible interaction of *E. coli* integration host factor (IHF) and the λpL upstream region (from −86 to −180), a λpL promoter carrying fragment that includes a longer patch sequences upstream to −10 and −35 regions may enhance transcription efficiency and finally increase the production of the recombinant protein [11]. Accordingly, in order to enhance the feasibility of the constructed vector, experiments are in progress to modify the constructed vector, focusing on the two above-mentioned elements (under preparation).

References

1. Armitage J.O. Emerging applications of recombinant human granulocyte-macrophage colony-stimulating factor. *Blood*, **92**(12): 4491-4508 (1998).
2. Besa E.C. Myelodysplastic syndromes (refractory anemia). A perspective of the biologic, clinical and therapeutic issues. *Med. Clin. North. Am*., **76**: 599-617 (1992).
3. Burgess A.W., Begely C.G., Johnson G.R., Lopez A.F., Williamson D.J., Mermod J.J., Simpson R.J., Schmaltz A., and Delmarter J.F. Purification and properties of bacterially synthesized human granolocyte-macrophage colony stimulating factor. *Blood*, **69**: 43-51 (1987).
4. Borjaliloo S., Zomorodipour A., Yakhchali B., and Shojai S. The comparison of T7 and lac-based systems for periplasmic expression of human granulocyte macrophage colony stimulating factor in *Escherichia Coli*. *Iraninan J. Biotechnol*., **1**(2): 101-108 (2003).
5. Burnett V. and Springer D. High level expression of human histon H_4 in *E. coli*. *Biotechniques*, **26**: 30-34 (1999).
6. Cantrell M.A., Anderson D., Cerretti D.P., Price V., McKreghan K., Tushiski R.J., Muchizuki D.Y., Larsen A., Arbestin K., Gillis S., and Cosman D. Cloning, Sequence and expression of human granulocyte-macrophage colony stimulating factorProc. *Natl. Acad. Sci. USA*, **82**: 6250-6254 (1985).
7. Curless E., Pope J., Loredo L., and Tsai B. Effect of preinduction specific growth rate on secretion of granulocyte macrophage colony stimulating factor by *E. coli*. *Biotechnol. Prog*., **10**: 467-471 (1994).
8. Elvin C.M., Thompson P.R., Argall M.E., Hendry P., Stamford N.P.J., Lilly P.E., and Dixon N.E. Modified bacteriophage lambda promoter vectors for overproduction of proteins in *E. coli. Gene*, **87**: 123-126 (1990).
9. Fatemi S.S.A., Yakhchali B., Towfighi Darian J., Shojaosadati S.A., Zomorodipour A., Karkhaneh A.A., and Rastgar Jazii F. Selection of a Suitable Strain from Recombinant *Escherichia coli* Strains with the same Genetic Structure Expressing Periplasmic hGM-CSF. *J. Biosci. Bioeng*., **96**(6): 578-580 (2003).
10. Ge A.Z., Pfister R.M., and Dean D.H. Hyperexpression of a Bacillus thuringiensis δ-endotoxin-encoding gene in *E. coli*: properties of the product. *Gene*, **93**: 49-54 (1990).
11. Giladi H., Koby S., Gottesman M.E., and Oppenheim A.B. Supercoiling, Interaction host factor, and a dual promoter system, participate in the control of the bacteriophage λpL promoter. *J. Mol. Biol*., **224**: 937-948 (1992).
12. Gupta C.J., Jaisani M., Pandey G., and Mukheijee K.J. Enhancing recombinant protein yields in *Escherichia coli* using the T7 system under the control of heat inducible λpL promoter. *J. Biotechnol*., **68**: 125-134 (1999).
13. Hua Z.C., Jie L., and Zhu D. Expression of a biologically active human granolocyte-macrophage colony stimulating factor fusion protein in *Escherichia coli. Biochem and Mol Biol Int.*, **34**(3): 621-626 (1994).
14. Hui A., Jhurani P., and de Boer H.A. Directing ribosomes to a single mRNA species: a method to study ribosomal RNA mutations and their effects on translation of a single messenger in *Escherichia coli*. *Methods. Enzymol*., **153**: 432-452 (1987).
15. La Londe J.M., Hanna L.S., Rattoballi R., Berman H.M., and Voet D. Crystallization and preliminary X-ray studies of recombinant human granulocyte-macrophage colony stimulating factor. *Mol. Biol*., **20**(205): 783-785 (1989).
16. Lammli U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**: 680-685 (1970).
17. Lee F., Okota T., Gemmel L., Larson N., Luh J., Arai K.I., and Rennick D. Isolation of cDNA for human granolocyte-macrophage colony stimulating factor by functional expression in mammalian cells. *Pro. Natl. Acad. Sci. USA*., **82**: 4360-4364 (1985).
18. Lee J. and Ramirez W.F. Mathematical modeling of induced foreign protein production by recombinant bacteria. *Biotechnol. Bioeng*., **39**: 635-646 (1992).
19. Levine E.G. and Bloomfield C.D. Leukemias and myelodysplastic syndromes secondary to drug, radiation, and environmental exposure. *Seminars in Oncology*, **19**: 47-84 (1992).
20. Libby T.R., Braedt G., Kronheim R.S., March J.C., Urdal L.D., Chiaverotti A.T., Tushinski J.R., Mochizuki Y.D., Hopp P.T., and Cosman D. Expression and purification of native human granulocyte-macrophage colony-stimulating factor from an *Escherichia coli* secretion vector. *DNA*, **3**: 221-229 (1987).
21. Love A., Lilly E., and Dixon E. Stable high-copy-number bacteriophage λ promoter vectors for overproduction of proteins in *E. coli*. *Gene*, **176**: 49-53 (1996).
22. Makrides S.C. Strategies for achieving high-level expression of genes in *E. coli*. *Microbiol. Rev.*, **60**: 512-538 (1996).
23. Mieschendahl M. and Hill M.B. F-Coded, temperature-sensitive λ CI_{857} repressor gene for easy construction and regulation of λ promoter dependent expression systems. *J. Bacterial.*, **Dec.**: 1366-1369 (1985).
24. Miyajima A., Otsu K., Schreurs J., Bond M.W., Abrams J.S., and Aria K. Expression of murine and human granolocyte-macrophage colony stimulating factors in *S. cervisiae*: mutagenesis of the potential glycosylation sites. *EMBO. J.*, **5**: 1193-1197 (1986).
25. Okita B., Arcuri E., Turner K., Sharr D., Del Tito B., Swanson J., Shatzman A., and Zabriskie D. Effect of induction temperature on the production of malaria antigen in recombinant E. coli. *Biotechnol. Bioeng*., **34**: 854-862 (1989).
26. Oloomi M., Bouzari S., and Rechinsky V.O. Purification and characterization of the cloned human GM-CSF gene expressed in *Escherichia coli*. *Iranian Biomed. J.*, **12**: 353-357 (1999).
27. Remaut E., Stanssens P., and Fiers W. Plasmid vectors for high-efficiency expression controlled by the pL promoter of coliphage lambda. *Gene*, **15**: 81-93 (1981).
28. Remaut E., Stanssens P., and Fiers W. Inducible high level synthesis of mature human fibroblast interferon in Escherichia coli. *Nucleic Acids Res.*, Jul 25, **11**(14): 4677-4688 (1983).
29. Sambrook J. and Rucell D. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory

Press, Cold Spring Harbor, New York (2001).
30. Schmidt M., Babu K.R., Khanna N., Marten S., and Rinas U. Temperature-induced production of recombinant human insulin in high-cell density cultures of recombinant *Escherichia coli. J. Biotechnol*., Feb 5, **68**(1): 71-83 (1999).
31. Seo J.H. and Bailey J.E. Effects of recombinant plasmid on growth properties and cloned gene product formation in *Escherichia coli. Biotechnol. Bioeng.*, **27**: 1668-1674 (1985).
32. Shatzman A.R. and Rosenberg M. Efficient expression of heterologous genes in *Escherichia coli*. The pAS vector system and its applications. *Ann. N.Y. Acad. Sci.*, **478**: 233-248 (1986).
33. Tabandeh S., Shojaosadati S.A., Zomorodipour A., Khodabandeh M., Sanati M.H., and Yakhchali B. Heat-induced production of human growth hormone by high cell density cultivation of recombinant *Escherichia coli*. *Biotechnol. Lett.*, **26**: 245-250 (2004).
34. Tabor S. and Richardson C. A bacteriophage T7 RNA polimerase/promoter system for controlled inclusive expression of specific genes. *Proc. Natl. Acad. Sci. USA*, **82**: 1074-1078 (1985).
35. Welte K., Platler E., Lu L., Gabrilove J.L., Levi E., Mertelsmann R., and Moore M.A.A. Purification and biochemical characterzation of human pluripotent hematopoetic colony-stimulating factor. *Proc. Natl. Acad. Sci. USA*, **82**: 1526-1530 (1985).
36. Wong G.G., Witek J.S., Temple P.A., Wilkens K.M., Leary A.C., Luxenberg D.P., Jones S.S., Brown E.L., Kay R.M., Orr E.C., Shoemaker C., Glde D.W., Kaufman R.J., Hewick R.M., Wang E.A., and Clark S.C. Human GM-CSF: Molecular cloning of the complementary DNA and purification of the natural and recombinant proteins. *Science*, **228**: 810-815 (1985).