

High level expression of recombinant human growth hormone in *Escherichia coli*: crucial role of translation initiation region

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Abstract

For high-throughput production of recombinant protein in *Escherichia coli* (*E. coli*), besides important parameters such as efficient vector with strong promoter and compatible host, other important issues including codon usage, rare codons, and GC content specially at N-terminal region should be considered. In the current study, the effect of decreasing the percentage of GC nucleotides and optimizing codon usage at N-terminal region of human growth hormone (hGH) cDNA on the level of its expression in *E. coli* were investigated. Mutation in cDNA of hGH was performed through site-directed mutagenesis using PCR. Then, the mutant genes were amplified and cloned into the expression vector, pET-28a. The new constructs were transformed into the BL21(DE3) strain of *E. coli* and chemically induced for hGH expression. At the final stage, expressed proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), scanning gel densitometry, and western blot. SDS-PAGE scanning gel densitometry assay and western blot analysis revealed higher expression level of hGH by using the two new expressions constructs (mutant genes vectors with decreasing GC content and optimized-codon usage at N-terminal of cDNA) in comparison with wild gene expression vector. Obtained results demonstrated that decreasing the GC nucleotide content and optimization of codon usage at N-terminal of the hGH cDNA could significantly enhance the expression of the target protein in *E. coli*. Our results highlight the important role of both 5' region of the heterologous genes in terms of codon usage and also GC content on non-host protein expression in *E. coli*.

Keywords: GC content; Codon usage; *Escherichia coli*; Recombinant human growth hormone; pET-28a

INTRODUCTION

Human growth hormone (hGH) is a small, single chain peptide hormone encoded by the GHN gene located at chromosome 17q22–24 and is produced by the anterior pituitary gland (1,2). Growth hormone (GH) is also locally synthesized by lymphoid cells in regional lymph nodes (3-9).

The mature structure of hGH contains 191 amino acid residues with a molecular mass of 22 kDa.

Since the production of hGH from human cadaver tissue has been prohibited, the implementation of recombinant DNA

technology has been considered as a safe and high throughput route for production of therapeutic hGH (10).

A fundamental capability of industrial recombinant DNA technology is the low cost and high efficient production of pharmaceutical proteins. To date, *Escherichia coli* (*E. coli*) has been more frequently used as an expression host among various heterologous systems for production of recombinant proteins (11). Genome of *E. coli* is known better than other microorganisms (12).

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In addition, rapid growth of *E. coli* (13), low-cost substrates for its culture, availability of a large number of compatible vectors with strong inducible promoters such as λ pl, λ pr, trp, and T7 and simple expression induction with either chemical stimulus or thermal shock, are other unique properties of *E. coli* that make it desirable for production of recombinant proteins (14,15).

As the native form of hGH is a single chain, non-glycosylated protein, recombinant production of hGH has been extensively performed in *E. coli* (8,16,17). Regardless of the environmental conditions, various parameters are crucial in high throughput production of recombinant proteins (especially with eukaryotic origin) in *E. coli* comprising efficient expression vector along with a compatible host, a strong promoter, rare codons content, codon usage and percentage of GC nucleotides in a foreign gene which are important factors affecting transcription and translation efficacy (18,19). In this regard, it was demonstrated that coding region modification of a gene affected its heterologous expression level in *E. coli* (20). It was previously proved that optimizing codon usage could increase expression levels of heterologous genes in numerous organisms including *E. coli* (21-23), yeast (24,25) and mammalian cells (26). In most genes, a substitution of low usage or rare codons with synonymous more optimal codons is neutral or even increases expression level (27-29). In contrast, for some genes, however, it has been demonstrated that such a substitution causes reduction in expression yield (30,31).

Another variable for high expression level is the modification of the translation initiation region for efficient ribosome assembly (32). Initiation sites are most desirable if they fold to an unstable sequence motif (33). In the current study, expression of hGH in *E. coli* was evaluated by two different approaches to enhance the gene expression. The expression levels of hGH yielded by a wild type or two N-terminally modified genes, either where the low usage codons were substituted with optimal codons or where GC content of 20 first codons were reduced from 64.6% to 41.3% according to codon usage of host were quantitatively analyzed.

MATERIALS AND METHODS

Construction of template vector (pET-hGH1)

A 663 bp of human somatotropin cDNA was synthesized by Takapouzist Co. (Tehran, Iran). The synthetic gene was digested by *Xho*I and *Nhe*I restriction endonucleases and introduced into the same digested PET28a expression vector (Novagen, USA). The recombinant plasmid was then transferred into *E. coli* BL21 (DE3) strain. The transformed bacteria were grown in LB medium (1% tryptone, 1% yeast extract, 1% NaCl) containing 25 mg/L kanamycin.

Primer design, PCR amplification, and construction of expression plasmids

We fabricated two N-terminally modified hGH-cDNA using two different long length forward primers.

The software used for codon optimization (20 first codons) was an online codon adaptation tool (<http://www.jcat.de/>). The GC content reduction of 20 first codons was done randomly with replacement of third bases of each codon from G or C to A and T. Finally the prepared sequences were inserted to Primer Premier Software and the restriction enzyme sites were considered for both ends. The reverse primer was also designed using Primer Premier Software.

Table 1 represents the designed forward and reverse primers. Primers were synthesized by Takapouzist Co. (Tehran, Iran). The alignment of two N-terminally modified cDNAs vs intact cDNA of hGH was performed using Cell Biology Unit Web Server tool (<http://xylian.igh.cnrs.fr/bin/align-guess.cgi>). Obtained results are demonstrated in Table 2.

The template used for all amplifications was pET-hGH1 construct which contains intact cDNA of hGH that was inserted into pET-28a vector.

Taq polymerase (Fermentas, USA) and recommended protocol was used during PCR. The PCR products were analyzed and quantified by agarose gel electrophoresis. Subsequently, the amplified DNA fragments about 680 bp with *Xho*I and *Nhe*I restriction enzyme sites at 5'/3' of the cDNA and coding sequences for hbFGF were purified from gel.

The extracted genes as well as pET28a expression vector were digested using *XhoI* and *NheI* restriction enzymes.

Following the digestion reaction, DNA fragments and linear vector were separated from digestive enzymes using gel extraction kit (Thermo Fisher Scientific, Nepean, Canada) and then ligated together and transformed into chemically competent *E. coli*

DH5α cells (Invitrogen, Carlsbad, USA) for amplification. The final ligation product were transformed into chemically competent *E. coli* DH5α cells (Invitrogen, Carlsbad, USA) and grown in kanamycin containing LB agar. After incubation, several colonies were randomly isolated and analyzed by colony-PCR and double restriction digests (by *XhoI* and *NheI*) on their purified plasmids.

Table 1. Sequences of the designed primers implementing in site directed mutagenesis process.

Descriptions	Name of primer	Sequence	GC%	T _m
Sequence of wild type gene (20 first codon)	W	5'- AT GCTAGCTAG CTA GCA TGG CTG CAG GCT CCC GGA CGT CCC TGC TCC TGG CTT TTG GCC TGC TCT GCC TGT CC -3'	64.6%	76.4 °C
Sequence of the designed primer for codon usage optimization at 20 first codon	CO	5'- AT GCTAGCTAG CTA GCA TGG CGG CGG GTT CTC GTA CCT CTC TGC TGC TCG CGT TCG GTC TGC TGT GCC TGT CT -3'	61.5%	75.2 °C
Sequence of the designed primer for GC content reduction at 20 first codon	GC	5'- AT GCTAGCTAG CTA GCA TGG CTG CAG GTT CTC GTA CTT CTC TTC TTC TTG CTT TTG GTC TTC TTT GTC TTT CT -3'	43.1%	69.2 °C
Reverse primer	R	5'-GAG GGC AGC TGT GGC TTC TAG CTC GAG GC CTCGAG TA-3'	65.5%	68.5 °C

Table 2. Alignment results of the first 20 codons of the two N-terminally modified cDNAs of hGH versus intact cDNA of hGH using Cell Biology Unit Web Server tool (<http://xylian.igh.cnrs.fr/bin/align-guess.cgi>).

Alignments	Output of Cell Biology Unit Web Server
Wild cDNA vs codon optimized cDNA	scoring matrix: , gap penalties: -12/-2 80.8% identity; Global alignment score: 194 10 20 30 40 50 60 165699 ATGCTAGCTAGCTAGCATGGCTGCAGGCTCCCGGACGTCCCTGCTCCTGGCTTTTGGCCT _ATGCTAGCTAGCTAGCATGGCGGCGGGTTCTCGTACCTCTCTGCTGCTCGCGTTCGGTCT 10 20 30 40 50 60 70 165699 GCTCTGCCTGTCC _ GCTGTGCCTGTCT 70
Wild cDNA vs. GC% content reduced cDNA	scoring matrix: , gap penalties: -12/-2 80.8% identity; Global alignment score: 194 10 20 30 40 50 60 395641 ATGCTAGCTAGCTAGCATGGCTGCAGGCTCCCGGACGTCCCTGCTCCTGGCTTTTGGCCT _ATGCTAGCTAGCTAGCATGGCTGCAGGTTCTCGTACTTCTTCTTCTTCTTGGTCT 10 20 30 40 50 60 70 395641 GCTCTGCCTGTCC _ TCTTTGTCTTCT 70

Expression of recombinant proteins

Transformed cells were cultivated at 37 °C on LB agar plates containing 25 µg/mL kanamycin for plasmid selection. Positive clones were cultured in LB medium until the optical density of the cells reached 0.7 at 600 nm. Each recombinant hGH (rhGH) genes was induced using Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Fermentas, EU).

Expression levels were analyzed under different temperatures and IPTG concentrations. At concentration of 1 mM IPTG, 37 °C temperature and 5 h shaking (200 rpm), the high expression levels were obtained. After the incubation time, the cells were collected at 4000 g centrifugation for 10 min. The cell palettes were re-suspended in 200 µL lysis buffer containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM MgCl₂, 1 mM EDTA, and 250 µg/mL lysozyme. Then the cell suspension was probe sonicated (3 times, 30 sec each time) on ice using an ultrasonic processor (Misonix, Framingdale, NY).

Protein assay

The concentration of protein in cell lysates was determined using Bradford method (34), where bovine serum albumin was used as standard. The target protein expression was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and confirmed by western blotting.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Samples (cell lysates) were mixed with an appropriate volume of 6× loading buffer, incubated at 90 °C for 5 min, cooled and loaded onto 12% polyacrylamide gels at 120 V for 2 h. Protein bands were stained by coomassie brilliant blue and Quantity One software (BioRad, USA) was used for densitometric analysis of protein bands on electrophoretic gel. Quantity One software calculates the mean pixel intensity for each horizontal line in the selected region and displays the resulting intensity.

Western blot analysis

The electroblotting was carried out on polyvinylidene difluoride (PVDF) membrane

using semi-dry method (Bio-Rad, USA) at 12 V for 45 min. The membrane was blocked overnight at 4 °C with 5% skim milk in PBS and then incubated with polyclonal goat anti-hGH antibody (Abcam, USA) for 2 h at room temperature. Then, membrane was thoroughly washed in PBS and then incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) enzyme (Sigma-Aldrich, Germany). Using 0.5 mg/mL 3,3-diaminobenzidine (DAB) and 0.01% H₂O₂ the peroxidase activity of HRP and consequently the immune-reaction was measured.

RESULTS**Site-directed mutagenesis**

The agarose gel electrophoresis of the PCR products illustrated 663 bp DNA fragment related to rhGH-cDNA (Fig. 1).

After ligating the two N-terminally modified cDNAs to the pET-28a expression plasmid, the new constructs comprising pET-rhGH2 (N-terminally modified cDNA with codon optimization) and pET-rhGH3 (N-terminally modified cDNA with GC content reduction) transformed into BL21(DE3) strain. Then, several colonies of each transformant grown in medium containing kanamycin were randomly isolated and analyzed by PCR colony (Fig. 2). The plasmids of positive transformants were also purified and double digested with *Xho*I-*Nhe*I enzymes (Fig. 3). A 663 bp band in agarose gel electrophoresis for both experiments verified the cDNA insertion into pET-28a expression plasmid. DNA sequencing also confirmed the correct insertion and modification of rhGH cDNA.

Expression and detection of rhGH

Expression of rhGH was analyzed under different temperatures and IPTG concentrations. Optimum condition for the expression was attained at 37 °C and 1 mM IPTG concentration. The expressed proteins were confirmed by SDS-PAGE, SDS-PAGE gel densitometry, and western blotting. As shown in Fig. 4, SDS-PAGE gel analysis represented a single sharp band with a molecular mass of about 22 kDa for expressed rhGH.

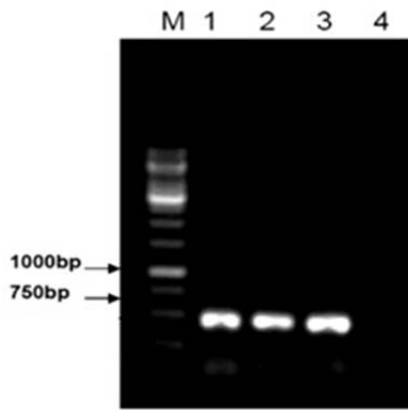


Fig. 1. Mutant N-terminally modified hGH cDNA amplification by PCR. (M) 1kb DNA ladder; (lane 1) intact cDNA; (lane 2) N-terminally modified cDNA with codon optimization; (lane 3) N-terminally modified cDNA with GC content reduction; and (lane 4) blank sample.

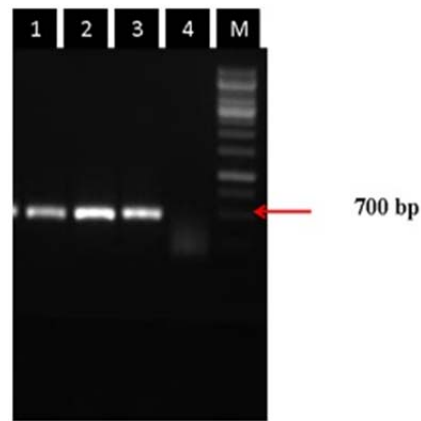


Fig. 2. Gel electrophoresis of PCR colony reaction of transformants. (lane 1) transformant with pET-rhGH1; (lane 2) transformant with pET-rhGH2 (N-terminally modified cDNA with codon optimization); (lane 3) transformant with pET-rhGH3 (N-terminally modified cDNA with GC content reduction); and (M) 1Kb marker.

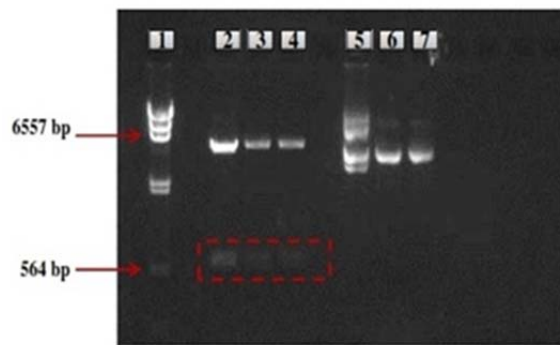


Fig. 3. Gel electrophoretic analysis of double digestion reactions of (lane 2) pET-rhGH1; (lane 3) pET-rhGH2; and (lane 4) pET-rhGH3 with XhoI-NheI enzymes. Lanes 5, 6, 7 illustrate the non-digested pET-rhGH1, pET-rhGH2, and pET-rhGH3 constructs, respectively. Lane 1 demonstrates the 1kb DNA ladder.

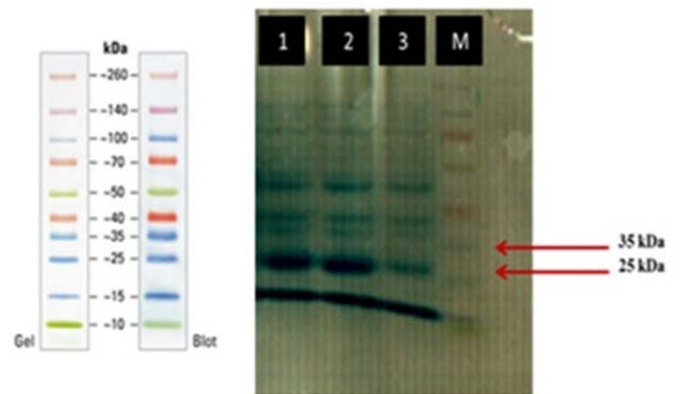


Fig. 4. SDS-PAGE analysis of expressed rhGH. (Lane 1) induced BL21(DE3) containing pET-rhGH2 construct; (lane 2) induced BL21(DE3) containing pET-rhGH3 construct; (lane 3) induced BL21(DE3) containing pET-rhGH1 construct; and (M) protein ladder.

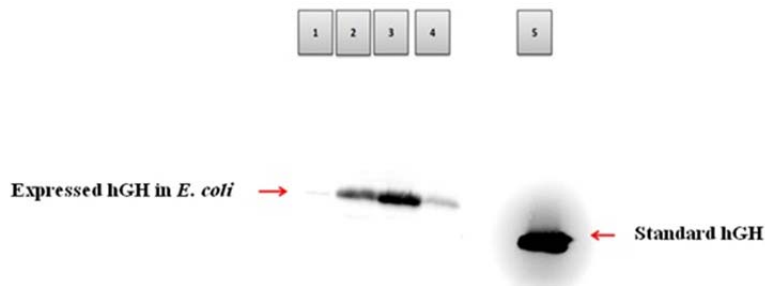


Fig. 5. Western blot analysis of rhGH. (Lane 1) BL21(DE3) cell lysate as negative control; (lane 2) induced BL21(DE3) containing pET-rhGH2 construct; (lane 3) induced BL21(DE3) containing pET-rhGH3 construct; (lane 4) induced BL21(DE3) containing pET-rhGH1 construct; and (lane 5) standard rhGH.

Table 3. Density scanning of the electrophoretic bands as shown in SDS-PAGE lane.

Expression system	Ratio of rhGH in SDS-PAGE lane	Expressed hGH in 1 L culture medium (mg/L)
pET-rhGH1 / BL21(DE3)	11.3%	31.27
pET-rhGH3 / BL21(DE3)	33.2%	294.18
pET-rhGH2 / BL21(DE3)	27.66%	269.94

Density scanning of the electrophoretic bands in SDS-PAGE gel (Table 3) and western blotting analysis (Fig. 5) showed a marked expressed protein quantity for both developed constructs (pET-rhGH2 and pET-rhGH3) in comparison with template vector (pET-rhGH1). According to scanning SDS-PAGE gel densitometry results, by decreasing the percentage of GC nucleotides at N-terminal of the hGH cDNA, the highest protein expression could be achieved.

DISCUSSION

In this study, we have analyzed the heterologous expression of wild type and two variations of hGH cDNA in *E. coli*. The obtained results provided crucial information in choosing strategies for expression of human genes in *E. coli*. Firstly, existence of rare codons at N-terminal of a gene does not necessarily correlate with expression levels and also does not disregard *E. coli* as a host for expression.

Our results contradicted all previous findings which reported the N-terminal optimization of rare codons in a gene negatively affects expression levels in *E. coli* (35). In agreement with our results, numerous examples of increased protein expression via codon optimization are available and introduce this methodology as a common strategy for high heterologous protein expression (36,37).

Despite the reported advantage for codon optimization in rhGH gene (38-40), it was found that the optimization of just first 20 codons (translation initiation region) was enough in order to achieve its high expression level in *E. coli*. Translation initiation region (TIR) is a key factor in prokaryotes determining the availability and efficiency of translation initiation.

As the eukaryotic genes do not have an active TIR, changes in nucleotide sequence of

eukaryotic genes TIRs can affect their expression in *E. coli* (31). There are some codon-related factors affecting the expression of foreign proteins in *E. coli*, such as secondary structure of mRNA, ramp hypothesis, and presence of some special codons in some regions.

According to the “ramp hypothesis”, low expression can be caused by presence of rare codons in the first 30–50 positions. Such codons are low-adapted by tRNAs and decrease the speed of ribosome movement in this region (41,42).

At least two possible explanations can be noted for obtained results. Firstly, mutations for codon optimization may increase the ribosome movement in this region and, thereby, increase the activity of the mRNA in initiation complex formation. Secondly, mutations applied for GC content reduction could decrease the structure stability of the mRNA.

In this case, the translation initiation site would become more accessible possibly resulting in higher expression level. Thus, reducing GC content and exposing the stability of secondary structure at translation initiation region could improve expression level.

Previously, Zomorrodipour, *et. al* have shown that an alteration in hGH N-terminal coding region causes to an over-expression of hGH in the bacteria (38).

According to our results, by GC content reduction at TIR of hGH cDNA from 64% to 43%, the highest level of protein expression was achieved. The total free energy at the 5' end of the hGH gene in pET-rhGH3 construct increased from -18.23 to -11.20 kcal/mol while the total free energy at the 5' end of the hGH gene in pET-rhGH2 construct remained constant at -19.40 kcal/mol. Obtained results imply that GC content reduction and unstable RNA secondary structure at 5' end eventually caused mRNA efficiency for translation.

CONCLUSION

In the current study, appropriate software was used to design the two N-terminally modified hGH cDNA with codon adaptation index (CAI) or GC content reduction in the N-terminal sequence of the gene as a silent mutation without changing in amino acid sequence. Both constructs (pET-rhGH2 and pET-rhGH3) which contain N-terminally modified cDNA showed an increase in hGH expression level while pET-rhGH3 which contained cDNA with N-terminally GC content reduction expressed the highest amount of target protein.

Thus, in recombinant DNA technology, expression trials of N-terminally modified genes with GC content reduction according to codon usage of host is highly recommended as a considerable solution preventing problems encountered by rare codon or specific mRNA secondary structure formation.

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REFERENCES

- Ghasemi F, Zomorodipour AR, Shojai SH, Ataei F, Khodabandeh M, Sanati MH. Using L-arabinose for production of human growth hormone in *Escherichia coli*, studying the processing of gIII: hGH precursor. Iran J Biotechnol. 2004;2(4):250-260.
- Zhan X, Giorgianni F, Desiderio DM. Proteomics analysis of growth hormone isoforms in the human pituitary. Proteomics. 2005;(5)5:1228-1241.
- Sommese L, Donnarumma G, Cipollaro de l'Ero G, Marcatili A, Vitiello M, Galdiero M. Growth hormone modulates IL- α and IFN- γ release by murine splenocytes activated by LPS or porins of *Salmonella typhimurium*. J Med Microb. 1996;45:40-47.
- Doessing S, Heinemeier KM, Holm L, Mackey AL, Schjerling P, Rennie M, et al. Growth hormone stimulates the collagen synthesis in human tendon and skeletal muscle without affecting myofibrillar protein synthesis. J Physiol. 2010;588(Pt 2): 341-351.
- Vance ML, Mauras N. Growth hormone therapy in adults and children. N Engl J Med. 1999;341(16):1206-1216.
- Carrel AL, Myers SE, Whitman BY, Allen DB. Benefits of long-term GH therapy in Prader-Willi syndrome: a 4-year study. J Clin Endocrinol Metab. 2002;87(4):1581-1585.
- Shin NK, Kim DY, Shin CS, Hong MS, Lee J, Shin HC. High-level production of human growth hormone in *Escherichia coli* by a simple recombinant process. J Biotechnol. 1998;62(2):143-151.
- Tabandeh F, Shojaosadati SA, Zomorodipour A, Khodabandeh M, Sanati MH, Yakhchali B. Heat-induced production of human growth hormone by high cell density cultivation of recombinant *Escherichia coli*. Biotechnol Lett. 2004;26(3):245-250.
- Cassidy SB, Driscoll DJ. Prader-Willi syndrome. Eur J Hum Genet. 2009;17:3-13.
- Rezaei M, Zarkesh-Esfahani SH. Optimization of production of recombinant human growth hormone in *Escherichia coli*. J Res Med Sci. 2012;17(7).
- Martial JA, Hallewell RA, Baxter JD, Goodman HM. Human growth hormone: complementary DNA cloning and expression in bacteria. Science. 1979;205(4406):602-607.
- Sørensen HP, Mortensen KK. Advanced genetic strategies for recombinant protein expression in *Escherichia coli*. J Biotechnol. 2005;115(2):113-128.
- Sezonov G, Joseleau-Petit D, D'Ari R. *Escherichia coli* physiology in Luria-Bertani broth. J Bacteriol. 2007;189(23):8746-8749.
- Makrides SC. Strategies for achieving high-level expression of genes in *Escherichia coli*. Microbiol Rev. 1996;60(3):512-538.
- Negahdari B, Shahosseini Z, Baniasadi V. Production of human epidermal growth factor using adenoviral based system. Res Pharm Sci. 2016;11(1):43-48.
- Goeddel DV, Heyneker HL, Hozumi T, Arentzen R, Itakura K, Yansura DG, et al. Direct expression in *Escherichia coli* of a DNA sequence coding for human growth hormone. Nature. 1979;281:544-548.
- Ikehara M, Ohtsuka E, Tokunaga T, Taniyama Y, Iwai S, Kitano K, et al. Synthesis of a gene for human growth hormone and its expression in *Escherichia coli*. Proc Natl Acad Sci U S A. 1984;81(19):5956-5960.
- Mir Mohammad Sadeghi H, Rabbani M, Rismani E, Moazen F, Khodabakhsh F, et al. Optimization of the expression of reteplase in *Escherichia coli*. Res Pharm Sci. 2011;6(2):87-92.
- Beheshti M, Abedi D, Mir Mohammad Sadeghi H. Expression of genes encoding (3-hydroxyalkanoate) synthases from *Pseudomonas aeruginosa* PTCC1310 in *Escherichia coli* and optimization of condition for production of these enzymes. Res Pharm Sci. 2012;7(5):S445.
- Gustafsson C, Minshull J, Govindarajan S, Ness J, Villalobos A, Welch M. Engineering genes for predictable protein expression. Protein Expr Purif. 2012;83(1):37-46.
- Hale RS, Thompson G. Codon optimization of the gene encoding a domain from human type 1

- neurofibromin protein results in a threefold improvement in expression level in *Escherichia coli*. *Protein Expr Purif*. 1998;12(2):185-188.
22. Hannig G, Makrides SC. Strategies for optimizing heterologous protein expression in *Escherichia coli*. *Trends Biotechnol*. 1998;16(2):54-60.
 23. Zhang MY, Schillberg S, Prins M, Fischer R. Optimizing expression of a rare codon-rich viral protein in *Escherichia coli* using the IMPACT system. *Anal Biochem*. 1999;271(2):202-204.
 24. Brocca S, Schmidt-Dannert C, Lotti M, Alberghina L, Schmid RD. Design, total synthesis, and functional overexpression of the *Candida rugosa* lipase gene coding for a major industrial lipase. *Protein Sci*. 1998;7(6):1415-1422.
 25. Cormack BP, Bertram G, Egerton M, Gow NA, Falkow S, Brown AJ. Yeast-enhanced green fluorescent protein (yEGFP): a reporter of gene expression in *Candida albicans*. *Microbiology*. 1997;143(Pt 2):303-311.
 26. Kim CH, Oh Y, Lee TH. Codon optimization for high-level expression of human erythropoietin (EPO) in mammalian cells. *Gene*. 1997;199(1-2):293-301.
 27. Makoff AJ, Oxer MD, Romanos MA, Fairweather NF, Ballantine S. Expression of tetanus toxin fragment C in *E. coli*: high level expression by removing rare codons. *Nucleic Acids Res*. 1989;17(24):10191-10202.
 28. Rangwala SH, Finn RF, Smith CE, Berberich SA, Salsgiver WJ, Stallings WC, *et al*. High-level production of active HIV-1 protease in *Escherichia coli*. *Gene*. 1992;122(2):263-269.
 29. Zahn K. Overexpression of an mRNA dependent on rare codons inhibits protein synthesis and cell growth. *J Bacteriol*. 1996;178(10):2926-2933.
 30. Griswold KE, Mahmood NA, Iverson BL, Georgiou G. Effects of codon usage versus putative 5'-mRNA structure on the expression of *Fusarium solani* cutinase in the *Escherichia coli* cytoplasm. *Protein Expr Purif*. 2003;27(1):134-142.
 31. Jiang X, Li S, Zhou A, Li F, Xu X, Zhu D. Translation initiation region plays an important role in the expression of human thrombopoietin in *Escherichia coli*. *Biochem Mol Biol Int*. 1996;39(6):1109-1113.
 32. Kozak M. Initiation of translation in prokaryotes and eukaryotes. *Gene* 1999;234(2):187-208.
 33. Ishida M, Oshima T. Effective structure of a leader open reading frame for enhancing the expression of GC-rich genes. *J Biochem*. 2002;132(1):63-70.
 34. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976;72:248-254.
 35. Mirzahoseini H, Mafakheri S, Soltan Mohammadi N, Enayati S, Mortazavidehkordi N. Heterologous proteins production in *Escherichia coli*: an investigation on the effect of codon usage and expression host optimization. *Cell J(Yakhteh)*. 2011;12(4):453-458.
 36. Kleber-Janke T, Becker WM. Use of modified BL21 (DE3) *Escherichia coli* cells for high-level expression of recombinant peanut allergens affected by poor codon usage. *Protein Expr Purif*. 2000;19(3):419-424.
 37. Li A, Kato Z, Ohnishi H, Hashimoto K, Matsukuma E, Omoya K, *et al*. Optimized gene synthesis and high expression of human interleukin-18. *Protein Expr Purif*. 2003;32(1):110-118.
 38. Zomorrodipour A, Yakhchali B, Khodabandeh M, Deezagi A, Hosseini Mazinani SM, Valian Borujeni S, *et al*. The over-expression of biologically active human growth hormone in a T5-based system in *Escherichia coli*, studying temperature effect. *J Sci I. R. Iran*. 2004;15(1):27-32.
 39. Levarski Z, Šoltýsová A, Krahulec J, Stuchlík S, Turňa J. High-level expression and purification of recombinant human growth hormone produced in soluble form in *Escherichia coli*. *Protein Expr Purif*. 2014;100:40-47.
 40. Bulmer M. Codon usage and secondary structure of MS2 phage RNA. *Nucleic Acids Res*. 1989;17(5):1839-1843.
 41. de Smit MH, van Duin J. Control of translation by mRNA secondary structure in *Escherichia coli*. A quantitative analysis of literature data. *J Mol Biol*. 1994;244(2):144-150.
 42. Sprengart ML, Fatscher HP, Fuchs E. The initiation of translation in *E. coli*: apparent base pairing between the 16srRNA and downstream sequences of the mRNA. *Nucleic Acids Res*. 1990;18(7):1719-1723.