Over expression of a synthetic gene encoding interferon lambda using relative synonymous Codon usage bias in *Escherichia coli*

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Abstract: Interferon Lambda (IFN-λ) is a type III interferon which belongs to a novel family of cytokines and possesses antiviral and antitumor properties. It is unique in its own class of cytokines; because of the specificity towards its heterodimer receptors and its structural similarities with cytokines of other classes. This renders IFN-λ a better choice for the treatment against many diseases including viral hepatitis and human coronavirus (HCoV-EMC). The present study describes a computational approach known as relative synonymous codon usage (*RSCU*); used to enhance the expression of IFN-λ protein in a eukaryotic expression system. Manually designed and commercially synthesized IFN-λ gene was cloned into pET-22b expression plasmid under the control of inducible T7-lac promoter. Maximum levels of IFN-λ expression was observed with 0.4 mM IPTG in transformed *E. coli* incubated for 4 hours in LB medium**.** Higher concentrations of IPTG had no or negative effect on the expression of IFN-λ. This synthetically over expressed IFN-λ can be tested as a targeted treatment option for viral hepatitis after purification.

Keywords: Relative synonymous codon usage (RSCU) Interferon Lambda, over-expression.

INTRODUCTION

IFNs were first characterized in 1957 by Isaacs and Lindenmann (Isaacs and Lindenmann, 1957) and were so called "Interferons (IFNs)" for their ability to "interfere" with viral replication. This conferred resistance to infection transferred from virally infected chick cells into uninfected cells (Thiebaut *et al.,* 2005). The therapeutic potential of IFNs in viral infection was first demonstrated through its ability to inhibit respiratory viral infections (Isaacs and Lindenmann, 1957). Since then IFNs have been proven clinically effective antiviral and antineoplastic therapeutic agents for a variety of disorders (Borden *et al.,* 2007). They were also the first cytokines to be purified to homogeneity, cloned, completely sequenced and produced in recombinant form and in extensive clinical applications (Pestka., 1994, Billiau *et al.,* 2006). With the discovery of isoforms, IFNs have been classified into three distinct group based on amino acid sequence and recognition by specific receptors (Li *et al.,* 2009).The proteins of IFN family are the most admired biopharmaceuticals against both RNA and DNA viruses and they have proved themselves as prototypic biological response modifiers for various tumors like hairy cell leukemia, AIDS related Kaposi's sarcomas and malignant melanomas (Li *et al.,* 2009). IFNs have furthermore established reliable efficacy profile in suppressing manifestations of multiple sclerosis also (Haghjooy Javanmard *et al.,* 2012). Due to its effective therapeutic roles, the family of IFN proteins has now reached the potential envisioned by its discoverers, but still much is left to be learned (Li *et al.,* 2009, Chelbi-Alix and Wietzerbin, 2007, Pestka, 2007, Borden *et al.,* 2007).

Synthetic IFN is rapidly degraded by human body i.e. 12-24 hours after injection, hence reducing patient compliance; therefore Pegylation (a process that attaches polyethylene glycol [a biologically inert compound] strands to the protein) was introduced. This made IFN less likely to be cleared from the bloodstream and thus sustained virological response (SVR) increased considerably due to the increased concentrations of IFN levels in the body for a prolong period of time (one dose per week); which constantly suppressed the virus (Baker, 2001). In 2001, FDA approved pegylated IFN-α-2b (PEG-IFN- α-2b) for the first time and it is the gold standard antiviral therapy against hepatitis to date. (Franciscus, 2010, Baker, 2001).

A year later, in 2002, pegylated interferon alpha-2a (PEG-IFN- α-2a) was also approved by FDA and was included in a combined therapy with ribavirin. It remained applicable with 30-70% of the SVR (Barnard, 2001, Fried *et al.,* 2002, Trimoulet *et al.,* 2004). This combination remained unchallenged until 2010, with many other polymerase/protease inhibitors, Immunomodulators, toll like receptors (TLR) Agonists, micro RNAs, Agonists to A3 adenosine receptor (A3AR), botanicals, Thiazolides and Vaccines to enter the race of successful antiviral therapies against hepatitis C, but the two most efficacious

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products passing the clinical trials were Boceprevir and Telaprevir (Galati, 2011, Alkhouri and Zein, 2012). They showed promising results in combination with IFNs and ribavirin as they increased the SVR against various genotypes (Franciscus, 2008 and Bekisz *et al.,* 2010). Researchers all over the World are trying to improve IFN therapy against HCV; nevertheless two research groups, led by Sergei Kotenko and Wayne Kindsvogel, came up with their new and immaculate research in year 2002. This added another class into the classification of IFNs i.e. Interferon lambda (IFN-λ1, λ2, λ3 and λ4) (Kotenko *et al.,* 2003, Sheppard *et al.,* 2003, Prokunina-Olsson *et al*., 2012). IFN-λ showed redundancy and initiated all antiviral pathways of type I or type II IFNs, whereas IFN-λs were structurally more similar to IL-10 cytokine family (Gad *et al.,* 2009 and Ole J. Hamming 2010).

Type III IFNs is emerging as an exciting area of research due to three main reasons. primarily, IFNs are one of the fundamental components of the innate immune system and are the backbone of the antiviral therapy against hepatitis (Borden *et al.,* 2007). Secondly, the genome-wide association studies of IFN-λ have ascertained it to have a key role in viral control, both in the context of antiviral therapy and the setting of spontaneous resolution of HCV, which is being said to be associated with the polymorphism in IFN-λ genes (Buti *et al.,* 2005, Ge *et al.,* 2009, Thomas *et al.,* 2009). Lastly, the heterodimer receptor of IFN-λ is thought to play a key role in minimizing the side effects of IFN therapy (Witte *et al.,* 2009). There are many other factors involved in the success stories of drugs and one of them is the low cost of productivity. We hereby have used a bioinformatics tool called "Relative synonymous codon usage (RSCU)" to over express the protein IFN-λ in Escherichia coli (*E. coli*). Codon usage bias is the term coined to differentiate the frequency of occurrence of synonymous codons in any coding DNA (Ghosh TC, 2000). Codon is a triplet of nucleotides which either encodes a specific amino acid residue in a polypeptide chain or is a signal for RNA to terminate the translation. Out of 64 different codons, 61 codons encodes just 20 different amino acids and the other three are said to be non-sense codons, as they stop the translation. Yet the point to be stressed is that the surplus codons allow many amino acids to be encoded by multiple codons (James Norman Davidson, 1993). Preferences in the selection for one of the several options available of codons or codon usage bias, that encodes the same amino acid has been seen in different organisms, i.e. frequency of one favorite codon will be greater than that expected by chance codon (Grantham *et al.,* 1981, Grantham *et al.,* 1980, Gouy and Gautier, 1982, Ikemura, 1985). An assortment of bioinformatics and statistical techniques have been used to analyze codon usage bias (Comeron and Aguade, 1998, Cancilla *et al.,* 1995, Gharbia *et al.,* 1995), which includes "Codon adaptation index (CAI)" and "Frequency of optimal codon (Fop)" for prediction of gene expression levels. Calculations like "Effective Number of Codons (ENC)" was used to measure evenness in codon usage (Ikemura, 1985, Sharp and Li, 1987a, Sharp and Li, 1986a, Sharp and Li, 1986b, Sharp and Li, 1987b, Sharp and Matassi, 1994, Sharp *et al.,* 1993, Ikemura, 1981). Many other factors on which the codon usage bias or the gene expression levels depend were also considered and were optimized, which includes ''Percentage G+ C composition'', GC of silent 3rd codon, A3s, T3s, C3s, G3s and GC of silent 3rd codon (strand specific mutational bias) (Sharp and Li, 1987a, Sharp and Li, 1986a, Gouy and Gautier, 1982, Robinson *et al.,* 1984, Sorensen *et al.,* 1989, Xia, 1998). We have used *E. coli* in this study, which are fast growing micro-organisms and optimal codon depends on the composition of their genomic RNA pool (Ikemura, 1981, Robinson *et al.,* 1984). Optimal codons are related to over expression of genes through faster translation rates retaining high accuracy (Ikemura, 1981).

MATERIALS AND METHODS

Sequence selection

The complete gene sequence of IFN-λ was extracted from the NCBI [Accession No. BC126183.1, NM_172140, AY_336716, BC_074985 and BC_117482]. The sequence similarity was enough to design a synthetic gene with similar translation using codon usage bias. We carried on with the following bioinformatics techniques:

Relative synonymous codon usage (RSCU)

To examine synonymous codon usage without the confounding influence of amino acid composition of different gene samples, the values of RSCU of different codons in each sequence were calculated for every amino acid (Robinson *et al.,* 1984, Sorensen *et al.,* 1989, Gouy and Gautier, 1982, Sharp and Li, 1986b, Xia, 1998). All of the 64 possibilities are not compulsory in the context of the analysis as stop codons are eliminated from analysis and in addition to this, TGG (codon for tryptophan) and ATG (codon for methionine). Codon bias is not exhibited in tryptophan and methionine; as they are only associated with a single codon.

Effective number of codons (ENC)

The ENC of a gene is generally used to quantify the codon usage bias of a gene, which is essentially independent of gene length (Robinson *et al.,* 1984, Sorensen *et al.,* 1989, Wright, 1990). The ENC value of the gene was calculated using Codon W 1.4 (http://codonw.sourceforge.net/).

Codon adaptation index (CAI)

CAI is used to predict the expression of any gene and is a measurement of the relative adaptivity of the codon usage of a gene towards the codon usage of highly expressed genes (Jansen *et al.,* 2003). CAI was calculated using

Codon W 1.4.

T3s, A3s, G3s, C3s, GC and GC3S contents

T3s, A3s, G3s and C3s are the number of these nucleotides on the third position of any codon in any gene and according to universal pattern; T3s and A3s are favored for higher expression of any gene. GC content should be between 30 to 70% in any gene to highly expressed (Nussinov., 1981). GC3S is the frequency of the nucleotide G+C at the synonymous third position of the codons, excluding Met, Trp, and the termination codons. It is a good indicator of the extent of base composition bias furthermore codons that contain the CG dinucleotide were avoided (low-usage codons) (Nussinov, 1981).

Software implementation

CodonW 1.4 (http://codonw.sourceforge.net/) and Genscript (https://www.genscript.com/cgi-bin/tools/rare_codon_analysis) were used for calculating the indices of codon usage.

Cloning and expression of synthetic INFλ1 into pET-22b vector

Synthetic INF-λ1 gene was designed and was delivered in pIDTSMART-KAN/IL-29 vector by Integrated DNA Technologies (IDT) (2009).*EcoR*1 and *Xho*1 restriction enzymes (Fermentas, USA) were used for restriction digestion. Plasmid confirmed after digestion was transformed into BL21 DE3 codon+ cells following the standard transformation protocol (Ditiatkin and Il'iashenko, 1979).

The successfully transformed *E. coli* BL21DE3 was picked from a single colony and was cultured in fresh Luria-Bertani mediacontaining ampicillin. To optimize the culture conditions and maximum expression conditions, IFN-λ expression was induced by adding 4mM IPTG to the transformed *E. coli* and the bacteria were incubated at 37 ˚C for a period of 2, 4, 6, 8, 10 or 12 h, respectively. The degree of expression was evaluated by 12 % of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970).SDS-PAGE gel was trans-blotted onto a Nitrocellulose membrane (Amersham, Bukinghamshire, UK) using standard protocol (Renart *et al.,* 1979, H Towbin, 1979). The membrane was washed with Tris buffered saline (TBS) and blocked with the blocking solution (TBS) containing 0.2% Tween 20 and incubated at room temperature for 1 hour in blocking solution followed by the overnight incubation at 4˚C in the primary antibody solution. The membrane was washed in TBS (containing 0.2% Tween 20) and incubated for 30 min in the enzyme conjugate solution followed by washing using TBS (containing 0.2% Tween 20). The membrane was washed again using TBS to remove tween 20 from the membrane and visualized.

RESULTS

Synthetic and Natural Gene

Codons of the natural genes were replaced by the codons used frequently by an *E. coli* but there was no change in the protein sequence [The complete gene sequence of synthetic IFN-λ is available at NCBI; Accession No. KC347303]. Results of the computational analysis for comparing the physical and chemical properties of the modified gene are as follows:

Computational analysis

A comparison of expression of NCBI reported IFN-λ gene and the synthetic IFN-λ gene was done by comparing the CAI and ENC. Scientists agree that if the preferred codon is used, the expression is always more than the routine yield (Ikemura, 1981). Now, for the calculated values of CAI and ENC of both genes included in this study, results showed that it would be remarkably high for the synthetic gene (i.e 0.87 and 20 respectively, (table 1), whereas for the natural gene, it was found to be 0.57 and 44.33 respectively. When *ENC* value is 20 (equal to the number of amino acids); it means that the bias is at a maximum and only one codon is used from each synonymous codon group, whereas it can go up to 61 (the number of sense codons) which indicates no codon-usage bias.

Table 1: Values of Codon Usage tools for synthetic gene various parameters of Natural and synthetic genes were compared and the results are as follows

Title	Natural Gene Values	Synthetic Gene Values
T3s	0.1585	0.4268
C3s	0.4939	0.2805
A3s	0.1265	0.1386
G3s	0.4110	0.3436
Codon Adaptive Index	0.57	0.87
GC	0.63	0.570
GC of silent 3rd codon	0.759	0.523
Effective number of codons	44.33	20
Frequency of codon index	0.451	1.000
Codon bias index	0.093	1.000

Codon Usage Frequency

Codon usage frequency refers to differences in the frequency of occurrence of synonymous codons in any coding gene or DNA. We can calculate codon adaptive index of any gene by using different tools. Fig. 1 shows the CAI of both natural and synthetic genes.

Codon Frequency Distribution (CFD)

Real codon frequency distribution is an important parameter to calculate codon usage bias of any gene and fig. 2 shows the CFD of both natural and synthetic gene.

GC Content Adjustment

Expression of any gene depends on the GC content of that gene and the average GC content of synthetic gene was much closer to the acceptable overexpression range as shown in fig. 3 (Nussinov, 1981).

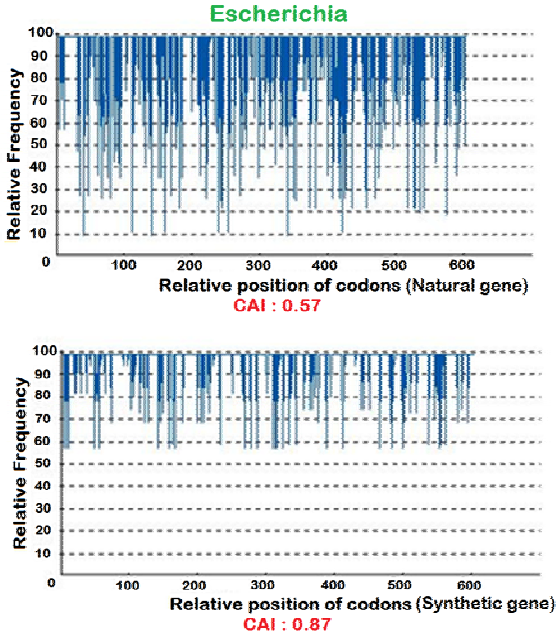

Fig. 1: The distribution of codon usage frequency along the length of natural and synthetic genes to be expressed in *E. coli*. The relative frequency of the natural gene was too low at different positions throughout the gene length, whereas in synthetic gene, codons having low usage frequency were avoided. Codon adaptive index was also improved for the same reason.

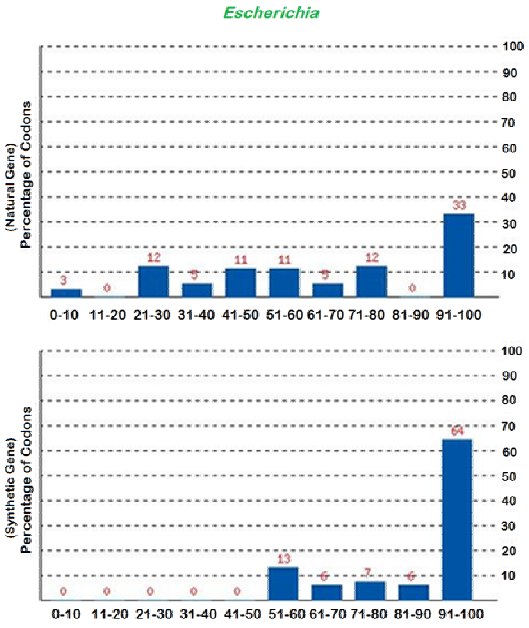

Fig. 2: The percentage distribution of codons in computed codon quality groups. The value of 100 is set for the codon with the highest usage frequency for a given amino acid in the *E. coli* expression system. Low usage codons were avoided to limit the codon frequency distribution to the highest possible values.

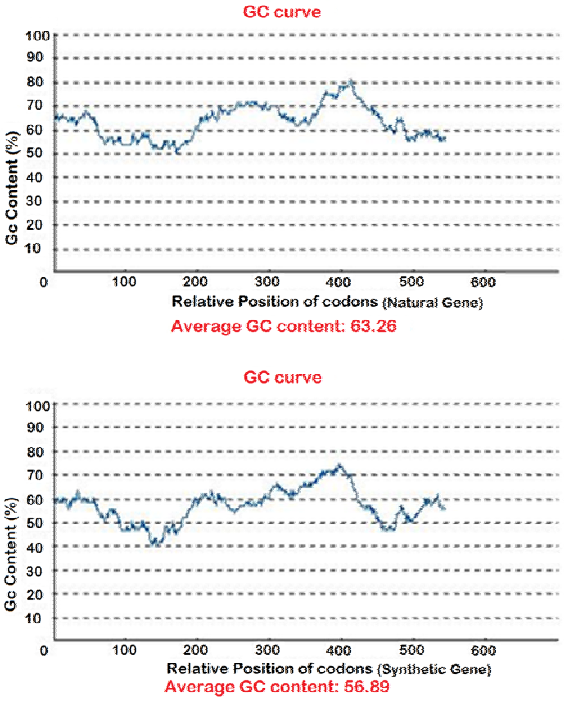

Fig. 3: Average GC contents of any gene has a great impact on the expression of that particular gene and the ideal percentage range of GC content for *E. coli* is between 30% to 70%. Graph shows the average GC content to be lowered in the synthetic gene, as well as it shows that the GC curve is touching the 70% border line in case of synthetic gene, which will enhance its expression.

Cloning and expression of synthetic gene

Cloning of synthetic IFN-λ Gene

Modified synthetic Interferon Lambda (IFN-λ) gene was transformed in Top-10 E. *coli* strain and then a single colony was cultured and plasmid was isolated. Positive clones were confirmed by plasmid isolation and double digestion (fig. 4).

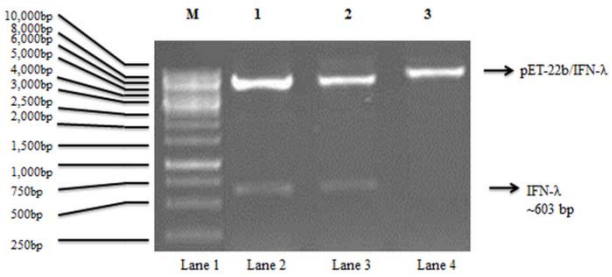

Fig. 4: Double digestion of recombinant PET-22b/IFN-λ vector. Lane 1: 1kb DNA ladder. Lane 2-3: double digested pET-22b/IFN-λ vector and Lane 4: undigested PET-22b/IFN-λ vector.

Expression of IFN-λ Gene in E. coli

To express synthetic IFN-λ protein, the bacterial BL-21 DE3 codon+ expression system was used (fig. 5).

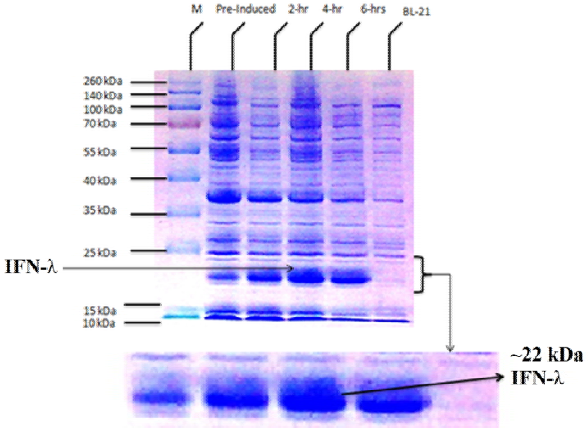

Fig. 5: Expression of IFN-λ in *E. coli* at different time intervals. lane 1: Protein ladder, Lane 2: un-induced BL-21 DE3 codon+ cells, Lane 3: 2 hours post-induction sample, Lane 4: 4 hours post-induction sample, Lane 5: 6 hours post-induction sample, Lane 6: un-induced BL-21 DE3 codon+ cells.

Immuno-blotting

For Western blot analysis, proteins were electro-blotted onto nitrocellulose membranes and immune-stained with different antibodies (fig. 6).

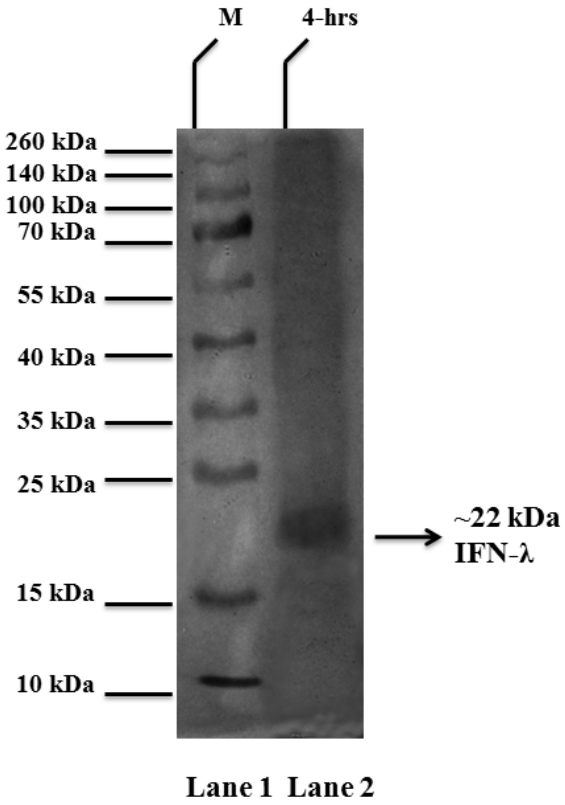

Fig. 6: Western blotting analysis: Lane 1: Protein ladder electro-blotted onto nitrocellulose membranes, Lane 2: ~22 kDa IFN-λ protein immune-stained with specific antibody.

Table 2: Yield in milligrams per liter of natural and synthetic genes (Li and He, 2006)

Natural Gene	Synthetic Gene
60mg/liters of bacterial culture	140mg/liters of bacterial culture

DISCUSSION

Type III IFNs share a few common characteristics with type 1 IFNs, including antiviral, immune-regulatory, anti-proliferative and *in-vivo* anti-tumor activity (Li *et al.,* 2009). Their unique feature includes the specificity of their private chain in the heterodimer receptor complex, which is present on few cells type only. The presence of this heterodimer receptor in liver cells makes it a specified target against hepatitis C Virus (Sommereyns *et al.,* 2008), similarly they are believed to be the primarily barrier for various viruses entering through epithelium (Kindler *et al.,* 2013). These factors lead to a general interest on the possibility of finding out whether IFN-λ can be directly used as a cure for patients with viral hepatitis? IFN-λ is deposited in the inclusion bodies, which is a common theme in recombinant technologies and mostly its formation is a consequence of higher expression rates (Lilie *et al.,* 1998). Many studies of the synonymous codon usage have exposed information on molecular evolution of individual genes of different organisms, which provides directions to improve gene recognition procedures (Robinson *et al.,* 1984, Sorensen *et al.,* 1989, Xia, 1998). On the basis of the information available, a modified IFN-λ gene was synthesized using RSCU and was expressed using prokaryotic expression system i.e. *E. coli*. Different factors have been expected to be related to codon usage bias, including gene expression level (reflecting selection for optimizing translation process by tRNA abundance), %G+C composition (reflecting horizontal gene transfer or mutational bias), amino acid conservation, transcriptional selection, RNA stability, optimal growth temperature and hypersaline adaptatione (Gouy and Gautier, 1982, Ikemura, 1981, Ikemura, 1985, Sharp and Cowe, 1991, Sharp and Li, 1986a, Sharp and Li, 1986b, Sharp and Li, 1987a, Sharp and Matassi, 1994, Sharp *et al.,* 1993, Sorensen *et al.,* 1989, Nussinov, 1981).

Rules for determining the choice of optimal codons in unicellular organisms includes that tRNA availability will be the first condition applied to get the optimal yield, second is the preference for A over G when thiolateduridine or 5-carboxymethyl are at the anticodon wobble position (Ikemura, 1985). The third rule states the preference for T and C over A when inosine is at the anticodon wobble position and in our synthetic gene

(Moriyama and Powell, 1997). GC_{3S} is the fraction of codons that are synonymous at the third codon position, which have either a guanine or cytosine at that third codon position. The ideal percentage range of GC content is in-between 30% to 70%. Any peak outside of this range will negatively affect transcriptional and translational efficiency (Nussinov, 1981, Hildebrand *et al.,* 2010). The universal pattern of codon usage states that "Codons that contain the GC dinucleotide are universally avoided" and the G+C content of any gene is the frequency of nucleotides that are guanine or cytosine in the gene and it has an impact on the codon selection of tRNA (Hildebrand *et al.,* 2010). Present study shows that the GC content of the synthetic gene has been reduced to 0.570, which actually help us to overexpress the synthetic gene. After optimizing the concentrations of IPTG and the optimum time for maximum expression of gene in *E. coli*, a protein of approximately 22 kDa size was observed. According to the results of present study, the yield increased and approximately double of the reported (60 mg/l) amount was obtained from 1 L of bacterial culture (Li and He, 2006, table 2).

CONCLUSION

Type III interferons are an interesting new addition to the interferon family. They undoubtedly induce an antiviral response in cells which is highly similar to that of type I IFNs, although they share several structural features with the IL-10 family. It is now clear that only a limited subset of cells respond to IFN-λ. As tissues which experience high frequencies of viral infections seem to respond well to IFN-λ. It is tempting to view this new cytokine family as a first line of defense against viral infections, with hepatitis being the chief subject of interest. The results of our study showed that the expression of IFN-λ gene can be enhanced using different computational techniques, so it will be beneficial in the production of different biopharmaceuticals; readily and cost-effective.

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