

## Molecular Cloning, High Level Expression and Activity Analysis of Constructed Human Interleukin-25 Using Industrially Important IPTG Inducible *Escherichia coli* BL21(DE3)

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### Abstract

Interleukin-25 (IL-17E) is a novel Th2 pro-inflammatory cytokine belongs to the member of IL-17 cytokine family. In the present study, bioactive recombinant human IL-25 (rhIL-25), the cDNA of mature IL-25 was synthesized using nested PCR and codon bias of prokaryotic host *Escherichia coli*. The desired template was cloned into the MCS region of expression vector pET28<sup>+</sup>. The recombinant vector was transformed into maintenance host *Escherichia coli* DH5a and the transformants were selected by kanamycin resistance marker. Expression was carried out using IPTG inducible *Escherichia coli* BL21(DE3) in different media like LB, terrific broth and M9 media. Among all, terrific broth was used for the enhanced production of rhIL-25. SDS-PAGE analysis shows 31 kDa proteins against low molecular weight protein marker. Refolding of inclusion bodies with denaturation buffer (25 mM Tris-HCl [pH 7.2], 5 M Urea, 20 mM β-ME and 200 mM NaCl) yields the rhIL-25 at a concentration of ~ 86 mg/L at 37 °C, where it is high when compared with the expression at 20 °C (~ 16.5 mg/L). Western blot analysis was carried out using anti human IL-17E/IL-25 antibodies. Biological activity of rhIL-25 was determined by the release of IL-6 from PBMC cells. For the first time, under the conditions of current good manufacturing practice (cGMP), bioactive recombinant IL-25 was produced at large scale in soluble form using industrially feasible bacterial host *Escherichia coli* BL21(DE3).

**Keywords:** Interleukin-25, pET28<sup>+</sup>, *Escherichia coli*, nested PCR, soluble form

### 1. Introduction

Interleukin-25 (IL-25) is a recently identified member of IL-17 cytokine family and also known as IL-17E, has the ability to influence innate and adaptive immunity. It possesses lowest degree of homology to IL-17A and does not share common biological properties with the other members of IL-17 cytokine family [1]. IL-25 was also useful to regulating the Th2 cell type immune responses. Different studies were conducted in human and animal models proved that IL-25 has anti-tumor and anti-inflammatory activities [2, 3, 4]. Some studies proved the IL-25 was largely found in polarized Th2 cells [5], lung epithelial cells upon allergic response [6], eosinophils and basophiles upon stem cell factor stimulation [7, 8], alveolar macrophages [9] and primary bone marrow-derived mast cells upon IgE cross-

linking [10]. IL-25 was expressed in brain microglia and brain capillary endothelial cells [11] and maintains blood brain barrier integrity [12].

The secreted IL-17E was known to bind to receptors viz., IL-17RA and IL-17RB (IL-25R) and activates the NF $\kappa$ -B pathway and it is involved in Th2 cell promoting cytokine [13]. IL-25 drives and induces the production of other cytokines including IL-4, IL-5 and IL-13 in multiple tissues, thereby contributing to allergic diseases and stimulates the expansion of eosinophils. In biological system, the drawbacks of IL-17E include symptoms of allergic asthma, such as eosinophil infiltration, airway hyper-reactivity and high circulating levels of IgE [14]. Even though the understanding of IL-25 was increasing progressively, nevertheless regulation of IL-25 was still unclear.

In the pathogenesis of autoimmune diseases, IL-25 and IL-17 seems to play opposing roles. In recent studies, a novel anti-inflammatory role of IL-25 as a key factor in the attenuation of IL-17 mediated inflammation, such as in colitis, encephalomyelitis and diabetes mellitus was studied [15]. IL-25 can kill some types of breast cancer cells and its expression studies were mostly carried out in prokaryotic hosts [16]. IL-25 produced in *E. coli* was a homo-dimeric, non-glycosylated polypeptide chain containing 146 amino acids with a molecular weight of 33.7 kDa. The expressed human IL-25 was purified using proprietary chromatographic techniques. Progressive research on IL-25 in the control of specific immune responses was recognized. IL-25 can promote the immune responses that cause allergic diseases and attenuate the detrimental inflammation associated with debilitating human pathologies, such as IBD, diabetes and multiple sclerosis (MS).

The dual role of IL-25 seems to be linked its ability to control the function of various immune and non-immune cells with the downstream effect of either amplifying Th2-immunity or suppressing Th1/Th17 cell responses [17]. Further experiment trails was needed to confirm these novel observations and also the therapeutic approaches targeting IL-25 are useful in the management of immune-inflammatory disorders. Meanwhile, we can speculate that blockade of IL-25 could help facilitate the resolution of the inflammation associated with asthma and allergy, but paradoxically the same therapeutic strategy could enhance the risk of developing/exacerbating autoimmune diseases. By contrast, IL-25 based therapies aimed at dampening Th1/Th17 driven inflammation can exacerbate the course of allergic disorders [18].

*E. coli* is one of the most widely used host for the production of heterologous recombinant proteins because of its well known genetic nature and it was better characterized than any other micro organisms, even though it contains drawbacks like post translational modifications. Notable differences were found in codon usage of prokaryotes and eukaryotes, especially for genes encoding recombinant proteins. Therefore, codon bias is often a serious problem while expressing eukaryotic proteins in prokaryotic hosts. Reports suggest that clusters of codons viz., AGG/AGA, CUA, AUA, CCA or CCC can reduce the quantity and quality of the protein. The low expression of some eukaryotic genes explained either by the limited pool of the t-RNA or by the competition of these clusters with the Shine-Dalgarno (SD) sequence. Lot of reports suggest codon bias helps in increasing the expression levels of eukaryotic genes in *E. coli*, sometimes up to 40-fold.

IL-25 gene sequence was codon optimized by replacing the translational bottleneck codons using *E. coli* preferred codons. In this study an attempt was made to express the human Interleukin 25 in prokaryotic expression host *E. coli* using industrially feasible vector pET28<sup>at+</sup>. The primary advantage of this system is the presence of T7 RNA polymerase on pET28<sup>at+</sup>, which helps in improving the transcriptional efficiency.

## 2. Materials & Methods

### 2.1. Media, Bacterial Strains and Plasmid

Media viz., Luria Bertani (Tryptone – 10 g/L, Yeast extract – 5 g/L, and Sodium chloride – 5 g/L), Terrific Broth (Yeast extract – 24 g/L, Tryptone – 12 g/L, Glycerol – 0.4%, KH<sub>2</sub>PO<sub>4</sub> – 2.31 g/L, and Na<sub>2</sub>HPO<sub>4</sub> – 12.54 g/L) and M9 media (K<sub>2</sub>HPO<sub>4</sub> – 6 g/L, KH<sub>2</sub>PO<sub>4</sub> – 3 g/L, NH<sub>4</sub>Cl – 1 g/L, Yeast extract – 5 g/L, Dextrose – 5 g/L, 1M MgSO<sub>4</sub> – 2 mL, TMM – 1 mL (Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>. 7H<sub>2</sub>O – 10 mg/L, CuSO<sub>4</sub>. H<sub>2</sub>O – 2 mg/L, H<sub>3</sub>BO<sub>4</sub> – 1 mg/L, MnCl<sub>3</sub>. 4H<sub>2</sub>O – 20 mg/L, NiCl<sub>2</sub>. 6H<sub>2</sub>O – 1 mg/L, Na<sub>2</sub>MoO<sub>4</sub>. 2H<sub>2</sub>O – 50 mg/L, ZnSO<sub>4</sub>. 7H<sub>2</sub>O – 50 mg/L, FeSO<sub>4</sub> – 50 mg/L) were used in the present study. All media ingredients were analytical grade and were procured from Merck India. The maintenance host *E. coli* DH5 $\alpha$  and expression host *E. coli* BL21(DE3) were purchased from MTCC, Chandigarh, India. The expression vector pET28<sup>+</sup> and kanamycin were procured from Novagen. Custom oligos were procured from Sigma-Aldrich, Bangalore, India.

### 2.2. Gene Amplification

Human IL-25 gene was isolated from human cDNA as template using forward primer 5' **CGCGGATCC**ATGAGGGAGCGACCCAG 3' and reverse primer 5' **CCC**GAATTC****TTAGCCCATCACACGGGG 3'. The PCR program was set to be initial denaturation at 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 61 °C for 1 min, 72 °C for 1 min and final extension of 72 °C for 10 min. The amplified wild type sequence was used as template to have codon optimized sequence.

Nested PCR was carried out using six oligos designed for the synthesis of codon optimized gene of human IL-25 and the oligos were designed to have an overlap of 10 to 12 nucleotides. The sequences of the codon optimized oligos are tabulated below.

Primer	Sequence
FP – 01 94 MER	5'CGCGGATCCATGTACTCTCACTGGCCGTCTTGCTGCCCGTCTAAAGGTCAGGACACCTCTGAAGAAGTCTGCTGCGTTGTCTACCGTTCCGGTTC3'
RP – 01 72 MER	5'CGTCTTCAGACGCACGGCAAGATTCGGGTGACGGTTCGGACGCGCCGGTTCAGCGGCGGAACCGGAACGG3'
FP – 02 92 MER	5'CGTCTGAAGACGGTCCGCTGAACTCTCGTGCGATCTCTCCGTGGCGTTACGAACTGGACCGTGACCTGAACCGTCTGCCGACGACCTGTAC3'
RP – 02 84 MER	5'AGAGTTACCACGCGGGTCCATGTGAGAACCGGTCTGCAGAGAAACGCAGTGCAGGACAGGCAACGCGCGTGGTACAGGTCCTG3'
FP – 03 98 MER	5'CGTGGTAACTCTGAACTGCTGTACCACAACCAGACCGTTTTCTACCGTCCGTGCCACGGTGAAAAAGGTACCCACAAAGGTTACTGCCTGGAACG3'
RP – 03 79 MER	5'CCC <b>GAATTC</b> TTAACCCATAACACGCGGACGAACGCAACGCACGCCAGAGAAACACGGTACAGACGACGTTCCAGGCAG3'

PCR was performed in two steps. In the first step, a template was generated by nested PCR with six oligomers by mixing all the oligomers in equal ratio using phusion DNA polymerase (pfu polymerase) and dNTPs. The mixture was incubated for 30 min in thermal cycler, preheated to 60 °C. The second step of PCR was to amplify the template with complete codon

optimized sequence using the gene specific forward and reverse primers having restriction sites for *Bam*HI and *Eco*RI at their 5' end (<http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=168927>). These two sites were used to introduce the gene into pET28<sup>+</sup> vector. PCR was carried out using the initial denaturation at 94 °C for 1 min, 25 cycles at 94 °C for 40 sec, 55 °C for 45 sec and 72 °C for 1 min and final extension of 72 °C for 5 min.

### 2.3. Construction and Expression of Recombinant Human IL-25

The codon optimized gene was purified using Qiagen Gel extraction kit method according to manufacturer instruction and digested with *Bam*HI and *Eco*RI. Desired fragment was gel eluted using standard protocol described in Sambrook *et al.*, 1989 [19]. Ligation of desired construct with double digested pET28<sup>+</sup> was carried out using T<sub>4</sub> DNA ligase. The recombinant plasmid was further transformed into maintenance host *E. coli* DH5 $\alpha$ . Ligation was confirmed using ligation confirmation PCR with gene specific forward and vector specific reverse primer and followed by DNA sequencing.

The host cell capability to express the proteins in soluble fraction was also depends on the total metabolic load. In general, soluble form of protein was achieved using the factors like expression at low temperature, genetically modified *E. coli* strains, modification of media composition and co-expression of molecular chaperones.

### 2.4. Inducer Concentration

*Escherichia coli* BL21(DE3) competent cells were transformed with recombinant plasmid [pET28<sup>+</sup>/IL-25]. The culture was grown overnight in a medium containing antibiotic [at a concentration of 50  $\mu$ g/mL] on a rotary shaker at 37 °C. The culture was used for the inoculation in LB medium. These cultures were grown at 37 °C until OD<sub>600</sub> reaches to 0.8 – 1.0. Culture was induced with different concentrations of IPTG viz., 10 mM IPTG, 1 mM IPTG, 0.1 mM IPTG, 0.01 mM IPTG and 0.001 mM IPTG respectively and was allowed to incubate at 37 °C for 5 hours. After incubation time, samples were collected and analyzed on 12 % SDS-PAGE.

### 2.5. Media Optimization

The effect of media composition on expression of rhIL-25 was confirm using different media, viz. LB, TB and M9 having the antibiotic concentration as described above [20]. Induction was carried out using 100  $\mu$ M IPTG, when OD<sub>600</sub> reaches to 0.8 – 1.0. Post induction samples were collected and analyzed on 12 % SDS-PAGE.

### 2.6. Induction Temperature

Protein expression in *E. coli* growing at low temperature has demonstrated its success in improving the solubility of a number of difficult proteins [21]. Generally, expression at low temperature conditions leads to the increased stability and correct folding patterns, which is due to the fact that hydrophobic interactions determining inclusion body formation are temperature dependent. Apart from this, any expression associated toxic phenotype observed at 37 °C incubation conditions, gets suppressed at lower temperatures. It has been observed that heat shock proteases induced during over-expression are poorly active at lower temperature conditions. Therefore, growth at a temperature range of 15–23 °C, leads to a significant reduction in degradation of the expressed protein.

## 2.7. Purification of Inclusion Bodies

Cells were harvested by centrifugation 6000xg for 10 min. Sometimes, media components were interfere with downstream process. In the process of purification, 1 L of cell pellet was washed in TN buffer (25 mM Tris-HCl [pH 7.4], 0.14 M NaCl). After complete resuspension, the cells were subjected to centrifugation at 6000xg for 10 minutes. The pellet was resuspended in lysis buffer (25 mM Tris-HCl [pH 7.4], 0.14 M NaCl, 2 mM  $\beta$ -mercaptoethanol). Cells were lysed by sonication. The cell lysis was confirmed by OD<sub>600</sub> and turbidity of the solution. The crude lysate was centrifuged at 12000 X g for 30 min at 4 °C. After centrifugation the inclusion bodies containing pellet was used to purify the protein.

The inclusion bodies were washed using resuspension buffer (20 mL of Tris-HCl [pH 7.4]) containing SDS (0.03%) to remove the impurities and cell debris. After resuspension, the solution was centrifuged at 12000 X g for 15 minutes at 4 °C. Washing was repeated twice and re-suspend the pellet in 30 mL of denaturation buffer (25 mM Tris-HCl [pH 7.2], 5 M Urea, 20 mM  $\beta$ ME and 200 mM NaCl). For complete denaturation, the sample was stirred at slow rpm (~ 50) at 4 °C for 1 hour.

## 2.8. Refolded of rhIL-25

Refolding was carried out at 4 °C for about 6 hrs using rapid dilution method. Before addition, the protein concentration was measured at a final concentration of 1 mg/mL. Addition was carried out with the help of very thin needle into 600 mL of re-suspension buffer (25 mM Tris-HCl [pH 7.2], 200 mM NaCl, 10 % Glycerol, 1 mM GSH, 10 mM GSSG, 0.5 M arginine and 2 mM EDTA). The protein sample after refolding was filtered by 0.45  $\mu$  filter using vacuum to remove any visible precipitates. Further protein purification was done by anion exchange chromatography and IMAC. The protein sample was loaded on 5 mL of DEAE chromatography in gravity based column. The filtrate was washed with 50 mL of 25 mM Tris-HCl [pH 7.2] and 200 mM NaCl buffer. Protein was eluted with 25 mM Tris-HCl [pH 7.2] and 400 mM NaCl buffer.

The eluted fraction further purified by 2 mL of Ni-NTA chromatography (Ni-NTA Resin from QIAGEN) loaded on to Ni-NTA buffer, pre equilibrated with 25 mM Tris-HCl [pH 7.2], 400 mM NaCl buffer. After loading, column was washed with 25 mL of wash buffer 25 mM Tris-HCl [pH 7.2] and 200 mM NaCl buffer containing 20 mM imidazole. Later, protein was eluted using Ni matrix with 10 mL of 25 mM Tris-HCl [pH 7.2] and 200 mM NaCl buffer containing 200 mM imidazole. At this stage, 40-50% of total protein was loss in the form of precipitation.

## 2.9. Western Blot Analysis

The protein bands were separated on 12 % SDS-PAGE and the gel was trans-blotted onto a nitrocellulose (NC) membrane with a buffer containing 25 mM Tris- HCl, 192 mM glycine, and 20 % methanol (pH 8.3). Transfer was done for 2 hrs at 100 V. After transfer, the NC membrane was incubated in 15 ml PBST (phosphate- buffered saline 10 mM, Tween 20 - 0.05%) containing 5 % skimmed milk powder for 90 min. This step was to block additional protein binding sites. Following the incubation, the NC membrane was washed with TBST twice for 5 min. After washing, the membrane was incubated with 20 mL anti-Human IL-17E/IL-25 Antibody (monoclonal mouse IgG, 1mg/mL obtained from R&D systems, USA) for 2 hrs at room temperature with gentle shaking. The membrane was washed thrice for 10 min in 15 ml PBST and then incubated the membrane in 15 mL Goat anti-mouse-IgG polyclonal antibody conjugated to horseradish peroxidase (dilution 1:1000 obtained from R&D systems USA) at room temperature for 2 hrs with gentle shaking. Washing was

repeated as mentioned above. Bands were developed by adding 1X TMB-H<sub>2</sub>O<sub>2</sub> for localization (1mL of 10X TMB-H<sub>2</sub>O<sub>2</sub> in 9 ml of distilled water).

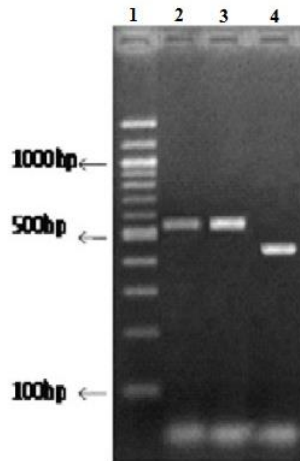
### **2.10. Biological Activity of rhIL-25**

The biological activity of rh IL-25 was determined by the amount of IL-6 released from IL-25 treated with PBMC (Peripheral Blood Mononuclear Cells). Various studies proved that IL-25 induces the production of IL-6. Dose dependent study was carried out using the human PBMC, by treating the cells with various IL-25 concentrations to analyze the IL-6 levels. Human PBMC were isolated from the fresh human blood. 10 mL whole blood was centrifuged at 900xg for 10 min at room temperature. Dilute remaining blood plasma to 30 mL in DPBS. 10 mL of ficoll hypaque solution was added to 50 mL Falcon tube. Gently overlay diluted blood on top of ficoll hypaque. Spin tubes 400xg for 30 min at room temperature. Collect PBMC from inter phase of PBS/ficoll with a transfer pipette and transferred to new 50 mL tube and add 5 mL RBC lysis solution to pellet and incubate with mixing for every 5 min at room temperature. Dilute the cells to 10 mL with DPBS and centrifuge at 240xg for 8 min at room temperature. Decant supernatant and resuspend the cells in 10 mL DPBS. Count the cells with the haemocytometer and determine the cell viability. Resuspend the cells in 10 mL DPBS for final wash and centrifuge at 240xg, 8 min at room temperature. Transfer the cells to 1 mL cryovial and store in liquid N<sub>2</sub> or use directly for analysis. 5 x 10<sup>5</sup> PBMC cells were plated into 96 well plates in 100 µL of culture medium. Recombinant human IL-25 purchased from R&D systems, USA used as standard. PBMC cells were treated with various concentrations of human IL-25 to generate the proper standard curve. PBMCs were treated with descending concentrations viz., 1000 ng/mL, 500 ng/mL, 250 ng/mL, 125 ng/mL, 62.5 ng/mL, 31.25 ng/mL, 15.6 ng/mL, 7.8 ng/mL and 3.9 ng/mL of rh IL-25 for 72 hours. Sample was added to the cells in 100 µl of culture medium. After 72 hrs of incubation, the cells with the medium were collected, boiled for 2 minutes at 90 °C and then centrifuged at 2000 rpm for 5 minutes. Then the lysate was used to analyze the IL-6 levels by ELISA method using the human IL-6 ELISA kit from Ray Biotech. The minimum detectable dose of IL-6 is < 3 pg/ml.

## **3. Results & Discussion**

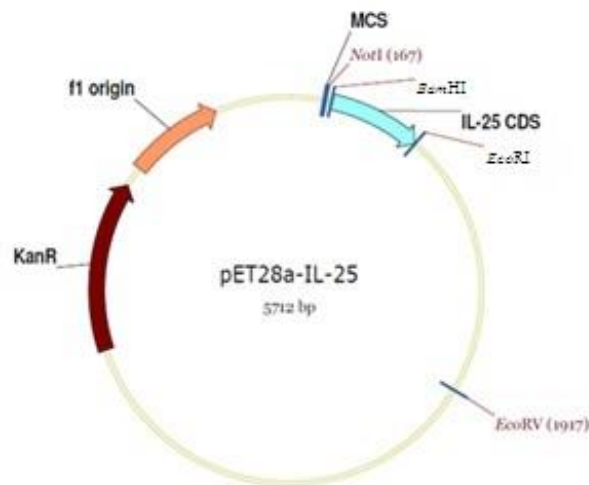
### **3.1. Construction of Plasmid pET28<sup>+</sup>/IL-25**

The human IL-25 gene (441 bp) was amplified using nested PCR and was column purified using the Qiagen PCR purification kit. The purified human IL-25 gene was confirmed on 1.2 % agarose gel against low range DNA ladder (Figure – 1). The digested gene was ligated into the MCS region of pET28<sup>+</sup> vector. The recombinant plasmid was showed in the figure – 2. DNA Sequencing was done to confirm the nucleotide sequence identity with the expected sequence. Alignment of amino acids sequence with various genbank sequences was done confirmed the 100 % identity at amino acid level.



- 1 = Low range DNA ladder
- 2 = Amplification of IL-25 at 60 °C (534 IL-25 along with signal peptide)
- 3 = Amplification of IL-25 at 63 °C (534 IL-25 along with signal peptide)
- 4 = Amplification of IL-25 at 63 °C (441 bp matured IL-25)

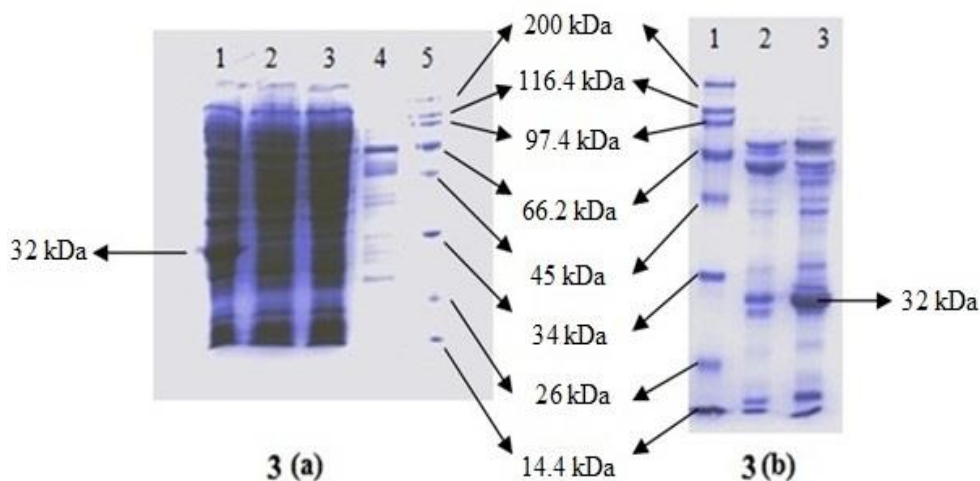
**Figure 1. PCR Amplification of Human IL-25 Gene**



**Figure 2. Recombinant Plasmid (pET28<sup>+</sup>/IL-25)**

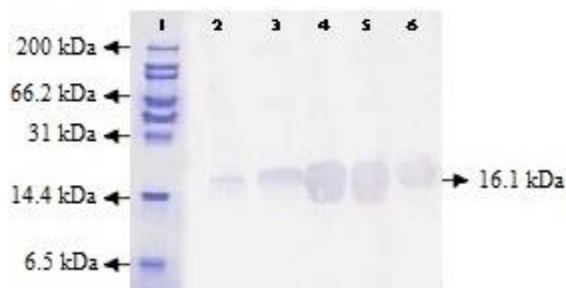
### 3.2. Expression of IL-25

For expression studies, IPTG inducible *Escherichia coli* BL21(DE3) was used. The rDNA IL-25/pET28<sup>+</sup> was extracted from DH5 $\alpha$  and transformed effectively into competitive BL21 (DE3) cells. Cells containing IL-25/pET28<sup>+</sup> plasmid was grown up to OD 0.8–1.0 and induced with 1 mM IPTG for 4 hrs at 37 °C. The recombinant protein expression profiles were resolved on 12 % SDS-PAGE (Figure – 3a). The IL-25 was visualized on the gel at the position corresponding to the standard protein. Western blot analysis was also confirmed the molecular weight of the rhIL-25 (Figure – 3b).



3 = SDS PAGE analysis of uninduced culture at 37 °C;  
4 = SDS PAGE analysis of uninduced culture at 20 °C;  
5 = Marker protein (200 kDa, 116.25, 97.4, 66.2, 45, 34, 26, 14.4 kDa).  
Figure – 3 (b): 1 = Marker protein (200 kDa, 116.25, 97.4, 66.2, 45, 34, 26, 14.4 kDa);  
2 = 6X histidine tag purification and refolding of IL-25 at 37 °C;  
3 = 6X histidine tag purification of IL-25 at 20 °C

**Figure 3. SDS PAGE Analysis of IL-25**



1 = Marker protein (200 kDa, 116.25, 97.4, 66.2, 45, 31, 14.4, 6.5 kDa).  
2 = Purified IL-25 (0.5 µg)  
3 = Purified IL-25 (1 µg)  
4 = Purified IL-25 (2 µg)  
5 = Purified IL-25 (3 µg)  
6 = IL reference protein (1.5 µg)

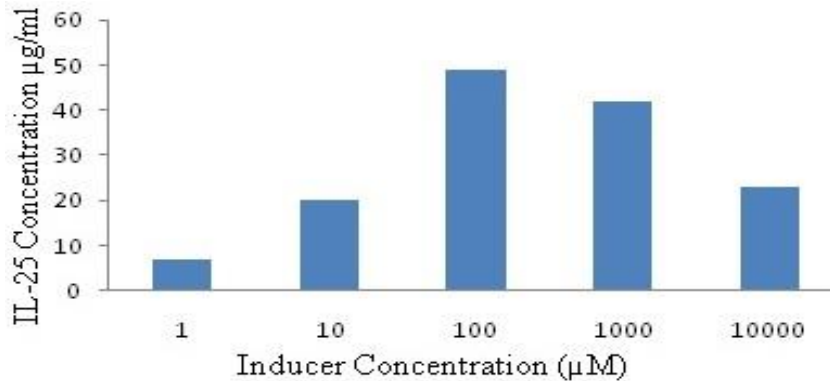
**Figure 4. Western Blot Image Showing the Immunoreactivity of Protein Compared with Reference Protein**

### 3.3. Effect of Inducer Concentration on Production of rhIL-25

Recombinant *E. coli* BL21(DE3) was grown in LB medium and then various inducer concentrations were used to know the optimal inducer concentration as mentioned in the material and methods. Expression studies of rhIL-25 was observed at two different post induction temperatures like 37 °C and 20 °C. Isopropyl β-D-1-thiogalactopyranoside (IPTG)



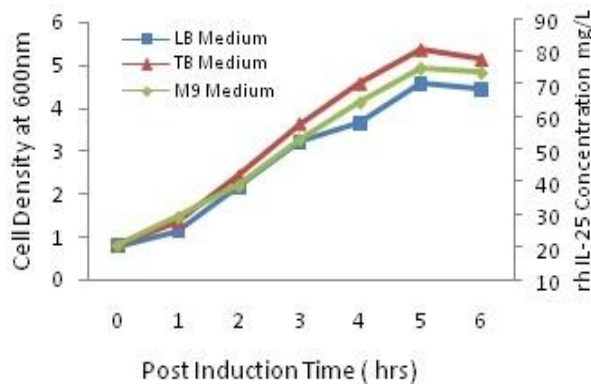
is a molecular mimic of allolactose, a lactose metabolite that triggers transcription of the lac operon. Unlike allolactose, the sulfur (S) atom creates a chemical bond which is non-hydrolyzable by the cell, preventing the cell from degrading the inductant; therefore the IPTG concentration remains constant. Post induction samples were analyzed using 12 % SDS-PAGE. 100  $\mu$ M IPTG produces 49 mg/L of rhIL-25 (Figure – 5). In some studies, 1 mM IPTG is enough to produce the recombinant proteins [22]. On the other hand, the production of IL-25 was 16 % higher when compared to the production at 1mM IPTG used as inducer.



**Figure 5. Expression Levels of rhIL-25 at Different Inducer Concentrations**

### 3.4. Effect of Media Composition on Production of rhIL-25

The recombinant *E. coli* BL21(DE3) cells were grown in various media viz., LB, TB and M9 medium, till the OD<sub>600</sub> reaches to 0.8 – 1.0. Then the cells were induced with 100  $\mu$ M IPTG. It was observed that OD and dry cell weight was peak at 5<sup>th</sup> hr of post induction and the expression of rhIL-25 was also observed maximum at 5<sup>th</sup> hour of post induction. The cell densities were measured at 600 nm. The cell densities after 5 hrs of post induction were determined as 4.56, 5.35 and 4.91 in LB, TB and M9 media respectively at 37 °C (Figure – 6). The expression levels of purified recombinant human IL-25 using LB, TB and M9 media were 51 mg/L, 86 mg/L and 61 mg/L respectively at 37 °C (Figure – 6), where the purified rhIL-25 production was very low (16.5 mg/L) at 20 °C because of soluble nature of protein. Compared with LB and M9 media, the expression was appreciable using TB medium.



**Figure 6. Cell Densities of Recombinant *E. coli* BL21(DE3) at Various Time Intervals in Different Media and the Expression Levels of rhIL-25 at 37 °C**

### 3.5. Effect of Post Induction Temperature on Production of rhIL-25

Most of the recombinant proteins were expressed in the form of inclusion bodies at 37 °C [22]. Induction at low temperatures is alternative method to improve the soluble expression using prokaryotes. Cell density was very low at 20 °C and the expression levels were slightly low when compared to 37 °C. The soluble form of protein was increased at 20 °C when compared with the induction at 37 °C.

On the other hand, total expressed proteins at 37 °C was comparatively high when compared to induction at 20 °C. (Figure-7). At 20 °C, 70 % of the expressed proteins were in soluble form. The overall expression levels of IL-25 at 37 °C was twofold higher when compared to the expression of soluble of protein at 20 °C.

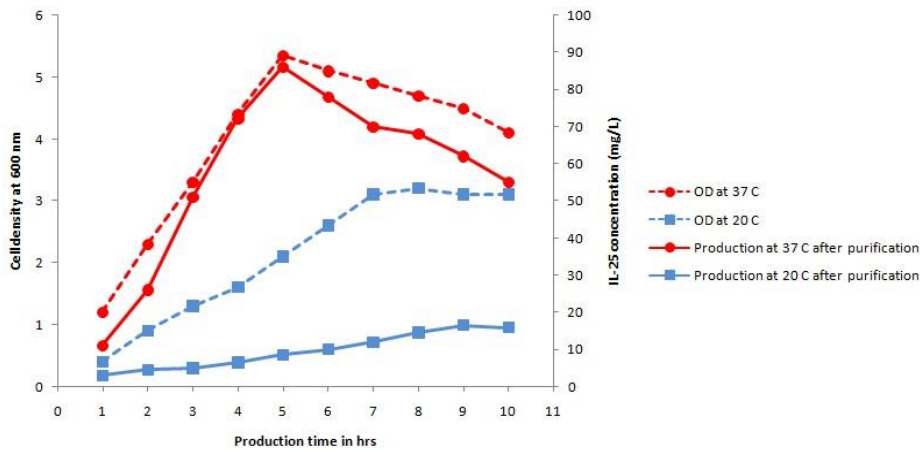


Figure 7. Growth Profile and Production of rhIL-25 at Different Temperatures

### 3.6. Biological Activity Assay

Biological activity of human IL-25 was tested as per the standard protocol using human PBMC. The IL-6 levels were estimated in the IL-25 treated cells were shown in the figure – 8. Correlation was observed between the IL-25 concentration and the secreted IL-6 levels.

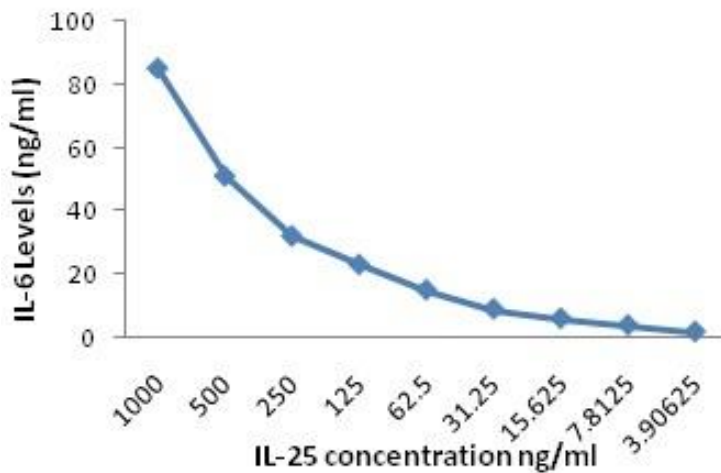


Figure 8. Estimation of IL-6 Levels Using PBMC Cells Treated with IL-25

## 4. Conclusion

In the pathogenesis of autoimmune diseases, IL-25 plays a critical role. Based on its importance, IL-25 has been successfully produced using prokaryotic host IPTG inducible *Escherichia coli* BL21(DE3). The aim of this study was to express the human IL-25 in IPTG inducible *E. coli* with enhanced expression levels. To enhance the yield, codon optimization method (replacement of less frequently used codons by the *E. coli* preferred codons) and vector having strong promoter for over expression of recombinant protein was desirable. In the codon optimized sequence approximately 48 % codons were replaced. Out of 177 codons, 85 codons were replaced with *E. coli* preferred codons. An additional parameter for improving expression was media composition and post induction temperature. It is the basic principle used to generate the high cell densities in optimum conditions. In this study, we obtained a final recovery of 35% in soluble form from inclusion bodies after refolding and purification. On the other hand, 67% of final recovery was achieved in soluble form devoid of refolding. The production levels were high when compared to production using *Pichia pastoris* strain X-33 [23]. This study will be useful to produce the rhIL-25 at large scale using cost effective process.

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