



# Cloning and Expression of Human Interleukin 2 (IL-2) in *E. coli* and its Antitumor Activity

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## ABSTRACT

Interleukin 2, also known as T cell growth factor, brought a great revolution in the field of immunology. IL-2 is a pleiotropic cytokine and one of the cytokines that is approved by FDA for cancer immunotherapy particularly kidney and skin cancer. Interleukin-2 (IL-2) is a 4-helix-bundle type I cytokine possessing a cytokine receptor chain essential for the immune response. The current study presents a vector/host combination (pET28a(+)/IL-2) and optimization of expression in LB, TB and M9 media with varying concentrations of inducers (IPTG and lactose) and post induction time. Maximum expression of IL-2 was achieved in LB with 0.5 mM IPTG for 6 hours post induction time. A denaturing purification scheme (Immobilized metal affinity chromatography) was employed for the purification of IL-2. The refolding of the purified IL-2 was achieved by stepwise dialysis method using urea gradient (8M-0M). Human IL-2 was recovered from 1 litre culture to a purity level of ~95%. In vitro cytotoxic potential of IL-2 on HepG-2 and MCF-7 cell lines revealed that it possess sufficient cytotoxic potential and can inhibit the growth of these cell lines directly. Thus, refolded IL-2 had activity identical to that of authentic IL-2 and enhanced the anti-tumor activity of HepG-2 as compared to MCF-7 cells. These conclusions suggest the potential use of the refolded cytokine as immunotherapeutic agent for treatment of hepatocellular carcinoma.

## Article Information

Received 22 August 2022

Revised 31 August 2022

Accepted 30 July 2022

Available online 15 February 2023  
(early access)

Published 12 April 2024

## Authors' Contribution

MS conceived the idea. QAA performed the experiments. SB helped in cytotoxic effect studies. HA helped in graphical presentation and write up. AT generated stable cell lines. RG supervised, reviewed and edited the manuscript. All the authors reviewed and approved the final manuscript.

## Key words

Interleukin 2, Antitumor activity, Cytotoxicity

## INTRODUCTION

It has long been established that immune system can be used to attack neoplastic cells directly. IL-2 was the first cytokine used for the treatment of cancer. IL-2 is a 15.5 kDa glycoprotein that is synthesized as 153 amino acid precursor and after processing converted to mature form composed of 133 amino acids. The mature IL-2 is comprised of four alpha helices. Mainly antigen-simulated CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, natural killer (NK) cells, and activated dendritic cells (DC) which produce IL-2 (Skrombolas and Frelinger, 2014). The major function of IL-2 is activation of CD4<sup>+</sup> T cells, CD8<sup>+</sup> T-cells as well as NK cells (Littman

and Rudensky, 2010). IL-2R is a trimeric complex which is composed of three subunits: IL-2R $\alpha$  (CD25), IL-2R $\beta$  (CD122), and IL-2R $\gamma$  (CD132). The trimeric complex has highest affinity as compared to dimeric and monomeric forms (Krieg *et al.*, 2010). There are many examples where IL-2 exhibited role in tumor regression, but its use is limited due to toxicity at high dose. It was found less effective in improving survival rate of patient at high dose (Jiang *et al.*, 2016). Holcar *et al.* (2015) have reported that human Treg cells from healthy controls and patients with type 1 diabetes are specialized to sense very low concentrations of IL-2; about tenfold high concentration of IL-2 is required for inducing phosphorylation of STAT5 and other lymphocytes. High doses of IL-2 were used for the treatment of cancer metastasis in mice. High dose can promote expansion of cytotoxic T cells and NK cells with very effective response in metastatic skin and kidney cancer (Rosenberg *et al.*, 2014).

The present study aimed at production of human IL-2 in *E. coli* expression system which may be used as immunotherapeutic agent either by inhibiting growth of tumor cells or as an adoptive therapy by activation of T cells.

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0030-9923/2024/0003-1263 \$ 9.00/0



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## MATERIALS AND METHODS

### *Cloning of IL-2 gene in pTz57R/T*

The gene specific forward and reverse primers having restriction sites (*EcoRI* and *XhoI*) at 5' end were designed using software Primer 3.

IL-2F: GAATTCATCATCATCATCATCATATGTACAGG

IL-2R: CTCGAGCTATCAAGTCAGTGTGAG

PCR amplification was performed under the following conditions: 1 cycle of 95°C for 3 min, 30 cycles of 95°C for 2 min, 65°C for 45s, and 72°C for 1 min., followed by a final extension of 72°C for 10min. The PCR products were purified using the Agarose Gel DNA Extraction Kit (cat. k0691) and ligated to pTz57R/T vector, followed by transformation into *E. coli* DH5 $\alpha$  competent cells. The positive recombinant clone was then selected using an Amp/IPTG/X-Gal agar plate. The recombinant plasmid was identified by PCR under the aforementioned conditions, digested with restriction enzymes *EcoRI* and *XhoI*, and fractionated on 1% agarose gel. DNA sequencing was also conducted.

### *Expression of IL-2 in E. coli BL21 codon plus cells*

Cloned plasmid i.e. pTz57R/IL-2 following double digestion with *EcoRI/XhoI* restriction enzymes was purified and ligated to similarly digested pET28a (+) to generate pET28a (+)/IL-2 expression vector. After the confirmation of positive transformants by restriction digestion and colony PCR, recombinant expression plasmids (initially maintained on *E. coli* DH5 $\alpha$  strain) were used to transform *E. coli* BL-21 (Codon Plus) strain. Small scale experiments were setup to optimize the effect of media conditions (LB, TB and M9), types of inducer (IPTG, Lactose) and post induction time on the expression of IL-2. For the large scale cultivation, 1 litre batch was setup and seeded with the primary inoculum and cultures were grown to exponential phase with good shaking conditions (150rpm) at 37°C.

To check soluble expression of *E. coli* BL-21 (Codon Plus) cells harbouring recombinant plasmid (pET28a(+)/IL-2) were harvested and disrupted by sonication in the lysis buffer (50mM TrisCl, 100 mM NaCl, 5mM EDTA and 1mM PMSF). Typically 1g wet cell mass was resuspended in 10 ml lysis buffer and shearing was performed on sonicator (Soniprep, Sanyo) by using 30  $\times$  30s bursts with 30s pause between two successive pulses. Molecular mass and % expression were analysed on 15% SDS gel followed by densitometry scanning of the gel using ImageJ software and Syngene gel documentation system (Syngene, UK).

### *Purification of IL-2 by Ni<sup>+</sup> affinity chromatography*

As IL-2 was expressed as IBs so a denaturing IMAC

(Immobilized metal affinity chromatography) protocol was employed for the purification. The IBs from the insoluble fractions were first washed with 0.1% Triton X-100 followed by two successive washings each with Tris-HCl buffer (pH: 8.0) and distilled water to remove the traces of detergent completely. Washed IBs were solubilized in the solubilisation buffer (50mM Tris.Cl pH:8.0, 1mM EDTA pH:8.0, 100mM NaCl, 8M urea, 1mM PMSF). Typically, the IBs pellet from 1 g dry cell weight was solubilized in 100 ml solubilisation buffer and incubated at 37°C for 10-12 h with gentle shaking. A clear supernatant was obtained by centrifugation (6,500 g, 4°C for 25 min). To purify IL-2, denaturing IMAC protocol was followed and the column was pre-equilibrated with 50mM Tris-HCl pH 8.0, 100mM NaCl.

### *Refolding of IL-2 by stepwise dialysis*

The refolding of the purified protein was conducted using dialysis tube (12 kDa pore size) by dialyzing gradient from 6 M-0M urea solutions in Tris-HCl buffer (pH 8.0) at 4°C mini cold lab. Dialyzed sample was lyophilized and resuspended in Tris-HCl (pH 8.0). The protein was quantified by using the Bradford assay with bovine serum albumin (BSA) as standard and NanoDrop spectrophotometer (Thermo Scientific) according to the manufacturer's instructions. The purified proteins were stored at -20°C for further analysis.

### *Western blotting*

In order to check the specificity and stability of the purified and refolded protein, western-blot analysis of IL-2 was performed. Briefly, the purified IL-2 was separated by 15% SDS-gel and transferred on nitrocellulose membrane at 25V for 1.5 h. Non-specific binding sites of the membranes were blocked by incubating for 1 h at 37°C in 1X PBS (containing 5% skim milk). Then the membrane was incubated with mouse anti-His-Tag antibody (Abcam) diluted 1:10,000 in 1 x PBS (containing 5% skim milk) for 2 h at 37°C. After washing 3 times with 20 ml 1X PBS, 5ul of mouse alkaline phosphatase conjugated IgG secondary antibody in 20 ml 1X PBS (containing 5% skim milk) was poured and incubated at 37°C for 1 h with continuous shaking. After washing off an unbound secondary antibody, the specific antigen-bound antibody was visualized using BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/ nitro-blue tetrazolium) substrates for 5 to 15 min. As the colour appeared the reaction was terminated by rinsing with distilled water.

### *In vitro growth inhibition assay for IL-2*

Cells ( $1 \times 10^4$  cells/cm<sup>2</sup>), with good morphological characters, were plated in 96 well plates. When properly

attached in monolayers, they were seeded with fresh medium, treated with different concentrations of IL-2 (250-10,000 ng/ml) and incubated for 48 h at 37 °C in a humidified chamber containing 5% CO<sub>2</sub>. Post-treatment cells were harvested by brief incubation (1–2 min) with Trypsin/EDTA, washed with 1 × PBS and resuspended in fresh medium contained in 96-well plates. The cell proliferation/viability was assessed by cell counting on Neubauer chamber hemocytometer using Eclipse TS 100 inverted microscope (Nikon, USA) and also by estimating the reduction of 3-(4,5Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) to soluble formazan crystals followed by absorbance measurements at OD<sub>570</sub>, as per the manufacturer's instructions (Thermo Fisher Scientific, USA). The graph was plotted between the viability % and the different concentrations of IL-2. Cultures for morphological changes were monitored by a phase contrast microscope after 24 and 48 h, and images were recorded. All experiments with cell lines were performed at least in triplicates and the results presented are as mean ± standard deviation (SD).

The viability % was calculated according to the formula:

$$\text{O.D. of treated cells} / \text{O.D. of control} \times 100$$

Where control=O.D. of cells without treatment.

A curve was prepared to check the cytotoxic effect of IL-2.

#### Statistical analysis

GraphPad Prism 5 was used to analyze the data statistically. To calculate LC<sub>50</sub>, log dose-response were assessed using non-linear regression with variable slope. The results were shown as mean± standard deviation.

## RESULTS

#### IL-2 gene clone in expression vector

Amplification of 492bp band of IL-2 was attained at 62°C and 3 mM MgCl<sub>2</sub> (Fig. 1A). The PCR amplified and gel purified IL-2 was ligated into pTz57R/T. Competent cells of *E. coli* DH5α cells were transformed with recombinant DNA which was later confirmed by colony PCR, restriction analysis (Fig. 1B) and sequence analysis showing 99.1% similarity with the IL-2 (Accession No. BC066257). IL-2 gene was later sub cloned in expression vector pET28a (+) (Fig. 1C) and confirmed by restriction analysis (Fig. 1D).

#### Expression of IL-2 gene in *E. coli* BL21 codon plus cells

##### IPTG Induction

BL21 codon plus cells with expression vector (pET28a

(+)/IL-2) were induced with 0.5mM IPTG for 2, 4, 6, 8 and 10 h (Fig. 2A). Maximum expression was attained after 6 h in LB having ~20% expression of the total cell protein. In TB media, maximum expression was attained after 10 h with ~7.5% expression of the total cell protein and in M9 media maximum expression was obtained after 4 h of induction with ~3.1% expression of the total cell protein as determined by densitometric scanning of gel.

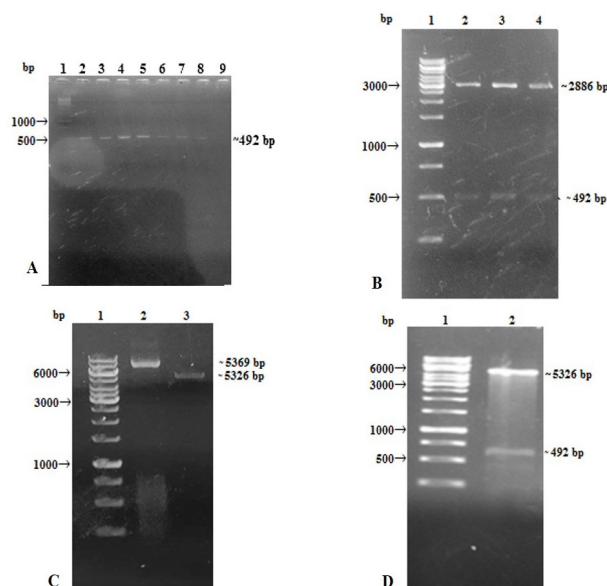


Fig. 1. Cloning of IL-2 gene in expression vector. A, amplification of IL-2 gene by PCR with different MgCl<sub>2</sub> conc. and temperature gradient, Lane 1: 1kb DNA ladder, Lane 2-5: 1.5, 2.0, 2.5 and 3.0mM MgCl<sub>2</sub> at 62°C, Lane 6-9: 1.5, 2.0, 2.5 and 3.0 mM MgCl<sub>2</sub> at 65°C. B, restriction digestion of pTz57R/IL-2 recombinant plasmid, Lane 1: 1kb DNA ladder, Lane 2-4: Double digested plasmid with *EcoRI/XhoI* restriction enzymes. C, restriction digestion of pET28a(+) isolated from DH5α, Lane 1: 1kb DNA ladder, Lane 2: Uncut pET28a(+), Lane 3: Cut pET28a(+) by using *EcoRI* and *XhoI* restriction enzymes. D, restriction digestion of recombinant pET28a (+)/IL-2, Lane 1: 1kb DNA ladder, Lane 2: Restriction digestion showing two fragments i.e. ~5326 bp pET28a and ~492bp IL-2.

BL21 codon plus cells bearing expression vector (pET28a+)/IL-2) were induced with varying concentrations of IPTG (0.1, 0.3, 0.5, 0.7 and 0.9 mM) (Fig. 2B). In LB, maximum expression was obtained with 0.5mM IPTG having ~20% expression of the total cell protein. In TB media, maximum expression was attained with 0.1mM IPTG with ~13.2 % expression of the total cell protein. In case of M9 media, maximum expression was attained with 0.1mM IPTG concentration with ~5.2% expression of the total cell protein.

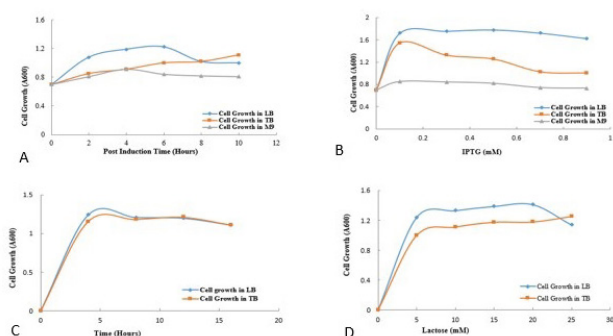


Fig. 2. Effect of different parameters on growth of *E. coli* (BL21 codon plus) cells transformed with pET28a (+)IL-2. A, Effect of post induction time after induction with 0.5mM IPTG in different growth media. B, Effect of IPTG conc. in different growth media. C, Effect of time in different auto inducing media after induction with 5mM Lactose. D, Effect of lactose concentration in different auto-inducing media after induction with 5, 10, 15, 20 and 25mM Lactose concentrations, the curves represent growth patterns in different media, ♦ (LB), ■ (TB) and ▲ M9.

#### Lactose induction

In order to optimize expression of IL-2 in BL21 codon plus cells, concentration of lactose was also optimized in different media. For optimization of time, 5mM lactose was added into all culture media (LB and TB) and harvested after 4, 8, 12 and 16 h (Fig. 2C). During optimization of time in LB, maximum expression was attained after 4 h induction and expression at that time was ~5.5% of total cell protein. Effect of time with lactose on expression of IL-2 in TB medium resulted in maximum expression after 12 h with ~4.5% expression of total cell protein. Optimization of concentration of lactose in LB media resulted in maximum expression at 20mM lactose concentration with ~7% expression of the total cell protein. Optimization of lactose concentration in TB media for gene expression indicated that maximum expression was attained at 25mM with ~7.5% expression of the total cell protein (Fig. 2D). Optimization of time and concentration of lactose in M9 medium showed no significant expression.

#### Western blot analysis

To confirm the presence of recombinant IL-2 protein, Western blot analysis was performed. A ~17kDa band confirmed the presence of recombinant IL-2 by using anti His tag antibody as shown in Figure 3.

#### IL-2 purified by immobilized Ni<sup>2+</sup> affinity

IL-2 protein showed 20% optimized expression upon 6 h induction at 0.5mM IPTG concentration in LB medium. 1 liter culture media was used for purification.

Upon sonication most of the protein was found as inclusion bodies so a denaturing immobilized metal affinity chromatography (IMAC) protocol was followed. Denaturation was carried out in the presence of 8M urea.

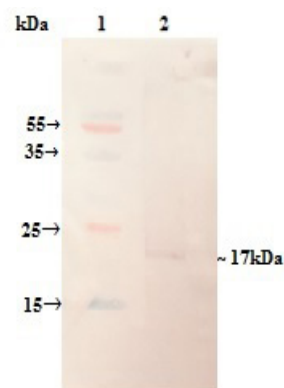


Fig. 3. Western blot analysis of IL-2 expression in *E. coli* (BL21 codon plus). Lane 1: Pre-stained protein marker, Lane 2: Pellet.

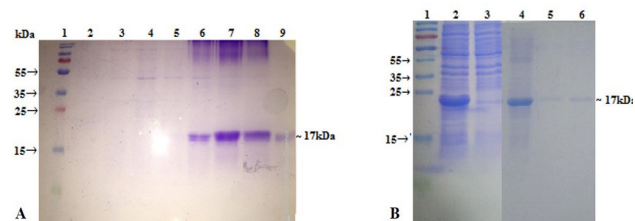


Fig. 4. 15% SDS gel showing. A, fractions of denaturing immobilized metal affinity chromatography (IMAC); Lane 1: Pre-stained protein marker, Lane 2-9: 50- 500mM fractions. B, stages of purification of IL-2; Lane 1: Pre-stained Protein marker. Lane 2, Pellet; Lane 3, Supernatant; Lane 4, Solubilized inclusion bodies (IBs); Lane 5, IMAC purified IL-2; Lane 6, Renatured IL-2 after dialysis.

**Table I. Summary of percentage recovery of recombinant IL-2 at every step of purification.**

Step	Total protein (mg/L)*	Recovery (%)	Purity (%) <sup>b</sup>
Crude	482	100	30
Solubilized IBs	150	31	25
IMAC purified	78	16	80
Purified renatured protein	30	6	95

<sup>a</sup> Protein concentration was determined by absorbance measurements at OD<sub>280</sub>. <sup>b</sup> The %age purity was determined by densitometry analysis of 15% SDS-gel.

Inclusion bodies obtained after sonication were washed with Triton X-100, solubilized and denatured with

8M urea and then purified by Ni<sup>2+</sup> affinity chromatography under denaturing conditions. 50-350 mM imidazole dilutions were used for the elution of protein and all the fractions were analyzed on 15% SDS PAGE as shown in Figure 4A. Fractions 6, 7 and 8 were pooled and dialyzed against 20mM TrisCl buffer (pH 8.0) for gradual removal of denaturing agent. The samples at every purification step were analyzed on 15% SDS PAGE (Fig. 4B). IL-2 was purified to a purity of ~95% with ~6% recovery (Table I).

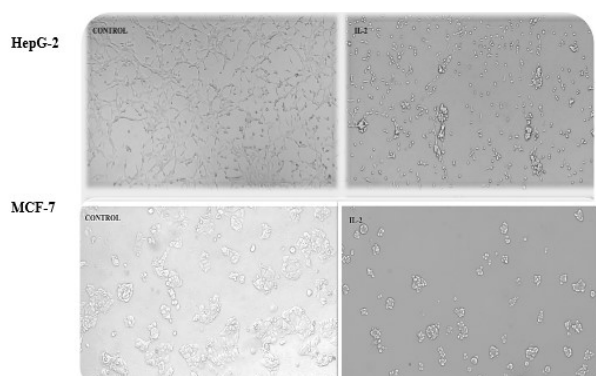


Fig. 5. Change in morphology of HepG-2 and MCF-7 cells after treatment with varying concentrations of IL-2 (250ng-10,000ng) for 48 h. (Magnification=10X, Resolution= 200um).

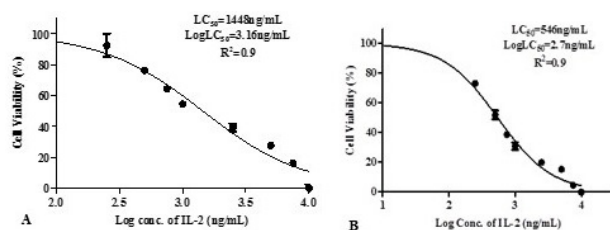


Fig. 6. MTT assay results showing. A, viability (%) of HepG2 cell lines after treatment with varying concentrations of IL-2 (250ng-10,000ng) for 48 h. B, viability (%) of MCF-7 cell lines after treatment with varying concentrations of IL-2 (250ng-10,000ng) for 48 h.

#### Growth inhibition by IL-2

To analyse the anti-tumour activity of the purified IL-2, different dilutions ranging 250-10,000ng were applied on HepG-2 and MCF-7. MTT cell viability assay was performed. Microscopic observation after 48 h indicated change in morphology of HepG-2 and MCF-7 cells after treatment with IL-2 (Fig. 5). MTT assay results revealed a remarkable decrease in the viability of HepG-2 cells (Fig. 6A) and MCF-7 (Fig. 6B) with increasing concentrations of IL-2. The LC<sub>50</sub> values calculated from MTT assay

were 1448ng/mL and 546ng/mL for HepG-2 and MCF-7, respectively.

## DISCUSSION

The cytokines discovered so far are Chemokines, Interleukins, Interferons, Lymphokines and Tumour necrosis factors (Turner *et al.*, 2014). Interleukin 2 is an immunomodulatory cytokine that belongs to type I, common  $\gamma$  chain receptor family that induce cell apoptosis either directly or via activation of T lymphocytes that kill cells by apoptotic mechanism. For the efficacy of T-cell based therapy, there should be sufficient number of activated T cells that respond to tumour cells. But therapeutic use of IL-2 in high dose is limited due to activation of Treg cells (Ahmadzadeh and Rosenberg, 2006).

IPTG is required for the induction of expression with T7 promoters. IPTG can influence cell mass as well as yield of the protein (Grabski *et al.*, 2005). Induction of expression in some proteins is slow with lower IPTG concentrations while others require high IPTG concentrations (Sambrook and Russell, 2001). Expression of IL-2 protein was optimized by using different concentrations of IPTG at different time intervals. The recombinant protein selected in this study showed maximum expression levels of 20% (0.5mM IPTG) for IL-2 in LB media. According to previous studies, maximum expression of mouse IL-2 was achieved with 0.2mM IPTG (Abdi *et al.*, 2019).

IPTG, due to its high cost and toxicity is not suitable for large scale production of therapeutic proteins (Jana and Deb, 2005). So, lactose was used as inducer for protein expression. During optimization of expression of IL-2 by auto induction in different media; maximum expression level of IL-2 was 7.5% (25mM lactose) in TB.

In this study, the cells were disrupted by sonication and conditions were optimized for maximum lysis of cells. One problem with heterologous proteins is that they are unable to fold properly and expressed in the form of inclusion bodies (Marston, 1986). These inclusion bodies could be covalent or non-covalent due to the presence of three cysteine residues (Sengupta *et al.*, 2008). In case of IL-2, the protein was expressed as aggregates that depicted that the protein was not folded properly.

Conditions were optimized to get maximum soluble proteins. The IBs were extracted from *E. coli* and contaminants were removed. The purified IBs contained 31% of ~17kDa IL-2 protein as determined by densitometry scanning of SDS gel. Solubilization of the IBs was achieved by chaotropic agent like 8M urea that is a critical step to get maximal amount of expressed proteins without any degradation. Solubilization of the recombinant proteins in SDS, CTAB and sarkosyl is already reported

(Sengupta *et al.*, 2008).

The solubilized proteins were subjected towards purification and then refolding. The protein purification techniques developed so far are required for biotechnology advancements. Protein purification techniques range from one step purification to multiple procedures (Ahmed, 2011). In the current study protein purification was achieved by nickel affinity chromatography under denaturing conditions followed by refolding by stepwise dialysis, and finally lyophilisation.

Refolding is a sensitive process by which excess denaturing agent is removed in a controlled manner so that protein can attain its native conformation. There is not a defined protocol of refolding for all the proteins. The most commonly used methods for proper refolding of the proteins are dialysis, gel filtration and immobilization to a solid support (Singh and Panda, 2005). Following purification and refolding 95% pure IL-2 was obtained. These results were comparable to mrhIL-2 purified from *Pichia pastoris* with ~97% purity (Ahmed, 2011). The purity and integrity of the proteins were analysed by 15% SDS gel. Western blotting was used for confirmation using anti-His Tag antibody. Overall recovery for IL-2 was 6%. The yield of IL-2 protein as determined by Bradford reagent assay was 48mg/L. Previous results reported 3mg/L recombinant human IL-2 expressed in *E. coli* (Sengupta *et al.*, 2008).

Human cancer cells were found to express proteins for IL-2 and IL-2R constitutively *in vivo* and *in vitro* and growth inhibition was observed by delivery of exogenous IL-2 in high dose (Reichert *et al.*, 2000). Therapeutic potential of purified protein (IL-2) was evaluated against tumor cell lines i.e., HepG-2 and MCF-7 using *in vitro* cytotoxicity assay. To check the cytotoxic effect, varying concentrations of purified IL-2 were used ranging from 250ng-10,000ng. IL-2 exerted a prominent decline in the viability of cells and at 10,000ng decline was at its peak and the effect was higher in HepG-2 as compared to MCF-7 cells. According to previous findings, IL-2 can reduce the proliferation, viability and mobility of HepG-2 cells *in vitro* (Ji *et al.*, 2018).

## CONCLUSION

The study demonstrated that the recombinant plasmid could express IL-2 in *E. coli* expression system effectively. The purification of expressed protein was accomplished by Ni<sup>2+</sup> affinity chromatography by denaturing method and successfully refolded by dialysis. IL-2 was purified from 1Liter culture with 95% purity. The cytotoxic activity of IL-2 on HepG-2 and MCF-7 cell lines showed that protein possess prominent cytotoxic effect at high dose and this

cytotoxic effect is higher in HepG-2 as compared to MCF-7 cells.

## ACKNOWLEDGMENT

Authors are grateful to Dr. Asima Tayyab (SBS, PU) for permission to use their cell culture laboratory and for her help in cell culturing experiments.

### Funding

The study received no external funds.

### Ethical statement

This article does not contain any studies with human participants or animals, performed by any of the authors.

### Statement of conflicts of interest

The authors declare no conflicts of interest.

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