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Increased yield of high purity biologically active recombinant human granulocyte-macrophage colony-stimulating factor from *Escherichia coli*

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Abstract

Human Granulocyte-Macrophage Colony-Stimulating Factor (hGM-CSF) is being produced by recombinant DNA technology and has been used as a therapeutic protein for different conditions. Different strategies have been reported to express and purify therapeutic grade hGM-CSF in different host systems including *Escherichia coli*. However, the reported yields are comparatively low and involve multiple steps of purification. In this present paper, we report a novel method of periplasmic secretion system for high expression, with relatively simple purification method using reversed phase chromatography and refolding technique. The final purity of the rhGM-CSF was more than 98 % with increased yield of ~300 mg per 35 g of wet weight cell pellet per litre of culture, which is relatively higher than the previous reports. Moreover, the final purified rhGM-CS proteinF has exhibited specific biological activity (1.1×10^7 IU/mg) in TF-1 cell proliferation assay similar to standard protein.

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Introduction

Human granulocyte-macrophage colony-stimulating factor (hGM-CSF) is a cytokine produced by T-lymphocytes, macrophages, mast cells, fibroblasts and natural killer cells (1, 2) in response to inflammatory or immune stimuli. Granulocyte, subsets of differentiated myeloid cells and monocyte precursors are target cells for GM-CSF (3). Many target cells require GM-CSF for survival. GM-CSF induces proliferation and is crucial for differentiation of many hematopoietic cells. GM-CSF activates functions of myeloid cells, thereby linking adaptive and innate immunity boosting anti-tumor immunity (4) and thus has the potentiality to be used as therapeutic target for inflammatory diseases (5, 6). Thus, there is huge commercial demand for the recombinant hGM-CSF (rhGM-CSF) as it has lot of clinical interest. hGM-CSF consists of 127 amino acids with four cysteine

residues that form two intra molecular disulphide linkages linking the first and third (Cys54 –Cys 96) and second and fourth Cys residues(Cys 88-Cys 121) and has two *N*-glycosylation sites and several O-glycosylation sites (7).

Production of recombinant hGM-CSF in several heterologous expression systems such as bacterial (8,9), yeast (10-12) and mammalian cells (13-15) have been reported so far. It is generally expressed in the cytoplasm, either as a soluble or in soluble form of inclusion bodies (IBs). Expressing as a IB form has a many disadvantages requires more number of steps for processing the inclusion bodies in the downstream, poor in vitro refolding with less efficacy. Methionine at its N-terminus stimulates the immune response and influences its efficacy in humans (17, Sletta et. al., 2007)). Recently even changing of kinetic parameters for the fed batch culture of h GM-CSF toxic protein not improved much (16).

Different strategies and genetic tools have been reviewed for the expression of highly toxic proteins in *E. coli* (18). Based on previous research it can be conclude that cytoplasmic expression for GM-CSF protein not succeeded well. The reason behind this might be.

Toxicity of the GM-CSF gene by nature since this gene contains high GC rich region 57.8% in cDNA (calculated by http://www.endemo.com/bio/gc.php) with 42.97% hydrophobicity of the protein *i.e.*,due to toxicity and protein accumulation in the cytoplasm which leads to retardation of cell growth (19-21) and with relatively low yields (7 mg/L) in *E. coli*, where they did not report the wet weight of culture obtained (22). To avoid problems associated with cytoplasmic expression of toxic genes, periplasmic space was chosen where the protein expressed in the cytoplasm is imported to periplasmic space and would be helpful in reduction of host cell proteins, DNA and endotoxins in downstream processing. Attempts are being made to express rhGM-CSF in periplasmic space with different signal peptides (23-25) but comparatively low yield and involve multiple steps of purification.

In the present study, to increase the yield, purity and specific activity of rhGM-CSF, a new strategy has been introduced where *hGM-CSF* gene was cloned into pET22b with pelB (pectate lyase B) signal sequence. This strategy helps to get a mature active form of rhGM-CSF by cleaving more than 90 % of precursor rhGM-CSF in the expressed total proteins by increasing the in *vitro* activity of signal peptidases in presence of hydrophilic non ionic surfactant Triton X-100 this innovative technology helps for utilizing the complete expressed protein by processing the precursor rhGM-CSF to mature rhGM-CSF, thus increasing overall recovery. Further, the mature rhGM-CSF is purified from its precursor by a single step and the final purified protein shows 98.0 % purity with specific activity of 1.0×10^{7} IU/mg. By using the above strategy, we have achieved ~300 mg/per 35g of wet cell pellet of liter with 21 % recovery, which is very high comparable to a recent report (22).

Materials and Methods

pET-22b (+) vector, *E. coli* host strains DH5α and Rosetta (DE3) were purchased from Novagen (Darmstadt, Germany), restriction endonucleases, polymerases and dNTPs were purchased from MBI Fermentas. Primers were synthesized at Eurofins Genomics India.

Source 30™ reverse phased chromatography matrix, *Sephacryl S-200* and Superdex 75™ size exclusion column were purchased from GE Healthcare Life Sciences (India).The *BioLogic DuoFlow*™ chromatography system was purchased from Bio-Rad Laboratories (Hercules, CA). TF-1cell line from American Type Culture Collection (Manassas,VA). rhGM-CSF reference standard (NIBSC code:88/646) was procured from The *National Institute* for *Biological Standards and Control*. All other chemicals were analytical grade and purchased from Merck, India.

cDNA synthesis and gene amplification

Total RNA was extracted from human peripheral blood T-lymphocytes (14) after stimulating with

Con A (5 µg/ml) for four hour (h). RNA was isolated, and poly(A) mRNA was prepared by oligo(dT) chromatography as described previosuly (26). hGM-CSF cDNA (NCBI Accession: NM_000758.3) was prepared by amplifying the mRNA using *cDNA* synthesis kit (Thermofisher). The GM-CSF gene (381bp) was amplified from cDNA using the following gene specific primers: forward primer: 5′-CATGCCATGGCACCCGCCCG CTCGCCCAGC (underlined sequence: *Nco*I site), reverse primer: 5′-GGCGAATTCTCACTCCTGG ACTGGCTCCCA (underlined sequence: *EcoR*I site).

Following program was used for amplification of the GM-CSF gene. Initial denaturation at 94°C for 5 min, 25 cycles of 94 °C for 50 sec, annealing at 56 °C for 60 sec, extension at 72 °C for 60 sec and with a final extension for 10 min at 72 °C followed by the final hold at 15°C.

Recombinant hGM-CSF plasmid construction

The amplified hGM-CSF gene product and pET-22b (5.4 kb) were digested with the *Nco*I and *EcoR*I, purified by using gel extraction kit and products were ligated by T4 DNA ligase. The ligated product (5.8 kb) was transferred into the host cells (*E.coliDH5α*) by heat shock and the cloned cells bearing the ligated product (transformed cells) were confirmed by restriction digestion analysis and DNA sequencing. The pGM-CSF was isolated and transferred into expression host (Rosetta -DE3) for further expression studies.

Gene expression studies at lab scale

A single colony was isolated from the transformed agar plate and inoculated into 15 ml Luria Bertani (LB) medium with ampicillin (100 µg /ml). The flasks were incubated at 37 °C in an orbital shaker at 250 rpm until the $O.D_{600}$ reached to 0.8. One ml of culture is collected and labeled as uninduced sample (0 h). The rest of the culture was induced with 0.5 mM IPTG for 4 h and samples were collected at every one hour interval and analysed by SDS-PAGE. Master cell bank was prepared from a clone which is showing high expression of protein and stored at –80°C.

Gene expression studies at large scale using fermentor

Seed inoculum was prepared from master cell bank in a 250 ml LB medium and cultured at 250 rpm at 35 ^{o}C for 2-3 h and transferred into modified LB medium (1 % peptone, 0.5 % yeast extract, 1 % NaCl, 0.3 % K_2HPO_4 and 0.6 % KH_2PO_4, pH 7.2) in a 3 L Biostat-C plus fermentor (Sartorius, Germany). The cells were grown for 6 h to reach the exponential phase ($O.D_{600}$ 5.8) and induced with 0.5 mM IPTG for 4 h. The culture was maintained at pH 7.0 to 7.4 with 50 % ammonia solution at 250 rpm and the dissolved oxygen was above 20 % saturation. Samples were collected at every one hour after IPTG-induction and analyzed by SDS-PAGE. Before harvesting the culture O.D was 19.6.

rhGM- CSF sub cellular localization:

Subcellular parts were processed as described in (Libby *et al*., 1987). To find out the distribution of precursor and mature form at different extracts (total cellular, periplasmic and cytoplasmic extracts), a small amount of pellet isolated from shake flask and batch scale and estimated the protein concentration by BCA method followed by SDS-PAGE analysis for comparing the localization of precursor and mature forms of rhGM-CSF. 20 µg of total proteins of each isolate were loaded on 14% SDS PAGE followed by Coomassie staining. The molecular weights of precursor and mature forms of rhGM-CSF compared with molecular weight marker and the gel images were captured using image J 1.48 software.

Isolation of cell lysate

Harvested culture was centrifuged at 4000 rpm for 10 min at 4 ^{0}C; supernatant was discarded and ~105 g of wet cell pellet (from 3 L culture i.e 35g/L harvested broth).contains ~17.334 g of total cell

proteins (calculated based on dry cell mass) was collected .

1050 ml of buffer A (20 mM Tris and 5 mM EDTA pH 8.0) was added to 105 g of cell pellet and homogenized for 2 min with ultra sonic homogenizer (Model No : 150VT from Biologics, Inc.) on ice now the volume of homogenous mixture volume is ~ 1000mL. From 1000mL taken first 200 ml of homogenous mixture and added 200 ml of ice cold 0.1 mm glass beads (BioSpec Products, Inc. OK, Cat. # 11079101) and processed for 15 cycles, each cycle at 2 min on and 2 min off. The same was repeated for 5 times. After bead beating the total cell suspension including the beads the volume was around 2L was collected in to the 5L beaker to this added 600 ml of ice cold buffer A and kept for 1 h for settling of beads. After settling of beads without disturbing the beads cell lysate suspension was transferred in to a centrifuge tubes and centrifuged at 12000 rpm for 20 min at 4 °C (Sorvall 6C plus, SA 800 rotor). Supernatant was discarded and ~ 63g (contains ~ 12.4g of total cell proteins) of total wet cell lysate pellet was collected and washed with 630 ml of buffer B (buffer A containing 100 mM NaCl, pH 8.0) using a mechanical homogenizer and centrifuged at 12,000 rpm for 30 min at 4 °C to remove the less dense materials, membrane bound fragments and low molecular weight soluble proteins.

After first washing the wet cell lysate pellet shown was ~ 54g (contains ~ 10.8g of total cell proteins).

Solubilization of rhGM-CSF protein

The washed wet cell lysate pellet ~ 54g (contains ~ 10.8g of total cell proteins) was solubilized in 500mL of buffer C (2 M urea in 50 mM Tris and 5 mM EDTA, 0.05 % Triton X 100 pH 8.0) with continuous stirring for 18 h at 2-8 °C and centrifuged at 12000 rpm for 30 min at 4 °C. The supernatant was collected and total protein concentration was measured by bicinchonic acid method by using BSA as a standard and total protein was ~8.1g.

Purification of rhGM-CSF protein by RP-HPLC (Purification)

2M Urea sol supernatant sample (~8.1 g of 500mL added 500µL of TFA) was loaded on to a Source - 30 (30 µm polystyrene /divinyl benzene) RP – HPLC preparative column (78 cm×36 mm; ~789 ml of bed volume) which is previously equilibrated with 10 bed volumes of buffer A (0.1 % TFA in Water for Injection) with a flow rate of 20 ml/min with peristaltic pump.

Column was washed with 2 bed volumes of buffer after sample loading .Now the connected to preparative HPLC.(BioLogic Duo Flow chromatography system, make Bio-Rad laboratories). at a flow rate of 20 ml/min and the elution was monitored at 214 nm. Fractions were collected and analyzed by SDS-PAGE.

Refolding, ultra filtration and diafiltration

The pure RP fractions from 38 to 51% of buffer B(~340mL elution volume) were pooled and estimated at 280nm by using UV Spectrophotometer considering 1 O.D =1mg/mL (extinction coefficient value is 0.963 for rhGM-CSF) 50% acetonitrile as a blank and the total protein.

Step No	Gradient	Buffer A(0.1%TFA in WFI)	Buffer B (0.1%TFA in Acetonitrile)	Time (minutes)
1	Isocratic	100%	0%	0-30
2	Linear	100-30%	0-70%	30-120
3	Isocratic	30%	70%	120
4	Linear	30-0%	70-100%	120-150
5	Isocratic	100%	0%	156
End Method				

Refolding, ultra filtration and diafiltration

The pure RP fractions from 38 to 51% of buffer B(~340mL elution volume) were pooled and estimated at 280nm by using UV Spectrophotometer considering 1 O.D =1mg/mL (extinction coefficient value is 0.963 for rhGM-CSF) 50% acetonitrile as a blank and the total protein solution was added to it. The solution was incubated at 37°C for 30 min and allowed to cool at room temperature. The absorbance of the solution was measured at 562 nm.

The protein concentration in sample was calculated by plotting the curve, standard protein concentration (20 to 100 µg/mL or 200 to 1000 µg/mL of human serum albumin) on X-axis and absorbance on Y-axis.

Total protein concentration by OD_{280} method:

RP eluted fractions, diafiltration and SEC elution samples were measured for optical density at 280 nm in UV Visible Spectrophotometry using different blanks respective steps (Shimadzu, Japan).

Characterization of GM-CSF protein by Immunoblot

The antigenicity of the protein was confirmed by western blot technique .First the protein undergoes to electrophoresis followed by transfer to membrane then bind to 1° ab (i.e rabbit polyclonal anti-human GM-CSF antibody from Chemicon International,Temecula CA) and reacts with 2° ab which is developed by NBT& BCIP as a substrate .

First, the standard and test protein samples were loaded on 14% SDS-PAGE followed by transferred to PVDF membrane (Invitrogen, Carlsbad, CA) for 30 min at 100 V. After transfer the membrane to blocking buffer (5% Non-Fat milk) for 2 h at RT and followed by three washes with 1X TBS-Tween 20 buffer (10 mM Tris, 100 mM NaCl with 0.1% tween -20) for 5 min. The membrane was incubated in 1^0 (1:5000) solution for 4 h at RT and again washed with 1X TBS-Tween 20 and reacted with goat anti-rabbit IgG-alkaline phosphatase conjugated 2^0 ab (1:20,000) for 1 h at RT followed by membrane washes with 1X TBS-Tween 20 buffer. The protein bands were developed with NBT/BCIP substrate solution and reaction was stopped with purified water.

rhGM- CSF purity analysis by analytical RP-HPLC

50 µg protein (0.5 mg/ml) was loaded on analytical HPLC (Shimadzu model : LC2010CHT) connected to reverse phase C_4 analytical column (150×4.6, 5 µ particle size, 30 nm pore size; Grace Vydac) using solvent A (0.1%TFA) and solvent B (90 % acetonitrile with 0.1 % trichloroacetic acid) with linear gradient of B composition (0 to 30 min: 36-55 % of B, 30 to 35 min: 56-100 % of B; 35 to 45 min: 100 % of B and 45 to 50 min: 100-36 %, 50 to 60 min 36 % with flow rate of 1.2 ml/min, samples were detected at 214 nm. Simultaneously standard GM-CSF was also analyzed to compare the purity of the sample.

Confirmation of size by analytical SEC-HPLC

To conform the size, first standard protein mixture (1mg/mL) from 6.5 kDa to 75 kDa was prepared by adding individual protein standards supplied as a part of LMW protein calibration kit (Supplied by GE healthcare) and 0.5mg/mL of test sample also prepared. Each 100 µL of standard and test samples were pre filtered and loaded on analytical Superdex^{TM} 75 (300×10 mm, From GE health care) column connected to Shimadzu HPLC (Model :LC20CHT) at a flow rate 0.5 ml/min of PBS buffer pH 7.2 used as a mobile phase and detected at 214nm.

Purity by analytical SEC-HPLC

To know the purity and low & high molecular weight aggregates, 10 µg of GM-CSF pure protein (0.1mg/mL) was loaded on analytical SEC column (used one for size conformation). and elute the protein with PBS buffer pH 7.2 at a flow rate 0.5mL/min and detected at 214 nm.

Potency

Determination of biological activity of protein solution is based on the stimulation of proliferation of TF1 cells by GM-CSF.The following method uses the conversion of trterazolium bromide (MTT) as a staining method.TF1 cells (ATCC CRL-2003) are incubated with varying dilutions of test and reference preparations .They are then incubated with a solution of MTT .This cytochemical stain is converted by cellular dehdrogenases to a purple formazan product. The formazan is then measured spectrophotometrically.

Added 50 µL of medium to all wells of a 96-well microtitre plate , additional 50 µL of medium to the wells designed for the blanks. Added 50 µL of the reference and test samples in triplicates at a concentration about 65 IU/mL, a serious of 10 twofold dilutions to 0.0625 IU/mL (As per WHO standard 10,000 IU/ µg of protein) then added 50 µL of a TF-1 cell suspension containing 3 × 105 cells /mL in to each well and maintained the cells in a uniform suspension during adding. Incubated the plate at 36.0-38.0 °C for 24 h in a humidified incubator at 5% CO2. , added 25 µ 0.5% sterile tetrazolium bromide R to each well

.Reincubated for 5h then removed the plates from the incubator and added 100 µL of a 24% solution of sodium dodecyl sulfate R to each well to dissolve the crystals and reincubated for overnight. Determined the relative quantity of purple formazan product by measuring the absrbance at 570 nm and at 690 nm with 96 well microtitre plate reader (Make: Microtek) taken. Subtracted the reading at 690 nm from the reading at 570 nm. Analysed the data standard and sample curves were fitted by using non linear sigmoidal (4 parameter model) regression (Sigma Plot TM.software, Systat Software Inc., Point Richmond, CA USA).

Results and Discussion

In view of the fact that rhGM-CSF protein is having a high therapeutic and market value. Due to the toxic nature of gene hGM-CSFgene and instability of proteins produced in the cytoplasm due to presence of cytoplasmic endoproteases in the host cells are the major obstacles for bioprocess development (16, 27, 28). *E. coli* is one of the conventional host cell to produce the recombinant protein remains a workhorse but requires suitable selection of expression vector, optimizations of medium compositions, time of induction and times for cell harvest for efficient production (29). So the present investigation has been focused to develop novel processing by the signal peptidases and purification strategy for the conversion of precursor form of rhGM-CSF into mature form and as well as improving the yield to make use of totally expressed rhGM-CSF protein in *E. Coli* cell machineries.

Cloning and expression

Amplified hGM-CSF gene was first time cloned in pET-22b secretion vector T7 promoter introduced to downstream of pelB signal peptide (2.5 kDa) with ampicillin as a marker gene. The pelB signal peptide is cleaved by host signal peptidases and the cleaved target protein GM-CSF would export to periplasmic space.

The constructed recombinant plasmid with T7 promoter and pelB (5.8 kb) was shown in figure 1A and its physical map of construction region was shown in figure1B. The recombinant clone was confirmed by restriction digestion analysis, DNA sequencing and by comparing the sequence from NCBI (Accession: NM_000758.3). Earlier similar attempts have been made to produce hGM-CSF in secretion vector using outer membrane signal peptide (ompA), under the control of tandem lipoprotein promoter, lactose promoter-operator (30). The levels of the expression with this secretion vector (ompA) was 20 mg/liter culture (31). The detail mechanism of translocation pathway was previously mentioned in Pugsley (1993). Briefly, secretory proteins are synthesized as pre-proteins in secretion vector with an N-terminal signal peptide. Type I signal peptidases are serine proteases remove these signal peptides during, or shortly after, pre protein translocation across the cytoplasmic membrane, there by releasing the mature proteins from the trans side of the membrane. The *pelB* signal peptide cleaved by host signal peptidases and the cleaved target protein, rhGM-CSF exports from cytoplasm to periplasmic space. In this study, the expressed rhGM-CSF protein was appeared as two forms (17.0 kDa: precursor and 14.5 kDa: mature active) on 14 % SDS-PAGE (Fig 2A lanes 5, 6, 7 and 8). rhGM-CSF protein expressed in *E. coli* cells demonstrated to be ~35 % of the total cell proteins measured by densitometry using ImageJ software. It was noted that the harvested density of the cells at $O.D_{600}$ was 1.3, in that 15 % of expression indicate mature form and the remaining 20 % indicate precursor rhGM-CSF, when compared with uninduced sample (Fig 2A lane 4 and Table 1). There was no protein

expression observed in the vector alone transformed cells collected at 0 and 3 h (Fig 2A lanes 1& 2) as controls.

Fermentation and harvesting

Small scale batch culture fermentation was performed in 3 L Biostat C fermentor as described in the methods. It has been observed that after IPTG induction (OD_{600} 5.8), 30 % of GM-CSF protein from total cell proteins was expressed when compared to uninduced samples analyzed by SDS-PAGE (Fig 2B).

Among the 30 % of total GM-CSF expression, 25 % constitute premature form (Fig 2B: lanes 2, 3, 4 and 5) while only 5 % is mature form (Fig 2B: lanes 2, 3, 4 and 5), and final harvested culture OD_{600} 19.6 (Table 1) was recorded. Additionally, we observed a higher percentage of mature form of rhGM-CSF in shake-flask cultures when compared with fermentation batch. The reason could be due to *E. coli* protein secretion machinery is only sufficient to process the precursor GM-CSF to mature GM-CSF at small scale culture but this couldn't be processed due to over expression of proteins in the fermentation of batch culture (Perez-Perez *et al*., 1994).

The highly produced precursor GM-CSF was deposited as insoluble aggregates in the cytoplasm, not recognized by the cellular enzymes due to the presence of pelB leader peptide-GM-CSF fusion protein which may mask the portion of the molecule for recognition and degradation by the *E. coli* proteases.

After expression analysis at different time points, the final bacterial culture was harvested by centrifugation at 6,000 *g* for 10 min at 4 °C, and the wet pellet was collected. The pellet was washed by gently suspending with 10-fold dilution (w/v) of buffer A (20 mM Tris and 5 mM EDTA at pH 8.0). Further, the washed wet cell mass (35 g/L) was collected by centrifugation at 16,000 *g* at 4 °C for 15 min and cell pellet was stored at –20 °C for further use.

Subcellular localization

Further to know the localization of expression, sub cellular rhGM-CSF was separated as reported earlier (30), quantified by measuring the precursor and mature forms in total cellular, periplasmic and cytoplasmic extracts isolated from recombinant *E. coli* shake flask and fermenter by SDS-PAGE analysis (Fig 3A). Highest expression of mature form (14.5 kDa) of GM-CSF was observed in total cellular and periplasmic fractions of shake flasks culture than fermenter. In addition, more intense band of precursor form (17 kDa) was observed in total cellular and cytoplasmic fractions from fermenter (Fig 3A). These results indicate that, GM-CSF expression was distributed in different cellular compartments and are similar to previous report (30). To utilize complete expressed protein it was developed the simple and rapid method by 0.1 mm beads for isolation of proteins from secretion vector for increased yields. For this, neither we used the osmotic shock method as earlier used (32) or described the methods for extraction of protein from periplasmic space (30).

Cell lysis and solubilization

To isolate protein from different sub cellular parts required more number of steps and it can lead to lesser yield of protein and difficult to optimize the downstream processing at industrial scale To reduce such a loss of expressed protein we have developed the simple and rapid methods for isolation of protein from secretion vector at higher yield other than the osmotic shock method (32) described for extraction of periplasmic space protein. About 105 g of wet cell pellet was lysed with 0.1 mm ice cold beads using Bead Beater and 63 g of wet cell lysate pellet was collected after centrifugation and pellet was washed with low concentration of salts for removing the cell debris, soluble host cell proteins, endotoxins and host DNA. After final washing, about 54g of wet cell lysate was obtained. SDS-PAGE analysis revealed that majority of the rhGM-CSF protein was present in the cell lysate after lysis (Fig 3B lane 1) and negligible amount of protein was present in

supernatant (Fig 3B lane 2) indicates that the expressed protein is in the insoluble form.

It is known that *E. coli* protein secretion machinery does not support always for over expression of proteins. In this study, we observed that under overexpressed conditions, the precursor form of protein is partially processed to mature form. Hence, to improve the *in vitro* host signal peptidase activity in-turn the release of the mature protein, we have optimized the conditions for cleavage with 2 M urea, 50 mM Tris, 5 mM EDTA, 0.05% Triton X-100 pH 8.0 for 16 h at 2-8 °C. Triton X-100 was used for optimal activity of peptidase as reported previously (33), where Triton X 100 significantly improved the cleavage process .we have observed the increased percentage of mature form of GM-CSF protein proportional to the time of treatment and maximum (75 %) was observed at 16 h of treatment with a proportional decrease in precursor form (Fig 3: lanes 3,4 and 5). No further increase in *in vitro* release of mature rhGM-CSF was observed up to 48 h (data not shown). This data indicates that the mature rhGM-CSF is produced by *in vitro* activity of signal peptidases in presence of urea and detergents.

Table.1 rhGM-CSF protein expression at two different culture systems

Type of culture	GM-CSF expression	Precursor form	Mature form	Induction O.D at 600 nm	Harvested O.D at 600 nm
Shake Flask	*35% (100%)	20% (57.8%)	15% (42.2%)	0.8	1.3
Fermentation	*30% (100%)	25% (83.1%)	5% (16.9%)	5.8	19.6

*The total expression of rhGM-CSF (35% or 30 %) is calculated considering 100% of total proteins. The percentages within the brackets are calculated considered rhGM-CSF expression as a 100%.

Table.2 Yields and purities of recombinant human GM-CSF (rhGM-CSF) achieved at each step of purification from one litre of *E. coli* culture

Step	Purification step	Total protein (mg)	rhGM-CSF (mg) [A]	Other proteins	Purity (%)	Yield [C] (%)
1	Whole cell*	5778	1394	4384	25 [B]	100
2	Cell lysis and Solubilization	2693	1003	1690	38 [B]	72
3	After RP Chromatography	765	689	76	90 [B]	49
4	Refolding and Buffer Exchange	410	390	20	95 [B]	28
5	Size exclusion-HPLC	297	291	-	98 [D]	21

* 35 g/L wet cell mass was taken for processing (data shown is an average of three different batches).

A. Total protein includes both precursor and mature rhGM-CSF
B. Purity was determined by densitometry from SDS-PAGE gels
C. Yield was calculated considering rhGM-CSF protein quantity as a 100% from step 1
D. Purity of rhGM-CSF was determined by analytical RP-HPLC

Table.3 Yields and purities of recombinant human GM-CSF (rhGM-CSF) achieved at each step of purification from 15 mL of *E. coli* shake flask culture (data shown is an average of three different batches).

Step	Purification step	Total protein (mg)	rhGM-CSF (mg) [A]	Other proteins	Purity (%) [B]	Yield (%) [C]
1	Whole cell*	300	105	195	35	100
2	Cell lysis and Solubilization	164	78	93	48	74
3	After RP Chromatography	64	57.5	5	90	55
4	Refolding and Buffer Exchange	33	30.5	2	95	30
5	Size exclusion-HPLC	28	27.5	2	98 [D]	26

* g/l wet cell mass was taken for processing

A. Total protein includes both precursor and mature rhGM-CSF
B. Purity was determined by densitometry from SDS-PAGE gels
C. Yield was calculated considering rhGM-CSF protein quantity as a 100% from step 1
D. Purity of rhGM-CSF was determined by analytical RP-HPLC

Figure 1: **A.** Construction of rhGM-CSF clone . Amplified hGM-CSF gene (384 bp) and pET-22b plasmid (5.4 kb) were digested with *Nco*I and *Eco*RI and hGM-CSF was ligated to upstream of T7 promoter next to pelB signal sequence. **B.** Physical map of part of the cassette of recombinant plasmid with signal sequence (pelB leader region) and hGM-CSF gene.

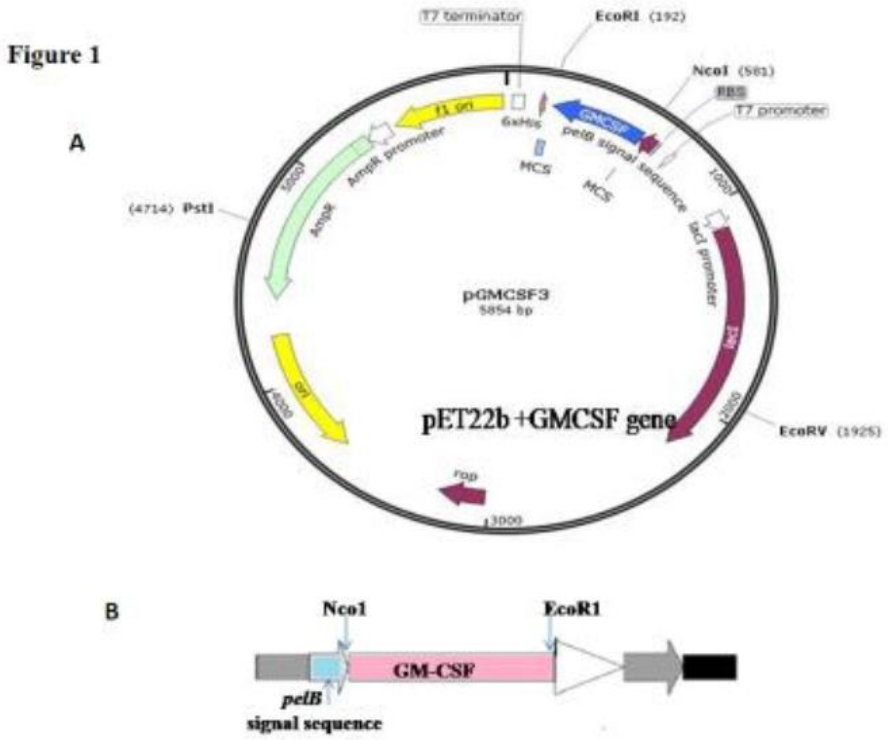


Figure 2: Expression of rhGM-CSF gene by pET-22b. **A.** SDS-PAGE analysis of shake flask expression. *E. coli* strain Rosetta (DE3) harbouring the recombinant plasmid were grown at 37 °C in Luria Bertani media and cells were-induced with 0.5 mM IPTG. Samples collected at before and after induction at 1, 2, 3 and 4 h. The whole cell extracts were subjected to 14 SDS-PAGE analysis. Lane 1 and 2, vector alone uninduced and IPTG-induced sample respectively; lane 4, uninduced sample at 0 h; lane 5-8, IPTG-induced samples collected after 1, 2, 3 and 4 h; lane 9, Protein Marker. Arrow indicates the mature rhGM-CSF after processing from premature form by signal peptidases, above this band premature form of rhGM-CSF was observed. **B**. SDS-PAGE analysis of fed-batch fermentation samples. Lane 1, uninduced sample at 0 h; lane 2-5, IPTG-induced samples collected after 1, 2, 3, and 4 h. Top arrow indicates the premature rhGM-CSF and bottom arrow indicated mature form of rhGM-CSF.

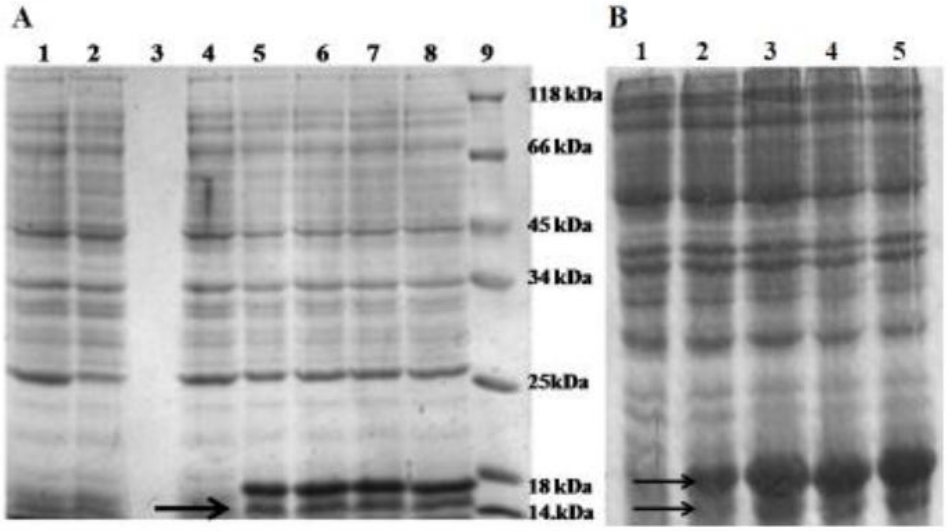


Figure 3: A: Subcellular localization of precursor and mature forms of GM-CSF by SDS-PAGE analysis. Total cellular, periplasmic and cytoplasmic extracts were isolated from *E. coli* shake flask and fermenter. The extracts were analysed on 14 % SDS-PAGE. **Lane 1-3:** Extractions from shake flask culture. Lane 4-6: Extractions from fermenter. TC: total cellular extracts, P: periplasmic extracts, C: cytoplasmic extracts. Arrows (top) indicates premature form (17 kDa) and bottom arrow indicated mature form (14.5 kDa) of rhGM-CSF. **B.** Analysis of samples after cell-lysis and solubilization at different time intervals samples were loaded on SDS-PAGE. Cell lysate was centrifuged at 12,000 rpm; pellet and supernatant were loaded on 14 % SDS-PAGE. The pellet was solubilized for 16 h and supernatant was subjected to SDS-PAGE at different time points. Lane 1, cell lysate pellet; lane 2, supernatant; lane 3, 4 and 5, are solubilized supernatant collected at 16, 8 and 0 h respectively. We observe that proportionately increased levels of mature rhGM-CSF with solubilization time. Arrow indicates the increased release of mature rhGM-CSF by *in vitro* signal peptidase action in presence of Triton X 100.

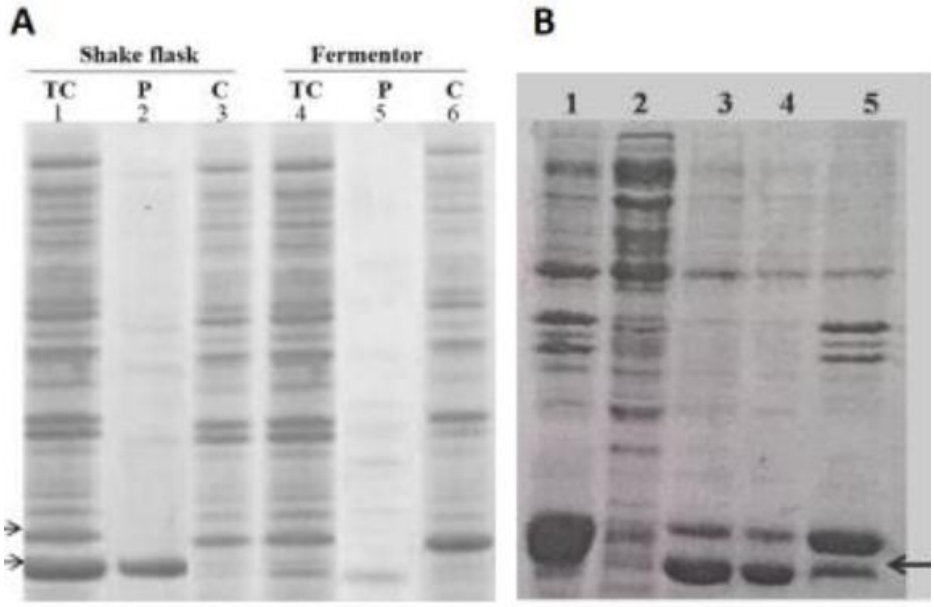


Fig 4: **A**. Reversed phase chromatography (RPC) purification. After 16 h of solubilisation, the clear supernatant was loaded on to Source30 C18 column and eluted with a linear gradient between buffer A (0.1 % TFA in Water for Injection) and buffer B (0.1%TFA in acetonitrile) with flow rate of 20mL/min. The pure rhGM-CSF protein was eluted between the 38-51 % (peak 2) of linear gradient. Peak 1 shows other *E. coli* cellular proteins and peak was premature rhGM-CSF. **B**: SDS-PAGE analysis of the pure fractions of rhGM-CSF. Lane 1, starting sample from RPC; lane 2, host cell proteins other than rhGM-CSF protein; lanes, 3 to 9 RPC eluted pure rhGM-CSF fractions; lane 10, protein marker. **C:** SDS-PAGE analysis of refolded rhGM-CSF protein. Pure fractions from RPC were pooled and refolded in 20 mM Tris and 5 mM EDTA buffer at pH 8.0 for 18 h at 2-8 °C and 5 µg of rhGM-CSF protein was analyzed in reducing and non- reducing conditions along with the standard. Lane 1, protein marker; lane, 2 and 3: rhGM-CSF in non-reducing and reducing conditions respectively; lane, 4 and 5: standard GM-CSF in reducing and non-reducing conditions respectively. Refolded protein migrated faster in non-reducing conditions than in reducing conditions.

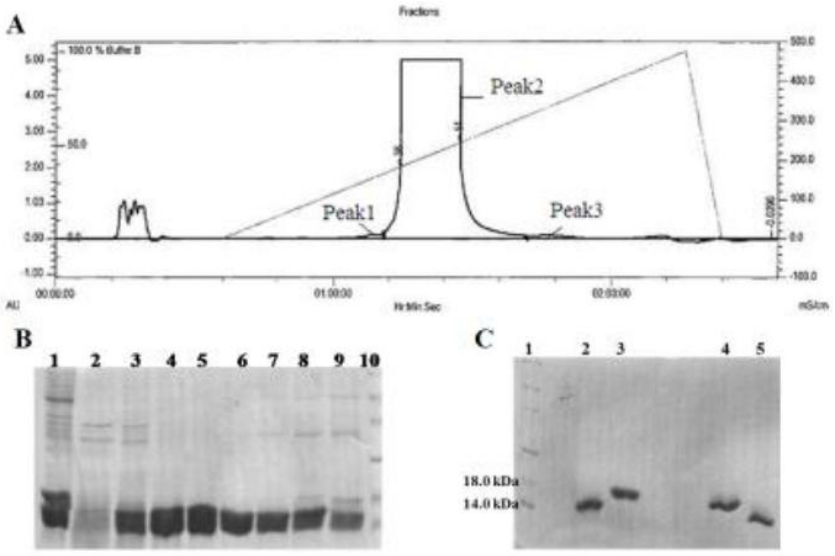


Figure 5. A: Purity analysis of rhGM-CSF protein by analytical size exclusion chromatography (SEC). Fifty micrograms of pure protein was loaded on Superdex 75^{TM} column and eluted with phosphate buffer pH 7.2 with flow rate of 0.5 ml/min and displayed a single main peak. **B**: Size analysis of rhGM-CSF protein by size exclusion chromatography (SEC). Peak 1, 2, 3, 5 and 6 represent standard molecular weight markers as described in the methods. Peak 4 represent rhGM-CSF protein eluted between the peak 3 (29.0 kDa) and peak 5 (13.7 kDa) and size of conformed to 14.5 kDa.

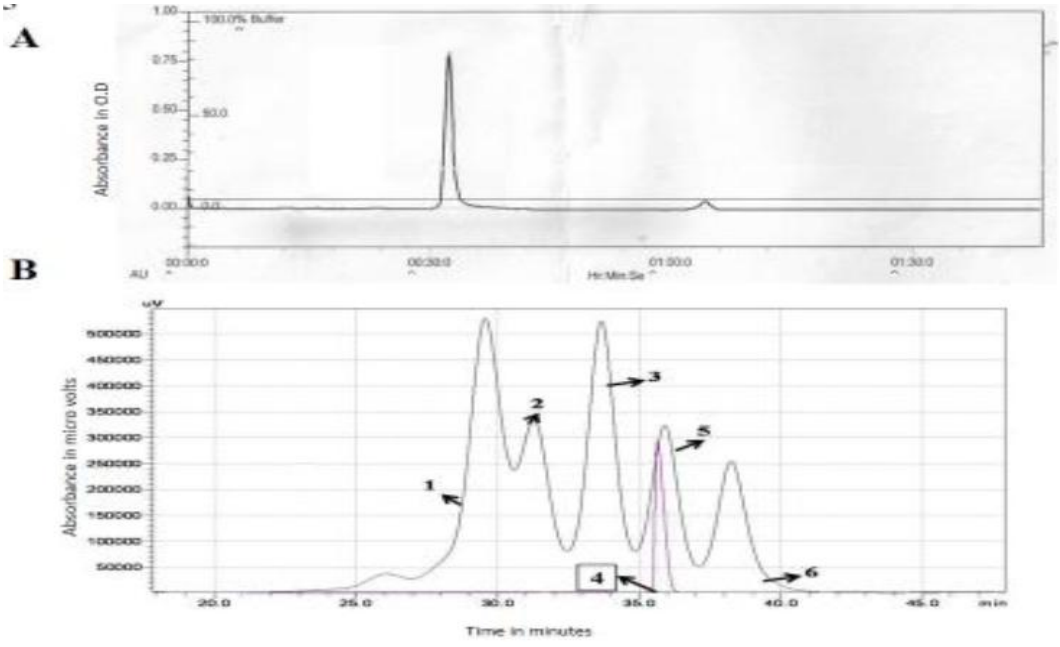
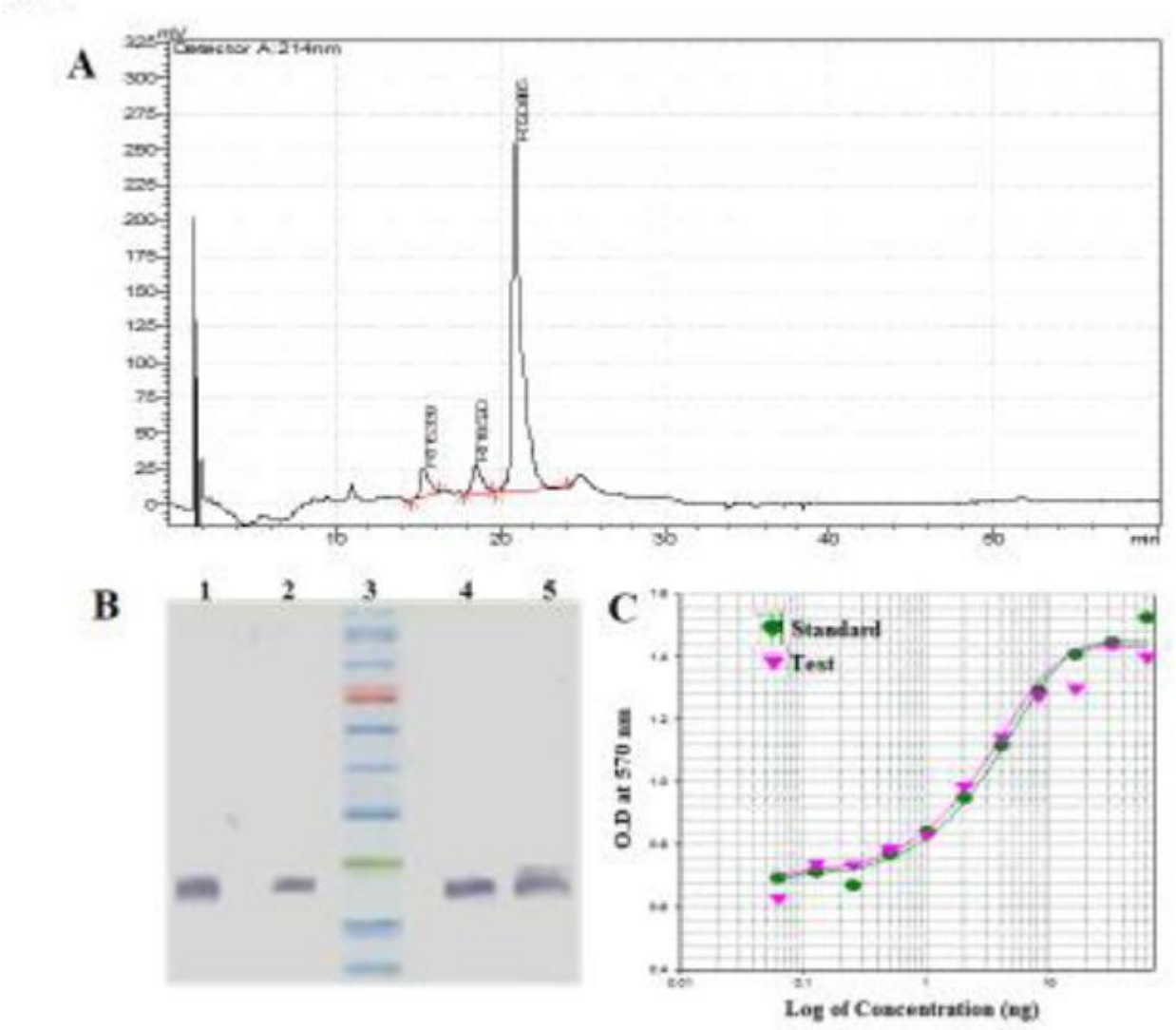


Fig 6 A: Reversed phase chromatography (RPC) analysis of purified rhGM-CSF. Final protein was loaded on the RPC column and percentage of each peak was analyzed. Peak 3 represent the rhGM-CSF and show more than 98 % pure and the remaining minor peaks are impurities represent less than 1.5 %. **B:** Immunoblot analysis of rhGM-CSF protein. Both standard and test protein samples were loaded on SDS-PAGE and transferred on PVDF membrane and probed with anti-GM-CSF antibody as described in the methods. Lanes 1 and 5, standard and test samples were run under non-reducing conditions respectively; lane 2 and 4, standard and test samples were run under reducing conditions. **C:** Cell proliferation assay. Ability of rhGM-CSF purified protein biological activity on cell proliferation was assessed against a NIBSC reference standard as described in methods. Color product was measured spectrophotometrically and obtained data was analyzed by fitting a sigmoidal dose response curves. The data in this figure is a representative of three different batches run in triplicates. Circles indicate NIBSC reference standard, triangles indicate purified rhGM-CSF.



Purification of rhGM-CSF by reversed phase chromatography (Preparative)

Reversed phase chromatography (RPC) was chosen to purify the rhGM-CSF protein since both forms of proteins having different in hydrophobic values and moreover this technique is easy to adopt at the industrial scale for increased yields (34, 35). The RPC elution profile showed three peaks (Fig 4A). The first peak represents *E. coli* host cell proteins eluted between 30-34 % of buffer B, peak 2 represents the pure rhGM-CSF protein eluted at 38-51% of buffer B and peak 3 represents precursor rhGM-CSF eluted between 58- 63 % (Fig 4A). The

different fractions collected were loaded on SDS-PAGE showing purified protein fractions (Fig 4B).

Refolding and dialysis

Based on the sub cellular localization only few GM-CSF molecules were processed completely to native form. Refolding step was chosen to bring the remaining in vitro processed GM-CSF molecules to native form and remove the acetonitrile content, aggregates and to diafiltration. Achieved complete refolding after 24 h of incubation at 2-8 °C in 20mM Tris and 5mM EDTA at 50 µg/ml of protein concentration and less than 5 % acetonitrile content. When increased the concentration of protein for refolding we noticed aggregation of the protein molecules. The refolded protein was concentrated first to 2 mg/ml and then diafiltered with 25 mM sodium phosphate buffer pH 7.2 several times to remove traces of acetonitrile and finally bring the protein conc to 7.7mg/mL . The final refolded purified protein was subjected to reduced and non-reduced conditions, loaded on to SDS-PAGE and observed that the refolded samples were migrated faster in non-reducing conditions than the reducing sample (Fig 4C lane 2 and 3) and same pattern was observed with the standard GM-CSF (Fig 4C lane 4 and 5). To achieve the highest purity the concentrated protein was loaded on Sephacryl S-200 HR column and pure single peak fractions were collected and dialyzed against the formulation buffer.

Characterization of purified rhGM-CSF

Analytical SEC analysis was performed to check the purity and size. The purified rhGM-CSF protein (10 µg) has shown a single peak at retention time of 30 min when loaded on Superdex 75^{TM} column with a flow rate of 1 ml/min indicated that there is no high and low molecular weight aggregates in the final purified protein (Fig 5A). Same column was used to determine the size of the purified rhGM-CSF. One hundred micro litres of pure protein was loaded on to the column with flow rate of 0.5 ml/min and peaks were detected at 280 nm. Relative molecular mass (M_r) range used was between 75.0 to 6.5 kDa. Peaks 1, 2, 3, 5 and 6 represents conalbumin (75.0 kDa), ovalbumin (44.0 kDa), carbonic anhydrase (29.0 kDa), ribonuclease A (13.7 kDa) and aprotinin (6.5 kDa) respectively. Peak 4 was rhGM-CSF protein eluted between the peak 3 and 5 and size was confirmed i.e ~14.5 kDa (Fig 5B). Analytical RPC was performed for checking the purity of rhGM-CSF protein using butyl silyl (C_4) silica gel. The purity was observed as 98 % and each individual impurity was identified less than 1.5 % (Fig 6A). The yields and purities of rhGM-CSF achieved at each step of purification from one litre of *E. coli* culture was shown in Table 2.

Immunoblot analysis of rhGM-CSF was performed for confirmation of antigenicity and compared with GM-CSF standard as described in the methods. .Test and standard GM-CSF samples were separated with non-reducing conditions (Figure 6B: lane 1 and 5) and in reducing conditions (Figure 6B: lane 2 and 4) indicate the same reactivity with anti-GM-CSF antibody and confirmed antigenicity of the purified protein which is similar to the standard GM-CSF.

Bioassay of rhGM-CSF purified protein was assessed against a NIBSC standard by stimulating the cell proliferation followed by MTT assay as described in the methods. The results obtained were plotted as sigmoidal dose response curves and activity was calculated by the method 4 parameter model. Similar technique was used for three more batches processed by similar purifications methods and results showed specific activity of 1.0×10^7 IU/mg. The calculated specific activities for the standard and test were observed respectively 1.2×10^7 IU/mg and 1.1×10^7 IU/mg. Figure 6C shows similar sigmoidal dose response for both reference standard (circle) and rhGM-CSF (triangle) indicate similar biological activities.

In conclusion, the present investigation has utilized periplasmic secretion system for increased expression and simple purification system for the biologically active hGM-CSF. In fed-batch culture it was achieved the production of ~300 mg/L/35g of pellet with a recovery of 21 % of the total

proteins. Such a large-scale production strategies might be helpful to meet the prevailing demand for therapeutically important proteins.

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