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An Optimized Process for Expression, Scale-Up and Purification of Recombinant Erythropoietin Produced in Chinese Hamster Ovary Cell Culture

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The DHFR mediated gene amplification employed for selection of recombinant Chinese hamster ovary (rCHO) clones was evaluated by single and multiple step selection of methotrexate (MTX). Multiple step selection of MTX resulted in cells with high amplified copies of erythropoietin (EPO) gene. Expression of EPO rapidly increased with increasing MTX concentration up to 1000 nM, further increased to 2000 nM does not affect the expression. After the MTX selection, cells grown in the presence of MTX were more stable and retained similar amounts of EPO expression & gene copies until 50 doublings. Whereas, cells grown in the absence of MTX were unstable and retained only 50% of initial EPO expression & gene copies at the 50th doubling. Scale-up from culture flask to wave bioreactor increases EPO yield, the novel wave bioreactor with a working volume of 1 liter could produce more than 0.56 g of EPO in 3 weeks, with a volumetric productivity of 24 mg/l/day and specific productivity of 5.93 µg/10^{6}cells/day. Simple two step chromatographic purification process was developed with relatively high yield and purity of EPO using blue sepharose affinity chromatography combined with Q-sepharose ion exchange chromatography. A single protein zone with molecular mass of 32-38kDa was appeared in SDS-PAGE analysis of the purified rHuEPO. Densitometric scanning of gels demonstrated, >90% purity of final EPO, giving a 42% recovery.

Keywords: MTX selection; amplified gene stability; wave bioreactor; repeated-batch; blue sepharose; isoelectric focusing (IEF)

The most widely used mammalian expression system is the gene amplification procedure offered by the use of dihydrofolate reductase-deficient (DHFR$^-$) CHO cells with DHFR-mediated gene amplification (Chung *et al*., 2003). Methotrexate binds and inhibits the DHFR enzyme, leading to cell death through the depletion of reduced folates (Hsieh *et al*., 2009). However, DHFR$^-$ CHO cells, which have taken up an expression vector containing the DHFR gene, can develop resistance to it by amplifying the DHFR gene. Since the gene of interest is integrated into same genetic locus as DHFR, the gene of interest is amplified as well, leading to increased production of the recombinant protein (Jun *et al*., 2005).

Gu *et al*. (1996) showed that increasing the foreign gene copy number using the DHFR gene amplification system results in severe growth rate reduction. Until now, the

major factor causing metabolic burden in DHFR gene amplification system has not been identified. For the production of recombinant proteins, not only the expression level but also the stability of amplified genes is critical. Instability of the amplified genes in the absence of selective pressure has been reported (Sinacore *et al*., 1995). Therefore, understanding the effect of MTX concentration on the cell growth and protein expression is important. It's also essential to investigate whether the rCHO cells expressing human EPO gene are stable or not.

Bioreactors are complex and expensive devices, yet they do not provide an ideal environment for mammalian cell growth due to high local fluid shear and bubble aeration. To avo1id problems associated with cell culture vessels, a new wave bioreactor was designed by Singh (1999). Wave bioreactors are an excellent choice for the elimination of need of cleaning, sterilization and associated validation requirements (Liangzhi *et al*., 2003). Batch (Yoon *et al*., 2004), fed batch (Bibila and Robinson, 1995), repeated batch (Takagi *et al*., 2001), continuous (Europa *et al*., 2000) and perfusion (Ryll *et al*., 2000) mammalian cell cultures are main modes of the industrial operation for large scale production of recombinant proteins for human therapy (Hu and Aunins, 1997; Kretzmer, 2002). Repeated batch culture is easy to operate and low risk of contamination (Yamamoto *et al*., 2000). However, there is no universal approach to optimize conditions for all animal cell culture systems. Each bioprocess must be optimized with respect to a specific set of parameters. These include cell growth, cell yield and specific productivity.

Methods of EPO purification have subsequently been developed (Broudy *et al*., 1988; Inoue *et al*., 1994; Ben-Ghanem *et al*., 1994), more efficient in terms of yield and specific activity, but still conceived for lab-scale preparations. Zanette *et al*. (2003) and Hu *et al*. (2004) described an improved, inexpensive industrial scale purification of EPO from mammalian cell cultures. Although resulting in good specific activity and yields, they cannot be easily modified to suit biopharmaceutical industry level purification for a number of reasons. The present study focuses on the aspects of MTX selection procedure, optimal MTX concentration, and stability of the amplified genes in presence and absence of MTX for the rCHO cells, followed by scale-up and down stream processing of the EPO.

Materials and methods

Cell culture: A cloned rCHO cell line (CHO-EPO) manipulated to secrete recombinant human erythropoietin was used in this work. These cells were kindly provided by Genomix Biotech Inc. (Atlanta, USA). Briefly, they were established by transfection of a vector containing DHFR and human EPO genes into DHFR deficient CHO cells. Excell 325 serum free medium (SAFC Biosciences, Kansas, USA) was used in this study, medium is supplemented with 4 mM glutamax (Gibco-Invitrogen) & 10μg/ml bovine insulin (Sigma).

In the multistep selection of MTX, selections were made sequentially in medium containing 50, 200, 500, 1000 and 2000 nM MTX. Exponentially growing cells (4×10^5 cells/ml) were cultured in 75 cm^2 flask containing 15 ml of Excel 325 medium. Medium was changed every 2-3 days for a period of 10 days, populations that reached normal growth at the chosen level of drug were used for the next round of selection, whereas in single step selection, cells were grown at a time in all the concentrations of MTX. Clones isolated at 500 nM MTX were grown in the absence and presence of MTX for the determination of amplified gene stability. Cells were always passaged upon

reaching 10^6 cells/ml by centrifugation and fed with fresh medium. Cell growth and protein production characteristics were monitored over a period of 50 doublings.

Scale-up in wave bioreactor: Cultures were carried out in wave 2-L perfusion cell bag (GE Health Care, USA) using the Amicon cell culture system (Amicon, MA, USA). Exponentially growing cells (2×10^5 cells /ml) in the T-flask were transferred directly in to 2-L cell bag with 200 ml of medium containing 500 nM MTX. Aeration (mixture of air & 5% CO_2) and agitations were maintained initially at 0.1 lpm (litre per minute) and 20 rpm (rocks per minute), further increased to 0.2 lpm and 30 rpm, depending on the glucose consumption and cell growth, fresh medium was added.

Purification

Clarification and diafiltration: Serum-free harvest collected from the wave bioreactor over a period of 10 harvests was centrifuged at 3000 rpm for 10 minutes, supernatant was filtered through 0.45 µm membrane using a Pellicon system (Millipore) to remove the remaining cells and debris. After the clarification, filtrate was concentrated by TFF (tangential flow filtration) system using a 10 kDa cut-off membrane (Millipore) to about 2 fold volume reductions. Conductivity was adjusted to 10-15 mS/cm with phosphate buffer.

Affinity chromatography and buffer exchange: A XK26 glass column (Pharmacia, 30mm internal diameter and 80mm length) was packed with 50 ml of Blue sepharose 6 fast flow matrix (Amersham). The column temperature and flow rate were maintained at 20°C and 10 ml/min respectively throughout the process. Column was equilibrated with 4 column volumes of PBS and regenerated with PBS containing 1.4 M NaCl and 8 M urea. Column was washed with PBS before loading with concentrated supernatant, again washed with 3 column volumes of PBS until absorbance at 280 nm of the eluate was nearly zero. EPO protein was eluted with 1.5 M NaCl, peak containing the active fractions was pooled and buffer exchanged against 20mM Tris (pH - 8.0) by TFF using 10 kDa cut off membrane (Millipore).

Ion-exchange chromatography: C26/40 Pharmacia column (30 mm diameter and 90 mm length) was packed with 65 ml of Q-sepharose matrix (Amersham), column temperature was approximately 20°C and flow rate was maintained at 10ml/min. Column was equilibrated with water and charged with 20mM Tris containing 1.4M NaCl. After re-equilibrated with 20mM Tris (pH-8.0), desalted blue sepharose elute was loaded on to the column. Column was washed with acid buffer (6M urea, 1mM glycine, 20µM $CuSO_4$, pH-4.7) followed by second wash with 20 mM Tris. EPO protein was then eluted with 20 mM Tris, 140 mM NaCl (pH-8.0). Fraction containing EPO was sterilized by passing through a 0.22 µm pore size filter.

Analytical Methods:
Viable and total cell concentrations were determined by the trypan blue exclusion method using a haemocytometer (Patterson, 1979). Secreted EPO concentrations were quantified by ELISA (EPO.96, MD Bioscience, USA), as for manufacturer's instructions. Glucose concentration was determined by enzymatic method (GOP/POD method, Excel diagnostics, India). Doubling time (Td) was calculated by the following formula, $Td= (t_2-t_1) \times \log (2)/\log (q_2/q_1)$, [$q_1$ - growth quantity at t_1 (time 1) and q_2 - growth quantity at t_2 (time 2)].

SDS-PAGE analysis: A discontinuous SDS-PAGE performed according to Laemmli (Laemmli, 1970) using the Bio-Rad X cell sure

lock vertical slab gel unit and gel documentation systems**.** Electrophoresis was carried out in 4-20% precast gels (Invitrogen, USA) and gels were silver stained (Blum *et al*., 1987).

Purified EPO samples, subjected to SDS-PAGE, transferred to nitrocellulose membrane and stained with anti-EPO primary antibodies (1:1000 dilution, Santa Cruz Biotechnology, USA) for 2 hrs at 4^{o}C, after washing with PBS containing Tween 20 (0.05%), blots were probed with 1:2000 dilution of goat anti-rabbit horseradish peroxidase conjugated second antibodies (Santa Cruz Biotechnology, USA) for 1 hr at RT. The enhanced chemiluminescent detection (Roche) was used to develop blot and fluorescence was detected by using Vilbert chemicapt.

IEF analysis of EPO: Isoelectric focusing was performed on a readymade immobilized pH gradient strips (IPG pH 3-10, Bio-Rad) using Bio-Rad PROTEIN IEF cell. IPG strips were rehydrated in the presence of urea using Resolyte (Merck ltd.). Samples containing purified EPO was applied to the strip and then subjected to gradient voltage (250 – 3500V) for 3 h at 20°C. After IEF, the strip was then subjected to SDS-PAGE and western blotting.

Statistical Analysis: The experiments were carried out in triplicate and results are expressed as mean±SD. Significant differences (P<0.05) were determined by the Student *t* test.

DNA extraction and PCR

For the determination of amplified gene copies, genomic DNA was isolated from cells using the method described by Sharma *et al*. (1993). DNA was quantitated spectro-photometrically (Nanodrop, DuPont, USA) and equal amounts of templates were amplified using EPO gene specific primers (Forward-5' TCA CTG TCC CAG ACA CCA AA 3' and Reverse-5' CAC TGA CGG CTT TAT CCA CA 3') and GAPDH primers (Forward-5'TGG CAA AGT GGA AGT TGT TG 3' and Reverse- 5' GAT CTC GCT CCT GGA AGA TG 3'). Equal volume of PCR products were analysed by agarose gel (0.8%) electrophoresis using Bio-Rad gel documentation system.

Results and discussion

Effect of MTX on CHO cell growth

To isolate higher resistant clones, we first performed multistep selection of MTX. As shown in figure 1(A), total viable cells in the control flask at the end of cultivation was 98.2±1.9×10^{6}, whereas 66.3±2.9×10^{6} (50nM), 61.9±3.3×10^{6} (200nM), 54.7±2.3×10^{6} (500nM), 25.2±2.6×10^{6} (1000nM) and 8.7±0.25×10^{6} (2000nM) at various levels of MTX, respectively. The first step in this selection (50nM) appears to be 32.5% of growth inhibition than the control population. Cells treated between 50 to 500 nM drug concentrations had negligible growth inhibition. Whereas, more than 50% growth inhibition was observed by 1000 nM MTX compared to 500 nM. Cell concentration was rapidly decreased at highest drug concentration (2000nM), this may be due to the greater increase in cytotoxicity. Viability is very similar in 50 nM, 200 nM & 500 nM cultures, although at the end, viability reached almost to that of control, a significant difference is noticed in first two days. Rapid drop in viability was seen by both 1000nM & 2000nM MTX [Fig: 1(B)].

Figure 1(D) &(E) shows typical CHO cell growth during the single step selection. There is a rapid suppression of cell growth was observed at 200 nM (37.8%, Student's t-test p<0.0002) and 500 nM (56.7%, Student's t-test p<0.0012) than at 50 nM drug concentration. The viability count decreased drastically at concentrations of 200 nM and 500 nM of the drug. No significant cell concentration and

viability was observed at higher MTX concentrations (1000 &2000nM). It has been well documented that selective gene amplification is one mechanism by which cultured mammalian cells become resistant to cytotoxic drugs (Brown *et al*., 1983). As a first step to develop the effective selection strategy, two selection processes were compared. There are, to date, no experimental data is available to directly compare them for CHO cells. In the selection based on gradual MTX increments, it took more than 40 days to obtain clones with high resistant. Cells were sensitive to MTX initially during the every increment in MTX concentration up to 1000 nM. Decrease in cell concentration and viability (52%) was observed at a concentration of 2000 nM of MTX, hence this concentration was avoided for the further analysis. Culturing cells at very high MTX concentration, will effect the cell growth, secretion efficiency, and intracellular degradation may outweigh the beneficial effect of enhanced protein productivity (Kim *et al*., 1998).

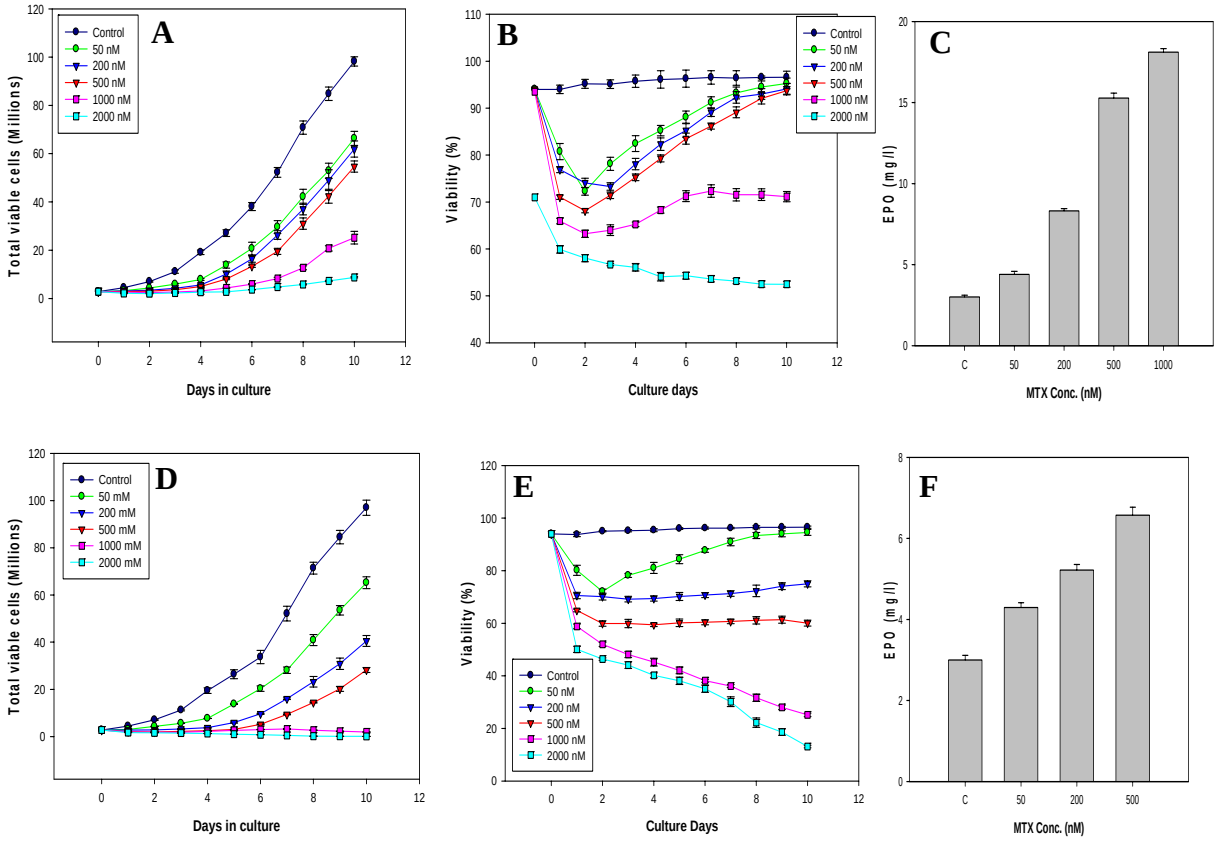

Figure 1. CHO cell concentration, viability and EPO expression at various levels of MTX concentration. (A) cell concentration, (B) viability, (C) EPO yields at gradual increase in MTX concentration. (D) cell concentration, (E) viability, (F) EPO yields at direct increase in MTX concentration. Bars indicate standard deviation.

Since, the gradual selection procedure is time taking, we have used the direct selection procedure to select the cells against MTX. The cell concentration was rapidly decreased with increased amounts of MTX by this process and the cell viability was started decreasing even at 200 nM MTX. Cells were not even grown at the higher levels of MTX (1000 &

2000nM). The present study suggests multi step selection of MTX for recombinant CHO cells, in order to achieve high EPO gene copies and optimal production of the protein.

Gene amplification and Protein expression

EPO gene amplification and increased protein expressions were determined during the both selections, MTX selected cells were cultured in T-flasks containing the corresponding level of MTX. Supernatants were analyzed for protein expression by ELISA, it was observed that the EPO expression was rapidly increased with increasing levels of MTX up to 1000 nM by the gradual selection [Fig: 1(C)]. However, further increase of MTX level up to 2000 nM did not increase EPO expression (data not shown), this may be due to poor cell growth and viability. EPO expression was not significantly increased with increasing level of MTX by direct selection [Fig: 1(F)]. Higher level of MTX concentrations (1000 & 2000nM) were eliminated from this analysis due to poor cell growth.

To investigate whether the increased EPO expression was related to changes in the EPO gene copy numbers, genomic DNA isolated from CHO cells at each MTX level was characterized by PCR. Increasing intensity of EPO gene bands were clearly observed by gradual selection of MTX [Fig: 2(A)], this indicates EPO gene amplification following MTX treatment (Pendse *et al*., 1992). Densitometric tracing of the bands indicated no significant increase in intensities was observed by direct MTX selection [Fig: 2(C)]. Very similar band intensities were observed for GAPDH gene (control) in all samples of gradual and direct selections [Fig: 2(B) & 2(D)].

PCR analysis revealed that the enhancement of EPO protein was related to the extent of EPO gene amplification. Finally, the EPO expression was increased 6 folds (at 1000nM) higher than the control cultures by the gradual increase in MTX levels. Eventhough EPO production was increased significantly at 1000 nM MTX, but the cell viability was not reached more than 72%, this indicates, cells did not get fully resistance at this concentration of MTX. Sialic acid content is essential for the glycoproteins to maintain circulatory lifetime, desialylation of glycoproteins is caused by sialidases released from dead cells, especially during the later phase of cell culture (Ferrari *et al*., 1998). So it is essential to maintain the high viability during the production of glycoproteins. Hence, it is suggested that 500 nM MTX concentrations is optimal for the production of recombinant EPO by CHO cells.

Amplified gene stability in the absence of MTX

MTX selected (500nM) cells were cultivated in the absence as well as in the presence of corresponding level of MTX. Cell growth rate without MTX was higher for most part than with MTX [Fig: 3(A)], this improvement was probably as a result of no cytotoxicity and gene loss (Kim *et al*., 1998). This could be possible because high producing clones are more susceptible to environmental changes and display lower cell growth rate than low or nonproducing clones due to the effect of DHFR and foreign gene amplification (Gu *et al*., 1994). In the absence of MTX, significant decrease in EPO production was seen [Fig: 3(B)]. In the initial doublings (up to 5^{th}), very similar expression levels were obtained in both cases, furthermore, it was decreased (15^{th} doublings onwards) in the absence of MTX. Cells grown in the presence of MTX were more stable and retained the similar amounts of EPO production in the entire cultivation.

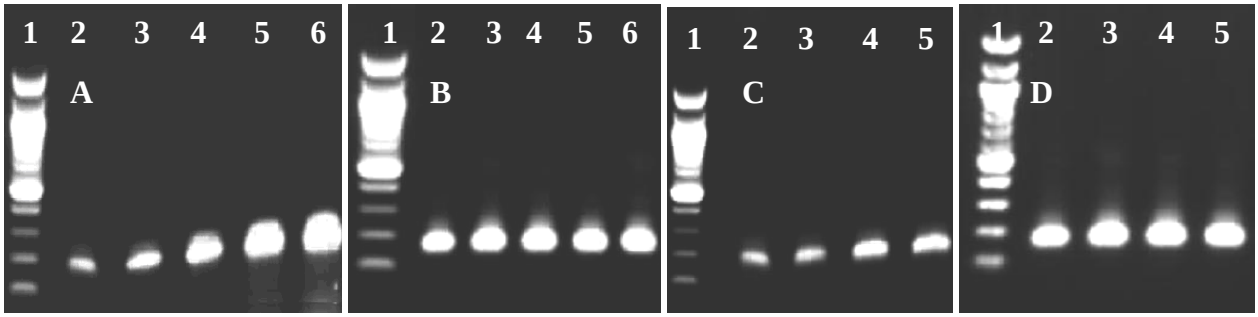

Figure 2. PCR analyses of EPO and GAPDH genes. (A) EPO, (B) GAPDH during the gradual selection (Lane 1: 100bp marker, Lane 2: Control, Lane 3: 50nM, Lane 4: 200nM, Lane 5: 500nM & Lane 6: 1000nM MTX conc.). (C) EPO, (D) GAPDH during the direct selection (Lane 1: 100bp marker, Lane 2: Control, Lane 3: 50nM, Lane 4: 200nM, Lane 5: 500nM MTX conc.).

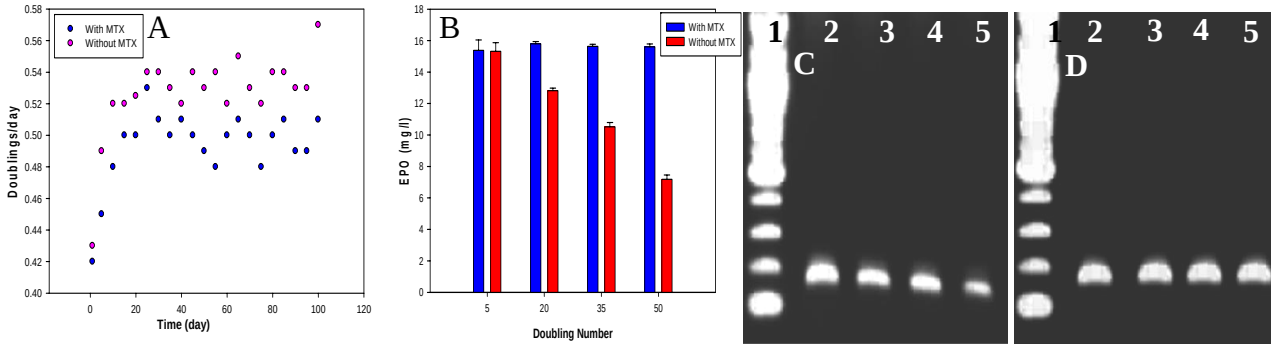

Figure 3. (A) Growth and (B) EPO production characteristics of CHO cells during the culture in the presence and absence of MTX (500nM). PCR analysis of EPO gene during the long term cultivation of CHO cells in the absence (C) and presence of MTX (D). Lane 1: 100bp marker, Lane 2: at 5th doubling, Lane 3: at 20th doubling, Lane 4: at 35th doubling and Lane 5: at 50th doubling. Cell growth was expressed as population doublings per day.

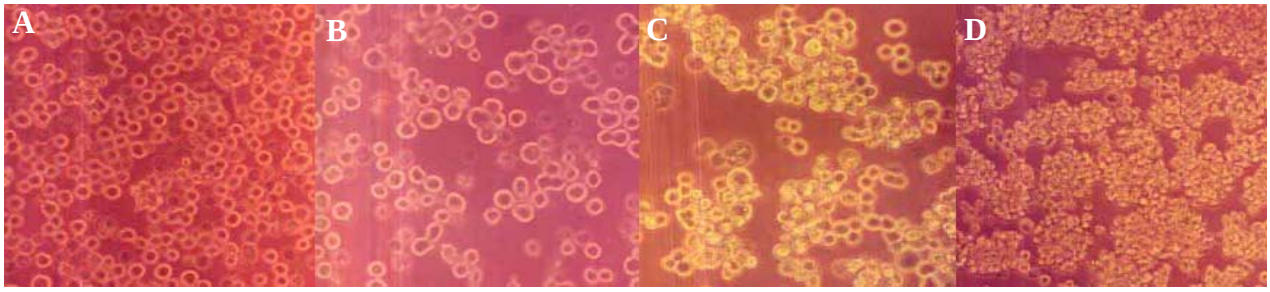

Figure 4. Growth of rCHO cells during the repeated batch cultivation in 2-L wave bioreactor. (A) day 2, (B) day 8, (C) day 13 and (D) day 23.

Changes in gene copy number were examined by comparison of PCR products on agarose gels. Cells retained most gene copies in the presence of MTX during the entire cultivation [Fig: 3(D)], whereas, only 50% of initial gene copies were retained by the cells in the absence of MTX [Fig: 3(C)]. When the genes are unstable, they reside on extra chromosomal elements, such elements replicate in the cell cycle and unequally distribute in to daughter cells, resulting in their instability (Schimke, 1984). As observed, decreased EPO production in the absence of MTX displayed the most significant loss of gene copies. It was clearly observed that the decreased level of protein expression was almost 50% lower in the absence of MTX than in presence. Similar to the protein expression, only 50% of initial EPO gene copies were retained by the cells in the absence of MTX. This is in correlation with the elevated DHFR levels that are characteristically unstable, when cells are grown under the absence of MTX for short time periods (up to 50 doublings) (Kaufman and Schimke, 1981; Weidle *et al*., 1988).

Table 1. Repeated batch process in wave bioreactor (harvest details)

Harvest No	Harvest Day	Cell viability (%)	Viable cells (per ml)	Glucose content (mg/l)	EPO (mg/l)
1	5	96.1±0.60	$1.01\pm0.09\times10^6$	1.69±0.09	29.90±0.46
2	7	96.4±0.52	$2.10\pm0.10\times10^6$	1.60±0.10	21.20±1.20
3	9	96.6±0.12	$3.18\pm0.14\times10^6$	1.30±0.04	35.36±0.80
4	11	96.0±0.62	$4.19\pm0.13\times10^6$	1.25±0.03	49.20±0.87
5	13	95.7±0.90	$5.12\pm0.12\times10^6$	1.19±0.02	65.10±1.10
6	15	*	*	1.11±0.09	75.00±1.20
7	17	*	*	1.22±0.09	77.27±0.50
8	19	*	*	1.33±0.05	75.10±0.90
9	21	*	*	1.42±0.03	69.10±0.96
10	23	*	*	1.64±0.05	63.20±0.50

Same volume of fresh medium was added after every harvest.

***** indicates the 'no cell count and viability' was measured due to cell aggregates

Scale-up of EPO expression in wave bioreactor

The repeated-batch parameters were optimized for the scale-up of EPO production in 2-L wave bioreactor. Initially cells were grown up to approximately 1.0×10^6 cells per ml and the culture was diluted with fresh medium to get a density of 5×10^5 cells/ml. When the maximum working volume (1000ml) reached in wave cell bag, 95% of the culture supernatant was drawn through perfusion filter and replaced the drawn culture volume with fresh medium to initiate subsequent production cycles. Depending on the glucose consumption and medium colour changes (red to light yellowish), medium was replaced (harvested) with fresh medium as required. Initial glucose concentration was maintained at 3.2 g/l up to first 4 harvests and further it was increased to 4.2 g/l to compensate the glucose depletion at higher cell density. Batch was stopped after the 23rd day of cultivation (10th harvest), where the cell viability, glucose consumption and EPO production were started decrease significantly.

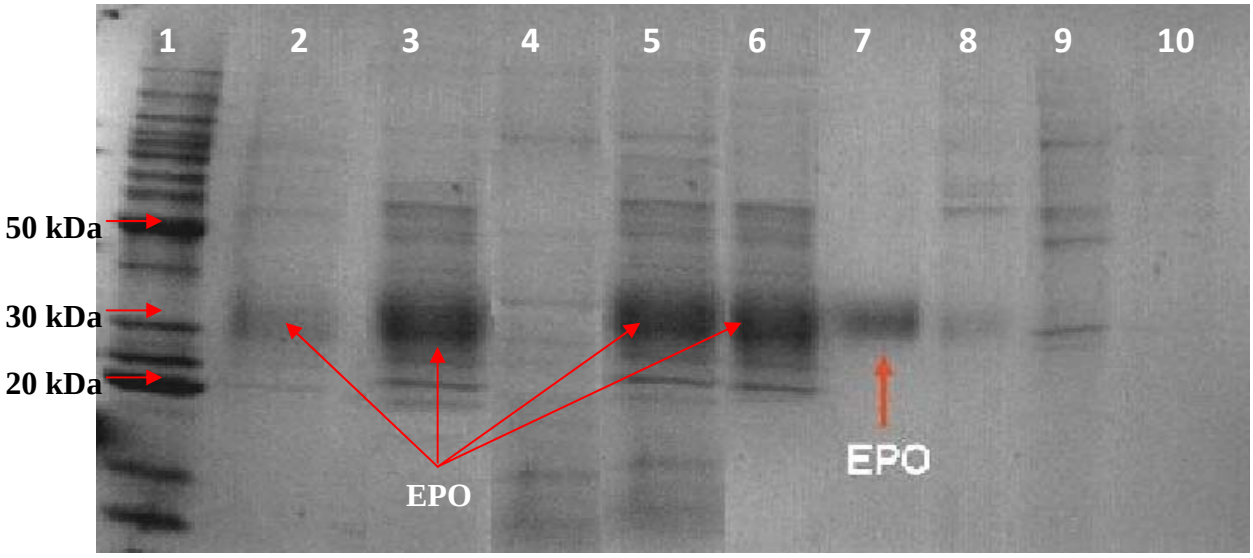

Figure 5. SDS-PAGE of fractions from blue sepharose (Lane 2,3,4) & Q-sepharose chromatography (Lane 7,8,9,10). EPO eluted with various concentrations of NaCl was analyzed by 4-20% SDS-PAGE. Lane 1: protein marker (Cat no: 10747-012, Invitrogen, Carlsbad, CA), lane 2: eluate at 1M NaCl, lane 3: eluate at 1.5 M NaCl, lane 4: eluate at 2 M NaCl, lane 5: concentrated cell culture supernatant, Lane 6: blue sepharose eluate (before loading to Q-sepharose), lane 7: eluate at 140 mM NaCl, lane 8: eluate at 250 mM NaCl, lane 9: eluate at 500 mM NaCl and lane 10: eluate at 1.4 M NaCl.

Cell concentration was gradually increased to 5.1±0.11×10^6cells/ml and viability was maintained above 95% up to 13th day of culture (Table: 1). Aggregates (≈25 cells/aggregate) were observed at this high cell density and cell counts were not taken properly due to cell aggregations (Fig: 4). Large aggregates (≈70-100 cells/aggregate) were seen at the end of cultivation, this may be due to the high cell density (Yamamoto *et al*., 2000). Small aggregates (≈5-6cells/aggregate) were disintegrated using sterile pipette and cells were counted at initial days of cultivation, but large aggregates were not disintegrated by the pipette and gave false results on cell number and viability.

Glucose consumption was increased gradually up to the 15th day and slightly decreased from 17th to 23rd day (Table: 1), decrease in glucose consumption (17th to 23rd) could be due to decrease in cell growth and increased aggregates (Han *et al*., 2006). EPO yields were increased gradually up to 17th day (Table: 1) and slightly decreased from the 19th day, this correlates well with the glucose consumption, where it was started decrease at 17th day. The novel wave bioreactor with a working volume of 1 litre could produce more than 0.56 g of EPO in 3 weeks, with a volumetric productivity of 24 mg/l/day, the volumetric productivity was calculated by dividing the total product concentration by the culture time. Specific productivity of 5.93 µg/10^6cells/day was obtained between 5th to 13th days of culture. The overall specific production rate of EPO was determined from the gradient of the plot of the accumulated EPO production against the integral from time zero of cell number-time (Renard *et al*., 1988). Increasing protein yields and thereby reducing production costs is a major biotechnology target (Simone *et al*., 2003).

However, major accumulation of product requires a high cell density, high cell density can be carried out by repeated batch cultivation (Takagi *et al*., 2001) and batch-refeed process can be the most efficient route to a highly productive process (Hammill *et al*., 2000). In the present study, highest cell density was obtained on 13th day of repeated batch cultivation, due to the large aggregate formation, cell counts were not taken during further cultivation. When cell density reached more than one million cells per ml, CHO cells tend to form aggregates during the repeated batch cultivation (Yamamoto *et al*., 2000). Similarly high viability was seen up to the day 13. Glucose consumption and EPO productions were increased gradually up to 17th day and slightly decreased from the 19th day to end of the cultivation. The decreased glucose consumption rate indicates the decrease in cell growth (Bibila *et al*., 1994).

To maintain the protein quality, it was decided to stop the repeated batch at 23rd day, even though rapid decrease in glucose consumption and EPO yields were not seen. Proteases (Gramer and Goochee, 1993) and sialidases (Elliott *et al*., 2003) released into culture medium from the dead cells during the cultivation of CHO cells, the general concern of these proteins activity may affect the quality of the recombinant product. Harvesting the product before extensive cell lysis occurs can readily reduce the extent of extracellular desialylation (Ferrari *et al*., 1998). The total volumetric productivity of EPO by the repeated batch using wave bioreactor could increase to gram quantity by scaling-up in large wave bioreactors.

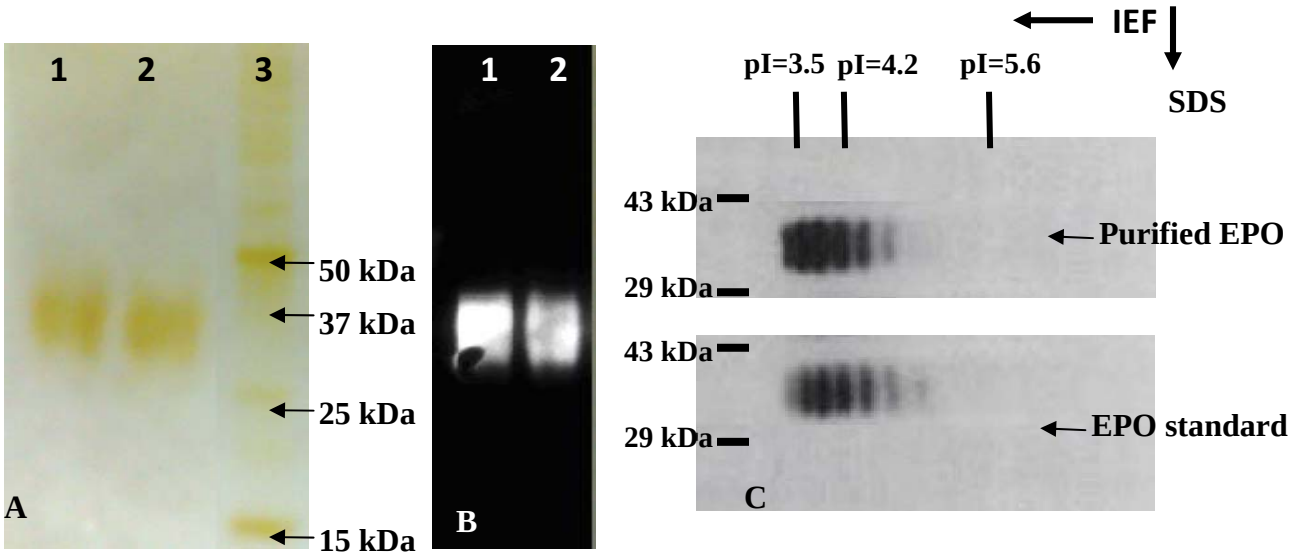

Figure 6. Analysis of purified EPO, (A) 2µg of both purified and commercial standard EPO was analyzed by 12% SDS-PAGE and detected by silver stain, lane 1: EPO standard (EPREX-4000, Johnson & Johnson, Switzerland), lane 2: purified EPO and lane 3: protein ladder (Cat no: 161-0374, Bio-Rad, Hercules, CA). (B) 2µg of Purified EPO was separated by SDS-PAGE (12%), then protein was transferred onto nitrocellulose membrane and was detected with anti-EPO antibody, lane 1: EPO standard and lane 2: purified EPO. (C) Typical profile of a two dimensional gel separation of EPO isoforms. 4µg of both purified and commercial EPO standard was analyzed by 2-D electrophoresis, separated by pI (ampholytes pH 3-10) in the first dimension followed by SDS-PAGE in the second dimension and detected by a western blot.

EPO purification from culture supernatant

Selection of elution buffer for affinity chromatography: The criterion for the most suitable elution buffer is one that dissociates EPO from the column but which minimize impurities and protein loss. Serum-free CHO cell culture supernatant (1L from wave bioreactor) was filtered through 0.45µm membrane filter and then directly loaded onto Blue sepharose column (50ml). To eliminate non-specific adsorption caused by weak interactions, the column was washed properly with 1X PBS. Different elution conditions were subsequently tested i.e., step elutions with 1, 1.5 and 2 M NaCl in 1X PBS. EPO eluted with 1.5 M NaCl was recognized as a main band corresponding to EPO molecular weight on SDS-PAGE (Fig: 5). However, some contaminant protein bands with high and low molecular weights were present. Faint EPO band was obtained at 1 M NaCl and no EPO band was seen at 2 M NaCl, however impurities were observed in both elutions.

As shown in table 2, higher final EPO recovery was obtained with 1.5 M NaCl as compared with other concentrations of NaCl, 60% of EPO was eluted with 1.5 M NaCl, whereas only 10% was eluted by 1 M NaCl and no EPO was recovered at highest concentration of salt in elution buffer. Hence, 1.5 M NaCl was selected for the elution of EPO from the blue sepharose column. Approximately 20% of EPO did not bind to the column and remained in the supernatant. The EPO binding capacity was not increased by repeated loading to the column. It is possible that the remaining EPO has lower carbohydrate content, which could not bind properly to blue sepharose.

Table 2. EPO recovery from affinity & ion-exchange chromatography

Samples	Total EPO (mg)	Percentage of EPO (mg/l)
Blue sepharose chromatography		
Before loading (culture supernatant)	58.00	100.00
After loading (flow through)	11.80	20.30
1 M NaCl eluate	5.95	10.20
1.5 M NaCl eluate	34.80	60.00
2 M NaCl eluate	--	--
Q-sepharose chromatography		
Before loading (blue sepharose eluate)	33.60	100.00
After loading (flow through)	2.10	6.25
140 mM NaCl eluate	23.00	68.40
250 mM NaCl eluate	6.10	18.15
500 mM & 1.4 M NaCl eluate	--	--

EPO concentrations in all fractions were determined by ELISA

Selection of elution buffer for Ion-exchange chromatography: Desalted blue sepharose eluate was applied on to a Q-sepharose column (65ml). Various elution conditions were tested for high purity and recovery, i.e., step elutions with 140, 250, 500 mM and 1.4 M NaCl in 20 mM Tris (pH-8.0). EPO eluted with 140 mM NaCl was recognized as a main

band corresponding to EPO molecular weight on SDS-PAGE (Fig: 5). Other contaminants were not observed in this eluate, whereas, faint EPO band was seen with 250 mM NaCl eluate along with some other contaminant protein bands. EPO band was not observed in 500 mM and 1.4 M elutes, however impurities were observed in both elutions.

As shown in table 2, higher final EPO recovery was obtained with 140 mM NaCl as compared with other concentrations of NaCl, 68% of EPO was eluted with 140 mM NaCl, whereas only 18% was eluted by 250 mM NaCl and no EPO were recovered by higher concentration of elution buffer. 180 mM & 220 mM NaCl elutions were also performed but very similar results to 250 mM NaCl elution was obtained (data not shown). Hence, 140 mM NaCl was selected for the elution of EPO from the Q-sepharose column. Approximately 8% of EPO not recovered in any fractions, this loss was could be due to the desalting.

Table 3. Purification of EPO from 2 liters of cell culture supernatant

Samples	Total EPO (mg)	Percentage of EPO (mg/l)
Culture supernatant	116.0	100.00
Ultra filtration	106.0	91.30
Blue sepharose	75.6	65.10
Desalting	70.0	60.30
Q-sepharose	49.0	42.20

EPO concentrations in all fractions were determined by ELISA

Overall purification process: The final scheme of the EPO purification process has described here, includes two UF/DF steps and two chromatography steps. To optimize the purification process for EPO, 2 liters of conditioned medium was clarified and concentrated to about 2 fold volume reductions, concentrated supernatant was loaded on to blue sepharose and blue sepharose eluate was desalted, and then applied on to Q-sepharose column (overall process explained as purification in materials & methods). The EPO fractions from the Q-sepharose were buffer exchanged with protein storage buffer (Sodium phosphate buffer-10 mM, Tris-20 mM and 0.1% of Tween 20) and filtered using 0.2µm syringe filter and the final solution was stored at 2-8°C. SDS-PAGE of elutes from each chromatography column showed increasing purity of the rHuEPO and a single protein zone with an apparent molecular mass of 32-38 kDa. Densitometric scanning of the SDS-PAGE gels demonstrated that the purity of final rHuEPO was >99%. The overall recovery of rHuEPO was 42% (Table: 3).

Protein characterization

Purified EPO was characterized by SDS-PAGE for the purity and molecular weight determination, western blot analysis was performed for the specific determination of EPO by anti-EPO antibody. Isoelectrofocusing was performed for the determination of isoforms. The SDS-PAGE analysis has shown [Fig: 6(A)] a good level of purity, a single protein zone with an apparent molecular mass of 34-38 kDa. EPO migrates with an apparent size of 34-38 kDa on SDS-PAGE gels (Jelkmann, 2004). Densitometric

scanning of the silver stained gel demonstrated that the purity level of final purified EPO was >99%. Western-blot analysis showed that purified rHuEPO has native immunogenicity [Fig: 6(B)]. IEF analysis [Fig: 6(C)] showed identical profiles for both commercial EPO and purified EPO, most glycoforms are found in the 3.5-5.6 pI range.

The degree and type of glycosylation of EPO is the key factor for *in-vivo* biological activity (Takeuchi *et al*., 1989). If rHuEPO partially deglycosylated during separation, the half-life of EPO in the blood circulation is very much shortened, which includes a significant or complete decrease in *in-vivo* bioactivity (Tsuda *et al*., 1990). Recombinant EPO expressed by CHO cells, displays physicochemical properties very similar to those of native EPO (Davis *et al*., 1987). Blue sepharose affinity chromatography was successfully used for the purification of EPO by Diana *et al*. (1995) and Mellado *et al*. (2007). Less expensive (Mellado *et al*., 2007), higher binding efficiency and higher recovery of EPO (Diana *et al*., 1995) facilitates the more specific use of blue sepharose rather than other affinity ligands for the purification of EPO.

In order to develop a simple, inexpensive, high-yield method suitable for both small and large scale purification of EPO from culture supernatant, blue sepharose as pseudo affinity chromatography followed by Q-sepharose ion exchange chromatography was used. Various step elution conditions were tested for the recovery of EPO by blue sepharose column, major band of the EPO was observed with the 1.5 M NaCl along with some other impurities. More than 50% of purity and 70% of recovery was achieved by this chromatography, whereas only partial purification of EPO was achieved in the previous studies (Diana *et al*., 1995).

EPO eluted with 140 mM NaCl was recognized as a main band in Q-sepharose column, other contaminant proteins were not seen in this elution condition, further increase in salt concentration could not elute EPO and most of the impurities were eluted. Eventhough, the significant EPO band was observed at 250 mM NaCl concentration, 140 mM NaCl was selected for the higher recovery and purity of EPO. An acid wash (containing 6 M urea, pH-4.7) was performed before eluting the EPO from Q-sepharose for the removal of unwanted isoforms (lower sialylation) and unrelated impurities.

Due to extensive glycosylation, EPO is a highly heterogeneous protein, i.e., a complex mixture of different, closely related glycoforms (Rush *et al*., 1995). Such heterogeneity explains the multiple bands observed when purified EPO is analyzed by IEF (Gokana *et al*., 1997). IEF analysis of purified EPO showed identical profiles for both commercial EPO and purified EPO, most glycoforms are found in the 3.5-5.6 pI range. Six distinct bands were obtained in the purified EPO, whereas seven bands were seen in commercial EPO sample. As expected most glycoforms are found in the 3.0-5.5 pI range. This corresponds to a highly glycosylated and sialylated protein with a profile showing at least six to seven distinct isoforms (Restelli *et al*., 2006). In the previous reports, the recovery and purity of EPO from the CHO cell supernatant was 30% & 97% (Zanette *et al*., 2003) and 38% & 99% (Hu *et al*., 2004) respectively. Moreover, they used three chromatographic steps to achieve the final recovery and purity. In present study, more than 42% of recovery and >99% of final purity was achieved by the simple two step purification process.

Conclusions

The selection procedure based on gradual increase in MTX concentration rather than direct increase in MTX is favored for establishment of high producing rCHO cells, although it is more time consuming and labor intensive. Gradual selection process with 500 nM MTX was achieved with the stable and high expression of recombinant proteins in CHO cells. Amplified genes in the CHO cell lines are not stable in the absence of MTX. Hence, it is essential to add MTX during the entire cultivation to maintain the stable and constant protein expression. A repeated batch process using the novel wave bioreactor including perfusion filter was successfully developed for high efficient production of EPO by CHO cells. First the wave bioreactor is successfully applied in repeated batch culture of CHO cells. This offers an alternative approach for producing gram quantities of protein from the industrial cell lines.

All these optimized conditions facilitate the higher recovery and yields of EPO from the cell culture supernatant. Characterization of purified EPO showed a very similar identical profile compared with commercial EPO standard. Hence, it could be used for therapeutic and commercial purpose. It is believed that besides efficient and reliable EPO purification, the process described here might have an even more general interest, as Blue sepharose based strategies might yield interesting results in large scale purification of other commercially important glycoproteins. This work may be useful for further study on a large scale process for highly efficient production of proteins toward industrial applications. The information obtained in this work can be applied to other cell cultures, which have wide utilization and application.

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