

# High Expression of Methylotrophic Yeast-Derived Recombinant Human Erythropoietin in a pH-Controlled Batch System

Ahmad Maleki<sup>1,3</sup>, Farzin Roohvand<sup>2\*</sup>, Hosnieh Tajerzadeh<sup>1</sup>, Hossein Khanahmad<sup>3</sup>,  
Maryam B. Nobari<sup>4</sup>, Ahmad Beirut<sup>3</sup>, and Abdolhossein Rouholamini Najafabadi<sup>1</sup>

1. Faculty of Pharmacy, Tehran University of Medical Science, Tehran, Iran

2. Hepatitis & AIDS Department, Pasteur Institute of Iran, Tehran, Iran

3. Research and Production Plant, Pasteur Institute of Iran, Karaj, Iran

4. Open University of Zanjan, Zanjan, Iran

## Abstract

To accomplish the worldwide demand for recombinant human erythropoietin (rHuEpo) as a therapeutic, application of cost-efficient expression system of methylotrophic yeast *Pichia pastoris* (*P. pastoris*) rather than mammalian cells is indispensable. Herein, a report on high levels secreted-expression of *Pichia*-derived rHuEpo by batch fermentation in a pH stabilized format is presented. The full length cDNA of rHuEpo was inserted into pPICZ $\alpha$ A vector under control of *AOX1* promoter, downstream of the secretion- $\alpha$ -factor and electroporated into *P. pastoris* strain X33. The highest expression transformant was selected by screening among the colonies surviving high concentration of Zeocin (1.0 mg/ml), followed by comparative small scale expression analysis by ELISA. Stabilization of pH around 6.0 by adding phosphoric acid into the culture media during induction period, improved the yield of expression to 150 mg/l of the media. Single-step Nickel-affinity chromatography was employed for purification of rHuEpo-6xHis to 80% purity. Analyses by SDS-PAGE, Western blot and N-terminal protein sequencing confirmed the authenticity of the 33 kDa expressed rHuEpo with a native N-terminal indicating the proper cleavage of secretion-signal. Results of this study, further confirmed the possibility of employing methylotrophic yeast for scaled up production aims of rHuEpo as a cost-efficient expression system when provided evidence for higher expression yields through application of pH-controlled systems.

### \* Corresponding author:

Farzin Roohvand, Ph.D.,  
Hepatitis & AIDS  
Department,  
Pasteur Institute of Iran,  
Tehran, Iran  
Tel: +98 21 66969291  
Fax: +98 21 66969291  
E-mail:  
rfarzin@pasteur.ac.ir  
farzin.roohvand@gmail.com  
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## Introduction

Erythropoietin (Epo), a 30-38 kDa glycol-protein hormone (exact Molecular Weight-MW depends on degree of glycosylation) is produced mainly by kidney as a 193 amino acids pre-mature protein which stimulates the growth of red blood cells and as a consequence increases haemoglobin levels<sup>(1, 2)</sup>. Administration of Epo as a therapeutic im-

proves the quality of life in patients with cancer, renal and heart failure anemia<sup>(3)</sup>.

To fulfil the high medical demand for Epo globally, large scale production of recombinant Human Epo (rHuEpo) was originally initiated in Chinese Hamster Ovary (CHO) cells<sup>(1)</sup>. Nonetheless, due to cost-efficiency considerations for scaled up production, more

efficient heterologous expression systems, rather than mammalian cells for this valuable drug are being explored.

To this end, production of Epo in different prokaryotic hosts like *Escherichia coli* (*E. coli*)<sup>(4)</sup> and *Bacillus brevis*<sup>(5)</sup>, as well as eukaryotic hosts such as *Saccharomyces cerevisiae* (*S. cerevisiae*)<sup>(6)</sup> and *Drosophila melanogaster*<sup>(7)</sup> was described. However, since proper glycosylation of Epo as a hormone is important for some of its functional properties such as biological half-life<sup>(8)</sup>, neither prokaryote systems lacking the ability to glycosylate proteins nor microbial eukaryotes like *S. cerevisiae* which hyperglycosylates proteins<sup>(9)</sup> could be considered as alternatives to currently exploited CHO cells for production of rHuEpo.

In recent years, a few yeast species capable of growing on methanol as sole carbon and energy source have been identified and their application in biotechnology for high level and cost-efficient production of recombinant proteins with proper post-translational modifications were established<sup>(10)</sup>. These so called methylotrophic yeasts belong to four genera; *Hansenula*, *Pichia*, *Candida* and *Torulopsis* and their most prominent representatives are *Hansenula polymorpha* (*H. polymorpha*), *Pichia pastoris* (*P. pastoris*), *Candida boidinii* and *Pichia methanolica*, respectively<sup>(11)</sup>. While *H. polymorpha* and *P. pastoris* are distinguished as industrial platforms for heterologous protein production, the other two methylotrophic yeasts are still in the research level of applications<sup>(12)</sup>.

Currently, the heterologous protein expression system of methylotrophic yeast "*P. pastoris*" is broadly used for production of many human and therapeutic proteins in biopharmaceuticals industries<sup>(13)</sup>. In fact with the advantages of both prokaryotic and eukaryotic systems and the availability of strong promoter for alcohol oxidase, *AOX1*, which is tightly regulated and induced by addition of methanol to the growth medium, *P. pastoris* provides the potential for producing correctly folded and properly glycosylated recombinant

proteins. It often allows the recombinant protein to be secreted in large quantities when it does not secrete significant amounts of intrinsic proteins, a phenomenon that facilitates downstream purification steps<sup>(14)</sup>.

However, to transport the expression profile of a new protein from research scale to industrial levels, the possibility of high level expression and purification yields should be proved. In this context, recently, expression of rHuEpo by *P. pastoris* in a batch culture system using baffled flasks is also reported<sup>(2)</sup>; but the expression level was shown to be as low as 5 mg/l. Therefore, for scaled up production purposes, there is absolute need to improve expression/culturing protocols for much higher yields of rHuEpo expression in methylotrophic *Pichia*.

Such optimizations may be achieved by exploring better vectors, host strains, and expression clones as well as optimizing the fermentation process to improve the yield and stability of expressed proteins by controlling the medium composition, pH, temperature, methanol induction and feed mode<sup>(15)</sup>. To this end, more recently higher production levels for *P. pastoris*-derived rHuEpo (130 mg/l) by Fed-batch methanol feeding strategy in the presence of co-substrate sorbitol was also described<sup>(16)</sup>.

In the present study, we attempted to investigate other fermentation strategies rather than Fed-batch methanol feeding and sorbitol utilization for high yield production of rHuEpo by *P. pastoris* when simultaneously selecting the highest-expressing clones. In this context, we proposed the application of acid feeding strategy to keep buffering and stabilization of the pH around 6.0 throughout the methanol induction period for the selected-high expression Mut<sup>+</sup> (Methanol Utilizing Positive) clone potentially containing multiple copies of the integrated vector. Results obtained in this study demonstrated the possibility of large scale production of rHuEpo in methylotrophic yeast by pH stabilization.

To our knowledge, this is the second report on high level expression of rHuEpo in meth-

ylotrophic yeast when the first report on optimization of fermentation process through stabilizing the optimum pH and also the first report on production of home-made yeast-derived rHuEpo in Iran.

## Materials and Methods

### Construction of expression vector for secretion of rHuEpo in methylotrophic yeast

The synthetic cDNA encoding for the 166 native amino acids of the homo sapiens Epo gene (NCBI accession no. NM\_000799) in tandem with the DNA sequence of six histidine amino acids (6xHis-tag) located at the C-terminal of Epo which was synthesized and cloned in pUC57 plasmid (Bio Basic Inc, Ontario, Canada) was used as template in PCR reactions throughout this study. For further direct cloning of PCR-amplified products in the *Pichia* expression vector pPICZ $\alpha$ A (Invitrogen), *Xho* I and *Xba* I restriction sites were considered in forward (F-EPO: 5' CTCGAGA AAAGAGCCCCACCACGCCTCATC3') and reverse (R-EPO: 5' TCTAGATTATCAATG ATGATGATGATGATGTCTGTCCCCTGT CCT 3') primers, respectively (restriction sites are underlined). This cloning strategy provided the *Kex2* cleavage site at the 5' ends of the DNA encoding Epo to locate the amplified fragment in frame with secretion sequence ( $\alpha$ -factor) under control of *AOX1* promoter while the 6xHis-tag and two termination triplets were located at the 3' ends in tandem (Figure 1).

DNA amplification was carried out through 30 cycles of denaturation (30 sec at 94°C), annealing (35 sec at 58°C) and extension (60 sec at 72°C), followed by a final elongation (10 min at 72°C) in a Perkin-Elmer thermocycler. Following digestion by *Xho* I and *Xba* I restrictions enzymes and gel purification (QIAquick Gel Extraction Kit, QIAGEN), PCR-amplified fragments and PICZ $\alpha$ A vector were mixed in the ligation reaction. The product of ligation reaction was used to transform *E. coli* TOP10 competent cells (Invitrogen) and developed Zeocin resistant bacterial colonies were screened for the presence of the proper recombinant construct. The presence and accuracy of the inserted gene within the expression cassette in the final recombinant PICZ $\alpha$ A-Epo construct was confirmed by both restriction analyses using *Xho* I and *Xba* I enzymes and DNA sequencing reactions using the 5'AOX1(5'-GACT GGTTC AATTGACAAGC-3') and 3'AOX1 (5'-GC AAATGGCATTCTGACATCC-3') primers (Invitrogen) which amplify the expression cassette together with the cloned Epo gene.

### Electro-transformation of methylotrophic yeast and selection of expression clones

Around 10  $\mu$ g of purified PICZ $\alpha$ A-Epo plasmids (using QIAprep Spin Miniprep Kit) were linearized with *Sac* I enzyme (Fermentas) and electroporated into the yeast *P. pastoris*, wild-type host strain X-33 (Invitrogen) in 0.2 cm cuvettes at 1.5 kV (25  $\mu$ F and

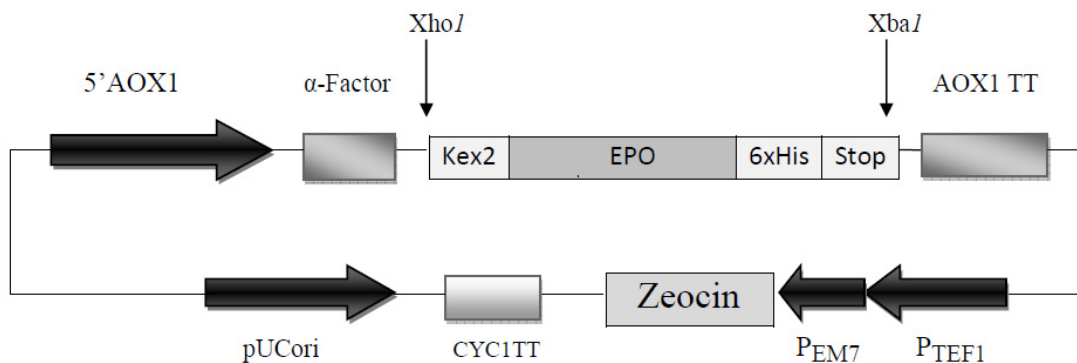


Figure 1. Schematic diagram of recombinant pPICZ $\alpha$ A harboring HuEpo gene (PICZ $\alpha$ A-Epo: 4.1 Kbp). *Xho* I and *Xba* I denote to the restriction sites employed for directional cloning of Epo gene into pPICZ $\alpha$ A under control of *AOX1* promoter, down stream of secretion signal ( $\alpha$ -factor) and cleavage sequence (kex2). *AOX1* TT, 6xHis and Stop denote to the transcription termination sequence, His tag and stop codons, respectively

200  $\Omega$ ), using a Bio-Rad Gene Pulser according to supplier's instructions (Easy Select *Pichia* Expression manual, Invitrogen).

Following electroporation, cells (200  $\mu$ l) were plated on YPDS medium (1% Yeast extract, 2% Peptone, 2% Dextrose, 1 M Sorbitol) supplemented with 100  $\mu$ g/ml Zeocin and incubated for 3-5 days at 30°C. Zeocin-resistant yeast transformants were further transferred on plates containing 1 mg/ml Zeocin. Finally, colonies that survived this concentration of Zeocin were screened for confirming the methanol utilization positive phenotype (Mut<sup>+</sup>) according to the supplier's instructions (Invitrogen).

Briefly, a single colony of each Zeocin resistant transformants was first streaked on a MM (Minimal Methanol) plate and then on a MD (Minimal Dextrose) plate and incubated 2 days at 30 °C. Mut<sup>+</sup> transformants were identified through their normal growth on both plates within this time (in these conditions Mut<sup>S</sup> strains only grow on the MD plate and show little or no growth on the MM). From screened Mut<sup>+</sup> transformants that survived a concentration of 1 mg/ml Zeocin, 10 colonies were randomly selected and confirmed for the presence of the corresponding Epo gene by PCR using AOX1 primers. Finally, confirmed Mut<sup>+</sup> transformants were analyzed for expression levels in a mini scale (50 ml conical tubes containing 10 ml of culture media) and the highest expression clone was selected on the basis of ELISA, Western blot and densitometric assays on the culture supernatants as described in the analyses and quantification section in the following.

#### **Optimization of the culturing conditions and purification of rHuEpo in methylophilic yeast**

A single colony from the highest expressing clones was inoculated into 5 ml of YPD (1% yeast extract, 2% peptone and 2% dextrose) + 100  $\mu$ g/ml of Zeocin medium and grown overnight at 28-30°C (200-250 rpm). The obtained seed culture was then used to inoculate 100 ml of BMGY medium (1% yeast extract, 2% peptone, 1.34% YNB, 4 ×

10<sup>-5</sup>% biotin, 1% glycerol and 0.1 M potassium phosphate, pH=6.0) in a 500 ml Erlenmeyer flask and growth allowed to proceed at 28-30°C (200-250 rpm) for 16 - 18 hr (OD<sub>600</sub> ~ 5-7). Subsequently, cells were harvested by centrifugation (3000 g for 4 min) and the pellet resuspended in 200 ml BMMY medium (1% yeast extract, 2% peptone, 1.34% YNB, 4 × 10<sup>-5</sup> % biotin, 0.5% methanol and 0.1 M potassium phosphate, pH=6) in 1l Erlenmeyer flasks to an OD<sub>600</sub> ~1 and incubated at 28-30°C (250 rpm) in a floor shaking incubator for 72 hr. To maintain induction, sterile 100% methanol was added to a final concentration of 0.5% every 24 hr during the expression (post-induction). The pH was kept constant at 6.0 throughout the expression time by checking pH every 24 hr and adjusting it with 0.1 M phosphoric acid.

After 72 hr, the medium was centrifuged at 10000 g for 10 min, the cell pellet was discarded and the supernatant was concentrated 10 fold and diafiltrated by LabScale TFF System (Millipore, USA) exploiting Pellicon XL50 ultrafiltration cassette equipped with Ultracel membrane (MWCO of 10 kDa). During diafiltration, the buffer of supernatant was exchanged with 20 mM sodium phosphate buffer pH=7, 0.5 M sodium chloride. Subsequently, expressed Epo was purified by the Ion Metal Affinity Chromatography (IMAC) columns charged with Ni<sup>2+</sup> according to the supplier instructions with some modifications (His-Trap HP affinity columns, GE Healthcare).

In brief, each diafiltrated sample was loaded on a prepacked column at a flow rate 0.8 ml/min and prewashed with 20-30 column volumes of equilibration buffer (20 mM sodium phosphate buffer pH=7, 0.5 M sodium chloride). Subsequently, purification column was washed with 10 column volumes of washing buffer (equilibration buffer plus 90 mM imidazole) and then bound proteins were eluted with elution buffer (equilibration buffer plus 250 mM imidazole). Finally imidazole was removed by ultrafiltration using the exchange buffer (50 mM sodium phosphate

pH=7, 150 mM sodium chloride, 10% glycerol) which simultaneously introduced the purified Epo into this final buffer. The purified proteins were stored at -80 °C for further analyses.

#### **Analyses and quantification methods of recombinant Epo protein**

Concentrations of the expressed Epo protein in the supernatants were quantified by human Epo ELISA kit (CIGB, Havana, Cuba) according to the supplier instructions. Purified proteins were quantified by both ELISA and the Bradford method<sup>(17)</sup>. Protein samples were analyzed by the standard Laemmli SDS-PAGE method<sup>(18)</sup> in a 12.5% separating gel and developed with silver staining. Further analysis of stained SDS-PAGE slabs for yield measurements was performed by densitometry using a GS-800 Bio-Rad Imaging Densitometer and employing Quantity One analyzer according to the software manual. Western analysis was performed by electroblotting of proteins from SDS-PAGE gel to Whatman nitrocellulose membranes (Schleicher & Schuell).

Tracking of the protein was achieved by employing the monoclonal (mouse) antibody against rHuEpo (CIGB, Havana, Cuba) and/or monoclonal (mouse) anti-His(C-term) antibody (Invitrogen) as the primary and horseradish peroxidase-conjugated goat anti-mouse IgG (Promega Corporation, Madison, WI, USA) as the secondary antibody. The bands were developed using DAB chromogenic substrate (Sigma-Aldrich). N-terminal amino acid sequencing was performed by Edman's degradation method on the purified sample electroblotted onto PVDF membrane (Institut für Chemie, Universität für Bodenkultur Wien, Austria). Cell concentrations were measured with a Perkin Elmer UV/VIS spectrophotometer (Lambda EZ 201) based on absorbance at 600 nm using a calibration curve.

Statistical analyses of the data were performed using Student's t test and one-way multivariate ANOVA to calculate p value. P<0.05 was considered statistically significant.

## **Results**

### ***PICZαA-Epo expression vector and selection of yeast transformants***

The human cDNA of Epo which was employed in this study encoded 166 native amino acids of the protein. In our cloning strategy, using *Xho* I and *Xba* I sites of pPICZαA, the cDNA of Epo was cloned in frame with alcohol oxidase (*AOX1*) transcription/ translational cassette. This strategy inserts the Epo cDNA downstream of the prepro-α-factor sequence and immediately after the *Kex-2* signal cleavage site to express and secrete Epo into the culture media with a native N terminus. Furthermore, two stop codons were placed immediately after the 6xHis-tag codons for preventing of any ribosomal-pass through (Figure 1).

The restriction analyses of the constructed recombinant Epo plasmid (Figure 2) and DNA sequencing analysis demonstrated the correct insert orientation and complete accuracy for the PICZαA-Epo construct without any unwanted mutations.

Due to the advantages of electroporation, such as high frequency of transformation (especially possibility of multi-copy insertion), this method was employed to transform yeast cells by the constructed plasmid (PICZαA-Epo).

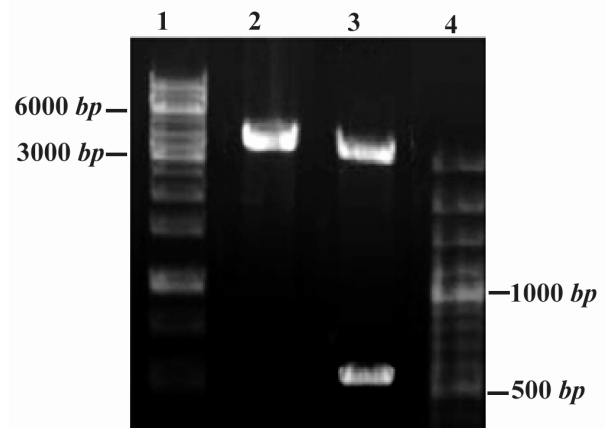


Figure 2. Restriction analysis of PICZαA-Epo construct (4.1 Kbp). DNA fragments were analyzed by 1% agarose gel electrophoresis. Lanes 1 and 4: DNA Ladder SM1163 and SM0321, respectively (Fermentas) Lane 2: Single digestion by *Xba* I (linear construct, 4.1 Kbp); Lane 3: Double digestion by *Xho* I and *Xba* I enzymes (linear pPICZαA and rHuEpo gene are identified as 3.6 Kbp and 540 bp fragments, respectively)

To reach the highest possible expression levels for our gene of interest (Epo), we primarily tried to select transformants that potentially may contain multiple copies of the integrated vector. For this purpose, transformants that appeared after 3-5 days on plates containing 100  $\mu\text{g/ml}$  Zeocin were further transferred on plates containing 1  $\text{mg/ml}$  Zeocin. Colonies that survive on such a high concentration of Zeocin, potentially may contain multiple copies of the cassette inserted into their genome<sup>(19)</sup>. Moreover, the highest expression clone for scale-up experiment was screened from 20 randomly selected Mut<sup>+</sup>-Zeocin resistant transformants (1  $\text{mg/ml}$ ) by culturing them in small scale (10  $\text{ml}$ ) for comparatively appraising the level of expression among them. It should be noted, however that multiple cassette insertions into the genome of selected transformants should be verified by methods like Southern blotting.

#### Optimization of media and buffering system of fermentation system for high level expression

Following the selection of best expression-clone, scaled-up expression of Epo in unbuffered media (simple: 1.34% YNB, 4  $\times 10^{-5}\%$  biotin, 0.5% methanol) and (complex: 1% yeast extract, 2% peptone, 1.34% YNB, 4  $\times 10^{-5}\%$  biotin, 0.5% methanol) was tried, but the expression levels were lower than 1  $\text{mg/l}$ . This low level expression may be explained by shocking levels of  $[\text{H}^+]$  concentration during yeast fermentation<sup>(20)</sup>. Shocking levels of  $[\text{H}^+]$  may be attributed to extreme high or low concentrations of  $\text{H}^+$  that could inhibit the expression of the protein during fermentation.

In fact, by the end of fermentation, utilization of unbuffered simple medium resulted in extreme acidic conditions (pH=3.5), due to apparently production of methanol oxidized metabolites when on the contrary to simple media, the pH for the unbuffered complex medium resulted in extreme basic conditions (pH=8.5) apparently due to the production of ammonium via metabolizing amino acids existing in yeast extract and peptone. This finding for extremely low expression levels of Epo in unbuffered media prompted us to

evaluate the effect of different pH values on the level of Epo expression.

In this context, by adjusting the pH value in a range of 5.0 - 7.0 (with 1.0 interval) we found that the expression of Epo in buffered methanol complex medium with a pH=6.0 was higher than other pH ranges (Figure 3A). Moreover we could show that stabilizing the pH at 6.0 throughout the expression time by addition of 0.1  $M$  phosphoric acid in a batch format every 24  $\text{hrs}$ , led to a considerable increasing of Epo expression level (150  $\text{mg/l}$ ) compared to unstabilized pH conditions (120  $\text{mg/l}$ ) (Figure 3B).

It should be noted that a perfect stabilization of pH may be achieved only through application of an automatically-controlled fermenter that adjust the pH through continuous measurement of  $[\text{H}^+]$  concentration and feeding acid in a fed-batch format. However, in shake-flask batch system, our observations indicated that up to 12  $\text{hr}$  of fermentation pH was around 6.5 and by 24  $\text{hr}$  of fermentation was around 7.0 (still below pH=8.5 and shocking levels of  $\text{H}^+$  concentrations as discussed above).

Therefore adjustment of pH every 24  $\text{hr}$  could relatively fulfill the required pH stabilization (around 6) in our system. A higher expression level of rHuEpo that obtained at pH 6.0 can be the result of improvement in the yeast growth and/or reduced activity of secreted proteases as previously proposed<sup>(19)</sup>.

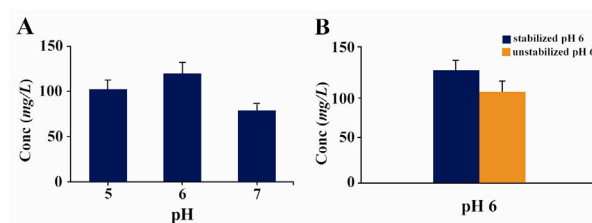


Figure 3. A) Effect of different pH values on expression/secretion level of rHuEpo. Data are the means  $\pm$  SD (n=3 each). B) Influence of pH stabilization at 6 throughout the expression time by addition of 0.1  $M$  phosphoric acid in a batch format every 24  $\text{hr}$ . Data are the means  $\pm$  SD (n=3 each, p-value = 0.014). Expression levels in all the trials were measured at the end of induction period (72  $\text{hr}$ ) by ELISA and Densitometry

### Purification and characterization of methylotrophic yeast-expressed rHuEpo

Due to the designed and inserted 6xHis-tag at the C terminal of the expressed rHuEpo in the body of PICZ $\alpha$ A-Epo construct, Ion Metal Affinity Chromatography (IMAC) was employed as a single-step purification of the expressed protein with a yield around 80% as determined by densitometry.

As shown in figure 4, yeast-derived rHuEpo migrated on the SDS-PAGE gel with an apparent mass of 33 kDa under denaturing conditions and was properly detected by monoclonal anti-rHuEpo antibody (Figure 4B) and monoclonal anti-His antibody (data not shown) in Western analyses.

To further identify and characterize the protein band corresponding to 33 kDa, N-terminal amino acid sequence analysis was performed on this 33 kDa band. The first 10 N-terminal residues in the 33 kDa band were found to be identical to the mature amino acid-deduced from cDNA sequence of rHuEpo, i.e. APPRLI? DSR (Cys7 couldn't be detected directly due to its engagement in disulfide bond formation) and showed complete processing and removal of prepro- $\alpha$ -factor sequence in this band.

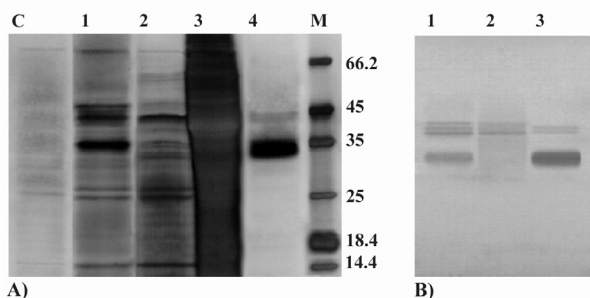


Figure 4. Analyses of *P.pastoris*-secreted rHuEpo by SDS-PAGE and western blotting. A) Silver stained SDS-PAGE view of different steps in purification of the rHuEpo by IMAC. Lane C: *P.pastoris* (wild type X-33) transformed with PICZ $\alpha$ A plasmid without the inserted Epo gene (control negative); Lane 1: loaded sample (concentrated supernatant); Lane 2: flow through of loading and prewashing; Lane 3: flow through of washing; Lane 4: Eluted (purified) *Pichia*-derived rHuEpo protein; M: Protein size marker SM0431 (Fermentas). B) Western blot view of crude and concentrated supernatant rHuEpo (Lane 1), flow through of loading and prewashing (Lane 2) and eluted (purified) *Pichia*-derived rHuEpo protein (Lane 3) using monoclonal anti-human Epo antibody

As it can be seen in figures 4A and 4B some notable bands in the region of 42-45kDa are also present in the purified fraction. With respect to the molecular weights of these bands and also their interaction with monoclonal anti-rHuEpo antibodies in Western blot analyses, these bands may indicate the unprocessed pro- $\alpha$ -factor/ Epo proteins that resulted from inefficient Kex2 endoprotease processing.

### Discussion

Although, expression of rHuEpo by *P. pastoris* in a batch culture system using baffled flasks was demonstrated <sup>(2)</sup>, their reported expression level was as low as 5 mg/l which is not an appreciated yield for scaled-up production aims.

The present study was undertaken to investigate possibility of application of other fermentation strategies rather than fed-batch methanol feeding and sorbitol utilization for high yield production of rHuEpo by *P. pastoris* (130 mg/l) which was reported recently <sup>(16)</sup> and to confirm possibility of scaled up production of rHuEpo by methylotrophic yeast. To our knowledge, this is the second report on high level expression of rHuEpo in methylotrophic yeast when the first report on optimization of fermentation process through stabilizing the pH and from a national point of view it is the first report on home-made production of yeast-derived rHuEpo.

While described capacities for recombinant protein production by *P. pastoris* varies from 1 to 10000 mg/l <sup>(15)</sup>, expression levels for rHuEpo reported in the present study (150 mg/l) provides sufficient deserve for future large scale plans on methylotrophic yeast-derived Epo production. In fact, by optimizing other factors involved in the fermentation process of methylotrophic yeasts such as temperature, induction mass, methanol concentration and feed mode through application of a controlled-continuous culturing system it may be possible to attain much higher yields for scaled-up applications <sup>(15)</sup>. Moreover, by employing other strategies

like addition of detergents such as different Tweens (20, 40, 60, 65 or 80) and/or addition of co-substrates such as sorbitol even higher yields of expression may be achieved<sup>(16, 21)</sup>.

Generally high level expression of a gene of interest depends upon the generation and detection of a recombinant strain that contains multiple copies integrated at the *AOXI* locus<sup>(22)</sup>. Because of the low frequency of multiple gene insertion events (between 1 and 10% of all selected Zeocin resistant transformants), there is a necessity of screening a large number of Zeocin-resistant transformants to locate these valuable clones.

In the previous report on rHuEpo expression in *P. pastoris*<sup>(2)</sup>, the Lithium chloride transformation method was used to transform yeast cells by the recombinant plasmid, where as application of electroporation in our study provided higher transformation frequencies as well as higher possibility of obtaining transformants with multi-copies of integrated vector (Easy Select *Pichia* Expression manual, Invitrogen). Therefore, providing a number of transformed colonies to be screened for their resistance to higher concentration of Zeocin (1 mg/ml), indicated the potential for the presence of multi-copies of expression cassette integrated into the genome<sup>(22)</sup> and still possibility for selection of the best expression clones by ELISA among a number of screened transformants in small scale expression trials, enabled us to find the highest Epo producing clone.

Moreover, in our investigation the level of Epo expression was improved noticeably through application of a batch culturing strategy by adding acid to maintain the pH at 6.0 (Figure 3B). These results are in line with many previous reports on the importance of the  $[H^+]$  concentration and specially pH stabilization at 6.0 during yeast induction period for the highest yields of expression of some other heterologous proteins<sup>(15, 23 - 26)</sup>. However, it is important to note that the optimum pH for obtaining the highest level of expression in *P. pastoris* is a protein-specific factor and greatly depends on physico-chem-

ical properties of the expressed protein that should be determined empirically and as a result, other optimum pH values have been already described for different proteins<sup>(27, 28)</sup>.

Celik et al<sup>(2)</sup> reported the apparent MW of 32 kDa for *P. pastoris* expressed rHuEpo on the SDS-PAGE gel which was 2 kDa heavier than the average MW of this protein (30 kDa) as determined by MALDI-TOF analysis. In agreement with these results, our purified *P. pastoris* expressed Epo with 166 amino acids and 6XHis-tag (which made it about 1 kDa heavier) had the apparent MW of 33 kDa under denaturing conditions.

As shown by SDS-PAGE and Western blot analyses (Figures 4A and 4B), in addition to the main rHuEpo band (33 kDa), some faint bands in the range of 42 - 45 kDa were also detected by both anti-His and anti-Epo antibodies. Incidence of these protein bands may be explained by occurrence of the uncleaved pro- $\alpha$ -factor/ Epo which were secreted into the culture media as fusion proteins. In this context, the variation in mobility between the bands in 42 - 45 kDa regions may reflect differential trimming of core oligosaccharides on the pro region segment; as this region contains three sites for Asparagine-linked glycosylation and complete or partial glycosylation of these sites may result to slightly different MWs<sup>(29)</sup>. The phenomenon of secretion of proteins in fusion with pro- $\alpha$ -factor into the media has been already reported and is the reflection of insufficient Kex2 endoprotease processing to remove the  $\alpha$ -factor secretory signal from protein<sup>(9, 30)</sup>.

Epo contains three complex type N-glycans located at asparagine (Asn) residues at positions 24, 38 and 83, and a mucin-type O-glycan located at Ser-126. Proper glycosylation of Epo is not required for its receptor binding but it is however quite essential for elongation of its biological half-life<sup>(8)</sup>. Although glycosylation pattern of CHO and *P. pastoris* may differ to some extent, but still glycosylation pattern of secreted proteins by *P. pastoris* is the most similar pattern to mammalian cells<sup>(13)</sup>.



In addition, according to recent reports on availability of engineered and humanized *P. pastoris* to secrete human glycoproteins with fully complex terminally sialylated N-glycans<sup>(31)</sup>, this expression system will become indispensable for the production of therapeutic proteins such as rHuEpo.

### Conclusion

In summary, results of this study indicated that with employing a proper cloning strategy and screening of transformants potentially containing multi-copies of the integrated expression cassette as well as improving fermentation conditions such as feeding acid in a fed-batch culturing strategy for stabilization of pH at 6.0, it is possible to reach yields of 150 mg/l or higher of methylotrophic yeast-derived rHuEpo. Data evidenced in this report demonstrated that *P. pastoris* has the potential for production of low-cost Epo and merits further scaled-up studies in more sophisticated and controlled production bioreactors.

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