



HETEROLOGOUS EXPRESSION AND CHARACTERIZATION OF HUMAN ERYTHROPOIETIN IN *PICHIA PASTORIS*

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ABSTRACT

Recombinant human erythropoietin (EPO) is a glycoprotein produced as a therapeutic agent for the treatment of anemia associated with severe kidney damage. The demand of this protein currently met by recombinant expression in mammalian cells. *Pichia pastoris* has become popular yeast based protein production systems to substitute mammalian expression systems. In this study, recombinant human EPO (rhEPO) protein obtained by expressing the human *epo* gene in methylotropic yeast *P. pastoris*, strain X33. The human EPO cDNA was inserted into pPICZαB vector, under the control of AOX1 promoter, and fused with a polyhistidine tag and c-myc epitope. Several clones were screened by Western blot analysis using polyclonal anti-EPO antibodies. The highest expressing clone was selected for subsequent study. The recombinant human EPO (rHuEPO) protein produced was approximately 37 kDa in size. Analyses by SDS/PAGE, Western blot, deglycosylation and internal amino acid sequencing confirmed the authenticity of the expressed rHuEPO protein.

KEYWORDS: Erythropoietin, EPO, *Pichia pastoris*, Yeasts



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INTRODUCTION

Erythropoietin (EPO) is a hematopoietic glycoprotein that stimulates the differentiation of late erythroid progenitor cells to mature red blood cells. EPO is a 30-38 kDa glycoprotein (exact molecular weight depends on the degree of glycosylation) in which sixty per cent of the molecule is an invariant 165 amino acid single polypeptide chain containing two disulphide bonds. The remaining 40% of the EPO mass consists of carbohydrate covalently attached at three N-linked sugar chain at Asn 24, 38 and 83, and one O-linked sugar chain at Ser126^{1,2,3,4}. In humans, EPO is mainly produced by hepatocytes in the fetus and in the peritubular cells in the kidneys of the adult. The glycosylation of EPO is extremely important for this molecule to perform its function. In particular, it has been shown that the addition of carbohydrate is required for secretion, stability, delay clearance and biosynthesis^{5,6}. However, this carbohydrate is not required for receptor binding⁷. In response to a decrease in tissue oxygenation, EPO synthesis increases in the kidney. The secreted hormone bind to receptor on the surface of red blood cell precursor in the bone marrow, leading to their survival, proliferation and differentiation, and finally increase in hematocrit^{8,9}. The use of EPO is highly effective at correcting the anemia, restoring energy levels, and increasing patient well being and quality of life^{10,11,12}. Recombinant human EPO (rHuEPO) has been frequently used clinically as an erythropoietic stimulating agent in the treatment of anemia associated with various pathologies (i.e. cancer, HIV, chemotherapy, chronic renal insufficiency). With the key patents for rHuEPO expired, the market has opened for biosimilar recombinant products from the same cell line with identical or improved pharmacokinetic properties and less immunogenic response. The production of recombinant human proteins has enabled great progress in healthcare and drug technology. With this, the availability of approved biopharmaceuticals has increased tremendously in the last several years.

Currently, the majority of successful recombinant biopharmaceutical ingredients used for the production of human medicines

are produced in CHO cell lines¹³. However, with its relatively high cost for complex growth media, recent innovations have revived the interest in other alternative systems. The first biopharmaceuticals to enter market such as insulin and growth hormone were produced in cloned bacteria. Bacteria grow quickly and can generate high quantities of recombinant protein. However, the majority of therapeutic proteins requires additional post-translational modifications, like N-glycosylation which prokaryotic cells can not provide¹⁴. N-glycosylation ensures protein structure, function and subsequent stability in human serum. Recently, the use of the methylotrophic yeast, *Pichia pastoris*, as a cellular host for the expression of recombinant proteins has become increasing popular. *Pichia* can be as easily cultured and genetically manipulated as *Escherichia coli* and has a eukaryotic modification machinery¹⁵. In addition, because the gene of interest is integrated into the *Pichia* genome rather than being contained in a plasmid, the stability of the expressed transformant can be maintained easily and reproducibly. This has enabled *P. pastoris* to provide the potential for stable and correctly folded recombinant proteins that have undergone all the post-translational modifications required for functionality¹⁶. *P. pastoris* has become a highly successful used system for the production of a variety of recombinant heterologous proteins for both academic and industrial purposes. This system is capable of metabolizing methanol as its sole carbon source by the enzyme alcohol oxidase, as the product of AOX1 and AOX2 genes. However, the largest fraction of alcohol oxidase activity by far, in the cell is attributable to the product of the AOX1 gene. Methanol is a requirement to fully induce transcription of AOX1 which implies a tightly regulated promoter. By taking advantage of the tight regulation of AOX1 promoter by methanol, the gene of interest encoding the desired heterologous protein is expressed¹⁷. Another advantage of this system is also from its ability to grow to a very high cell density on simple media. The availability of vector with the secretion signal of the α -mating factor

from *Saccharomyces cerevisiae* that causes the protein to be secreted into the growth medium greatly facilitates subsequent downstream processing of this system. Thus, the interest for using *P. pastoris* for production of recombinant proteins stems to a large extent from the tight regulation and efficient methanol-inducible promoters, capacity to perform post-translational modifications, its ability to grow to very high cell densities in simple media and pathways leading to recombinant products secretion and purification. In this study, we report the expression of rHuEPO in *P. pastoris*. Under the control of the AOX1 promoter and in-frame with α -mating factor secretion signal sequence, human *epo* gene was incorporated into the AOX1 locus, and protein synthesis was induced with methanol. Protein rHuEPO secreted into the culture medium was purified using immobilized metal affinity chromatography (IMAC) and peptide identification was performed on the purified protein. The gel shift analysis of the purified protein using PNGase F was also performed and discussed.

MATERIALS AND METHODS

(i) Strains and growth culture media

E. coli TOP10 (Invitrogen) was used as the host for plasmid cloning experiment and grown in LB medium (1% tryptone, 0.5% NaCl and 0.5% yeast extract, plus 2% agar in plates). For screening purposes, zeocin was added at standard concentrations for screening the bacteria and the selection of yeast transformant. *P. pastoris* strain X-33 (Invitrogen, San Diego). The yeast strain was cultured in the YPD medium (1% yeast extract, 2% peptone and 2% dextrose, plus 2% agar in plates). The media compositions for expression study were BMGY (1% yeast

extract, 2% peptone, 1.34% YNB, $4 \times 10^{-5}\%$ biotin, 1% glycerol and 0.1 M potassium phosphate, pH = 6.0) and BMMY (BMGY medium containing 0.5% methanol instead of glycerol). Yeast cultures were grown at 30°C.

(ii) Recombinant DNA techniques

DNA manipulations, such as PCR, bacterial transformation, restriction enzymes digestion, plasmid preparation, ligations and agarose-gel electrophoresis were performed as previously described¹⁸. The restriction enzymes were obtained from New England Biolabs (Beverly, MA, USA) or Fermentas (Lithuania).

(iii) Construction of the plasmid pPICZ α B-*epo*

The human *epo* gene contains inside KShEPO#2 plasmid (a kind gift of Dr. Emmanuel Payen, Laboratoire de Therapie Genique Hematopoietique, Paris, France) was amplified using PCR amplification. The forward primer (5'-CCGCTCGAGAAAAGAGAGGCTGAAGCTGCCACC ACGCCTCATC-3') contained 45 bases (the restriction site for *Xho* I is underlined). The reverse primer (5'-GCTCTAGAGCTCTGTCCCCTGTCCTGCAGGCCTC-3') was designed such that, at the 3'-end of the *epo* sequence, a stop codon and *Xba* I restriction site were present. The final insert DNA fragment contains *Xho* I restriction site, human *epo* gene, *Xba* I, c-myc epitope, 6x Histag, and stop codon (scheme in Fig. 1). The insert DNA was double-digested with *Xho* I and *Xba* I, purified and ligated with the vector pPICZ α B that has been cut with the same restriction enzymes. The ligation mix was used to transform *E. coli* TOP10 cells, according to the manufacturer's instructions. The resulting recombinant plasmid was named as pPICZ α B-*hEPO*.

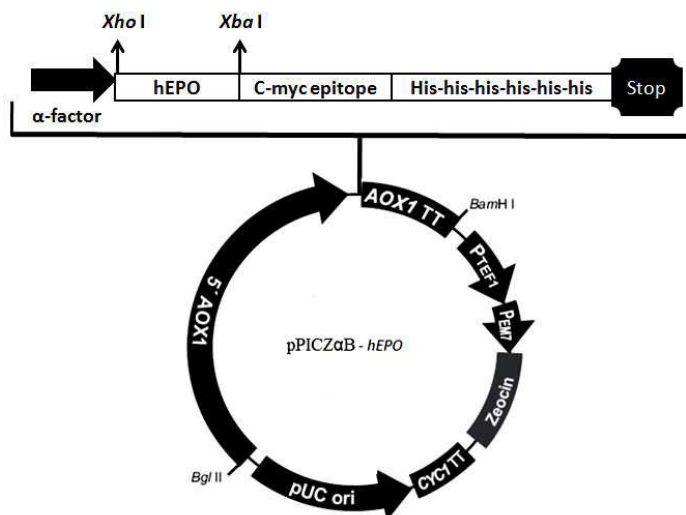


Figure 1

Schematic diagram of recombinant pPICZαB harboring human epo gene. Xho I and Xba I denote to the restriction sites employed for directional cloning of human epo gene into pPICZαB under control of AOX1 promoter, down stream of secretion signal (α-factor).

(iv) Transformation of *P. pastoris*

Purified pPICZαB-epo recombinant plasmid was linearized with *BstX I*. Following complete digestion and verification by agarose gel electrophoresis, the plasmid was used to transfect *P. pastoris* wild-type host strain X-33

using electroporation method. Transformation was performed using electroporator Biorad Gene pulser XCell according to supplier's instructions (Easy Select *Pichia* Expression manual, Invitrogen).

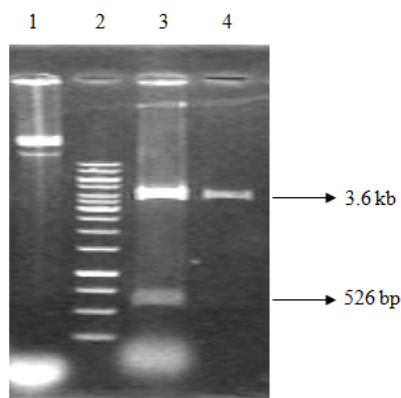


Figure 2

Restriction analysis of pPICZαB-epo construct. DNA fragments were analyzed by 1% agarose gel electrophoresis. Lane 1, uncut; lane 2, DNA Ladder (Catalog: #SM0311, Fermentas); lane 3, double digestion by Xho I and Xba I enzymes; and lane 4, single digestion by BstX I enzyme.

(v) Selection of the potential rHuEPO-producing strain

Selection of cell producing strain was performed based on expression level. Several clones were screened by Western blot analysis using human polyclonal anti-EPO antibodies. The highest expressing clone was selected for subsequent study. Each selected

colony on zeocin YPD plate was inoculated into 2 ml in BMGY at 30°C. Growth was allowed until OD₆₀₀ 2-6 (approximately 16-18 hours). The cells were harvested by centrifugation at 1500-3000 x g for 5 min at room temperature and gently resuspended in 2 ml BMMY media. The cells were incubated at 30°C for another 3 days and methanol was

replenished to a 0.5% (v/v) final concentration every 24 hr. After 3 days, the medium was centrifuged at 13000 x g for 10 min and the cell pellet was discarded. The supernatant was analyzed by Western blot. The transformant that gave the highest high level of expression was selected for production.

(vi) Production and purification rHuEPO

The transformant that gave the highest level of expression was used for this experiment. Protein production was carried out in batch culture with 250 ml Erlenmeyer baffled flasks containing 50 ml of production medium. Incubation was performed for 72 h in total, and every 24 h methanol was added to the medium to a 0.5% (v/v) final concentration. At the end of 72 hr, the media were collected by centrifugation at 13000 x g for 10 min, the cell pellet was discarded and the supernatant was concentrated with Centriprep 10 YM concentrators (Milipore, Bedford, MA). Subsequently, The polyhistidine-tagged rHuEPO was purified from the concentrated medium by the Ion Metal Affinity Chromatography (IMAC) columns charged with Ni²⁺ according to the supplier instructions (His-Trap HP affinity columns, GE Healthcare).

(vii) Deglycosylation of purified rHuEPO

Deglycosylation experiments were performed using PNGase F (Roche, Germany) in accordance with the manufacturer's protocols. For positive control experiments, recombinant human erythropoietin produced in CHO cells (CHOEPO) (Calbiochem, USA) was used. Bands were revealed by staining with Silver stain (Fermentas, Lithuania).

(viii) SDS-PAGE and Western blotting

The purity of the protein analyzed by SDS/PAGE performed as previously described¹⁹ in a 12% separating gel with a 5% stacking gel using the Mini-PROTEAN-3 apparatus (BioRad, Hercules, CA, USA). Following electroporation, proteins were transferred to Amersham Hybond ECL (GE Healthcare) by electroblotting. Western blots

were performed using polyclonal anti human EPO antibody (Sigma, St. Louis, MO, USA) as the primary antibody and anti rabbit IgG alkaline phosphatase linked whole antibody (Promega, Madison, WI, USA) as the secondary antibody. The bands were detected by BCIP/NBT color development substrate (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium) (Promega, Madison WI, USA).

(ix) Mass Spectrometry Analysis

Purified protein was analyzing using 15% SDS-PAGE for characterization. The protein band was sliced from gel and subjected to peptide sequencing that was performed by MALDI TOF/TOF (Proteomic International). In brief, coomassie blue stained gel pieces were digested by trypsin.

RESULTS

1. Expression study

Recombinant HuEPO protein was expressed and purified from the yeast *P. pastoris*. The human *epo* sequence (including C-terminal polyhistidine tag and myc-epitope) was subcloned into the yeast expression vector pPICZα B. The expression of the human *epo* was placed under the control of the *P. pastoris* AOX1 promoter and *S. cerevisiae* alpha-factor prepro-leader sequence. After linearized by *Bst*X I digestion, the construct was then used to transform *P. pastoris* strain X-33. Following induction of protein expression for 3 days at 30°C in the presence of 0.5% methanol, the rHuEPO is secreted into the medium. The media were collected by centrifugation at 13000 g for 10 min and the supernatant containing rHuEPO was collected while the cells were removed. Following purification, the sample was run on SDS/PAGE and detected with Western blot analysis using polyclonal anti human EPO antibody. The molecular mass of rHuEPO-Stop was detected at about 37 kDa while that of CHOEPO ranged from 35-38 kDa (Fig. 3).

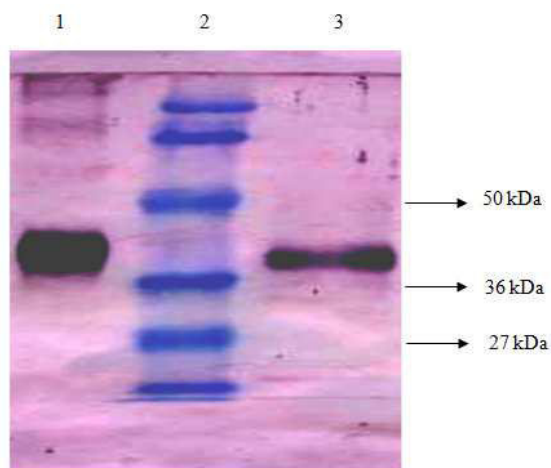


Figure. 3

Silver-stained SDS-PAGE analysis. Lane 1, human EPO produced by CHO cell (Calbiochem, Merck); lane 2, molecular-mass markers (*b*-galactosidase, 118 kDa ; BSA, 90 kDa ; ovalbumin, 50 kDa ; carbonic anhydrase, 36 kDa ; beta-lactoglobulin, 27 kDa ; lysozyme, 20 kDa); lane 3, polyhistidine tag affinity-purified rHuEPO from *P. pastoris*.

2. Deglycosylation of purified rHuEPO

To examine the difference in glycosylation of rHuEPO produced in *P. pastoris* and CHO cells, the two recombinant EPOs were treated with glycosidase (PNGase F) to release the N-linked carbohydrates. The molecular mass of the polypeptide backbone of CHO EPO was

ranged from 23-36 kDa (lane 4 Fig. 4). For rHuEPO, the molecular mass of the polypeptide backbone ranged from 27-38 kDa (lane 2 Fig. 4). The molecular size difference between CHO EPO and rHuEPO is not visible when the proteins are not treated with PNGase F (Fig. 3 and Fig. 4).

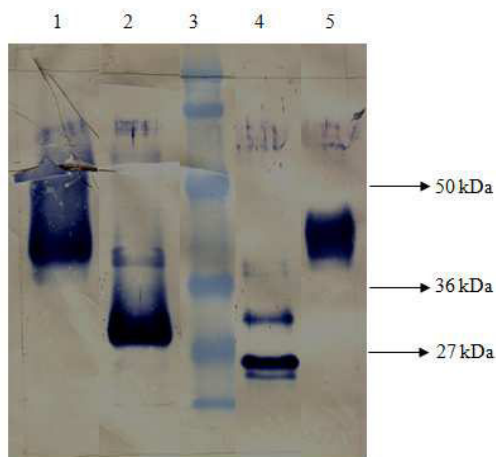


Figure 4

Western blot of purified protein before and after deglycosylation by PNGase F. Lane 1, rHuEPO from *P. pastoris*; lane 2, deglycosylated rHuEPO from *P. pastoris*; lane 3, molecular-mass markers (*b*-galactosidase, 118 kDa; BSA, 90 kDa; ovalbumin, 50 kDa; carbonic anhydrase, 36 kDa; beta-lactoglobulin, 27 kDa; lysozyme, 20 kDa); lane 4, deglycosylated CHO EPO; lane 5, CHO EPO.

3. Protein Identification

The expressed recombinant protein was purified and digested with trypsin and the peptides were analysed to generate peptide mass fingerprint. The predicted fragment products of the trypsin digestion reaction of

rHuEPO are shown in Fig. 5. The peptide fragments underlined in the figure were those identified. The peptides identified have sequence coverage about 40 % from the total sequence.

APPRLICDSRVLERYLLEAKEAENITTGCAEHC SLNENITVPDTKVNFYAWKRMEV
 GQQA VE V W QGLALLSEAVLRGQALLVNSSQPWEPLQLHVDKAVSGLRSLTLLRAL
 RAQKEAI SPPDAASAAPLRTITADTFRK LFRVYSNFLRGK LKLYTGEACRTGDR

Figure 5

Erythropoietin (EPO) amino acid sequence. The underlined amino acids are the ones detected in the mass spectrometry analysis. The bold asparagine residue indicates the glycosylation sites.

DISCUSSION

The advance of recombinant DNA technology and its application in the pharmaceutical industry has brought a growing market within the human medical biotechnology industry. Most of approved biopharmaceuticals are proteins such as: blood factors, anticoagulants, hormones, vaccines and monoclonal antibodies. However, 70% of them are N-linked glycoproteins, and therefore host cells for production must contain the relevant protein modification machinery for correct folding, biological activity and/or stability in the circulation. The methylotrophic yeast *P. pastoris* has a number of attractive characteristics for heterologous protein production, including the ability to perform post-translational modifications, such as N- and O-linked glycosylation, and secrete large amounts of recombinant protein. To explore expression system that would allow for rHuEPO expression, in this current study, the coding sequence of the therapeutically important human glycoprotein EPO was fused with a polyhistidine tag to enable easy, rapid purification, and the human *epo* gene was expressed in *P. pastoris* expression system. In our study, we obtained up to 4 mg/l media of purified rHuEPO at the end of 72 hour production period. Our result is comparable with previous study²⁰ where 5 mg/l of rHuEpo was obtained. However, using fed-batch methanol and sorbitol feeding strategy, as much as 130 mg/l of rHuEpo was obtained at 24 hour incubation time²¹. Interestingly, using simple shake-flask batch system and maintaining the pH at 6.0²² reported higher result where 150 mg purified rHuEpo/l media was produced at 72 hour incubation time. Recombinant protein production capacities by *P. pastoris* greatly vary from 1 mg/l²³ to 10 g/l

media²⁴. Overall, this all may reflect the different conditions of incubation including pH control, oxygen limitation, nutrient limitation and temperature fluctuation. Switching from a shaker to a fermentor could overcome most all those problems and can increase protein production by 140%²⁵. Since the concentration of the product in the extracellular medium is roughly proportional to the concentration of cells in the culture in many instances, cell growth is particularly important for secreted protein production. Since its incubation conditions can be controlled, the use of bioreactor allows more efficient production of the desired heterologous protein²⁶. Overall, the results suggest that, through optimization of medium and conditions, higher amounts of recombinant protein could be obtained.

Observed in the culture supernatant, Figure 3 shows the SDS-PAGE analysis of the expressed rHuEPO protein with a molecular weight of about 37 kDa. The increased size of the expressed protein was caused by the addition of C-terminal polyhistidine tag and cMyc-epitope to the human *epo* gene cloned in AOX1 locus. The size of the cMyc alone is approximately 2.5 kDa. From the clean band obtained with Western blot using polyclonal antihuman EPO antibody suggested that the protein has no visible degradation. Western blot analysis showed that in our study the rHuEPO had been expressed successfully. By using the strategy described in this study, 6× his-tagged rHuEPO can be produced and purified easily with a one step Ni-NTA agarose chromatography. Protein glycosylation is one of the most common structural modifications employed by biological systems to expand proteome diversity. The

prevalence of glycosylation is such that it has been estimated that 50% of all proteins are glycosylated. Functionally, glycosylation has been shown to influence a variety of critical biological processes at both the cellular and protein levels including its stability and long residence in body⁵. Structurally, glycosylation is highly complex due to the fact that there can be heterogeneity with respect to the site of glycan attachment (macroheterogeneity) and with respect to the glycan's structure (microheterogeneity). Some of these charged terminal glycans (e.g., sialic acid) have also been found to be critical in regulating the circulatory half-life of glycoproteins. This has led to the development of glycosylation profile as a novel strategy to improve the therapeutic efficacies of protein pharmaceuticals⁵.

EPO is a heavily glycosylated protein hormone. Mature EPO is composed of 165 amino acids single polypeptide chain containing two disulphide bonds. The molecular mass of the polypeptide backbone is estimated to be 60% of the total mass (by weight). The remaining 40% of the mass of the molecule is carbohydrate. PNGase F has been utilized extensively for demonstrating shifts in SDS/PAGE mobilities as an indication of N-glycosylation. In our study, enzymatic removal of the glycans moiety of EPO produced in *P. pastoris* with N-glycosidase resulted in a band with a molecular mass approximately corresponds to the molecular mass of the EPO polypeptide backbone which ranged from 27-38 kDa, indicating that the N-linked carbohydrate moiety was present in our expressed protein. Similar result was also found using CHO EPO which ranged from 23-36 kDa. The higher bands in lane 2 and 4 (Fig. 4) were probably due to incomplete deglycosylation. The broadness of the protein band noted on SDS/PAGE has also been observed and is believed to be due to heterogeneity of the carbohydrate moiety²⁷. The most prominent case of microheterogeneity for EPO is in the N-linked carbohydrate chains, where the oligosaccharides may contain two, three or four branches¹⁰. In order to further verify the protein under study, protein identification was performed. Protein sample was enzymatically digested by trypsin to produce fragmented

peptides. The predicted fragment products of the trypsin digestion reaction of rHuEPO are shown in Fig. 5. The identified peptides have sequence coverage of 37.5%. Combined with deglycosylation study, this protein identification data showed that the rHuEPO expression in *P. pastoris* worked successfully. Combined with previous studies, this result demonstrates the great potential of *P. pastoris* expression system for production of recombinant protein. However, the most critical issue still needs to be answered is whether glycosylated protein like erythropoietin expressed in *P. pastoris* can be used for human. Despite its highly successful system for production of recombinant protein due to its unique features, the glycosylation pattern of *P. pastoris* and CHO cells differ to some extent in that recombinant proteins produced in *P. pastoris* tend to be hypermannosylated which has the potential to be immunogenic. With this, production of therapeutic glycoproteins still relies on mammalian hosts. However, since many biopharmaceuticals are glycoproteins or have glycoprotein targets, the use of the methylotrophic yeast *P. pastoris* with its highly successful usage for heterologous proteins production can be tremendously beneficial for a reduction in the time and cost of production. To overcome this problem, *P. pastoris* with fully complex terminally sialylated N-glycans which can increase the half-life of glycoproteins and reduce or eliminate the risk of immunogenicity has been engineered²⁷. With this, the generation of such yeast cell lines will allow the production of therapeutic glycoproteins in a nonmammalian host.

CONCLUSION

P. pastoris has become a highly successful system for the production of a variety of recombinant heterologous proteins for both academic and industrial purposes. In this study, we describe the cloning, expression and analysis of rhEPO produced in the methylotrophic yeast *Pichia pastoris*. Analyses by SDS/PAGE, Western blot, deglycosylation using PNGase F and internal amino acid sequencing confirmed the authenticity of the expressed rHuEpo protein with the size

approximately 37 kDa. In our study, we obtained up to 4 mg/l media of purified rHuEPO at the end of 72 hour production period.

ACKNOWLEDGEMENT

The authors greatly appreciate Dr. Emmanuel Payen, Laboratoire de Therapie Genique

Hematopietique, Paris, France, for the human EPO clone. This work was supported by the Competitive Program of Indonesian Institute of Sciences (LIPI) (0076b/IPH.2/HK.03.01/2009) and the RUT Program of State Ministry of Research and Technology, Indonesia (07/Perj/Dep.III/RUT/PPKI/II/2007).

REFERENCES

1. Skibeli V, Nissen-Lie G, and Torjesen P, Sugar profiling proves that human serum erythropoietin differs from recombinant human erythropoietin, *Blood*, 98: 3626-3634, (2001).
2. Lai PH, Everett R, Wang F.F, Arakawa T, and Goldwasser E, Structural characterization of human erythropoietin, *J Biol Chem*, 261: 3116-3121, (1986).
3. Tsuda E, Goto M, Murakami A, Akai K, Ueda M, Kawanishi G, Takahashi N, Sasaki R, Chiba H, and Ishihara H, Comparative structural study of N-linked oligosaccharides of urinary and recombinant erythropoietins, *Biochemistry*. 27(15): 5646-5654, (1988).
4. Takeuchi M, Takasaki S, Miyazaki H, Kato T, Hoshi S, Kochibe N and Kobata A, Comparative study of the asparagine-linked sugar chains of human erythropoietins purified from urine and the culture medium of recombinant Chinese hamster ovary cells, *J Biol Chem*, 263(8):3657-3663, (1988).
5. Sola RJ and Griebenow K, Effects of glycosylation on the stability of protein pharmaceuticals, *J Pharm Sci*, 98(4): 1223-1245, (2009).
6. Huijuan Li and Marc d'Anjou, Pharmacological significance of glycosylation in therapeutic proteins. *Current Opinion in Biotechnology*, 20(6): 678-684, (2009).
7. Narhi LO, Arakawa T, Aokii KH, Elmore R, Rohde MF, Boone T and Strickland, T.W, The effect of carbohydrate on the structure and stability of erythropoietin, *J Biol Chem*, 266(34): 23022-23026, (1991).
8. Fried W, Erythropoietin and erythropoiesis, *Experimental Hematology*, 37:1007-1015, (2009).
9. D'Andrea AD, Lodish HF and Wong GG, Expression cloning of the murine erythropoietin receptor, *Cell*, 57(2): 277-285, (1989).
10. Egrie JC, Browne JK, Development and characterization of novel erythropoiesis stimulating protein (NESP), *Neophrol Dial Transplant*, 16: 3-13, (2001).
11. Esbach JW, Egrie JC, Downing MR, Browne JK and Adamson JW, Correction of the anemia of end-stage renal disease with recombinant human erythropoietin. Results of a combined phase I and II clinical trial, *N England J. Med*, 316: 73-78, (1987).
12. Evans RW, Rader B and Manninen DL, The quality of life of hemodialysis recipients treated with recombinant human erythropoietin, Cooperative Multicenter EPO Clinical Trial Group. *JAMA*, 263: 825-830, (1990).
13. Matasci M, Hacker DL, Baldi L and Wurm FM. Protein therapeutics recombinant therapeutic protein production in cultivated mammalian cells: current status and future prospects. In: *Drug Discovery Today: Technologies*, 5: 37-42, (2008).
14. Helenius A and Aebi M, Intracellular function of N-linked glycans, *Science*, 291(5512): 2364-2369, (2001).
15. Fischer R, Drossard J, Emans N, Commandeur U and Hellwig S, Towards molecular farming in the future: moving from diagnostic protein and antibody production in microbes to plants,

- Biotechnol. Appl. Biochem*, 30: 117-120, (1999).
16. Cereghino GPL and Cregg JM, Applications of yeast in biotechnology: protein production and genetic analysis, *Curr Opin Biotech*, 10(5): 422-427, (1999).
 17. Cereghino GPL and Cregg JM, Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*, *FEMS Microbiol Rev*, 24: 45-66, (2000).
 18. Sambrook J and Russell D, Molecular Cloning: A Laboratory Manual, 3rd Edn. New York: Cold Spring Harbor Laboratory Press, (2001).
 19. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature*, 227: 680-685, (1970).
 20. Celik E, Calik P, Halloran SM and Oliver SG. Production of recombinant human erythropoietin from *Pichia pastoris* and its structural analysis. *Journal of Applied Microbiology*, 103(6): 2084-2094, (2007).
 21. Celik E, Calik P and Oliver S, Fed-batch methanol feeding strategy for recombinant protein production by *Pichia pastoris* in the presence of co-substrate sorbitol. *Yeast*, 26(9): 473-484, (2009).
 22. Maleki A, Roohvand F, Tajerzadeh H, Khanahmad H, Nobari M, Beiruti A and Najafabadi AR, High Expression of methylotrophic yeast-derived recombinant human erythropoietin in a pH-controlled batch system. *Avicenna Journal of Medical Biotechnology*, 2(4): 197-206, (2010).
 23. Weiss HM, Haase W, Michel H and Reinlander H, Expression of functional mouse 5-HT_{5A} serotonin receptor in the methylotrophic yeast *Pichia pastoris*: Pharmacological characterization and localization, *FEBS*, 377: 451-456, (1995).
 24. Sreekrishna K, Nelles L, Potenz R, Cruse J, Mazzaferro P, Fish W, Fuke M, Holden K, Phelps D, Wood P and Parker K, High level expression, purification and characterization of recombinant human tumor necrosis factor synthesized in the methylotrophic yeast *Pichia pastoris*, *Biochemistry*, 28: 4117-4125, (1989).
 25. Chen Y, Krol J, Cino J, Freedman D, White C and Komives E, Continuous production of thrombomodulin from a *Pichia pastoris* fermentation. *Journal Chem Tech Biotechnol*, 67: 143-148, (1996).
 26. Patrick SM, Fazenda ML, McNeil B and Harvey LM, Heterologous protein production using the *Pichia pastoris* system, *Yeast*, 22: 249-270, (2005).
 27. Hamilton SR, Davidson RC, Sethuraman N, Nett JH, Youwei Jiang Y, Rios S, Bobrowicz P, Stadheim TA, Li H, Choi BK, Hopkins D, Wischniewski H, Roser J, Mitchell T, Strawbridge RR, Hoopes J, Wildt S and Gerngross TU, Humanization of yeast to produce complex humanization of yeast to produce complex terminally sialylated glycoproteins, *Science*, 313: 1441-1443, (2006).