EFFECT OF METHANOL INDUCTION AND INCUBATION TIME ON EXPRESSION OF HUMAN ERYTHROPOIETIN IN METHYLOTROPIC YEAST *Pichia pastoris*

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Abstract

Erythropoietin (EPO) is a glycoprotein hormone consists of 165 amino acids and has molecular mass of 30,400 Daltons. The large quantities of these hormone required to satisfy clinical demand are currently met by recombinant expression in mammalian cell, namely chinese hamster ovary (CHO). *Pichia pastoris* has become popular yeast based protein production systems to substitute mammalian expression systems. *P. pastoris* is capable to use methanol as sole carbon and energy source. In this study, recombinant human EPO (rhEPO) protein obtained by expressing the hEPO gene in methylotropic yeast *P. pastoris,* strain X33. The present work was carried out to study the optimal methanol concentration for induction and the incubation time to obtain rhEPO protein. To perform this study, the transformed *P. pastoris* was induced with various concentrations of methanol (0%, 0.5%, 1%, 2.5%, 5%, 10%, and 20%) and incubation times (0 hours, 24 hours, 48 hours, 72 hours, 96 hours, 120 hours, and 144 hours). The results demonstrate that the highest protein expression level occurred at concentration of 2.5% methanol induction, while the optimal incubation time was at 48 hrs.

Abstrak

Pengaruh Induksi Methanol dan Waktu Inkubasi terhadap Ekspresi Eritropoietin Manusia pada Khamir Metilotropik *Pichia pastoris*. Eritropoietin (EPO) adalah hormon glikoprotein yang terdiri dari 165 asam amino dan memiliki berat molekul sebesar 30.400 Daltons. Sebagian besar kebutuhan EPO didapatkan dari hasil sintesis pada sel mamalia *Chinese hamster ovary* (CHO). *Pichia pastoris* adalah sejenis khamir yang populer digunakan untuk menggantikan sistem ekspresi pada sel mamalia. *P. pastoris* dapat menggunakan metanol sebagai satu-satunya sumber energi karbon. Pada studi ini, protein rekombinan EPO (rhEPO) disintesis dengan cara mengekspresikan gen hEPO pada khamir metilotropik *P. pastoris* strain X33. Studi ini dilakukan untuk mengetahui konsentrasi metanol dan waktu inkubasi yang optimal untuk mensintesis rhEPO. Pada studi ini konsentrasi metanol yang digunakan adalah 0%, 0.5%, 1%, 2.5%, 5%, 10%, dan 20%. Sedangkan waktu inkubasi yang digunakan adalah 0 jam, 24 jam, 48 jam, 72 jam, 96 jam, 120 jam, and 144 jam. Hasil penelitian ini menunjukkan bahwa ekspresi protein yang tertinggi terjadi pada konsentrasi metanol sebesar 2.5% dan waktu inkubasi selama 48 jam.

Keywords: hEPO, methanol, Pichia pastoris, Western blot

1. Introduction

Erythropoietin (EPO), a glycoprotein hormone of 30 Dalton produced in the adult kidney and fetal liver, regulates the level of erythrocytes in response to the level of oxygen in the blood [1]. In response to a decrease in tissue oxygenation, EPO synthesis increases in the kidney. The secreted hormone bind to specific 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 [2-3]. It is highly effective at correcting anemia associated with cancer, HIV infection, myelodysplastic syndromes, prematurity, autologous blood donation, chronic renal failure, bone marrow transplantation, restoring energy levels and increasing patient well being and quality of life [4-8].

The large quantities of the hormone required to satisfy clinical demand are currently met by recombinant

expression in mammalian cell, namely Chinese hamster ovary (CHO). However, there are significant disadvantages of the use of mammalian cell cultures with respect to efficientcy and cost [9]. Mammalian systems are often hampered by disadvantages such as long production times, low protein titers, product heterogeneity and viral containment issues. This can impose serious challenges for large scale production of therapeutic glycoproteins when using mammalian cell lines [9-11]. Consequently, there is a continual search for alternative expression systems with improved performance.

Besides mammalian, another expression system frequently used is a prokaryotic system using bacteria. Some therapeutic proteins can be expressed in prokaryotic cells, like insulin in *Escherichia coli*. However, the majority of therapeutic proteins require additional post-translational modifications, like N-glycosylation which prokaryotic cells can not provide [12-14]. N-glycosylation ensures proper protein structure, function and subsequent stability in human serum. Thus, the use of bacterial system for production of EPO protein is not suitable. This makes alternative system beyond mammalian and bacterial systems is needed.

Pichia pastoris has become popular yeast based protein production systems to substitute mammalian and bacterial expression systems. Yeasts grow rapidly and produce proteins using a eukaryotic protein-synthesis pathway, and the costs of the media, equipment and infrastructure to culture yeast are lower than that of mammalian cells. Yeast can carry out glycosylation of the amide nitrogen of asparagine residues in a protein when found in the consensus sequence Asn-Xaa-Thr/Ser, providing N-linked glycosylation. Also, glycosylation of hydroxy groups of threonine and/or serine residues in proteins occurs in yeast cells, yielding O-linked type of glycosylation [15]. Practically, the advantages of this particular expression system can be attributed to several factors, most importantly: a) the simplicity of techniques, b) the ability of *P. pastoris* to produce foreign proteins at high levels, c) the capability of performing many eukaryotic post-translational modifications, and d) the ability of the cells to grow at very high density [16].

P. pastoris is capable to use methanol as sole carbon and energy source. The methanol is oxidized by the alcohol oxidases of this yeast. The alcohol oxidases are produced by the two AOX genes of *Pichia*: AOX1 and AOX2 [4]. Where as the protein coding regions of the functional genes and the protein products are 92% and 97% homologous, respectively, the promoter regions are distinct: AOX1 produces approximately 90% of the alcohol oxidase in the methanol induced state of the cells. The strong AOX1 promoter is used for

constructions of recombinant protein expressing cassettes which are integrated into the genome of the yeast, resulting strains in which the recombinant protein production is under the control of methanol concentration in the media [17-18]. Thus, methanol serves as the inducer of product formation as well as energy and carbon source.

Methanol induction and incubation time are both critical parameters for acquiring optimal results of protein expression in *P. pastoris* expression system. Therefore, here we used these two parameters, to know the effect of various methanol concentrations and duration of incubation times on the expression of hEPO gene in methylotrophic yeast *P. pastoris*.

2. Methods

Strain. The *E. coli* DH5alpha strain (Invitrogen, San Diego, USA) was used as a host for DNA manipulation and cultured in low-salt LB medium (1% tryptone, 0.5% NaCl and 0.5% yeast extract, plus 2% agar in plates). For screening purposes, ampicillin was added at standard concentrations for screening the bacteria and Zeocin for the selection of yeast transformant. *P. pastoris* strain X_{33} mut$^+$ (wild-type) from Invitrogen Co. (USA) was used. It was maintained on YPD agar plate (1% bacto yeast extract, 2% peptone, 2% dextrose (glucose), and 1,7% bacto agar).

Construction of the plasmid. hEPO clone (a kind gift of Dr. Emmanuel Payen, Laboratoire de Therapie Genique Hematopoietique, Paris, France) was subcloned into the *Xho* I and *Xba* I sites of yeast pPICZαB (Invitrogen, Carlsbad, CA, USA). This vector allows the gene of interest to be cloned into the the alcohol oxygenase locus of the methanotropic yeast *P. pastoris* which are integrated into the genome of the yeast, resulting strains in which the recombinant protein production is under the control of the methanol concentration in the media. Thus, methanol serves as the inducer of product formation as well as energy and carbon source. The resulting construct Epo/pPICZαB, was verified by DNA sequencing. To simplify expression analysis and purification, the expressed rhEPO protein includes C-terminal polyhistidine tag and Myc-epitope. The inclusion of this sequence led to increase in expressed protein size to become approximately 37 kDa. Positives clones were screened in Zeocin as described by manufacturer. Several clones were screened for level of expression of Epo protein by Western blot using polyclonal anti-EPO antibodies. The highest expressing clone was selected for subsequent study.

Transformation of *Pichia pastoris*. Purified pPICZalphaB/hEPO recombinant plasmid was digested with BstX I (Roche), and after verifying complete

digestion by agarose gel electrophoresis, the plasmid was used for transfection of *P. pastoris* wild-type host strain X-33 using electroporator. Transformation was performed using electroporator Biorad Gene pulser XCell (Biorad).

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

Recombinant protein production. *P. pastoris* transformant containing recombinant human EPO (rhEPO) was maintained in YPD medium (1% yeast extract, 2% peptone and 2% dextrose, plus 2% agar in plates). A single colony was inoculated into 2 mL in buffered complex glycerol medium (BMGY) containing 1% yeast extract, 2% peptone, 100mM Potasium phosphate pH 6.0, 1.34% yeast nitrogen base, 0.00001% biotin and 1% glycerol. Growth was allowed until OD_{600} 2-6 (approximately 16-18 hours). The cells harvested by centrifugation at 1500-3000 x g for 5 min at room temperature. The pellet was then resuspended in: 1) BMMY production medium containing 0%, 0.5%, 1%, 2.5%, 5%, 10% and 20% of methanol and 2) BMMY production medium containing 2.5% methanol was added to the culture after 24h, 48h, 72h, 96h, 120h and 144h incubation. After incubation times reached the completion, the supernatant was harvested by centrifugation at 3000 x g for 5 min.

Measurement of cell concentrations. Cell concentrations were measured using a UV-Vis Spectrophotometer (Beckman DU 65D) at λ_{600nm}. For cell counting, we refers to the Instruction Manual (Invitrogen) where at OD one at 600 nm = ~5 x 10^7 cells/mL.

SDS-PAGE and Western Blotting. SDS-polyacrylamide gel electrophoresis (PAGE) [19] was performed in a 12% separating gel with a 5% stacking gel using the Mini-PROTEAN-3 apparatus (BioRad, Hercules, CA, USA). Protein was transferred from the gel to Hybond-ECL Nitrocellulose membrane (Amersham Biosciences, Germany) by electroblotting. Immunodetection was achieved by using anti hEPO antibody (Sigma, USA) as primary antibody and anti-rabbit IgG (isolated from Goat) peroxidase conjugate (Promega, USA) as secondary antibody. The band was visualized by NBT-BCIP staining kit (Promega, USA).

3. Results and Discussion

The effect of methanol induction and incubation time on the number of *P. pastoris* cells. As previously mentioned, the overall goal of this research is to study the optimal methanol concentration for induction and the incubation time to obtain maximum amount of rhEPO protein production. As seen below (Table 1), induction of *P. pastoris* culture by various methanol concentrations strongly influences cell numbers. Overall, increasing methanol concentrations decreased the cell numbers. Meanwhile, study on incubation time (Table 2) showed that the longer incubation the higher the cell numbers and, finally, it peaked at 96 hours. The peak was followed by decrease in cell numbers at 120 and 144 hour incubation times.

The effect of methanol induction and incubation time on rhEPO expression as analyzed by Western blot. Western blot using antibody specific for rhEPO was used for analyzing the rhEPO expression levels in response to methanol induction and time of incubation. This analysis showed that the highest rhEPO expression level occurred at 2.5% methanol induction (Figure 1). The effect of methanol induction at 10 and 20% was the same as that of 0% methanol which gave no results in protein expression. Study of the effect of time incubation on protein expression (Figure 2) showed that, from 24 to 144 hours, the transformed *P. pastoris* showed positive results in Western blot analysis. However, the highest expression occurred at 48 hours of incubation time.

Table 1. The Effect of Methanol Concentration on the Number of *P. pastoris* cell. Absorbance was Measured at λ_{600nm}. OD One at 600 nm = ~5 x 10^7 Cells/mL

Methanol conc. (%)	Absorbance	Number of cells
0	2.95	14.75 x 10^7
0.5	2.99	14.95 x 10^7
1	2.77	13.85 x 10^7
2.5	2.57	12.85 x 10^7
5	2.38	11.90 x 10^7
10	2.27	11.35 x 10^7
20	2.15	10.75 x 10^7

Table 2. The Effect of Incubation Time on the Number of *P. pastoris* cell. Absorbance was Measured at λ_{600nm}. OD One at 600 nm = ~5 x 10^7 Cells/mL

Incubation time (hours)	Absorbance	Number of cells
0	2.48	12.40 x 10^7
24	2.55	12.75 x 10^7
48	2.59	12.95 x 10^7
72	2.68	13.40 x 10^7
96	2.81	14.05 x 10^7
120	2.59	12.95 x 10^7
144	2.55	12.75 x 10^7
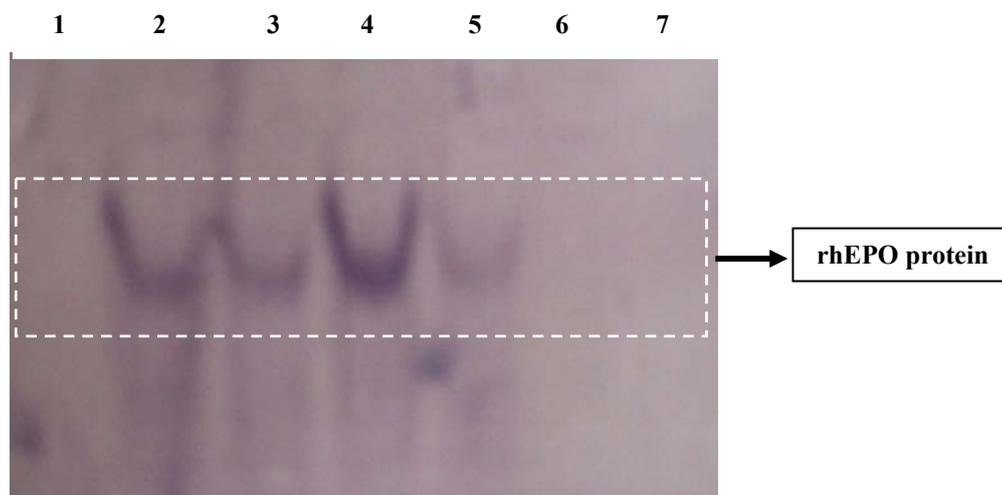
Figure 1. **Western Blot Analysis of Methanol Induction Effect on rhEPO Expression in *P. pastoris* Expression System. Polyclonal Antibody Specific for hEPO was used as the Primary Antibody. Inside the White Dotted Box is the Band that Represents the rhEPO Protein with the Size Approximately 37 kDa. Lane 1: 0% Methanol, Lane 2: 0.5% Methanol, Lane 3: 1% Methanol, Lane 4: 2.5% Methanol, Lane 5: 5% Methanol, Lane 6: 10% Methanol, and Lane 7: 20% Methanol**
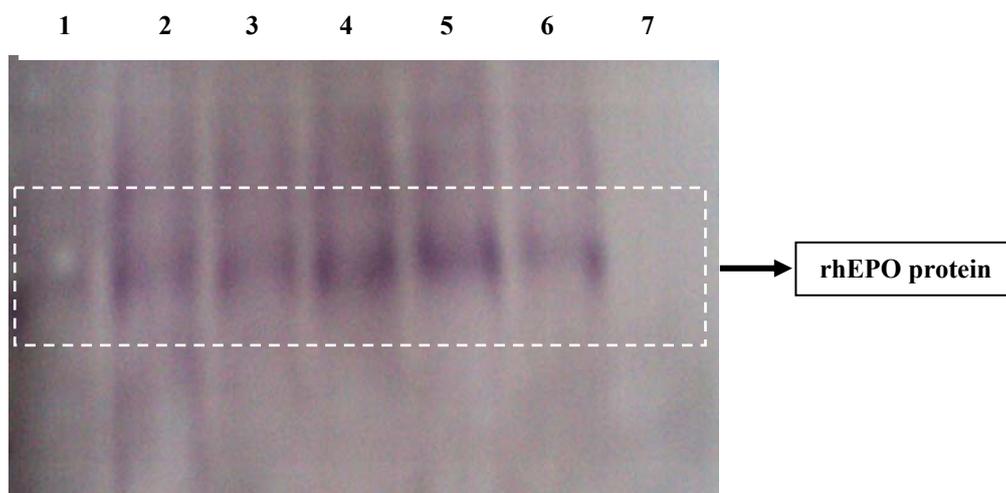
Figure 2. **Western Blot Analysis of Incubation Time Effect on rhEPO Expression in *P. pastoris* Expression System. Polyclonal Antibody Specific for hEPO was Used as the Primary Antibody. Inside the White Dotted Box is the Band Represents the rhEPO Protein with the Size Approximately 37 kDa. Lane 1: 144 hrs Incubation Time, Lane 2: 120 hrs, Lane 3: 96 hrs, Lane 4: 72 hrs, Lane 5: 48, Lane 6: 24hrs, and Lane 7: 0% hrs**

P. pastoris is a methylotropic yeast, capable of metabolizing methanol as its sole carbon source. The first step in the metabolism of methanol is the oxidation of methanol to formaldehyde using molecular oxygen by the enzyme alcohol oxydase. In addition to formaldehyde, this reaction generates hydrogen peroxide. To avoid the toxic effect of hydrogen peroxide, this metabolism occurs at a specialized cell organelle, called peroxisome. In this organelle, the toxic by-products sequestered away from the cell [17,20].

In this expression system, the hEPO gene was cloned under the control of alcohol oxidase 1 (AOX 1) promoter. This promoter is tightly regulated and induced by methanol which also serves as carbon source during induction. Thus, the expression level of the protein can be regulated simply by adjusting the amount of methanol concentration used in the media. Theoretically, higher methanol concentration led to higher expression level. While, low levels of methanol may not be enough to initiate transcription.

However, optimal concentration of methanol was necessary to have the balance between slightest toxic effect and highest protein expression [17,20-23].

To study the effect of methanol induction on the expression of hEPO protein, the hEPO gene was cloned under the control of the *P. pastoris* AOXI promoter. The construct was used to transform *P. pastoris* strain X-33. After induction, the use of *S. cerevisiae* alpha-factor prepro-leader sequence will target the protein to the secretory pathway and finally secreted into the medium for simple analysis [24-25]. In this study, information on optimal concentration of methanol induction as well as variation incubation times is presented. Western blot analysis was used to detect the presence of rhEPO expression using antibody specific for hEPO that raised in CHO cells.

Incubated under various concentration of methanol (Table 1), it was shown that the highest growth of *P. pastoris* cells expressing hEPO was at 0.5% of methanol concentration. The higher the methanol concentration applied to the culture, the slower the growth of the cells. We predicted that the slower growth of the cells was caused by the toxic effect of the methanol. This can be seen from the fact that without the addition of methanol (0% methanol induction), the cell growth was higher than that of 0.5%. Previous studies have reported that methanol induction above 0.5% of media can be toxic to the cells [26-28]. Interestingly, the highest growth of cell (at 0.5% of methanol concentration) was not followed by the highest protein expression level (Figure 1). In this study, the highest protein expression level occurred at concentration of 2.5% methanol.

Although, the number of the cells at 2.5% methanol induction was lower than that of 0.5%, the protein expression level at 2.5% methanol induction was still higher. While the number of cells was decreasing because of the toxic effect, yet the induction process was still increasing until it peaked at 2.5% of methanol. Interesting to note that this result contradicts the previous studies where above 0.5% of methanol induction can be toxic to the cells [27-28] and others). This study proved that methanol induction in *P. pastoris* can be higher than 0.5%. In rather similar study, expression of SAG2 (the major surface antigens of the intraculler protozoan parasite *Toxoplasma gondii*) in *P. pastoris*, [29] reported that 0.5–1% of methanol induction gave optimal expression result, while below 0.5% or above 2% of methanol induction slightly reduced the expression.

To further study the rhEPO expression study in *P. pastoris,* we were attempted to find the optimal incubation time for getting the highest protein expression level. Incubated under various incubation times (Table 2), it was shown that the highest growth of

P. pastoris cells expressing hEPO was at 96 hrs incubation time. The longer the incubation time the higher the number of the cells until it peaked at 96 hrs incubation time. Interestingly, the highest number of the cell at 96 hrs was not followed by the highest protein expression level. The highest protein expression occurred at 48 hrs (Figure 2). This result is in line with several previous studies that said that 48 hrs incubation time is the optimal time for incubation [30-32]. Thus, the number of cells is not correlated with the level of protein expression. To make sure that the cells have enough methanol, during incubation time, fresh methanol was added everyday to induce protein expression.

The fact that the number of cells that peaked at 96 hrs incubation time was not followed by the higher level of protein expression suggests that protein degradation may play role in this process. It is very possible that the longer the incubation time, the more proteolytic digestion occur. Theoretically, long incubation time will eventually result in complete degradation of the protein. However, in this study, Western blot analysis was still able to detect the presence of rhEPO protein even at incubation time of 144 hrs, suggesting that in our condition the proteolytic degradation was rather low. Previous studies had found that Recombinant proteins secreted by *P. pastoris* are often subjected to proteolytic degradation in the high cell density environment of bioreactor cultivation [26-28]. Intracellular vacuolar proteases from dead lysed cells are believed to be the major source of proteolytic activity in the culture medium because *P. pastoris* secretes small amounts of endogenous proteins. Hence controlling cell viability is important for controlling proteolytic degradation, most likely by increasing viability of cells and reducing activity of the proteases [27-28].

4. Conclusion

P. pastoris has many advantages of higher eukaryotic expression system. Since the gene of interest is cloned under the promoter regulating the production of alcohol oxidase, the level of the expressed protein can be manipulated by the induction on methanol. By combining the incubation time and the optimal concentration of methanol, we can obtain the balance between slightest toxic effect and highest protein expression. In the expression study of hEPO protein that was cloned in *P. pastoris* strain X33, we find that the highest protein expression level occurred at concentration of 2.5% methanol induction, while the optimal incubation time was at 48 hrs.

Acknowledgement

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