Evaluation of pH/buffering conditions effect on the optimization of Recombinant Human Erythropoietin expression in the methylotrophic yeast, *Pichia pastoris*

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ABSTRACT

Expression of recombinant proteins and drugs in *Pichia pastoris* has been in development since the late 1980s and the number of recombinant proteins produced in *P. pastoris* has increased significantly in the past several years. Unlike bacteria, this strain is capable of producing complex proteins with post translational modifications such as correct folding, glycosylation, proteolytic maturation. Most importantly the strain can secrete proteins to very high levels under the control of an efficient and highly regulated promoter of the alcohol oxidase gene, AOX1. In the present study by inserting the recombinant human erythropoietin (rHuEpo) gene downstream of the highly inducible AOX1 promoter and the pre-pro α-factor signal sequence, we were able to efficiently produce rHuEpo protein. Subsequently, we evaluated the role of the pH of *P. pastoris* growth medium and different types of buffers effects used in the expression-specific medium during the process of rHuEpo expression to optimize the production of rHuEpo. Our result indicated that the concentration of rHuEPO produced in the medium containing MES buffer (pH 6.0, 100 mM) is significantly higher than that in other pH/buffering conditions (P-value < 0.05). MES buffer with pH of 6.0 and 100 mM final concentration in the expression-specific medium regulated the pH/buffering conditions throughout the expression time with the significant increase in the concentration of expressed rHuEpo in comparison to the standard buffering protocol suggested by Invitrogen.

Key words: *Pichia pastoris*, Recombinant Human Erythropoietin (rHuEpo), pH/buffering conditions

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1. Introduction

Erythropoietin (EPO) is a glycoprotein hormone that chiefly regulates the production of red blood cells, and is produced primarily by the kidney in the adult and by the liver during fetal life (Jacobson et al., 1957; Zanjani et al., 1977). EPO is composed of 165 amino acids in its mature form, having an average molecular weight of 30 kDa.

Decreased production of EPO caused by kidney failure results in anaemia. The recombinant human EPO (rHuEPO) has been approved by the U.S. Food and Drug Administration (FDA), and is now widely used for treatment of anaemia associated with renal failure, cancer, prematurity, chronic inflammatory disease and human immunodeficiency virus infection (Jelkmann et al., 1992). The large demand for the hormone to satisfy clinical requirement is currently met by production via recombinant expression of the protein in mammalian cell Chinese hamster ovary (CHO) (Egrie et al., 1990).

Production of EPO in bacterial hosts has been reported for Escherichia coli (Leehuang et al., 1984; Bill et al., 1995) and Bacillus brevis (Nagao et al., 1997). Also the expression of the protein in a eukaryotic host has been reported for baker’s yeast, Saccharomyces cerevisiae (Elliott et al., 1989). However, expression of the recombinant protein in eukaryotes is of more interest relative to the bacteria regarding the former capability to glycosylate the proteins (Walker et al., 1998). Pichia pastoris has become popular as an alternative to the most commonly used S. cerevisiae strains for production of heterologous proteins. Expression of recombinant proteins in P. pastoris has been under development since the late 1980s and the number of recombinant proteins produced in P. pastoris has increased significantly in the past several years (Cregg et al., 1993; Sberna et al., 1996). P. pastoris is a desirable expression system because it grows to extremely high cell densities in very simple and defined media free of animal-deriven contaminants, and it can secrete expressed proteins up to 80% of total cellular protein (Cregg et al., 1993; Sberna et al., 1996). Unlike bacteria, it is capable of producing complex proteins with post translational modifications including folding correction, glycosylation, and proteolytic maturation (Sberna et al., 1996; White et al., 1994). Most importantly P. pastoris can secrete proteins to very high levels, under the control of the efficient and highly regulated promoter of the alcohol oxidase gene, AOX1 (Mok et al., 1997; Nagao et al., 1997; Walker et al., 1998). A specific advantage of P. pastoris as an expression system is that due to the strain very low levels of native protein secretion, separation and purification of heterogenous proteins becomes very effective (Jacobson et al., 1957; Cereghino et al., 2000).

The methylotrophic yeast P. pastoris is a highly successful system for the expression of heterologous genes encoding both intracellular and secreted products (Zanjani et al., 1977; Nagao et al., 1997; Walker et al., 1998). We selected P. pastoris as an expression host due to its ability to properly process and fold eukaryotic proteins while maintaining a fast growth rate (Jelkmann et al., 1992; J.M. Cregg et al., 1985). P. pastoris is capable of metabolizing methanol as a sole carbon source by inducing the production of alcohol oxidase. Although P. pastoris codes for two alcohol oxidase genes, AOX1 and AOX2, the AOX1 gene is responsible for 85% of alcohol oxidase activity in the yeast. The expression of the AOX1 gene is tightly regulated by the AOX1 promoter (Nagao et al., 1997; R.A. Brierley et al., 1998). In addition, the use of the secretion signal sequence from the S. cerevisiae α-factor for prepro peptide has been successful in the expression and secretion of heterologous proteins in the P. pastoris system. This system has been used for producing several proteins, with an expression level ranging from mg/L to g/L. For example, human serum albumin (4 g/liter) (Nagao et al., 1997; Tschopp et al., 1987) and insulin-like growth factor 1 (IGF-1, 0.6 g/liter) (Elliott et al., 1989, 1998; Tschopp et al., 1987) have been successfully secreted into the culture media. In addition, P. pastoris is different from S. cerevisiae in that it does not hyperglycosylate proteins (Grinn and Tschopp, 1989) and the highly immunogenic α-1, 3-mannose structure is not present (Cregg et al., 1993). P. pastoris generally secretes expressed proteins into the medium in a fairly pure form (30% to 80% of total secreted proteins) (Sberna et al., 1996) hence allows for relatively easy purification of the product. It is also capable of growing in a very wide pH range, from 3 to 7.

Unfortunately, the concentration of other cellular materials such as proteases increases in high-cell density culture as well. Several papers have described the problems with proteolysis of recombinant proteins in P. pastoris bioreactor cultures (Clare et al., 1991, 1998 Brierley et al., 1994, 1998). Strategies for overcoming proteolysis include the use of protease deficient strains (Gleeson et al., 1998), modification of the protein structure (Gustavsson et al., 2001), addition of protease inhibitors (Holmqvist et al., 1997), changing the pH (pH 3 to 7) of the culture medium and supplementation of the medium with casamino acids and peptone (Clare et al., 1991). The components and pH of the media can also make a difference to expression levels. When the media is buffered to pH values between 3.0 and 6.0, the amount of proteolysis of the recombinant protein is often reduced (Kang et al., 2000).

In the present study by inserting the rHuEpo gene downstream of the highly inducible AOX1 promoter and the pre-pro α-factor signal sequence we were able to efficiently produce rHuEpo protein. Subsequently, we evaluated the role of growth medium pH and the effects of different types of buffers used in the expression-
specific medium during the process of rHuEPO expression to optimize the production of rHuEPO in P. pastoris.

2. Material and Methods

2.1. Construction of expression vectors for rHuEPO

The pUC57 plasmid harboring a synthetic cDNA encoding 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 (hereafter: p57Epo) was constructed (Bio Basic Inc, Ontario, Canada). The Epo-encoding DNA synthesized by polymerase chain reactions (PCRs) via exploiting p57EPO as template. RHuEpo gene was amplified using F1-NAT (5’ CTC GAG AAA AGA GCC CCA CCA CCA CTC ATC3’) as the forward primer and B1-NAT (5’ TCT AGA TTA TCA ATG ATG ATG ATG ATG TCT GTC CCC TGT CCT3’) as the reverse primer. For further direct cloning of PCR-amplified product in the P. pastoris expression-secretion vector PICZαA (Invitrogen), XhoI and XbaI restriction sites were considered in forward and backward primers, respectively (Fig. 1). This cloning strategy provided the Kex2 cleavage site at the 5’ ends of the DNA encoding Epo while the 6xHis-tag and two termination triplets were located at the 3’ ends in tandem. PCR-amplified fragments and PICZαA vector following digestion by XhoI and XbaI restriction enzymes were gel-purified (QIAquick Gel Extraction Kit) and utilized in separate ligation reactions. The product of ligation reaction were used to transform E. coli TOP10 (Invitrogen) competent cells. Around 5-10 µg of purified plasmid was linearized with SacI (Fermentas) and electroporated into the yeast P. pastoris wild-type host strain X-33 (Invitrogen) according to supplier instructions (EasySelect Pichia Expression Kit, Invitrogen). The transformed cells (200 µl) were plated on YPDS medium (1% Yeast extract, 2% Peptone, 2% Dextrose, 1M Sorbitol) supplemented with 1mg ml⁻¹ Zeocin and incubated for 3-5 days at 30 °C. Zeocin-resistant transformants were screened for confirming the Mut+ phenotype according to the supplier instructions (Invitrogen). 10 Mut+ transformants that survived a concentration of 1mg ml⁻¹ Zeocin, were randomly selected and confirmed for the presence of the corresponding Epo gene by PCR. Finally, confirmed Mut+ transformants were analyzed for expression levels in 50 ml conical tubes containing 10 ml of culture media. The highest producing clones for each construct were selected on the basis of SDS-PAGE and densitometric results.

2.2. rHuEPO production

2.2.1. Standard expression condition

Standard protocol of expression (suggested by Invitrogen) was applied as the control condition for the further comparative evaluations. In brief, a single colony of rHuEpo-producing P. pastoris was inoculated into 5 ml of YPD medium containing (in g l⁻¹) peptone, 20; yeast extract, 10; glucose, 20; agar, 20 and 100 µg ml⁻¹ Zeocin. The culture was incubated overnight at 30 ˚C with mild shaking. The mentioned YPD medium (seed culture) containing the grown rHuEpo-producing P. pastoris was transferred to 200 ml of the buffered complex glycerol medium (BMGY) containing (in g l⁻¹) yeast extract, 10; peptone, 20; yeast nitrogen base (YNB), 13.4; biotin, 4 X 10⁻5 ; glycerol, 10 and 0.1 mol l⁻¹ of potassium phosphate buffer pH 6.0 in V= 1.0 l baffled Erlenmeyer flask and growth allowed to proceed overnight at 30 °C with 240 rpm shaking until OD600 was about 2-6. Cells from the medium were harvested by 3 minutes centrifugation at 3000 rpm and then the pellet resuspended in V= 1.0 l baffled Erlenmeyer flasks containing 200 ml BMMY expression-specific medium (containing 5 ml/l of methanol instead of glycerol), respectively. Incubation continued for 72 hours. Every 24 h methanol was added to the medium to a 5 ml l⁻¹ final concentration (5% V/V). At the end of 72 h, the medium was centrifuged at 1000 g for 10 min and the cell pellet was discarded (EasySelect Pichia Expression Kit, Invitrogen).

2.2.2. Modified expression condition

After the expression of rHuEpo in the standard condition, the expression process carried out with different buffer solutions and varied pH ranges in the expression medium. pH/buffering conditions of expression-specific media containing different buffer solutions with a variable pH range and final concentrations have been evaluated. The Buffers applied in this study have been potassium phosphate, sodium acetate and MES. The pH of buffer solutions was set according to the Henderson-Hasselbach equation via the known value of each buffer
According to the above-mentioned standard conditions. A variety of primary pH ranges of expression media were set and the potential of these conditions to regulate and maintain the pH in a suitable range for P. pastoris growth and expression of rHuEpo was evaluated. The other expression parameters were set according to the above-mentioned standard conditions.

### 2.3. Expression process control

During the standard and modified expression processes, pH and cell concentration of expression-specific culture medium were measured at the beginning of expression and 24 hour intervals. pH was adjusted at the standard primary value (pH 6) through addition of 0.1 N HCl. Cell concentrations were measured with a Perkin Elmer UV/VIS spectrophotometer (Lambda EZ 201) based on absorbance at 600 nm (OD<sub>600</sub>) using a calibration curve. The cultured media was used for serial dilutions and also as the blank. OD<sub>600</sub> was assayed as a control of rHuEpo-expressing P. pastoris growth in the culture medium.

### 2.4. SDS-polyacrylamide gel electrophoresis (PAGE)

rHuEpo expression and existence in the culture medium samples were confirmed after each standard and modified expression experiment by analyzing samples with SDS-PAGE before purification of rHuEpo. SDS-PAGE was performed in a 12% separating gel with a 5% stacking gel and proteins were silver-stained (John et al., 2002; Morrissey et al., 1981).

### 2.5. Densitometric analysis

Densitometric analyses were performed by a GS-800 Imaging Densitometer (Bio-Rad) employing Quantity One analyzer according to the software manual to measure the concentration of SDS-PAGE rHuEpo specific developed bands.

### 2.6. Statistical methods

Significance of differences in concentrations of rHuEpo measured during different expression experiments was determined by Student’s t-test.

### 3. Results and Discussion

#### 3.1 rHuEpo-expression vectors and selection of P. pastoris transformants

The human cDNA of EPO which was employed in this study encoded 166 native amino acids of the premature protein. In our cloning strategy, using XhoI and XbaI sites of pPICZαA, the cDNA of Epo were cloned in frame with alcohol oxidase (AOX1) transcription/translational cassette and downstream of the prepro-α-factor sequence immediately after the Kex-2 signal cleavage site in order 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 any ribosomal-pass through.

### 3.2 Analysis of Buffer Solution and pH Effect on the rHuEPO-Expression (pH/buffering conditions)

As previously mentioned, a variety of buffer solutions with different primary pH was analyzed to evaluate the effects of these factors on the optimization of rHuEpo expression. The effect of these factors will be reflected in the yield of expression and would be detected through different analyses such as SDS-PAGE and densitometric assessments of rHuEpo samples concentration. The growth of rHuEpo-Expressing P. pastoris and cell concentration are also influenced by the type of buffer solution and pH of expression-specific culture medium that would be analyzed through measurement of OD<sub>600</sub> of culture medium.

Buffer solution in the expression process as a main part of expression-specific medium should be accurately and specifically selected since the solubility and integrity of different proteins are variable in different buffers and pH ranges. Auto-proteolysis of secreted proteins is also an important yield-reducing reaction. Reduction of the auto-proteolysis rate thus can be considered as an optimization strategy in the production of recombinant proteins. The expression levels of rHuEpo in unbuffered simple media such as MM (1.34% YNB, 4 x 10<sup>-5</sup>% biotin, 0.5 % methanol) or unbuffered complex medium (1% yeast extract, 2% peptone, 1.34% YNB, 4 x 10<sup>-5</sup>% biotin, 0.5 % methanol) was as low as 1 mg l<sup>-1</sup> or below the detection limits which may be caused by shocking levels of [H<sup>+</sup>] concentrations (pH effect) (Su et al., 2009). The pH values in the MM medium reached to about 3.5 at the end of expression time when at the same time the pH for the unbuffered complex medium was about 8-8.5 (possibly due to the effects of ammonium the produced by metabolizing amino acids existing in yeast extract and peptone). It was found that expression of Epo in buffered methanol complex medium with a pH around 5.5-6 was clearly higher than other pH ranges.

According to the supplier instructions (Easy Select Pichia Expression manual, Invitrogen) potassium phosphate buffer pH 6.0 is the buffer used in the standard expression condition. There are some advantages in the application of this buffer as it is not toxic for the P. pastoris, pH changes and fluctuations are limited and this it is applicable for weeks at 4°C. Despite these advantages this buffer reacts with ions such as Ca<sup>2+</sup> and Mg<sup>2+</sup> and precipitates them as salts. This reaction restricts the culture enzymatic activity and reduces production capacity since the precipitated ions are co-enzymes of the important intra-cellular enzymes of mi-
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crobial cells (Chandra Mohan, Chalbiochem). In the present project, we tried to keep the pH value at 5.5-6 range during the expression time. A higher obtained expression level of rHuEpo at this pH range is in accordance with optimal pH values for the expression of many other proteins in *P. pastoris* (Demain and Vaishnav, 2009). This pH range supports the optimal growth of yeast and the limiting parameters such as activity of secreted proteases will be down-regulated and inhibited (Clare et al., 1991). In the present study different experiments analyzing the effect of type of buffer solution, primary pH of the buffer solution and the buffer final concentration in the expression-specific medium have been performed.

### 3.2.1 Potassium phosphate buffer, pH 4.0, 5.0, 6.0 and 7.0

Three different expression processes were performed to investigate the capacity of potassium phosphate buffer with three different primary pH values (4.0, 5.0, 6.0 and 7.0) in the regulation of expression medium pH and the probable influence of this pH ranges on *P. pastoris* growth and concentration of expressed rHuEpo. Final concentration of this buffer solution in the expression-specific medium was 100 mM in all these experiments. pH and OD$_{600}$ of expression medium were measured every 24 hours. The highest OD$_{600}$ which reflect the highest cell concentration was achieved through application of primary pH 7.0. The pH increase was also minimal throughout 72 hours of expression time applying this condition (Table 1).

**Table 1.** Expression experiments PP1-PP4 with four different primary pH values (4.0, 5.0, 6.0 and 7.0) of potassium phosphate buffer, pH changes and final OD$_{600}$ of expression media with PS3 is the standard buffer suggested by Invitrogen

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Primary pH</th>
<th>pH changes during the expression process</th>
<th>Final OD$_{600}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP1</td>
<td>4.0</td>
<td>4.7, 4.2</td>
<td>24</td>
</tr>
<tr>
<td>PP2</td>
<td>5.0</td>
<td>6.6, 5.1</td>
<td>30</td>
</tr>
<tr>
<td>PP3 (standard)</td>
<td>6.0</td>
<td>6.8, 6.8</td>
<td>38</td>
</tr>
<tr>
<td>PP4</td>
<td>7.0</td>
<td>7.3, 7.0</td>
<td>40</td>
</tr>
</tbody>
</table>

Following four mentioned experiments, supernatants of expression medium samples were analyzed by SDS-PAGE to detect the expressed rHuEpo and the electrophoretic pattern of this recombinant protein. Analyzing of samples at the end of the expression by SDS-PAGE and silver staining showed an intensive band around 33 kDa and a faint band at 66 kDa that can be attributed to Epo and Epo dimmer respectively. Additionally, in all samples some notable bands around 42-45 kDa were observed correspond to the unprocessed pro-α-factor/ 

Densitometric analysis to quantify the concentration of expressed rHuEpo in the experiments applying potassium phosphate buffer revealed that the highest amount of rHuEpo is produced with a primary pH of 6.0. As is indicated in the Table 2, at potassium phosphate buffer primary pH=6.0, the rHuEpo concentration has been 95 µg/ml which is significantly higher than the other measured concentrations corresponding to the primary pH of 4.0, 5.0 and 7.0 (P-value < 0.05). Although the results of quantifying rHuEpo concentration does not agree with the cell concentration or OD$_{600}$ measurement (that indicates the highest final cell concentration applying potassium phosphate buffer with primary pH 7.0), it would be concluded that the best primary pH of this buffer is 6.0 since the difference between cell concentrations is not significant in the experiments with primary pH 6.0 and 7.0 (P-value > 0.05).

**Table 2.** rHuEpo Concentration following different expression experiment with potassium phosphate buffer

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Final rHuEpo Concentration (µg/ml)$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP1</td>
<td>12</td>
</tr>
<tr>
<td>PP2</td>
<td>68</td>
</tr>
<tr>
<td>PP3</td>
<td>95</td>
</tr>
<tr>
<td>PP4</td>
<td>75</td>
</tr>
</tbody>
</table>

### 3.2.2 Sodium acetate buffer, pH 5.0, final concentration: 30, 40 and 50 mM

Three different expression experiments was performed to evaluate the effect of sodium acetate buffer with the primary pH of 5.0 and the final concentration of 30, 40 and 50 mM corresponding to (S1, S2 and S3, respectively). pH changes were measured every 24 hours to assess the capacity of the buffer three final concentrations to regulate the pH of expression medium, pH
changes where very limited in all the three assessed concentrations of Sodium acetate buffer (Table 3). In comparison to all other expression experiments, the concentration of the expressed rHuEpo was dramatically reduced by applying this buffer (Fig. 3). The expression was highly limited in all three concentrations of sodium acetate buffer and rHuEpo was detected in very low concentrations in the supernatant of production medium. Among these three expression experiments, the highest concentration of rHuEpo was detected in SA1 in which 30 mM final concentration of sodium acetate buffer with primary pH of 5 was used. In this experiment the yield of rHuEpo was found to be 21 µgml⁻¹ (Table 4).

Table 3. pH changes during S1, S2 and S3 expression experiments with sodium acetate buffer 30, 40 and 50 mM, respectively.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Buffer Concentration (mM)</th>
<th>pH during expression process</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24⁰ h</td>
</tr>
<tr>
<td>SA1</td>
<td>30</td>
<td>5</td>
</tr>
<tr>
<td>SA2</td>
<td>40</td>
<td>5</td>
</tr>
<tr>
<td>SA3</td>
<td>50</td>
<td>5</td>
</tr>
</tbody>
</table>

Figure 3. SDS-PAGE analysis of SA1, SA2 and SA3 expression experiments.

Table 4. rHuEpo final concentration in the supernatant of expression media SA1, SA2 and SA3.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Buffer Concentration (mM)</th>
<th>Final rHuEpo concentration (µgml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA1</td>
<td>30</td>
<td>21</td>
</tr>
<tr>
<td>SA2</td>
<td>40</td>
<td>9.3</td>
</tr>
<tr>
<td>SA3</td>
<td>50</td>
<td>6.9</td>
</tr>
</tbody>
</table>

3.2.3 Sodium acetate buffer, pH 6.0, final concentration: 30, 40 and 50 mM

Exactly similar expression process performed with sodium acetate buffers of 30, 40 and 50 mM and primary pH of 6.0. None of the final concentrations of sodium acetate were able to regulate pH range during the expression process at a primary pH of 5.0 and culture medium pH rapidly increased to 8-8.8. This pH rise severely limited the growth of rHuEpo-expressing P. pastoris as well as rHuEpo production. SDS-PAGE analysis identified no significant production of rHuEpo.

3.2.4 MES buffer, pH 6.0, final concentration: 10, 15, 30, 50, 70 and 100 mM

Expression experiments with BMMY medium containing MES buffers of 10, 15, 30, 50, 70 and 100 mM (MES1-MES6 experiments, respectively) with primary pH 6.0 (Table 5). pH regulatory properties of MES buffer with concentrations 10-50 mM were not satisfactory and failed to maintain the pH in an appropriate range. In contrast, higher concentrations of MES (70 and 100 mM) kept the pH at the optimal pH range for P. pastoris growth and rHuEpo expression. pH regulatory capacity of MES2 (final concentration 100 mM) expression medium agrees with SDS-PAGE and densitometry results (Table 6, Fig. 4). pH increase in the MES2 experiment was minimal and was modulated in a range that supports the growth of P. pastoris and rHuEpo expression. The highest level of rHuEpo expression was obtained in the MES2 experiment (using MES 100 mM).

Table 5. rHuEpo final concentration in the supernatant of expression media MES1 and MES2.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Buffer Concentration (mM)</th>
<th>Final rHuEpo concentration (µgml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MES1</td>
<td>70</td>
<td>227</td>
</tr>
<tr>
<td>MES2</td>
<td>100</td>
<td>282</td>
</tr>
</tbody>
</table>

Figure 4. SDS-PAGE analysis of MES1 and MES2 expression experiments.
In contrast to phosphate buffers, MES does not interfere with coenzymes of *P. pastoris* and other expression systems. Resistance to oxidation, high solubility in water and low solubility in other solutions are some other advantages of MES that would make it an efficient buffer for the optimization of rHuEpo expression (Good et al., 1966; Ferguson et al., 1980). The yield of rHuEpo production is influenced by the pH of the expression-specific medium and the pH changes and fluctuations intensively affect the expression level of this protein. Similar results have been concluded for other recombinant proteins expressed by *P. pastoris*. For instance the production of *Dactylium dendroides* recombinant galactose oxidase by *P. pastoris* has been shown to be influenced by the medium pH and to be labile in the pH values less than 6.0. Optimization of expression process to increase the pH of the culture medium up to 6.0 has led to significant higher concentrations of this protein (Whittaker and Whittaker, 2000).

In the present study, expression processes were performed using expression media containing three different buffer solutions and pH was adjusted at 4.0-7.0 with an interval of 1.

Different buffer solutions have some specific properties and characteristics that might interfere in some biological reactions and processes. These variable features should be considered as important factors in the selection of pH regulatory system. Expression process is highly sensitive to the pH/buffering conditions. The microorganism which is used as an expression system could grow in a defined pH with an almost narrow optimal range (Jahic et al., 2006; Xie et al., 2005; Macauley et al., 2005).

The activity of expressed protein may be severely affected by the pH of expression medium. Most of the protein structures and functions are tightly pH-sensitive since the function of the proteins depends on their three-dimensional structure and folding that would be formed in a specific pH range (Koganesawa et al., 2002).

The activity of the secreted extracellular proteolytic enzymes is a yield-decreasing factor that could be minimized through selection of an appropriate buffering system and adjustment of a proper pH (Macauley et al., 2005; Shi et al., 2003). As described in the Table 7 and according to the results of SDS-PAGE and densitometry, the highest level of rHuEpo is expressed with a pH around 5.0-6.0. In the expression experiments with potassium phosphate buffer, the concentration of the produced rHuEpo declines as the pH value rises. The concentration of produced rHuEpo was satisfactory when potassium phosphate buffer of 100 mM and pH=6.0 was used as expected and suggested by the manufacturer of *P. pastoris* expression system (Invitrogen).

### Table 6. pH changes during MES1 and MES2 expression experiments exploiting MES buffer 70 and 100 mM. Other experiments did not lead to a controlled pH range and *P. pastoris* growth was limited and stopped after a short period of expression.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Buffer Concentration (mM)</th>
<th>pH during expression process</th>
<th>24th h</th>
<th>48th h</th>
<th>72nd h</th>
</tr>
</thead>
<tbody>
<tr>
<td>MES1</td>
<td>70</td>
<td></td>
<td>6.1</td>
<td>6.7</td>
<td>6.7</td>
</tr>
<tr>
<td>MES2</td>
<td>100</td>
<td></td>
<td>6</td>
<td>6.4</td>
<td>6.6</td>
</tr>
</tbody>
</table>

### Table 7. Final concentration of expressed rHuEpo following the standard and optimized expression processes.

<table>
<thead>
<tr>
<th>Expression Experiment</th>
<th>Buffer</th>
<th>Final rHuEpo concentration (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard pH/buffering conditions</td>
<td>Potassium phosphate (100 mM, pH 6)</td>
<td>248</td>
</tr>
<tr>
<td>Optimized pH/buffering conditions</td>
<td>MES (100 mM, pH 6)</td>
<td>282</td>
</tr>
</tbody>
</table>

The concentration of produced rHuEpo in the medium containing MES buffer (pH 6.0, 100 mM) is significantly higher than that expressed in the other pH/buffering conditions (P-value < 0.05). MES has been applied as a biological and biochemical buffer since 1960 when identified as a Zwitterionic buffer. The pKa and the buffering range of MES is 6.15 and 5.5-6.5 respectively. MES buffering features the optimal pH range for *P. pastoris* growth and metabolism. Moreover, MES is highly soluble in water and up to 2 mol/liter concentration would be solved in water-based solutions and the culture media. These properties have led to widespread application of MES as a buffer in a variety of biological and biochemical processes (Good et al., 1966).

MES buffer with pH of 6.0 and final concentration of 100 mM regulated the pH/buffering conditions throughout the expression time while significantly increased the concentration of expressed rHuEpo as compared to the standard buffering protocol suggested by Invitrogen.

This optimization which resulted in the significant increase of the rHuEpo expression would be an efficient strategy for enhancing the concentration of the expressed glycoprotein. The MES based optimized protocol applied in this study with the mentioned pH and final concentration will lead to more considerable, cost-effective and commercially beneficial results in the large scale production of this widely-used recombinant drug.

### References


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