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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 requirementd 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 heterogenouse 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, 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 (Grinna 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 (Holmquist *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 CGC CTC ATC3') as the forward primer and B1-NAT (5' TCT AGA TTA TCA ATG 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 restrictions 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) using a Bio-Rad Gene Pulser. The transformed cells (200μl) 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.

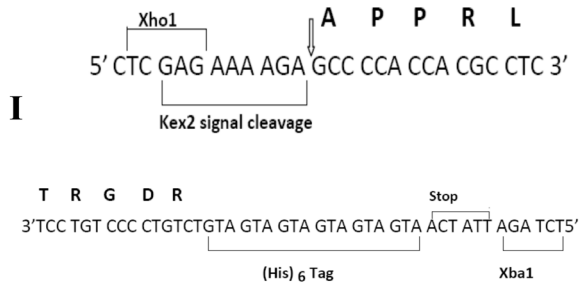

Figure 1. Design of forward (I) and backward (II) primers.

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

Pk_a (Po Henry *et al*., 2001). 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_{600}) using a calibration curve. The cultured media was used for serial dilutions and also as the blank. OD_{600} was assayed as a control of rHuEpo - expressing *P. patoris* 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_{600} 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^{-5}% biotin, 0.5 % methanol) or unbuffered complex medium (1% yeast extract, 2% peptone, 1.34% YNB, 4 x 10^{-5} % biotin, 0.5 % methanol) was as low as 1 mg l^{-1} or bellow the detection limits which may be caused by shocking levels of $[H^+]$ 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^{2+} and Mg^{2+} 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-

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

Experiment	Primary pH	pH changes during the expression process (24 / 48 / 72)	Final OD_{600}
PP1	4.0	4.2 / 4.7 / 4.9	23
PP2	5.0	5.1 / 6.61 / 6.63	30
PP3 (standard)	6.0	6.0 / 6.8 / 6.8	38
PP4	7.0	7.0 / 7.3 / 7.4	40

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/Epo (as fusion proteins) resulted from inefficient digestion of Kex2 protease (Fig. 2) (Julius, *et al.,* 1984).

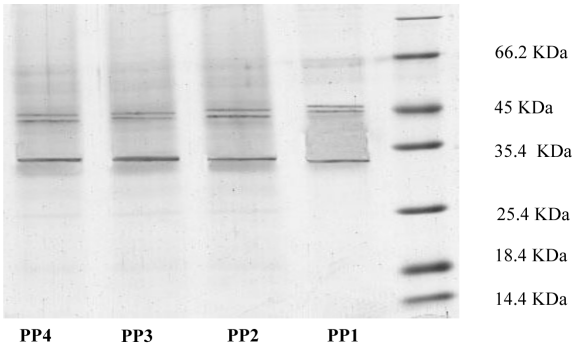

Figure 2. SDS-PAGE analysis of PP1, PP2, PP3 and P P4 expression experiments.

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 μgml^{-1} 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

Experiment	Final RHuEpo Concentration (μgml^{-1})
PP1	12
PP2	68
PP3	95
PP4	75

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.

Experiment	Buffer Concentration (mM)	pH during expression process		
		24th h	48th h	72nd h
SA1	30	5	5.1	5.1
SA2	40	5	5	5
SA3	50	5	5	5

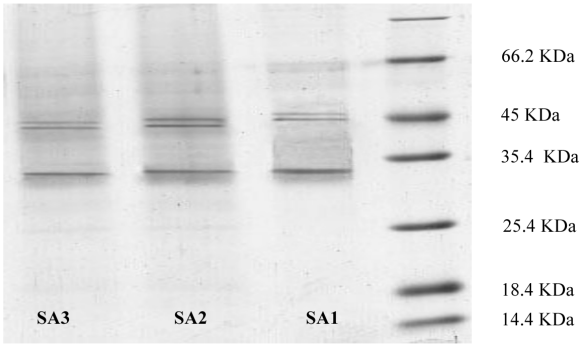

Figure 3. SDS-PAGE analysis of SA1, SA2 and SA3 expression experiments.

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

Experiment	Buffer Concentration (mM)	Final rHuEpo concentration (μgml^{-1})
SA1	30	21
SA2	40	9.3
SA3	50	6.9

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.

Experiment	Buffer Concentration (mM)	Final rHuEpo concentration (μgml^{-1})
MES1	70	227
MES2	100	282

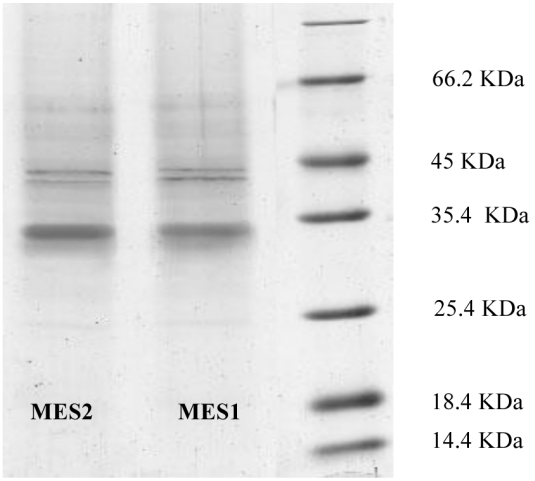

Figure 4. SDS-PAGE analysis of MES1 and MES2 expression experiments.

Table 6. pH changes during MES1and 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.

Experiment	Buffer Concentration (mM)	pH during expression process		
		24th h	48th h	72nd h
MES1	70	6.1	6.7	6.7
MES2	100	6	6.4	6.6

In contrast to phosphate buffers, MES dose 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 has been concluded for other recombinant proteins expressed by *P. pastoris*. For instance the production of *Dactylium dandroides* 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 highes 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 7. Final concentration of expressed rHuEpo following the standard and optimized expression processes.

Expression Experiment	Buffer	Final rHuEpo concentration ($\mu g ml^{-1}$)
Standard pH/buffering conditions	Potassium phosphate(100mM, pH 6)	248
Optimized pH/buffering conditions	MES (100Mm, pH 6)	282

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 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.

4.References

Cereghino JL, Cregg JM. Heterologous protein expression in the methylotrophic yeast pichia pastoris. *FEMS Microbiol Rev*. 2000; 24:45-66.

Cregg JM, Cereghino L, Shi J, Higgins DR. Recombinant protein expression in pichia pastoris. *Mol Biotech.* 2000; 16:23-52.

Cregg JM, Madden KR. Development of the methylotriphic teast, Pichia pastoris, as a host system for the production of foreign proteins. *Dev Ind Microbiol.* 1988; 29: 23-42.

Jacobson LO, Goldwasser E, Fried W. Role of the kidney in erythropoiesis. *Nature*. 1957; 179:633–4.

Zanjani ED, Poster J, Borlington H, Mann LI, Wasserman LR. Liver as the primary site of erythropoietin formation in the fetus. *J Lab Clin Med.* 1977; 89:640-44.

Jelkmann W. Erythropoietin: structure, control of production, and function. *Physiological Rev.* 1992; 72:449–89.

Egrie J, The cloning and production of recombinant human erythropoietin. *Pharmacotherapy*. 1990; 10:3S–8S.

Leehuang S, Cloning and expression of human erythropoietin cDNA in *Escherichia coli*. *P Natl Acad Sci Biol*. 1984; 81:2708–12.

Bill RM, Winter PC, McHale CM *et al*. Expression and mutagenesis of recombinant human and murine erythropoietins in *Escherichia coli. Biochim Biophys Acta*. 1995; 1261:35–43.

Nagao M, Inoue K, Moon SK, Masuda S, Takagi H, Udaka S, Sasaki R. Secretory production of erythropoietin and the extracellular domain of the erythropoietin receptor by *Bacillus brevis*: affinity purification and characterization. *Biosci Biotechnol Biochem.* 1997; 61:670–4.

Elliott S, Giffin J, Suggs S, Lau EPL, Banks AR. Secretion of glycosylated human erythropoietin from yeast directed by the a-factor leader region. *Gene.* 1989; 79:167-80.

Walker GM. Yeast: Physiology and Biotechnology. *NY: John Wiley & Sons, Inc*., 1998.

Mok SS, Sberna G, Heffernan D *et al*., Expression and analysis of heparin-binding regions of the amyloid precursor protein of Alzheimer's disease. *FEBS Lett.* 1997; 415: 303-7.

Hollenberg CP, Gellissen G. Production of recombinant proteins by methylotrophic yeasts. *Curr Open Biotechnol* 1997; 8:554-60.

Sreekrishna K, Brankamp RG, Kropp KE *et al*. Strategies for optimal synthesis and secretion of heterologous proteins in the methylotrophic yeast *Pichia pastoris*. *Gene* 1997; 190:55-62.

Makrides SC. Strategies for achieving high-level expression of genes in *Escherichia coli. Microbiol Rev.* 1996; 60:512-38.

Tschopp JF, Sverlow G, Kosson R, Craig W, Grinna L. Highlevel secretion of glycosylated invertase in the methylotrophic yeast *Pichia pastoris. Nature Biotechnology.* 1987; 5:1305–8.

Paifer E, Margolles E, Cremata J, Montesino R, Herrera L, Delgado JM. Efficient expression and secretion of recombinant alpha amylase in *Pichia pastoris* using two different signal sequences. *Yeast*. 1994; 10:1415–19.

Cregg JM, Barringer J, Hessler AY, Madden KR. *Pichia pastoris* as a host system for transformations. *Mol Cell Bio*. 1985; 5:3376–85.

Cregg JM, Vedvick TS, Raschke WC. Recent advances in the expression of foreign genes in *Pichia pastoris*. *Biotechnology (NY).* 1993; 11:905–10.

Bar KA, Hopkins SA, Sreekrishna K. Protocol for efficient secretion of HSA developed from *Pichia pastoris. Pharm Eng*. 1992; 12:48–51.

Brierley RA. Secretion of recombinant human insulin-like growth factor (IGF-I). In: Higgins DR, Cregg JM, eds. Meth¬ods in Molecular Biology. 2nd ed. *NJ: Humana Press.*, 1998: 149–177.

Tschopp JF, Brust PF, Cregg JM, Stillman CA, Gingeras TR. Expression of the LacZgene from two methanol-regulated promoters in *Pichia pastoris*. *Nucleic Acids Res.* 1987; 15:3859–76.

Tschopp JF, Sverlow G, Kosson R, Craig W, Grinna L. High level secretion of glycosylated invertase in the methylotrophic yeast *Pichia pastoris*. *Biotechnology (NY)*. 1987; 5:1305–8.

Gleeson MAG, White CE, Meininger DP, Komives EA. Generation of protease-deficient strains and their use in heterologous protein expression. *Methods Mol Biol.* 1998; 103:81-4.

Gustavsson M, Lehtio J, Denman S, Teeri TT, Hult K, Martinelle M. Stable linker peptides for a cellulosebinding domain-lipase fusion protein expressed in *Pichia pastoris*. *Protein Eng.* 2001; 14:711-5.

Holmquist M, Tessier DC, Cygler M. High-level production of recombinant Geotrichum candidum lipases in yeast *Pichia pastoris*. *Protein Expr Purif.* 1997; 11:35-40.

Clare JJ, Romanos MA, Rayment FB *et al*. Production of mouse epidermal growth factor in yeast: high-level secretion using Pi¬chia pastoris strains containing multiple gene copies. *Gene.* 1991; 105:205-12.

Kang HA, Choi ES, Hong WK *et al.* Proteolytic stability of recombinant human serum albumin secreted in the yeast *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol.* 2000; 53:575-82.

Po Henry N, Senozan NM. Henderson–Hasselbalch Equation: Its History and Limitations. *J Chem Educ*. 2001; 78:1499–503.

SDS Polyacrylamide Gel Electrophoresis of Proteins. In: Walker JM, ed. The Protein Protocols Handbook. *Humana Press Inc.,* 2002; 41:60-7.

Morrissey JH. Silver stain for proteins in polyacrylamide gels: a modified proceduredwith enhanced uniform sensitivity. *Analyt Biochem*. 1981; 117:307–10.

Su M, Xu T, Wang D, Zhou Y, Niu C, Yan W. High yield and purification of recombinant human apolipoprotein E3 in *Pichia*

pastoris. *Protein Expr*. *Purif*. 2009; 68:7-11.

Mohan C. Buffers: A guide for the preparation and use of buf¬fers in biological systems systems. *CA (La Jolla): Chalbiochem, Co.*, 1995.

Demain AL, Vaishnav P. Production of recombinant proteins by microbes and higher organisms. *Biotechnol. Adv*. 2009; 27:297-306.

Julius D, Schekman R, Thorner J. Glycosylation and processing of prepro-α-factor through the yeast secretory pathway. *Cell.* 1984; 36:309-18.

Julius D, Brake A, Blair L, Kunisawa R, Thorner J. Isolation of the putative structural gene for the lysine-arginine-cleaving endopeptidase required for processing of yeast prepro-α-factor. *Cell.* 1984; 37:1075-89.

Good NE, Winget GD, Winter W, Connolly TN, Izawa S, Singh RMM. Hydrogen Ion Buffers for Biological Research. *Biochemistry*. 1966; 5: 467–77.

Ferguson WJ, Braunschweiger KI, Braunschweiger WR. *Anal Biochem*. 1980; 104:300-10.

Whittaker MM, Whittaker JW. Expression of recombinant galactose oxidase by *Pichia pastoris. Protein Expr Purif*. 2000; 20:105-11.

Jahic M, Veide A, Charoenrat T, Teeri T, Enfors SO. Process technology for production and recovery of heterologous proteins with *Pichia pastoris*. *Biotechnol Prog*. 2006; 22:1465-73.

Xie J, Zhoub Q, Dub P, Ganb R, Yea Q. Use of different carbon sources in cultivation of recombinant *Pichia pastoris* for angiostatin production. *Enzy Microb Technol*. 2005; 36:210-16.

Macauley S, Mariana P, Fazenda L, McNeil B, Harvey LM. Heterologous protein production using the *Pichia pastoris* expression system. *Yeast*. 2005; 22:249-70.

Koganesawa N, Aizava T, Shimojo H *et al*. Expression and purification of a small cytokine growth-blocking peptide frome armyworm pseudaletia separata by an optimize fermentation method using the methylothrophic yeast *Pichia pastoris. Protein Expr Pur*. 2002; 25:416-25.

Shi X, Karkut T, Chamankhah M, Alting-Mees M, Hemmingsen SM, Hegedusa D. Optimal conditions for the expression of a single-chainantibody (scFv) gene in *Pichia pastoris. Protein Expr Purif*. 2003; 28:321–30.