ABSTRACT:
The pharmaceutical production of the key therapeutic protein erythropoietin is largely dependent on the establishment of a successful heterologous expression system. This study reports the successful expression of the recombinant human erythropoietin in the methylotrophic yeast *Hansenula polymorpha* at a significantly high level (265 mg/l). Although erythropoietin was previously expressed in a wide variety of mammalian, bacterial and fungal-based systems, this is the first time this key hormone is expressed in the industrially important *H.* polymorpha. The significantly high expression level and the convenience of this organism for the industrial production of recombinant proteins make the development of a stable *H.* polymorpha erythropoietin transformants an essential improvement, in the exploitation of this key protein by the pharmaceutical industry. The expressed protein was successfully purified and characterized; confirming its structural and functional properties, while an industrial scale development of the system is under development.

KEY WORDS:
EPO, Erythropoietin, *H.* polymorpha, Recombinant proteins, Glycosylated

INTRODUCTION:
Erythropoietin (EPO) is an essential human glycoprotein produced in the renal fibroblasts, the hepatocytes and the neuronal cells. It acts as the prime haematopoietic growth factor; as it plays the principle role in regulating red blood cells production. An imbalance in EPO levels can lead either to anaemia or polycythemia. The recombinant EPO is used widely for treating cancer and anaemia patients, especially those with chronic kidney disease and chemotherapy-related anemia (Henry and Spivak, 1995; Duh et al., 2008; Testa, 2010; Chateauvieux et al., 2011; Bunn, 2013).

Endogenous human EPO is a complex of 165 amino acids which forms a mature 30 kDa protein with four glycan side chains attached to it; three located at asparagine 24, 38, and 83, and the fourth is located at Ser-126. In total, the glycosylation elements comprise about 40% of the total mass of the protein as the polypeptide accounts for 18 kDa only (Jelkmann, 2004). Like other glycoprotein hormones, EPO exists as a mixture of isoforms differing mainly in their carbohydrate structures (Storring and Gaines Das, 1992). This is because the glycosylation of the polypeptide moiety is of a post-translational nature which is influenced by the type of the cell in which the protein is synthesized and the physiological factors at the time of the synthesis. In addition, the isoforms composition of an EPO preparation is usually affected by the purification method which can also affect its folding (Goto et al., 1988).

Homologously expressed EPO is often purified in minute quantities from very large volumes of the urine of the anaemic patients (Espada and Gutnisky, 1970) or of the plasma of the anaemic sheep (Goldwasser and Kung, 1971). In 1985, Jacobs et al. isolated the human EPO cDNA and demonstrated its heterologous expression in a mammalian host system. Since then, several patents protected the production of various EPO isoforms with similar pharmacological properties (Lin, 1990; Wellesley et al., 1990; Elliott and Byrne, 2007; Carcagno et al., 2009; Pecker, 2011; Calo-Fernández and Martínez-Hurtado, 2012).
Currently, the recombinant EPO biosimilars (genetically-engineered forms of erythropoietin) represent the largest market of a class of biopharmaceuticals; with global sales of over 13 billion USA dollars per annum (GBI Research, 2012; Kalorama Information, 2013). The key patents for the first generation of EPOs have expired in 2009 and 2010 which opened the market for many similar analogues to be produced. The principle step towards any commercial exploitation of EPO is to establish a reliable expression system to manufacture the protein at an industrially viable scale.

While most of the recombinant EPO is produced in mammalian cells-based systems (Lin et al., 1985), non-glycosylated EPO is produced in bacteria (Lee-Huang, 1984; Bill et al., 1995; Nagao et al., 1997). On the other hand, the baker’s yeast, the methylotrophic yeast and the soil fungi-based expression systems enabled the expression of recombinant EPO with glycosylation profiles different from the mammalian cell-produced forms (Elliott et al., 1989; Celik et al., 2007; Vats and Padh, 2007). In addition, erythropoietin cysteine analogs were recently expressed in Pichia pastoris (Maleki et al., 2012).

Naturally, eukaryotic expression hosts attach oligosaccharides to the side chain of the asparagine residues existing in specific order within the amino acid chain of the protein. Humanized mammalian host cells usually glycosylate heterologously expressed proteins by adding a tri-mannose linked to a galactose and a sialic acid, whereas yeasts usually hyperglycosylate the proteins by adding 100 or more of mannose sugars. Despite this, the yeast-based protein expression systems carry many advantages over the mammalian-based systems; including lower overall costs, easier growth conditions and much higher expression levels. Because of this, the yeast-based expression systems are usually preferred for the industrial production of EPO biosimilars as well as many other recombinant human proteins despite the over-glycosylation issue (Goeddel, 1990; Mattanovich et al., 2012).

In order to overcome the glycosylation differences between the mammalian expression systems and the yeast-based systems, metabolically engineered yeast strains were used, in which the yeast glycosylation pathway was altered to mimic the human glycosylation profile. Erythropoietin was expressed in engineered strains of the methylotrophic yeast Pichia pastoris where its heavy glycosylation genes were knocked out and the genes of the human glycosylation were introduced to confer a humanized glycosylation profile on the expressed proteins (Hamilton et al., 2006). Alternatively, in vitro post-purification processing methods could be used to tailor the yeast-expressed recombinant proteins to the profile of their mammalian counterparts (Maras et al., 1997).

Hansenula polymorpha is a GRAS organism (Generally Regarded as Safe) and belongs to a group of yeasts capable of using methanol as a sole source of carbon and energy and so are referred to as the methylotrophic yeasts (Gellissen, 2000). A multitude of expression vectors is available for use in H. polymorpha where the recombinant protein expression can be controlled via a carbon source dependent repression/derepression induction mechanism conferred by the inherent properties of the promoter used (Gellissen et al., 1992; Esser, 2003).

This yeast is relatively simple to cultivate and scale-up to thousands of litres in growth tanks. High yields of heterologously expressed proteins are achieved through high gene copy integration, fast growth to high cell density, and an exclusive library of strong promoters.

Hansenula polymorpha is also able to secrete the protein products directly in the culture medium, a fact that renders the whole process of downstream processing easier and less costly. The ability of this organism to survive in a wide pH range, from 2.5 to 6.5, makes it a versatile protein factory which is exploited in the production of various proteins, each of which requires a very different optimum pH value throughout the production process (Kunze et al., 2009). Overall, high yields of recombinant proteins can be produced in H. polymorpha at a low-cost. In fact, this organism holds the record in yeast-derived protein production, with a productivity of more than 13 g/l in phytase production (Mayer et al., 1999; Sudbery, 2003).

Amongst the methylotrophic yeasts, P. pastoris and H. polymorpha are particularly efficient for commercial-scale recombinant protein production. These two species are close relatives; in fact the AOX1 promoter of P. pastoris is the analogue of the H. polymorpha MOX promoter. However, although both promoters are responsive to methanol, in P. pastoris, alcohol oxidase is rapidly and strongly induced in response to methanol induction while in H. polymorpha, methanol oxidase may be induced either by methanol or by glycerol de-repression. This is a particularly significant advantage for using H. polymorpha in applications involving large-scale productions, since it circumvents the potential hazards associated with the use of methanol (Gellissen et al., 1992; Gellissen, 2000).

P. pastoris expression vectors are designed for homologous recombination and integration at the AOX or HIS4 locus, whereas
those used in *H. polymorpha* can integrate by non-homologous recombination at random loci. Expression vector copies integrated in *P. pastoris* are usually limited to <10, whereas up to 150 copies can integrate in *H. polymorpha*. Since protein expression is correlated with gene dosage in these organisms, it is possible to achieve significantly higher expression in *H. polymorpha* despite the fact that the AOX promoter of *P. pastoris* is the strongest promoter. It is also possible to achieve more control over the level of production by regulating the copy number.

When plasmids are integrated at the AOX locus, the gene encoding the alcohol oxidase is disrupted; once the cells are transferred to methanol their growth is significantly retarded. The growth rate of *H. polymorpha* remains rapid before and after induction. In addition, *H. polymorpha* is a more thermotolerant organism than is *P. pastoris*, easily withstanding temperatures up to 43°C (Sudbery, 2003).

In this study, we have generated stable *H. polymorpha* transformants expressing the recombinant human erythropoietin at levels significantly higher than those reported from other expression systems. The protein was purified and characterized confirming its biochemical and functional properties. Scaling up of EPO production in *H. polymorpha* in a fermenter-based set up is well underway.

**MATERIAL AND METHODS:**

**Strains:**

*Hansenula polymorpha* strain 201322 was obtained from the ATCC. *Escherichia coli* strain XL10-Gold® was obtained from Agilent.

**Constructs:**

Erythropoietin open reading frame construct was obtained in plasmid pReceiver-Y01 from GeneCopoeia Inc. Vector pGAPZα was purchased from Invitrogen.

**Reagents and equipments:**

Advantage® HD Polymerase Mix, used for polymerase reaction-based amplifications, was obtained from Clonetech. In-Fusion 2.0 Dry-Down PCR Cloning Kit used for cloning amplified PCR products was purchased from Clonetech. Veriti® 96-Well Thermal Cycler, Applied Biosystems, was used for thermal cycling of PCR and other enzymatic incubations. ReadyAagarose Gels and the ReadySub-Cell GT cell system and buffer reagents from Bio-Rad were used for nucleic acids electrophoresis. QIAquick PCR Purification Kit, QIAquick Gel Extraction Kit and Plasmid preparation kits from QIAGEN were used for PCR reaction purification, DNA gel extraction and plasmid preparations respectively. All restriction and modification enzymes were purchased from New England Bio-labs. Gene Pulser MXcell Electroporation System, Bio-Rad, was used for electroporations. Innova 4900 incubator shaker, New Brunswick, and SANYO MIR Standard Incubator were used for cultures incubations. Eppendorf centrifuges 5702 R and 5810R and Avanti® J-26 XP Centrifuge were used for routine centrifugation and culture harvest. Corning® 500 ml Erlenmeyer flask polycarbonate and, PYREX® Fernbach-Style Culture Flask with Baffles and Phenolic Screw Cap, from CORNING, were used for liquid cultures. BCA Protein Assay Reagent purchased from Thermo Scientific, was used for determination of protein concentration. Gogen® μScale TFF System, from Millipore, was used to concentrate EPO from supernatants. Criterion Precast Gel System and buffer reagents from Bio-Rad were used for protein electrophoresis. Protein Deglycosylation Mix, New England Biolabs, was used for protein deglycosylation. Factor Xα Cleavage Capture Kit, EMD4Biosciences, was used for removing the signal sequence from the expressed protein. Mouse Anti-Myc tag Monoclonal Antibody, HRP Conjugated, from abCam, was used in Western blot analysis. iBlot® Western Detection Chemiluminescent kit (anti-Mouse), Invitrogen, was used for Western blot analysis. Cell culture reagents were all purchased from Becton, Dickinson and Company (BD). All other laboratory chemicals were purchased as molecular biology grade from Sigma. Water used in reagents preparations, as well as cell culture was ultrapure grade obtained from Milli-Q Advantage A10 Ultrapure Water Purification System, Milliopre.

**EPO expression in *H. polymorpha*:**

Competent *H. polymorpha* cells were prepared and transformed according to Faber *et al.* (1994) using the Gene Pulser MXcell Electroporation System from Bio-Rad. In brief, a single cell grown on the non-selective YPD medium (1% Yeast extract, 1% Bactopeptone, 1% glucose, 2% agar) at 37°C, was used to grow a fresh overnight culture in liquid YPD at 37°C with 250 rpm orbital shaking. A 100-fold dilution of the growing cells was made in 200 ml fresh, pre-warmed (37°C) YPD medium. Cells were grown at 37°C with 250 rpm orbital shaking until the optical density of the culture reached 1.3 at 663 nm. Cells were then harvested by centrifugation at 3000 g for 10 minutes and cell pellet was resuspended in 50 ml of 50mM potassium phosphate buffer pH 7.5 containing 25 mM dithiothreitol (DTT). Re-suspended cells were incubated at 37°C for 10 minutes before washing twice in 200 ml of the electroporation buffer STM (270 mM sucrose, 10 mM Tris-HCl pH 7.5 and 1 mM MgCl2).

Cells were harvested again by centrifugation at 3000 g and finally re-suspended in 1 ml of the STM buffer. 100 ul
fresh aliquots were used for electroporation with 5 µg of the gel purified KpnI-linearized pGAPURAEP01 in a pre-chilled electroporation cuvette. The Gene Pulser MXcell was used to deliver a 5 mille second pulse using 7.5 kV/cm; 50 µF and 120 Ohm electric field to the cells/DNA mixture. Immediately following the pulse, 1 ml of room temperature YPD medium was added and the cells suspension was then incubated for 1 h at 37°C without shaking. Cells were harvested (5 min, 3000 g), washed once with 1 ml YND selective medium (0.67% Bacto yeast nitrogen base without amino acids, 1% glucose) then spread on selective YND plates (0.67% Bacto yeast nitrogen base without amino acids, 1% glucose+ 100 µg/ml Zeocin, 2% agar) and incubated at 37°C. Surviving transformants started to grow after three days and were left to grow further for another 2 days. The transformation frequency was 223 transformants/µg of DNA and 20 fast growing colonies were selected and re-streaked three times (each time 4 days at 37°C) on selective YND plates until 5 fastest growing transformants were finally selected for liquid culture expression.

A single cell colony from each of the selected fast growing transformants was used to inoculate a 10 ml YPD medium in a 500 ml Erlenmeyer flask for 18 hours at 37°C. This will facilitate the post-expression protein directly in the expression medium. The recombinant EPO expression construct: the secretion factor is in frame with the EPO culture expression. Design and manufacture of the recombinant EPO expression construct:

The OmicsLink pReceiver-Y01 construct (Fig. 1b) was used as a source for the EPO cDNA (ORF), where the fragment was released by an EcoRl/NotI double digest. The released fragment (522 bp) was gel purified using the QiAquick Gel Extraction Kit according to the manufacturer recommendations. The Invitrogen vector pGAPZaA (Fig. 1a) was also EcoRl/NotI double digested and gel purified before ligated with the EPO cDNA using T4 DNA ligase. Ligated vector was transformed into XL10-Gold® E. coli competent cells by heat shock and the competent cells by heat shock and the supernatant was collected and kept on ice for processing.

RESULTS AND DISCUSSION:
Design and manufacture of the recombinant EPO expression construct:

A range of auxotrophic H. polymorpha host strains is available which can be transformed by complementing their deficiencies (positive control). The ATCC H. polymorpha strain 201322 is a uracil deficient as it lacks the URA3 gene which encodes orotidine-5'-phosphate decarboxylase (ODCase), an enzyme required for the biosynthesis of uracil. Loss of ODCase activity leads to a lack of cell growth unless uracil or uridine is added to the media. Transforming a URA3 deficient H. polymorpha with plasmids containing the URA3 gene enables maintaining these plasmids in an autonomous multicopy state within the organism (Roggenkamp et al., 1986). In addition, the presence of a heterologous URA3 gene in the transformation plasmids favours multiple integration within the genome which results in stable transformants that can carry <100 copies of the transformation construct without maintaining the selection pressure (Gatzke et al., 1995; Brito et al., 1999; Esser, 2003; Sudbery, 2003).

The pGAPZa was selected (Fig. 1a), as the backbone for constructing an expression vector to produce the recombinant EPO in the auxotroph ATCC H. polymorpha strain 201322. Although this plasmid was originally designed for expressing recombinant proteins in P. pastoris, it carries elements which function equally well in H. polymorpha. The expression promoter was the glyceraldehyde-3-phosphate dehydrogenase promoter (PGAP). This is a high level constitutive promoter which was used successfully for recombinant protein expression in H. polymorpha. In fact, the expression levels obtained from PGAP were comparable to those obtained from the famous methanol oxidase promoter which is usually used for the recombinant protein expression in H. polymorpha (Heo et al., 2003). The alcohol oxidase element of the pGAPZa vector (AOX TT) also functions well in H. polymorpha since its transcriptional machinery correctly recognizes and utilizes the elements of the alcohol oxidase promoter (Raschke et al., 1996). In addition, pGAPZa uses Zeocin as a selection marker for both bacterial and yeast transformants. Zeocin selection has also proved to be successful in H. polymorpha (Salomons et al., 2000; Qian et al., 2009).

The OmicsLink pReceiver-Y01 construct (Fig. 1b) was used as a source for the EPO cDNA (ORF), where the fragment was released by an EcoRl/NotI double digest. The released fragment (522 bp) was gel purified using the QiAquick Gel Extraction Kit according to the manufacturer recommendations. The Invitrogen vector pGAPZaA (Fig. 1a) was also EcoRl/NotI double digested and gel purified before ligated with the EPO cDNA using T4 DNA ligase. Ligated vector was transformed into XL10-Gold® E. coli competent cells by heat shock and the transformants were selected on 100 µg/ml Zeocin LB agar plates (1% Bacto Tryptone, 0.5% Bacto Yeast Extract, 1% NaCl, 2% agar) then 10 stable transformants were minipreped and checked for the presence of the right size EPO cDNA fragment using EcoRl/NotI restriction digestion. The sequence and the in-frame alignment of the EPO construct was confirmed by DNA sequencing and the resulting expression construct was named pGAPZEP01 and was propagated in E. coli on Zeocin selection to prepare enough DNA using the QIAGEN Midi plasmid prep kit for subsequent creation of the final expression construct.

In order to add an extra selection criterion and encourage the generation of multiple genome-integrated constructs, in the H.
polymorpha expression transformants, we inserted the *S. cerevisiae* URA3 gene (Bonneaud et al., 1991) into the pGAPZEP01 construct at the BglII restriction site. Gatzke et al. (1995) showed that the copy number of the integrated plasmids in the transformed *H. polymorpha* is dependent on the plasmid marker used for the selection of the transformants. Since the URA3 gene of *S. cerevisiae* is expressed at a low level in *H. polymorpha*, the selection of transformants with the highest growth rate positively discriminates cells with multiple copies of this marker. In contrast, plasmids bearing the homologous *H. polymorpha* URA3 gene usually integrate into the genome in a single copy. In order to do this, we used the OmicsLink pReceiver-Y01x vector (Fig. 1B) as a template to amplify the *S. cerevisiae* URA3 segment using PCR. Forward and reverse oligonucleotide primers flanking the URA3 segment were designed carrying the BglII recognition site. The forward primer was designed so that an extra restriction site (KpnI) is located 3’ to the BglII site with the intention that it can be used for linearization of the final construct (Fig. 1C). Primers were synthesized and purified before used in the PCR amplifications. Using the Advantage® HD Polymerase Mix and the purified DNA of the vector pReceiver-Y01x, as a template, a 1.2 kbp URA3 fragment was amplified and gel purified using the QIAquick Gel Extraction Kit.

The In-Fusion 2.0 Dry-Down PCR Cloning Kit was used to clone this 1.2 kbp URA3 fragment into the pGAPZEP01 construct at the BglII site according to manufacturer recommendations. The generated plasmid was called pGAPZURAEPO1 and was transformed into XL10-Gold® *E. coli* competent cells by heat shock according to the manufacturer recommended procedure. The bacterial transformants were selected on 100 µg/ml Zeocin LB agar plates where 5 stable transformants were minipreped using the QIAGEN plasmid miniprep kit, according to the manufacturer recommendations, and checked for the presence of the right size URA3 segment by PCR using the same amplification primers. The resulting construct (Fig. 1C) was used in transforming *H. polymorpha* strain 201322 to express EPO as described in the following sections.

**Processing and characterization of the expressed EPO:**

The Cogent® µScale TFF system was used for protein collection and concentration from the ice-cooled clear supernatant according to manufacturer recommendations. This concentration process is dependent upon membrane filtration technology called tangential flow filtration (TFF). The process uses filtration membranes with specific cut off threshold to separate components in a liquid solution based on their molecular weight and size. A 10 kDa cut off was used to make sure that the concentration process collects all of the recombinant protein that was expressed in the culture medium. Figure 2 lane C, shows a silver stain of 20 µl of the concentrated supernatant run on 4-20% precast gradient Tris-HCl SDS-PAGE. Despite the presence of some background proteins, the predominant species is about 36 kDa in size which is likely to be the glycosylated EPO. The recombinant EPO construct (Fig. 1C) was designed to express in frame, at the N-terminus, with the myc epitope, as well as a 6 poly-histidin tag which will facilitate purification from the culture supernatant.
recommendations. Purified EPO was mixed with the Cleavage/Capture Buffer and diluted factor Xa, incubated at room temperature for 6 hours before factor Xa was removed by affinity purification of the kit Xarrest Agarose. The equilibrated agarose was mixed with the protease reaction with gentle agitation for 15 minutes before the agarose was separated from the clear buffered protein by centrifugation. Figure 2 lane B is showing the Ni-column purified EPO run on Coomassie-stained 4-20% precast gradient Tris-HCl SDS-PAGE. Despite showing a molecular weight slightly smaller than the predominant species in the un-purified culture supernatant (lane C), the purified EPO is showing a size of 34 kDa. The native mature EPO is known to be 30 kDa in size and the difference is probable due to over glycosylation, as well as the tags linked to the protein at C-terminus (Fig. 1C). However, the concentration of the purified protein was estimated by the BCA Protein Assay Reagent, according to the manufacturer recommendations. We estimated that assuming 100% purification efficiency via the recommendations. Purified EPO was mixed with the Cleavage/Capture Buffer and diluted factor Xa, incubated at room temperature for 6 hours before factor Xa was removed by affinity purification of the kit Xarrest Agarose. The equilibrated agarose was mixed with the protease reaction with gentle agitation for 15 minutes before the agarose was separated from the clear buffered protein by centrifugation. Figure 2 lane B is showing the Ni-column purified EPO run on Coomassie-stained 4-20% precast gradient Tris-HCl SDS-PAGE. Despite showing a molecular weight slightly smaller than the predominant species in the un-purified culture supernatant (lane C), the purified EPO is showing a size of 34 kDa. The native mature EPO is known to be 30 kDa in size and the difference is probable due to over glycosylation, as well as the tags linked to the protein at C-terminus (Fig. 1C).

However, the concentration of the purified protein was estimated by the BCA Protein Assay Reagent, according to the manufacturer recommendations. We estimated that assuming 100% purification efficiency via the concentration and the Ni-purification, EPO was produced in *H. polymorpha* at 265 mg/l of culture medium. This is about 50 fold the yield reported before in *P. pastoris* (Celik et al., 2007) and more than double EPO cysteine analsogs expression under the same AOX promoter in shake flasks (Maleki et al., 2012). This significantly high yield may be attributed to a construct multi-copy integration event which is known to be associated with the heterologous recombination of the URA3 marker into the *H. polymorpha* genome (Gatzke et al., 1995). *H. polymorpha* is known to be particularly suitable for large scale fermenter recombinant protein production (Mayer et al., 1999; Kunze et al., 2009; Crommelin et al., 2013). This high EPO expression level potentiates the likelihood of a successful industrial fermenter scale production.

After the factor Xa digestion, the purified EPO was deglycosylated under non-denaturing conditions by mixing it with the deglycosylation mix from NEB and incubated it for 4 hours at 37°C. The deglycosylated EPO was then passed through the Ni-columns to purify it from the deglycosylation mix. Figure 2 lane A is showing the deglycosylated purified EPO after factor Xa treatment. The SDS PAGE migration is reflecting the removal of the glycosylation as in lane B the signal-less recombinant EPO is migrating at about 35 kDa while after deglycosylation (lane A) the migration of the protein was shifted to just above 17 kDa. In order to confirm the identity of the expressed EPO, anti-myc antibodies were used to interrogate the protein using Western blotting. The recombinant EPO construct (Fig. 1C) was designed so that a myc epitope is fused to the mature protein C-terminus. Figure 3 is showing the X-ray exposure of a chemiluminescence Western blot of the α-signal-free EPO after and before deglycosylation. Despite some degradation, that is likely to result from post-expression processing and purification, the recombinant EPO is reacting positively to the anti-myc antibody detection which confirms the proper structure of the expressed protein as designed (Fig. 1C). The Western blot has also showed a predominant uniform band migrating at 18 kDa (lane A) and two closely located bands (lane B) migrating at 30 and 35 kDa, respectively.

![Fig. 2. SDS-PAGE of the recombinant erythropoietin expressed in *H. polymorpha.*](image)

- a: purified-deglycosylated EPO after removal of the signal sequence.
- b: purified EPO before deglycosylation.
- c: concentrated expression supernatant before EPO purification.

In order to confirm the identity of these bands a copy of the Western blot membrane was used to perform N-terminus protein sequencing for the three predominant bands (Fig. 3). The protein bands were electroblotted to the PVDF membrane which was then stained with Coomassie brilliant blue. The bands were carefully excised from the membrane and were sent for N-terminus protein sequencing service at the Proteome Factory AG. It was revealed that the 18 kDa bands on lane A (Fig. 3), as well as the two closely migrating bands on lane B are sharing the same N-terminus amino acid sequence of Met-Gly-Val-His-Glu-Cys-Pro-Ala-Trp-Leu-Trp. This result confirmed that the factor Xa digestion to remove the α-signal sequence from the mature EPO was efficient and the protein expressed is actually the recombinant human EPO. It is likely that the two closely migrating bands, on lane B, are two differently glycosylated isoforms of the recombinant EPO, since after the deglycosylation, the protein migrated as a predominantly single band on lane A.
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**Fig. 3.** Western blot of the recombinant erythropoietin expressed in *H. polymorpha* using anti-myc antibodies. a: purified-deglycosylated EPO after removal of the signal sequence. b: purified EPO before deglycosylation. The N-terminus amino acid sequence is shown next to each of the major bands.

**CONCLUSION:**

*Hansenula polymorpha* proved to be a suitable expression host for the production of the recombinant human erythropoietin under shaking flask conditions. Despite the over-glycosylation problem of the synthesized EPO, the high yield achieved using the URA3 integration construct with the PGAL promoter carries a great potential for large scale production due to the significantly high yield. The generated transformants were maintained under non-selective conditions which make them particularly suitable for producing this pharmaceutically essential protein under industrial fermentation conditions.

**REFERENCES:**


نتاج البروتينات الجزيئية المشتركة بمعدل مرتفع في هانسبيولا بوليمورفا

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يعتمد الإنتاج الجزيئي للبروتين العلاجي البروتين المشتركة بصورة كبيرة على تأسيس نظام الإنتاج الصناعي. من ناحية أخرى، فهذا النوع من البروتينات المشتركة قد يكون من فلسفة نشأة وكبيانية ووظيفة، لذلك يبدو أن هذا النوع من البروتينات المشتركة يكون من فلسفة نشأة وكبيانية، ولكن البروتينات المشتركة وراثيًا على نطاق صناعي يجعل تأسيس هذا النظام

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