Production of recombinant human granulocyte-colony stimulating factor by *Pichia pastoris*

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Abstract
Human granulocyte-colony stimulating factor (hG-CSF) cDNA was expressed in the methylotrophic yeast Pichia pastoris under the control of the alcohol oxidase (AOX1) promoter. An expression vector for hG-CSF secretion was constructed using vector pPIC9. Higher levels of hG-CSF was obtained using a P. pastoris Mut+ (methanol utilization fast) phenotype. The effects of environmental factors such as temperature and pH on the P. pastoris cell growth and hG-CSF production during fermentation were investigated. Cell growth and hG-CSF production were found to be optimal at 28°C and pH 6.0. A fed-batch fermentation process was also developed to obtain high cell density and higher levels of protein expression. Using a high cell density cultivation method, cell dry weight and hG-CSF concentration reached 100 g/l and 35 mg/l, respectively.
Keywords**:** Pichia pastoris; rhG-CSF; Fed-batch culture; Methanol.

INTRODUCTION

Granulocyte-colony stimulating factor (G-CSF) is a hematopoietic growth factor that stimulates the proliferation and differentiation of neutrophil precursor cells, enhancing some of the functional properties of mature neutrophils (Metcalf, 1988; Morstyn *et al*., 1988). The mature human G-CSF (hG-CSF) is a glycoprotein with 174 amino acids which is produced mainly by monocytes and macrophages upon activation by endotoxin (Rasko *et al*., 1994). The hG-CSF has become the most widely used hematopoietic growth factor because of its proven efficacy against different forms of neutropenia. It is also used in chemotherapy to induce leucopenia and mobilize progenitor cells for the purpose of autologous or allogenic transplantation (Frampton *et al*., 1994: Welte *et al*., 1996). Two forms of recombinant hG-CSF are currently available for clinical use. One form is derived from Chinese hamster ovary (CHO) cell cultures, and possesses a sugar chain on Thr133 (Kubota *et al.,* 1990). The other is expressed and purified from *Escherichia coli*, but is not glycosylated (Frampton *et al.,* 1994).

The methylotrophic yeast *P. pastoris* has emerged as an important host for heterologous proteins in both medical research and industrial biotechnology (Lin Cereghino *et al.,* 2000; Lin Cereghino *et al.,* 2002). As a eukaryotic organism, *P. pastoris* offers well known advantages for protein expression, such as efficient protein folding, disulfide bond formation and glycosylation. Very recently the capability of *P. pastoris* to produce complex human proteins has been demonstrated in a paradigmatic study (Hamilton *et al.,* 2003), indicating that this organism might well become the premier choice for production of pharmaceutical proteins. Specific advantages of *P. pastoris* are due to (a) the availability of an unusually tightly regulated promoter present in the methanol-regulated alcohol oxidase I (AOX1) gene (b) the system's efficient protein secretion, which when combined with the very low secretion levels of endogenous proteins, is a major advantage for their purification, and (c*)* the preference of *P. pastoris* to grow in a respiratory mode, thus reducing the excretion of fermentation by products such as ethanol or acetic acid and allowing exceptionally high cell densities to be reached (Sola *et al.,* 2004). Many eukaryotic proteins have been produced using the *Pichia* expression system (Tschopp J *et al.,*

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1987; Clare *et al.,* 1991; Lin Cereghino *et al.,* 2000), because it is more efficient, simpler and less expensive than the other eukaryotic expression systems.

The gene coding for rhG-CSF has been cloned and expressed in a strain of *P. pastoris* with the methanol utilization slow (Mut^s) phenotype (Lasnik *et al.,* 2001). However, a detailed investigation of the expression and secretion of rhG-CSF by high cell density cultures of *P. pastoris* has not been documented. In this paper we describe cloning, expression and a fed-batch fermentation strategy for hG-CSF production by a Mut^+ strain of *P. pastoris*.

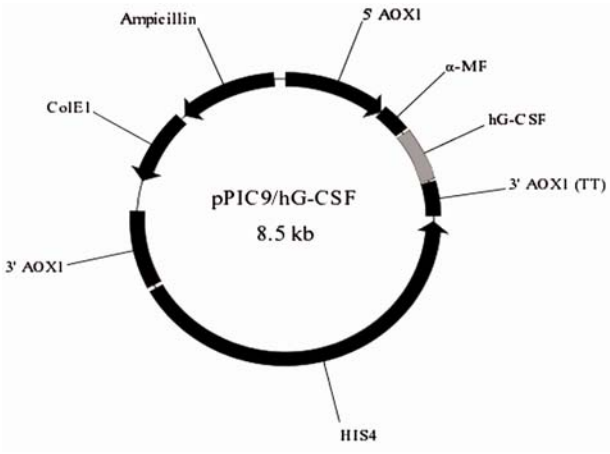

Figure 1. Structure of the pPIC9/hG-CSF expression vector. The hG-CSF cDNA was cloned into the pPIC9 plasmid under the control of AOX1 promoter.

MATERIALS AND METHODS

Bacteria, Yeast, and growth media: *Escherichia coli* strain TOP10F′ was used for the propagation of recombinant plasmid. *E. coli* transformants were selected on Luria-Bertani (LB) plates 0.5% (w/v) yeast extract, 1% (w/v) tryptone, 1% (w/v) NaCl, 1.5% (w/v) bacteriological agar supplemented with 100 µg/ml of ampicilin. *Pichia pastoris* strain GS115 (Invitrogen) was used for hG-CSF protein expression. The *P. pastoris* GS115 strain was cultured in yeast peptone dextrose (YPD) medium 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose plus 2% (w/v) agar in plates). *P. pastoris* transformants were selected on minimal dextrose medium 1.34% (w/v), yeast nitrogen base without amino acids, 4×10^{-5}% (w/v) biotin, 2% (w/v) dextrose, and 2% (w/v) agar, and minimal methanol medium 1.34% (w/v) yeast nitrogen base without amino acids, 4×10^{-5}% (w/v) biotin, 0.05% (w/v) methanol, and 2% (w/v) agar. The growth and induction media used were buffered minimal glycerol-complex medium (BMGY) 1% (w/v) yeast extract, 2% (w/v) peptone, 0.1 M phosphate buffer pH 6.0, 1.34% (w/v) yeast nitrogen base, 4×10^{-5}% (w/v) biotin and 1% (w/v) glycerol and buffered minimal methanol-complex medium (BMMY) same as BMGY except that glycerol was replaced by 0.5% (v/v) methanol, respectively.

Expression vector: The hG-CSF cDNA was amplified by real time polymerase chain reaction (RT-PCR) using the following primers: forward 5′-AAACTCGA-GAAAAGAACACCCCTAGGCCCTGC- 3′and reverse 5′-CGGAATTCTTACTAGGGCTGG GCAAGGTGG-3′. The forward primer contained a *Xho*I site and the reverse primer contained a *Eco*RI site together with two stop codons. Primers were designed to generate a 569 bp PCR fragment containing a hG-CSF open reading frame (ORF) lacking a signal peptide. The RT-PCR conditions were set up as described by Saeedinia *et al.* (2003). The hG-CSF cDNA was digested with *Xho*I and *Eco*RI restriction enzymes (Fermentas) and ligated into the pPIC9 vector (Invitrogen), under the control of AOX1 promoter. The resultant recombinant vector was named as pPIC9/hG-CSF (Figure 1). The α-MF present in the vector, upstream of the hG-CSF gene was used for the synthesis and secretion of the target protein into the medium. The recombinant vector was used to transform *E. coli* TOP10F′ cells. Plasmid DNA was extracted and analyzed for the presence of the hG-CSF fragment. The resulting recombinant plasmid was then sequenced by automated sequencer (ABI PRISM 310 genetic analyzer, PE Biosystems) using vector specific primers (5′ and 3′ AOX1 primers).

Transformation of *P. pastoris* with pPIC9/hG-CSF: *P. pastoris* strain GS115 cells were made electro competent following manufacturer's instructions (Invitrogen, CA, USA). Approximately 10 µg of recombinant expression plasmid pPIC9/hG-CSF was linearized by digestion with the *Bgl*II enzyme, resulting in *P. pastoris* GS115 transformants carrying the His^+ Mut^+ and His^+ Mut^s phenotypes. Electroporation was carried out by a Gene Pulser (Eppendorf, Germany). Transformants were plated onto MD plates and incubated at 30ºC for 4 days. The parent pPIC9 without the insert, linearized with *Bgl*II, was also transformed into *P. pastoris* and used as a negative control. Transformants carrying the methanol utiliza-

tion plus (Mut^+) phenotype were selected by growing on Minimal methanol medium (MM) and Minimal dextrose medium (MD) plates. Transformants bearing the chromosomally integrated copies of the pPIC9/hG-CSF were then detected by a genomic PCR assay using the 5′ and 3′ AOX1 primers.

hG-CSF expression in *P. pastoris*: The colonies that were found positive by the genomic PCR assay were selected for induction. Positive His^+ Mut^+ colonies were inoculated into 50 ml of BMGY medium in 250 ml conical flasks along with the negative control (*Pichia* transformed with pPIC9 without the insert) and were incubated at 28°C in a shaker incubator at 250 rpm, until the culture reached an optical density (OD_{600}) of 2-6. The cells were harvested by centrifugation at 3,000g for 5 min, at room temperature and the resulting cell pellet was suspended in BMMY medium to an OD_{600} of 1.0 in a conical flask. Induction was carried out by incubation at 28°C in a shaker incubator at 250 rpm. This culture was maintained for 6 days and supplemented daily with methanol (0.5% (w/v). Different induction periods ranging from 24-144 h were also tested along with different methanol concentrations in order to find the optimal expression conditions.

Western blot analysis: The expressed proteins were separated by electrophoresis on a 15% (w/v) SDS-PAGE gel. The secretory expression of hG-CSF protein was confirmed with a hG-CSF positive serum through western blotting. Briefly, proteins were transferred from the gel onto nitrocellulose membrane. After transfer, the membranes were probed sequentially with 1:500 dilution of the hG-CSF specific polyclonal antibody raised in rabbit against the commercial hG-CSF (Roche) and 1:1000 dilution of horse radish peroxidase (HRP) labeled goat anti-rabbit immunoglobulin G (IgG). Protein samples were visualized with the diaminobanzidine/hydrogen peroxide (DAB/H_2O_2) chromogen/substrate solution.

Enzyme-linked immunosorbent assay (ELISA): The expression level of recombinant hG-CSF was measured by ELISA. Briefly, a serially diluted samples of hG-CSF was coated onto a microtiter plate for two hours at 37°C. Following three washes with phosphate buffered saline (PBS), blocking buffer was added and incubated at room temperature for an hour. Anti-hG-CSF hyper immune serum was added to each well at a dilution of 1:500 in PBS and incubated for an hour at room temperature. A polyclonal goat anti-rabbit immunoglobulins/HRP (Dako cytomation, Netherlands) was diluted 1:2000 in PBS and added to each well and incubated as before. Then, a freshly prepared substrate-chromogen mixture (hydrogen peroxide and tetramethyl-benzidine) was added to each well. Incubation was carried out at room temperature for 15 min. The reaction was stopped by adding an equal volume of 1 N H_2SO_4 to each well. The standard curve covering 0 to 13 µg/ml of recombinant hG-CSF (Neupogen syringe, Amgen) was obtained. This curve is shown in Figure 2.

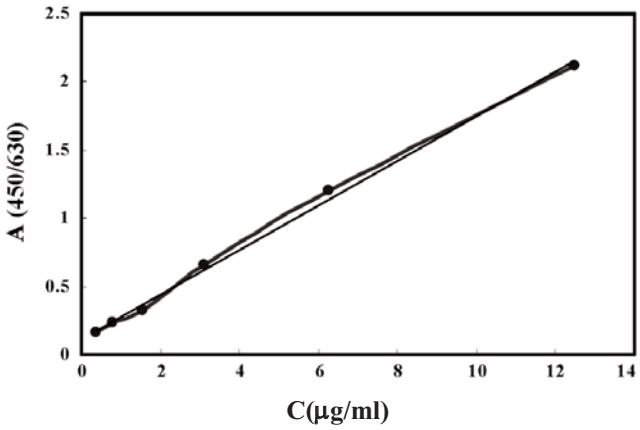

Figure 2. ELISA standard curve for quantification of hG-CSF in *P. pastoris* culture medium.

Cell density analysis: The optical density (OD) of cell suspension was measured at 600 nm. The wet cell weight (WCW, g/l) was used as a measure of cell density within the bioreactor. A 1.0 ml sample was centrifuged at 10,000 rpm for 3 minute. The supernatant was stored at 4°C and the pellet was weighed. The dry cell weight (DCW, g/l) was calculated using the following equation DCW = 0.35 × WCW which was obtained from a calibration curve prepared using 20 samples that were dried for 24 h at 105°C.

Glycerol and protein levels in the medium: Off–line analyses of glycerol were performed using an enzymatic kit (Boehringer Mannheim, Germany). The concentration of protein was measured according to Bradford (Bradford, 1976).

Fed-batch fermentation: The fermentation inoculum was prepared from a frozen cell stock vial (1 ml), in 50 ml of BMGY medium at 30°C and 200 rpm for 18 h. The inoculum was transferred into a 2 liter fermentor (Infors, Switzerland) containing 1 liter of the basal salts medium which consisted of (g/l): glycerol,

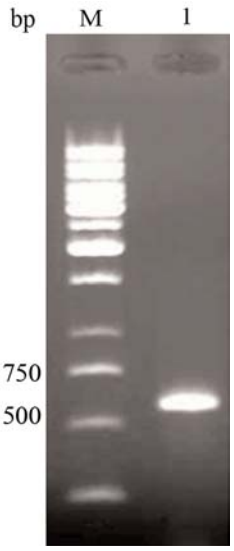

Figure 3. PCR amplification of hG-CSF cDNA. Lane 1 shows the band of 569 bp pertaining to the amplified hG-CSF cDNA; lane M is the DNA molecular weight size marker.

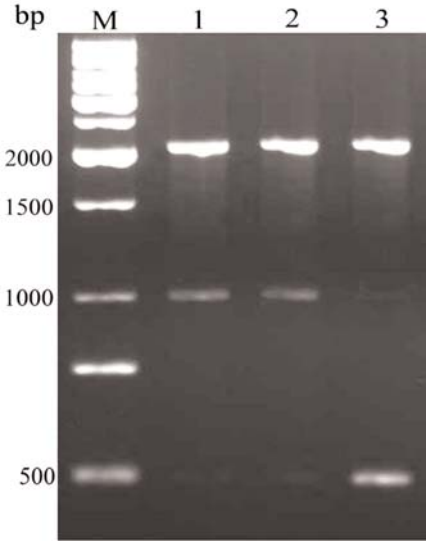

Figure 4. PCR detection of expression cassette in the transformed yeast by 5′ and 3′ AOX1 primers. Lanes 1, 2 reveals a PCR band of 1056 bp corresponding to the cDNA of hG-CSF. Lane 3 reveals a PCR band od approximately 492 bp corresponding to the empty vector (pPIC9). Lanes 1, 2, 3 have the additional 2200 bp of the AOX1 gene band indicating the Mut$^+$ phenotype. Lane M is the DNA molecular weight size marker.

40; K_2SO_4, 18; $MgSO_4 \cdot 7H_2O$, 14.9; KOH, 4.13; $CaSO_4$, 0.9 and H_3PO_4, 27(ml/l), plus 4.4 ml of a trace metal stock solution that consisted of (g/l): $CuSO_4 \cdot 5H_2O$, 6; KI, 0.09; $MnSO_4 \cdot H_2O$, 3; H_3BO_3, 0.02; $MoNa_2O_4 \cdot 2H_2O$, 0.24; $CoCl_2$, 0.5; $ZnCl_2$, 20; $FeSO_4 \cdot 7H_2O$, 65; biotin, 0.2 and H_2SO_4, 5.0 (ml/l) (Lee *et al.,* 2003). The pH of the medium was adjusted to 5.0 by using ammonium hydroxide solution (28%). The temperature was controlled at 28°C and the dissolved O_2 was then maintained at 1.5-2.2 mg/l (20-30% saturation) by controlling both air flow and stirrer speed. The cells were then grown in batch (~24 h) until the initial glycerol was depleted. This was followed by glycerol fed-batch phase in which a 50% solution of glycerol (w/w) was supplemented with 12 ml/l of trace elements. The initial feed rate was 12 ml/lh and was increased further in order to maintain a specific growth rate of 0.12 h^{-1}. After 4 hours, when the DCW was approximately 45 g/l, the glycerol feed was replaced with a feed containing 780 g/l of methanol and 12 ml/l of trace elements solution. The initial feed rate was 2 $ml/l^{-1}h^{-1}$ and was then increased up to 8 ml/l/h,which was kept constant until the end of induction.

RESULTS

Construction of expression vector (pPIC9/hG-CSF): The 569 bp fragment of the hG-CSF gene was isolated through PCR (Figure 3). The gene was cloned into the *Pichia* expression vector pPIC9, at *Xho*I and *Eco*RI sites and the resulting recombinant construct, pPIC9/hG-CSF was transformed into *E. coli* TOP10F′ for the purpose of mass production, prior to being used for transformation of *P. pastoris*.

Transformation of *P. pastoris*: The hG-CSF expressing construct, as well as the intact parent vector (pPIC9) were linearized by *Bgl*II and used to transform the *P. pastoris* GS115 strain. Yeast transformants with the Mut$^+$ phenotype were isolated after growth on both MM and MD media. The PCR amplification with AOX1 primers indicated that the hG-CSF gene had been integrated into the AOX1 locus on the chromosome of the transformed *P. Pastoris*. For Mut$^+$ clones, two expected bands were detected, One 1056 bp pertaining to the G-CSF expression cassette flanked by AOX1 sequences, and the other 2200 bp corresponding to the native AOX1 gene of the yeast genome (Figure 4).

Expression of recombinant hG-CSF protein in shake flask: The positive His$^+$ Mut$^+$ colonies that were found positive as a result of genomic PCR were selected for the purpose of inducing the expression of the target gene. Ten randomly selected clones were grown in shaking batch cultures. The expression level

of the recombinant protein was measured by ELISA and the best clone was selected. The results revealed the production of 2 mg of hG-CSF per liter of culture. Furthermore, the secretory expression of hG-CSF protein was confirmed using western blot analysis.

Effect of culture temperature and pH on cell growth and hG-CSF expression: In order to investigate the effects of pH and temperature on cell growth and hG-CSF production, batch cultures maintained at various pHs and temperatures were carried out for 3 days using the BMMY medium (containing 30 g/l of methanol), in 2 l fermentors. The culture temperatures varied within the range of 24-34°C at intervals of 2°C. The cell density (DCW, g/l) at 26-30°C was within a range of 10-11 g/l, but in the case of samples cultured above 30°C, the cell concentration was decreased. The hG-CSF expression was generally constant when culture temperatures increased up to 28°C, but decreased at temperatures exceeding 30°C.

Also the effects of pH on cell growth and hG-CSF production was investigated within a range of 4-7. Under these conditions the cell concentration ranged from 11 to 13 (DCW, g/l). The results showed that pH variation has no considerable effect on cell growth, but it had a significant effect on hG-CSF expression. According to the data, maximum hG-CSF 4 mg/l of hG-CSF was produced at pH 6.0.

Fed-batch fermentation strategies: The effects of induction time on *P. pastoris* cell growth and h-GCSF production in fed-batch fermentation are shown in Figure 5. Fed-batch fermentation is carried out by two strategies. In the first strategy, a culture cell density of 80 (DCW, g/l) was obtained at the end of the glycerol fed-batch phase. This was followed by the methanol fed-batch phase for a period of 100 h. At the end of the induction phase, the recombinant hG-CSF concentration reached to 14±2 mg/l. In the second strategy, the culture was induced at a DCW of 45 g/l. Using this strategy, after 100 h of methanol feeding both the culture cell density and hG-CSF concentrations increased to 90 (DCW, g/l) and 25 ± 2 mg/l, respectively.

Effect of peptone on hG-CSF production: In order to investigate the effect of an amino acid rich nitrogen source on cell growth and hG-CSF production in fed-batch culture, a 30% (w/v) peptone solution was fed in to the fermentor, at the feed rate of 0.25 g per g of methanol. After 70 h of induction, the final DCW and hG-CSF concentration of 105 g/l and 35 ± 2 mg/l were obtained, respectively (Figure 6). The SDS-PAGE and western blot analysis of supernatant samples are shown in Figure 7.

DISCUSSION

In this study, the hG-CSF gene was inserted downstream of the AOX1 promoter in the secretory expression vector pPIC9 and the chimeric construct was integrated into the host chromosome through homologous recombination. For the purpose of preparing the pPIC9/hG-CSF plasmid construct, the Glutamine-Alanine-Glutamine-Alanine(Glu-Ala-Glu-Ala) sequence was deleted and *Sna*BI restriction

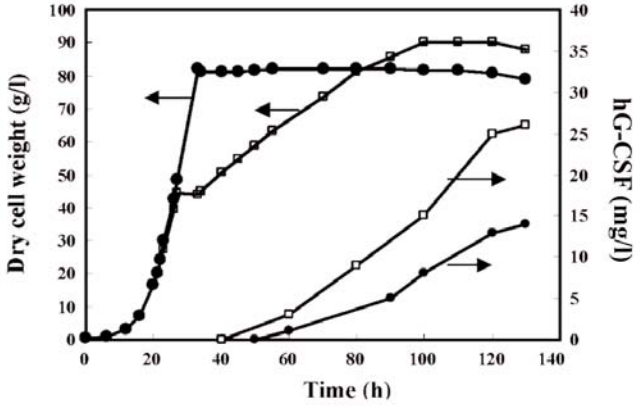

Figure 5. Effect of induction time point on the *P. pastoris* cell growth and hG-CSF production in fed-batch fermentation. Induction was carried out at two different cell concentrations. DCW of 45 g/l (□) and DCW of 80 g/l (●).

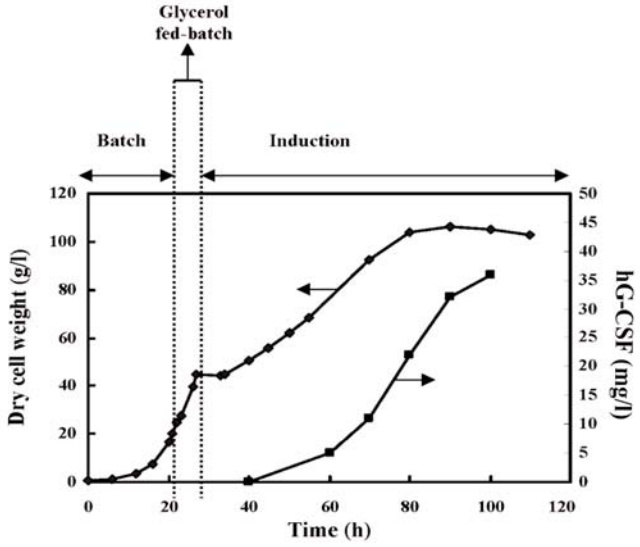

Figure 6. Effect of peptone on *P. pastoris* cell growth and hG-CSF production during fed-batch fermentation.

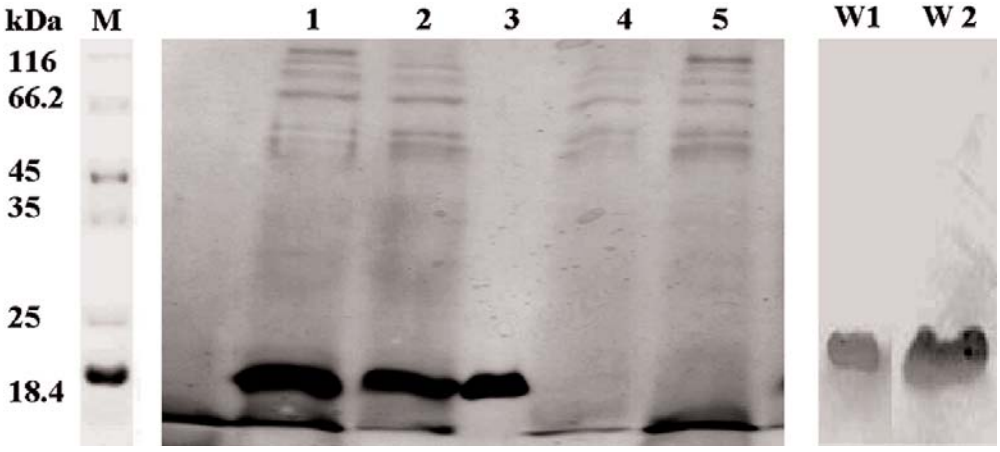

Figure 7. SDS-PAGE analysis (Lanes M to 5) and western blot analysis (Lanes W1 and W2) of culture supernatants of *P. pastoris* during fed-batch fermentation using a mix feed of methanol and peptone. Lane M is the standard molecular weight marker; lane 1 and lane 2 are culture supernatants after 70 and 50 h of methanol induction, respectively; lane 3 represents the commercial hG-CSF; lane 4 is the culture supernatant before methanol induction; lane 5 shows the culture supernatant of recombinant *P. pastoris* without the hG-CSF gene insertion (negative control); Lane W1 represents the commercial hG-CSF and Lane W2 shows the culture supernatant after 70 h induction.

site located after the Lysine-Arginine (Lys-Arg) sequence (it is originally present in pPIC9). It is known that when a foreign gene is fused to the α-MF prepro leader at the Lys-Arg processing site without the Glu-Ala spacer, it can lead to the formation of properly or non-properly processed protein. On the other hand, the presence of the Glu-Ala spacer between the Lys-Arg site and the N-terminus of the foreign gene can lead to a secreted protein, which can lead to cleaved Lys-Arg sequenceefficiently, whil the Glu-Ala spacer at the amino terminus remained (Lin Cereghino *et al.,* 2000; Zsebo *et al.,* 1986). Although all several positive colonies were selected for induction, two colonies showed higher expression levels of the target protein.

Expression levels reported in the literature for foreign proteins produced by *P. pastoris* are highly variable and range from the mg/l to g/l levels. The expression level for a given recombinant protein produced by *P. pastoris* seems to be decided largely by its inherent properties such as the amino acid sequence, the tertiary structure, the site of expression, codon usage and the culture conditions used for growth and induction (Lin Cereghino *et al.,* 2000; Lin Cereghino *et al.,* 2002). In the fermentor, higher transcription levels can be obtained as a consequence of the controlled methanol, aeration and pH (Gregg *et al.,* 1987).

In this study, high yield production of hG-CSF by recombinant *P. pasroris*, under various environmental conditions such as temperature, pH, carbon and nitrogen sources were investigated. It is documented in literature that a lower cultivation temperature increases the yield of recombinant proteins. This condition also increases cell viability and protein folding (Hong *et al.,* 2002; Li *et al.,* 2001). It has already been observed that temperatures above 32°C could be detrimental to protein expression and could lead to cell death (Lin Cereghino *et al.,* 2002). It is also well known that lower temperatures decrease protease activity in the fermentation media. From the data obtained in this study, it appears that temperatures of between 24 and 28°C are optimum for hG-CSF expression. Whereas, temperatures above 30°C are not appropriate for hG-CSF production.

P. pastoris is known to grow over a wide pH range, from 3 to 7, showing minimal effects on the growth rate. However, pH has been shown to significantly affect secreted recombinant proteins due to protease activity in the fermentation broth (Cregg *et al.,* 1993). However, in some cases, different pH values were found to be optimal with respect to recombinant protein stability. For example, pH 6.0 has been observed to result in the optimal production of recombinant mouse epidermal factor and human serum albumin (Kobayashi *et al.,* 2000; Clare *et al.,* 1991). Also, pH 3.0 was found to be responsible for the optimal production of insulin-like growth factor-I and cytokine growth blocking peptide (Brierley *et al.,* 1994; Koganesawa *et al.,* 2002). In order to find out the optimal pH for the expression of hG-CSF, we performed

experiments at pH 4 to 7 during batch cultures, in a 2 l fermentor. The highest yield of recombinant hG-CSF (4 mg/l) was observed at pH 6.0.

Generally, a three-stage fed-batch process is utilized for the production of foreign proteins in a fermentor using *P. pastoris*. At the first stage, the cells are cultured in a glycerol-containing medium, which is used as the carbon source to accumulate biomass. The second stage is the fed-batch transition phase in which glycerol is fed to the culture at a growth-limiting rate in order to further increase the biomass and to prepare the cells for induction. At the third stage, the induction phase is initiated by the addition of methanol at a slow rate. For increasing productivity of the recombinant protein in *P. pastoris*, it is desirable to achieve high cell density by fed-batch culture. The observations of this study specify that, methanol induction time is an important parameter in *P. pastoris* fed-batch fermentation (Figure 5). If the cell densities are too high (greater than 80 g/l) prior to induction, the fermentation process would be difficult to control, because too much foam will be produced in the fermentation broth, and too much antifoam has to be added, which will then make it difficult to precipitate the target protein from the fermentation supernatant during the down stream process (Jahic *et al.,* 2006). Furthermore, dissolved oxygen is the main limitation factor during high cell density fermentation. A cell density greater than 80 g/l (DCW) can result in the shortage of dissolved oxygen. The results of this study showed that the optimal cell density prior to induction should be 40-50 g/l (DCW).

In the *P. pastoris* fed-batch culture, ammonium sulfate is used both as nitrogen a source and pH adjuster. However, this pH control strategy results in shortage of ammonium during the the expression phase (Kobayashi *et al.,* 2000). In the nitrogen source starvation phase, the *P. pastoris* cells release proteases and other contaminating proteins into the culture supernatant and consequently decrease the quality of feedstock for purification processes (Brady *et al.,* 2001). In this study, the mixed feed of methanol and peptone improved hG-CSF expression. The higher yield and productivity of hG-CSF were obtained using such a feeding strategy (Figure 6). When the peptone and ammonium sulfate were used together as nitrogen sources, the final DCW and hG-CSF concentration was increased to 105 g/l and 35±2 mg/l, respectively. Currently the effect of codon usage for the purpose of increasing levels of hG-CSF expression by *P. pastoris* is under investigation.

Acknowledgments

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