The Effect of Temperature on Recombinant Human Granulocyte Colony Stimulating Factor Production by *Pichia pastoris* Expression System

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ABSTRACT

Granulocyte colony stimulating factor (G-CSF) is a haematopoietic growth factor that functions as specific stimulator of the survival, proliferation, and differentiation of precursor cells of the neutrophilic granulocyte cell lineage as well as an activator of mature neutrophil function. The main objective of this work is to compare the effect of different temperature on the production of extracellular recombinant G-CSF in *Pichia pastoris*. Cells were cultured for 72h in baffled shake-flasks at 20°C, 25°C, and 30°C in two different medium; buffered glycerol/methanol-complex medium (BMGY/BMMY) and buffered minimal glycerol/methanol (BMGH/BMMH) after methanol induction every 12h. Expressed recombinant hG-CSF in the methylotrophic yeast *P. pastoris* was analyzed with SDS-PAGE. The 23 kDa protein was secreted into the culture supernatant when induced with methanol. Production of recombinant G-CSF protein in *P. pastoris* at 30°C at 48h incubation after methanol induction every 12h is the highest in both complex and minimum medium.

Key words: granulocyte colony stimulating factor, complex and minimal medium, temperature, *Pichia pastoris*

INTRODUCTION

Granulocyte colony-stimulating factor (G-CSF) also known as colony-stimulating factor 3 (CSF 3) is a colony-stimulating factor hormone. G-CSF is a glycoprotein, growth factor and cytokine produced by a number of different tissues to stimulate the bone marrow to produce granulocytes and stem cells. G-CSF then stimulates the bone marrow to release them into the blood. G-CSF also stimulates the survival, proliferation, differentiation, and function of neutrophil precursors and mature neutrophils. The recombinant human G-CSF synthesized in an *Escherichia coli* expression system is called filgrastim (Neupogen). Another form of recombinant human G-CSF called lenograstim is synthesized in Chinese Hamster Ovary cells (CHO cells). G-CSF is produced by endothelium, macrophages, and a number of other immune cells. This natural human glycoprotein exists in two forms, a 174- and 180-amino-acid-long protein of molecular weight 18671.65 dalton and 19600 dalton. The more abundant and more active 174-amino acid form has been used in the development of

pharmaceutical products by recombinant DNA (rDNA) technology (Hongbao *et al*, 2011).

Human granulocyte-colony stimulating factor (hG-CSF) cDNA was expressed in the methylotrophic yeast *Pichia pastoris* under the control of the alcohol oxydase (AOX1) promoter [Bahrami *et al* (2007), Bahrami *et al* (2008)]. Bahrami *et al*. (2007) was using pPIC9 vector secretion constructed as an expression vector for hG-CSF. Higher levels of hG-CSF was obtained using a *P. pastoris* Mut+ (methanol utilization fast) phenotype. They also investigated the effects of environmental factors such as temperature and pH on the *P. pastoris* cell growth and hG-CSF production during fermentation. This study using pPICZαA vector as an expression vector for G-CSF. The host cell using *P. pastoris* SMD1168H.

Cereghino and Cregg (2000) state that *P. pastoris* have been developed as a highly popular expression system for the production of variety of therapeutic proteins. *P. pastoris* used preferred over eukaryotic expression systems because of its efficiency, simplicity and affordability. This host can typically be grown

to high cell density and is a potent expression system with great capability to secrete recombinant proteins into the medium (Cereghino *et al.* 2002). One of the most important features of yeast expression systems is their ability to perform eukaryotic post-translational protein modifications, such as N- and O-glycosylation, disulfide bond formation, and oligomerization. The majority of therapeutic proteins display one or more post-translational modifications (PTMs), and these PTMs are often essential for the functionality of these proteins (Corchero *et al.* 2012).

Recombinant protein in this study using *P. pastoris* as host cell which has been integrated with *gcsf* gene. This gene was inserted with the c-myc tag and six histidine tag (6× His) at C-terminal. It was inserted into pPICZαA vector (Invitrogen). For extracellular secretion of recombinant protein, some commercial expression vectors contain a sequence encoding the secretion an epitope tag for affinity purification or detection of the recombinant protein such as 6×His, FLAG, HA, and c-myc tags are generally included in frame with the gene of interest (Çelik, and Çalık, 2012).

Most processes with *P. pastoris* are run at a temperature of 30°C, which is optimum for growth. By decreasing growth temperature from 30 to 20°C, a 3-fold increase in specific recombinant protein productivity was obtained in chemostat cultures of *P. pastoris*, which was accompanied by a reduced flux through the TCA-cycle, reduced levels of proteins involved in oxidative stress response and lower cellular levels of molecular chaperones (Çelik, and Çalık, 2012).

Fickers (2014) reviewed that bioprocess parameters such as medium composition, pH, temperature, aeration rate, induction and feeding strategies are of utmost (very) importance since it affects directly the production yield. Those parameters vary according to the genotype of the producing strain and/or the recombinant protein. However, there are some guidelines that allow high productivity yields. The main objective of this work is to compare the effect of different temperature in two kinds medium on the production of extracellular recombinant G-CSF in *P. pastoris* SMD1168H. This study using pPICZαA vector as an expression vector for G-

CSF. Expressed recombinant *Pichia* strain is a strain that has been inserted gene recombinant *gcsf*-cmv. The gene is a synthetic gene which is the result of the construction of recombinant genes our previous study.

MATERIAL AND METHODS

Yeast strain and Plasmid

The EasySelect® *Pichia* expression system was used for genetic engineering experiments. The expression vector pPICZα and host cells of *P. pastoris* SMD1168H containing the AOX1 promoter, which allows rapid growth on methanol as the carbon source, were used for heterologous protein expression of G-CSF. The recombinant *P. pastoris* strain SMD1168H which has been inserted gene *gcsf*-cmv was obtained from our previous study.

Protein expression of *P. pastoris* transformants

Pichia pastoris recombinant which inserted *gcsf*-cmv 21 were cultured to evaluate recombinant protein expression according to manual instructions of *Pichia* expression kit (Invitrogen). One colony of *P. pastoris* recombinant was cultivated in 2mL of YPD medium (with zeocin) and was incubated at 30°C and 250rpm for 24h. This culture was used for production of G-CSF by using 25 ml of BMGY and BMG medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer pH 6.0, 1.34% YNB, 4×10⁻⁵ % biotin, 1% glycerol) and BMGH minimum medium (100mM potassium phosphate buffer pH 6.0, 1.34% YNB, 4×10⁻⁵ % biotin, 1% glycerol). Cultures were incubated at 30°C and 250 rpm for 24h. The cells were harvested aseptically by centrifugation before they were transferred into two different induction medium, 25mL each; which BMMY (1% yeast extract, 2% peptone, 100mM potassium phosphate buffer pH 6.0, 1.34% YNB, 4×10⁻⁵ % biotin, 0.5% methanol) and BMMH (100mM potassium phosphate buffer pH 6.0, 1.34% YNB, 4×10⁻⁵ % biotin, 0.5% methanol) with starting OD₆₀₀=5.0. Cultures were incubated in 100mL Erlenmeyer flask at 20°C, 25°C, 30°C and 250rpm for 3 days and methanol was added at 0.5% (v/v) into the culture every 12h to induce expression of the recombinant G-CSF gene. *P. pastoris* cells culture was collected

every 24 hours after methanol induction for growth and protein analysis. Twenty five milliliter cultures were harvested by centrifugation (10.000rpm, 15min, 4°C). Cell biomass was stored at -20°C and the cell-free culture medium (supernatant) was stored at 4°C for further protein analysis.

Protein assay

Recombinant protein (extracellular product) was analyzed using SDS-PAGE and BCA methods. The BCA Protein Assay Kit (Pierce, Bonn, Germany) was used according to the manufacturer's instructions using test-tube procedure. BCA's method was used with serum bovine albumin as protein standard. Cultures protein were harvested by centrifugation (10.000rpm, 15min, 4°C). Proteins were precipitated from cell-free supernatant samples by TCA solution (trichloro acetic acid) (Florens & Washburn, 2008). Sample results of TCA then resuspended in phosphate buffer at 25µL. Its used to BCA assay as much 12.5µL sample for the measurement. Then sample added 8× cv BCA working reagent and incubated at 37 for 30min. Once it is added to 500uL ddH₂O and read at a wavelength of 562nm.

RESULT AND DISCUSSION

A recombinant *P. pastoris* strain which can express protein G-CSF-cmyc was constructed in our previous study. Briefly, the *gsf-cmyc* gene bearing pPICZαA plasmid was introduced into *P. pastoris* strain SMD1168H to generated the SMD1168H/G-CSF-cmyc strain. The recombinant G-CSF-cmyc expression was confirmed by SDS PAGE and Western Blot analysis, as analyzed in other part of this research. Appeared on SDS PAGE as a protein of ~23 kDa (which corresponds with a theoretical molecular weight of 23 kDa).

Cos *et al.* (2005) and Li *et al.* (2007) stated that heterologous protein expression under control of pAOX1 allows for the design of defined controlled high cell density cultivation strategies for heterologous protein production, described in two main phases. First phase is to generate biomass utilizing carbon sources that lead to a high substrate-biomass yield, such as glycerol; the cells are grown until glycerol is depleted. The second phase, in the presence of methanol, is for induction of foreign protein

expression. Optimization of cultivation processes usually involve intermediate cultivation phase, where both carbon sources are present.

To optimize condition for the expression of G-CSF, *Pichia* transformant were cultured for 72h in baffled shake-flasks at, 20°C, 25°C, and 30°C in two different medium; buffered glycerol/ methanol-complex medium (BMGY/BMMY) and buffered minimal glycerol/ methanol (BMG/BMM). Every treatment was done in triplicate. Recombinant yeasts grew much faster and reach a higher cell density in the complex medium BMMY (OD₆₀₀ of 24.8 to 25.77) and in the minimal medium BMM (OD₆₀₀ of 16.6 to 17.39) (Table I). The highest OD on complex medium obtained in temperature 30°C, while in minimal medium in temperature 25°C. The rate of methanol conversion determines the available energy and carbon for maintenance, cell growth, product formation and product transport into the media. Overall productivities for cell mass and HSA (human serum albumin) showed similar dependence on methanol concentration as the specific rates (Kupcsulik & Sevilla, 2004).

The environmental condition, e.g. temperature and fermentation processes have close correlation with the cell growth, the internal secretion pathway machinery, the secretion levels of proteins, and their stability in the medium. Another parameter is media composition. The optimal culture medium depends on many factors, including host metabolism, potential inhibitory products, target protein, ect. (Hou *et al.*, 2012). *P. pastoris* considrable to to produce heterologous proteins. Production of heterologous proteins consisted of two phase; biomass production phase and recombinant protein production phase. In the first phase, as the sole carbon source glycerol is initially added to medium. During this phase, biomass is accumulated, but the gene expression of heterologous proteins is completely repressed. In the second phase, after the initial depletion of glycerol, there is a transition period in which glycerol is added to the culture at a limiting rate (nutritional stress). In many production heterologous protein with *P. pastoris* as a host cell, complex medium is required for growth and induction of protein expression (Aizemberg *et al.*, 2011).

Table I. Optical Density (OD600) of *Pichia pastoris* Culture in BMMY and BMM at 72h.

Temperature	Bach	BMGY	BMM
20°C	1	23.60	16.52
	2	25.96	17.76
	3	25.40	17.74
	mean	24.99	17.34
25°C	1	26.94	17.64
	2	24.75	17.45
	3	22.77	17.07
	mean	24.820	17.39
30°C	1	26.52	16
	2	26.64	17.81
	3	24.16	15.975
	mean	25.773	16.6

Figure 1. SDS PAGE for G-CSF-cmyc production analysis. (A) Complex medium BMMY and (B) Minimum medium BMM : 1. Marker; 2-4. Protein sample at 24h, t=20°C, t=25°C, t= 30°C; 5-7. Protein sample at 48h, t=20°C, t=25°C, t= 30°C ; 8-10: Protein sample at 72h, t=20°C, t=25°C, t= 30°C.

Complex medium is used for both growth and induction of protein expression in the field of recombinant protein production from *P. pastoris*. Complex media are excellent for providing the correct chemical environment required for *Pichia* cell growth, they have some limitations when considering larger-scale and industrial fermentations. The use of minimal media is expected to reduce production costs for a larger scale and industrial scale fermentation.

G-CSF was secreted from *Pichia* transformant after methanol induction every 12h at different temperature (20, 25, and 30°C) for 72h. Protein analysis was done after TCA precipitation of culture supernatant on 12% SDS-PAGE and visualized by commasie blue (Figure 1).

Strategies of combination cultivation-level (in complex and minimal medium towards temperature) are effective to obtained intact recombinant protein, it was produced by *P. pastoris* with minimum proteolytic degradation. The work described in this section demonstrates the necessity to optimize production/ cultivation process at all levels, not only to achieve maximum growth and protein production, but also to obtain maximal amounts of the desired protein in an intact and bioactive form (Aizemberg *et al.*, 2011). Most of the processes of recombinant protein production by *P. pastoris* are performed at 30°C which is the optimal growth temperature (Fickers, 2014) (Figure 1 and Table II) in complex medium the yield of recombinant protein in temperature condition 30°C at 48h is the highest than other condition

Table II. Concentration of total recombinant protein G-CSF quantified by BCA method and concentration of target protein was semiquantified with ImageJ software on different media and temperature

Medium	Time (hour)	Concentration Target Protein ($\mu\text{g/mL}$)		
		Temperature ($^{\circ}\text{C}$)		
		20	25	30
BMMY	24	17.14	20.94	50.66
	48	28.31	49.81	50.47
	72	19.02	21.76	24.58
BMM	24	1.37	2.02	2.27
	48	29.99	3.62	16.86
	72	6.31	6.88	23.36

(50.47mg/L). Lower cultivation temperature can also influence yields of recombinant protein, possibly due to poor stability of the recombinant protein at higher temperatures, release of more proteases from dead cells, and folding problems at higher temperatures (Aizemberg *et al*, 2011) as shown in Figure 1 in minimal medium the band of recombinant protein in temperature condition 20°C at 48h is the highest and the yield protein is 29.99 mg/L.

This study of expression of recombinant protein used complex medium (BMMY) and minimum medium (BMM). On both medium applied three different temperatures i.e. 20°C , 25°C , and 30°C . This is done to determine the best temperature conditions for the expression of recombinant proteins in each media and a preliminary study for the expression of recombinant protein, so it can be applied to larger volume/ fermentor. In heterologous protein production the pH of growth and induction media play an important role, while optimal pH range for different heterologous production varied. The pH of medium in this study adjusted at 6.0. The preference pH of around 6.0 is all crucial for efficient protein secretion and reducing degradation (Patrick, 2005). The components of BMMY, such as peptone, yeast extracts and steady pH had prominent effects on the growth and expression of recombinant proteins in *P. pastoris* (Ling, 2010). Curvers *et al.*, (2001) also study the optimization of pH and temperature during cultivation were favored to minimize proteolytic activity.

In this study on complex medium (BMMY), the yield of G-CSF-cmyc at 48h is the highest concentration in each temperature, and it all decreased at 72h. In this medium, at temperature 30°C concentration on target recombinant protein at 24h is closely with the concentration at 48h (50.66mg/mL and 50.47mg/mL) and it decreased at 72h (Table II). On minimum medium (BMM) the yield of G-CSF-cmyc is very diverse. At temperature 20°C on 48h occurred the highest concentration and decreased at 72h. Whereas at temperature 25°C and 30°C in 24, 48 and 72h protein recombinant concentration trends are increased, despite at temperature 25°C the increase not significant as in temperature 30°C . To produce recombinant protein as we have observed through the optimization experiment that temperature 30°C is the best condition for complex medium. While the best condition to produce recombinant protein for minimum medium in temperature 20°C . For those medium, the cultivation time are 48h. Li *et al.* (2007) state that there are several reports on expressing heterologous proteins in *P. pastoris* at low temperatures. Incubation temperatures of 30, 27, 25, and 23°C have been examined in attempts to minimize the proteolytic degradation of the recombinant protein in the culture medium, also can reduced rate of protein synthesis which allow more time for the nascent peptide chains to fold properly.

The recombinant G-CSF-cmyc protein after 72h of cultivation in complex medium was estimated as 19.02 to 50.47mg/L, while in

minimum medium approximately 6.31 to 23.36mg/L. Chien (2010) reported that gene expression in the protease resistant strain of yeast (SMD1168) the total recombinant G-CSF protein in shaking flask medium was estimated as 15mg/L. Expressed mGM-CSF in GS115, Jacobs *et al.* (2010) and Saeedina (2008) also reported have a final yield of ~18mg/L and 3.1mg/L respectively.

CONCLUSION

Recombinant hG-CSF produced in the methylotrophic yeast *Pichia pastoris* analyzed with SDS-PAGE. Protein was secreted into the culture supernatant when induced with methanol, the proteins have a size of 23 kDa. Production of recombinant G-CSF protein in *P. pastoris* at 30°C at 48h incubation after methanol induction every 12h is the highest in both complex and minimum medium.

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