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Research Article

Secretory Expression of recombinant human Interferon-alpha2b in methilotropic yeast *Pichia pastoris*

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ABSTRACT

Interferon is a cytokine secreted by eukaryotic cells as a response to virus, bacteria, and different type of macromolecules exposure. This research was purposed to obtain human interferon-alpha2b (hIFN-α2b) protein in methylotropic yeast *Pichia pastoris (P. pastoris)*. The isolated and amplified open reading frame (ORF) was produced as 522 bps that corresponding to its theoretical *hifn-α2b* ORF size. The ORF was cloned into pPICZαB shuttle vector and transformed into *Escherichia coli* (*E. coli)*. Nucleotide sequence analysis confirmed that the recombinant plasmid contained correct sequence of *hifn-α2b* ORF. The recombinant plasmid pPICZαB-*hifn-α2b* was linearized by using *Bstx1* enzyme and transformed into *P. pastoris* genome. High expression screening of transformants that performed by using 2000 µg/mL zeocin as a marker resulted in thirteen transformants. All transformants produced 24.05 kDa protein bands in extracellular compartment by using 0.5% methanol as inducer. Slot blot analysis confirmed that the band was hIFN-α2b with the highest expression achieved by clone number 12. To conclude, hIFN-α2b protein was successfully obtained as secretory protein with 24.05 kDa in size containing polyhistidine tag and c-myc epitope in its C terminus.

Keywords: hIFNα-2b; secretory expression; methilotropic yeast and *Pichia pastoris*

INTRODUCTION

Interferon is a cytokine secreted by almost all eukaryotic cells that exposed to virus, bacteria, mitogen and antigen. The secreted interferon stimulates surrounding cells to produce other protein, which regulate viral replication, immune response, cell growth and other cell functions. Interferon can be exactly detected after viral infection locally or systematically. Based on its receptor at cell surface, it can be classified into two types, type 1 that consists of α, β, ω and τ and type 2 that consists of γ (Kontsek and Kontsekova, 2002). Human interferon alpha (hIFN-α) is a family protein that has wide biological activity including antiviral, antiproliferative and immunomodulatory (Maeyer and Maeyer, 1994; Goodbourn et al., 2000). The hIFN-α2 locus comprises three allelic variants, hIFN-α2a, hIFN-α2b and hIFN-α2c. hIFN-α2b is 21.550 kDa glycoprotein in size which consists of 188 amino acids (23 residues are signal peptide and 165 residues are mature protein) and O- glycosylation at threonine position 106. It has been approved by the U.S. Food and Drug Administration on June 1986 as a therapeutic protein for chronic hepatitis B and C and several cancers treatments. Currently recombinant hIFN-α2b is produced by using several expression systems, such as *Escherichia coli*, *Saccharomyces cerevisiae, Bacillus subtilis, Streptomyces lividans* and *P. pastoris*. Previous research that reported by Shi *et al* (2007) used *P. pastoris* GS115 as an expression host (Srivasta et al., 2005; Hitzeman et al., 1981; Pulido et al., 1986; Breitling et al., 1989; Shi et al., 2007).

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P. pastoris is a methylotropic yeast that genetically engineered to express target protein. The advantages of using *P. pastoris* as a host are easiness to manipulate at molecular level, ability to perform many of eukaryotic protein modification such as glycosylation, easiness of doing scaling-up, lower production cost than mammalian cell culture, and simplicity of transformation and selection comparing to bacteria (Cregg et al, 2000). In addition the existence of inducible *AOX1* promoter that regulates gene expression of alcohol oxidase in methanol metabolism can easily control the expression of recombinant proteins. The secretory system of *P. pastoris* also offers very high level of recombinant protein expression. It has α-factor prepro sequence that can be used to secret heterologous protein. Production of mature α factor from the precursor molecule initiates by *KEX2* gene product cleavage at the site after Lys-Arg residues. Then it is followed by *STE13* gene product for the Glu-Ala repeat (Skoko et al, 2007).

This study was aimed to construct ORF encoding hIFN-α2b and to obtain its gene product as secretory protein in *Pichia pastoris*. We used X-33 strain that is wildtype and Mut^{+} as a host to attain the highest expressing transformant.

METHODS

Strain, vector, and media: *E. coli* XL1 blue was used for cloning purpose while *P. pastoris* X-33 (Invitrogen, USA) was used on protein expression. pPICZαB (Invitrogen, USA) was used as cloning and expression vector. Media to cultivate bacteria was low salt LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) containing zeocin (25 µg/mL). Cultivation, screening and expression media for yeast in sequence were YPD (1% yeast extract, 2% peptone, 2% dextrose), YPDS (1% yeast extract, 2% peptone, 2% dextrose, 1M sorbitol), BMGY (1% yeast extract, 2% peptone, 100mM potassium phosphate pH 6, 1.34% YNB, 1% glycerol, 0.2% Biotin), and BMMY that consist of 1% yeast extract, 2% peptone, 1.34% YNB, 0.2% Biotin and 0.5% methanol, respectively.

RNA extraction and RT-PCR: Total RNA was extracted from whole blood of Indonesian people and used as RT-PCR template. The primers were (F-IFN): 5 'CCG CTC GAG AAA AGA GAG GCT GAA GCT TGT GAT CTG CCT CAA 3' that contains *Xho* I site and reverse primer (R-IFN) : 5'GCT CTA GAG CTT CCT TAC TTC TTA AAC T 3' that contains *Xba* I site. RT-PCR was carried out with this following condition: denaturation at 94°C for 1 min, 42°C reverse transcription for 60 min and then 30 cycles of denaturation 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min. The amplified cDNA was characterized on 1% agarose gel.

Construction of pPICZαb-hifnα2b and Transformation into *P. pastoris*: cDNA product was digested by *Xho* I and *Xba* I then ligated into pPICZαB. The ligation product was transformed into *E. coli* XL1 blue. Recombinant plasmid was isolated and characterized by migration and restriction analysis. The inserted ORF was sequenced by using 5'AOX and 3' AOX primers. The recombinant plasmid that contained correct sequence of ORF was linearized by *Bst*X I restriction enzyme. 10 µg of linearized plasmid was transformed into *Pichia pastoris* cells (X-33) by electroporation. The transformation product was grown on YPDS medium containing 100 µg/ml of zeocin at 30°C for 24 h.

Screening and Expression: All single colonies on YPDS were screened to obtain multi copy integration of transformants. The screening was conducted using YPDS containing 2000 µg/ml of zeocin. Survival transformants were used for expression study. A single colony of each transformant was cultivated in 2 ml BMGY for at 30°C and 250 rpm. The pellet was collected, resuspended in 2 ml BMMY (OD=1) and cultivated at previous condition. Methanol (0.5%) was added at 24 h cultivation time. Pellet was harvested by centrifugation at 1500 x g for 5 min. Supernatant was collected and characterized by SDS- PAGE and slot blot methods to observe expression level and verify protein identity. The highest expression level was determined based on densitometry method by using ImageJ software.

RESULT AND DISCUSSION

Isolation and amplification of *hifn*-α2b ORF produced a 522 bps fragment in size (Fig.1 lane 2) which was similar to its theoretical size. The cDNA product containing *Xho* I and *Xba* I restriction sites was ligated into pPICZαb shuttle vector as shown in schematic map of Fig.2. Transformation into *E. coli* XL-1 blue resulted in hundred of transformants and eight of them were characterized. Migration and restriction analyses (data not shown) informed that all recombinant plasmids had correct size (4115 bps). These results strongly indicated that the recombinant plasmids were harboring *hifn-α2b* ORF. Nucleotide sequence analysis using *5' AOX1* and *3'AOX1* primers confirmed that the ORF inserted exactly and had correct size (data not shown).

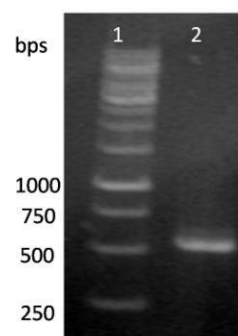

Figure 1: RT-PCR product on 1% agarose gel. Lane 1= DNA marker and 2 = *hifn-α2b* cDNA

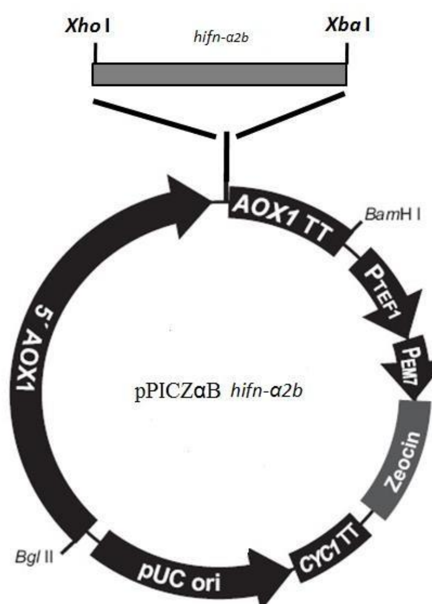

Figure 2: Schematic map of pPICZαB hifn-α2b recombinant plasmid

The recombinant plasmid was linearized by *BstX* I enzyme before transformed into *P. pastoris* cells. Linear

vector DNA can generate stable transformants through homologous recombination between sequences of vectors and homologous sequences in *AOX1* locus of host genome (Pulido et al., 1986). There are three approaches that can be applied to generate multi copy transformant : the first is constructing a vector with head to tail copies of an expression cassette, the second is using expression vector that contains *P. pastoris* HIS4 and kanamycin resistance gene and the third is using vector that contains zeocin resistance gene (Pingzuo et al., 2007). We used the third approach to obtain multi copy integration of transformants. The screening was carried out using increasing concentration of zeocin (from 500 to 2000 µg/mL). Thirteen of transformants were survive on highest concentration which were further used on expression study.

The strain used to express *hifnα-2b* was X-33 which is Mut^+ phenotype and can be used for positive selection of transformation using zeocin. This strain also can grow rapidly on media containing methanol and quickly produce a foreign protein. Other reports used GS115 strain which has mutation in the histidinol dehydrogenase gene (his4) that prevents it to synthesize histidine. So, It allow the selection of expression vectors containing HIS4 upon transformation (Shi et al., 2007; Ghosalkar et al., 2008; Li et al., 2007). X-33 is very responsive for methanol since high concentration of methanol can inhibit cell growth. Optimal concentration of methanol in the media was ranging from 0.4% to 0.5% (Shi et al., 2007; Cregg et al., 2000; Skoko et al., 2003). In this study, we used 0.5%, 24 hours induction time point and 48 hours production time for protein expression as reported by our previous research in erythropoietin expression (Santoso et al., 2012).

Analysis of the expression level of hIFN-α2b was performed by using SDS-PAGE. Thirteen transformants were characterized. As shown in Fig. 3, all samples produced 24 kDa bands that corresponding to rhIFNα2b size that contain polyhistidine tag and c-myc epitope in its C terminus. Each sample had different intensity of expression level. This strongly indicated that there was various copy number of expression vector. Cregg (2009) reported that disadvantage of using drug resistance gene as the selectable marker was various vector copy number. After selection, Most of the transformants still carrying only single copy although they were resistant to highest concentration of drug. So further analysis was important to verify vector copy number and expression level (Cregg et al., 2009).

In this study, the expression level was determined based on densitometry method by using ImageJ software (Fig. 4). The expression level percentage was calculated by comparing area under curve of each sample into the highest one. The result informed that the highest expression was achieved by clone number 12 which had slightly different level with clone number 9.

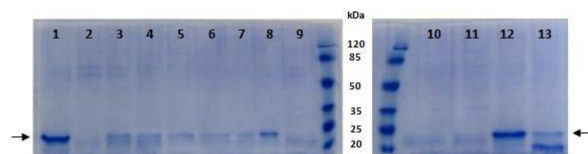

Figure 3: Characterization of rhIFN-α2b from transformants by using SDS PAGE. Lane1 to 13 in sequence were transformants number 1 to 13

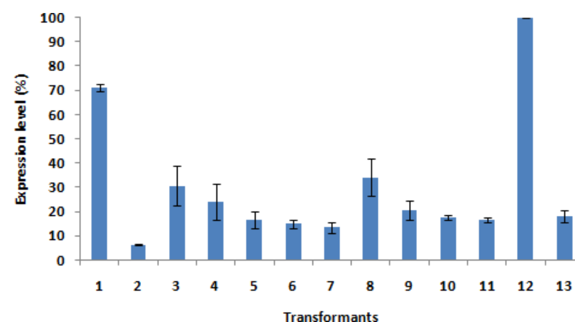

Figure 4: hIFN-α2b expression level determination by using ImageJ

We performed further analysis to verify protein identity by slot blot method. All transformants had positive result on immunology principle detection (Fig.5). This result strongly designated that the protein was rhIFNα-2b.

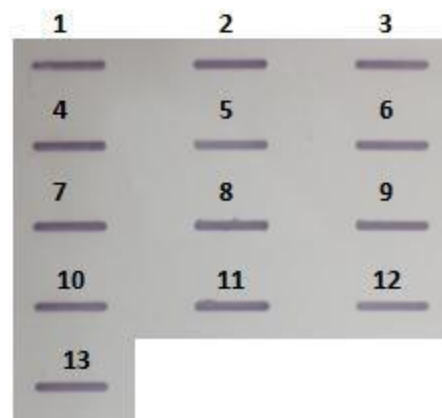

Figure 5: Protein characterization by slot blot method. Number 1 to 13 in sequence were transformants 1 to 13

CONCLUSIONS

This study successfully obtained rhIFN-2b as secretory protein in methylotropic yeast *P. pastoris*. Our rhIFNα-2b protein containing polyhistidine tag and c-myc epitope in its C terminus.

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