Human interferon alpha 2b production from *Pichia pastoris*

Cloning of human huIFNα2b gene and construction of expression vector

P. pastoris codon optimized huIFNα2b variant gene (huIFNα2b gene with single N-glycosylation site) was ligated into XhoI and NotI digested plasmid pPICZαA to generate pPICZαA-huIFNα2b variant. A native huIFNα2b encoding gene fragment was generated by site-directed mutagenesis PCR and was ligated into XhoI and NotI digested plasmid pPICZαA to generate pPICZαA-huIFNα2b native. T4 DNA ligase was used for ligation reactions and $CaCl_2$ competent cells of *E. coli* TOP10F' strain was used for transforming ligation mixtures. Positive clones were verified by restriction digestion and Sanger sequencing. huIFNα2b native and its variant gene in pPICZαA encompass a N-terminal α-factor signal peptide for extracellular export in *P. pastoris* and a C-terminal 6X Histidine tag for facilitating affinity purification. During the extracellular export, Kex2 protease trims away the α-factor signal peptide in *P. pastoris*.

Transformation of P. pastoris strains and screening for recombinant strain

The schematic representation of recombinant events in *P. pastoris* is depicted in Figure 1. Transformation of *P. pastoris* was performed as per the standard protocol of electroporation as described in Easyselect™ *P. pastoris* manual.

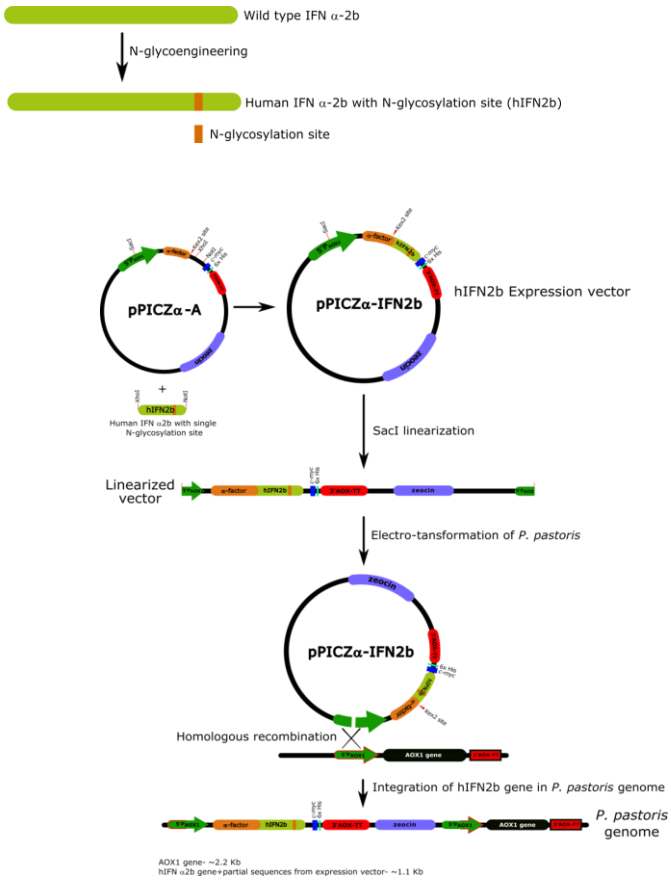

Figure 1: Schematic representation of N-glycoengineering approach and recombination events in *P. pastoris*

Wherein competent *P. pastoris* X33 and SuperMan5 (*pep4Δ prb1Δ*) cells (80 µL) were mixed separately with 5µg of *SacI*-linearized pPICZαA-huIFNα2b-variant and pPICZαA- huIFNα2b-native and were

transferred into an ice-cold 0.2-cm electroporation cuvette and incubated in an ice bath for 5 min. Immediately after electroporation (at 2 kV, 25 μF, 200 Ω), 1 mL of ice-cold 1 M sorbitol was added to the cuvette and incubated for 1 h at 30˚C without shaking. After incubation the contents were centrifuged and dissolved in fresh YPD medium (350 μL) and plated onto YPDS plates supplemented with 100 μg/mL Zeocin and incubated at 30˚C for 2 days. The transformants bearing the genomic integrants of the pPICZαA-huIFNα2b were confirmed by colony PCR using the primers 5′-AOX1: 5′-GACTGGTTCCAATTGACAAGC-3′, and 3′-AOX1: 5′-GCAAATGGCATTCTGACATCC-3′. The huIFNα2b gene from the positive clone was gel extracted and sequence verified.

Protocol for protein production & purification

Strain

A Glycoswitch® *P. pastoris* SuperMan5 (glycoengineered, protease deficient and Mut^+ strain), expressing glycosylated human interferon alpha 2b extracellularly under the control of the AOX1 promoter, was used in this study. The stock culture was maintained at – 80˚C, in YPD media containing 20% (v/v) glycerol.

Inoculum preparation

YPD medium was used in starter culture preparation with a composition of 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) dextrose. The medium with composition of 1% (w/v) yeast extract, 2% (w/v) peptone, and 1% (w/v) glycerol was used for inoculum preparation for the reactor study.
A loop full of recombinant *P. pastoris* culture from glycerol stock vial stored at -80°C was used to inoculate 5 mL of YPD broth for starter culture, and incubated at 30°C and 220 RPM for 48 h. The starter culture was used to prepare the inoculum for reactor studies by inoculating in 170 mL of YPG media and incubated at the afore mentioned conditions for 24 h. 10% (v/v) inoculum of an optical cell density (OD_{600}) of 4 was used for the reactor study.

High cell density fermentation for human interferon alpha 2b production

The entire process of high cell density fermentation (batch fermentation) consisted of three phases: glycerol growth phase, transition phase, and methanol induction phase. The bioreactor study was performed using the optimized basal salt medium. Glycerol growth phase commenced with 1.7 L of optimized basal salt medium having the **composition (in g/L): 48.84 glycerol, 18.2 K_2SO_4 , 7.28 $MgSO_4$, 4.13 KOH, 0.93 $CaSO_4.2H_2O$, 8.42 $(NH_4)_2SO_4$ and 26.7 mL/L 85% orthophosphoric acid** supplemented with 4.4 mL/L PTM4 salt (added after autoclaving) was also used. The PTM4 salt solution contained (in g/L): 2 $CuSO_4.5H_2O$, 0.08 NaI, 3 $MnSO_4.H_2O$, 0.2 $Na_2MoO_4.2H_2O$, 0.02 H_3BO_3, 0.5 $CaSO_4.2H_2O$, 0.5 $CoCl_2$, 7 $ZnCl_2$, 22 $FeSO_4.7H_2O$, 0.2 biotin and 1 mL/L H_2SO_4 98% (v/v). The temperature was maintained at 30°C during the glycerol growth phase. A sharp rise in the dissolved oxygen (DO) indicated the end of the glycerol phase. Following this phase, a transition phase was started with the addition of 1.5 g/L of methanol which allowed the cells to adapt and utilize the methanol during the induction phase. Once the cells were completely transitioned and adapted to methanol, the methanol feed (100% methanol + 12 mL of PTM4 per liter) commenced in order to initiate the methanol induction phase. The temperature during the methanol phase was maintained at 28°C, which was optimized previously. DO spike method based pulse feeding of the methanol feed (1.4 %, v/v) was employed in this study. Agitation rate was maintained at 800 RPM and pH was maintained at 5.4 by adding 25% (v/v) Ammonia solution and 30% (v/v) H_3PO_4 as neutralizing agents. Foaming during fermentation was controlled using silicone oil as an antifoaming agent.

Sampling and Analysis

Samples were collected at regular time intervals during the entire fermentation process, and analyzed for biomass, glycerol and methanol concentration, and huIFNα2b titer as described below. Cell concentration of the collected samples was estimated by measuring the optical density at 600 nm, using UV-visible spectrophotometer (GE Healthcare, UK). For dry cell weight (DCW) estimation, 1 mL of the culture broth was centrifuged at 10000 g for 10 min, in a pre-weighed 1.5 mL centrifuge tube, and the supernatant was separated and stored at –20°C for further analysis. The pellet was washed twice with distilled water, re-centrifuged and dried to constant weight at a temperature of 80°C. One OD_{600} corresponds to 0.16 g DCW/L. The concentrations of glycerol and methanol in the samples were analyzed by HPLC (Shimadzu Ltd., Tokyo, Japan), using a Rezex RHM- monosaccharide H+ Column (Phenomenex Inc, California, USA). 5 mM sulfuric acid was used as the mobile phase with an isocratic flow of 0.6 mL/min at 50°C. Peaks were detected using a refractive index detector and quantified based on the peak height. The concentration of huIFNα2b in the samples was determined by enzyme linked immunosorbent assay (ELISA) analysis using huIFNα2b ELISA kit (Mabtech AB, Sweden).

Purification of human interferon alpha 2b

During the end of high cell density cultivation, the pH of the culture broth was adjusted to 7 and the culture supernatant was collected by centrifugation at 4,500 x g for 10 min. The supernatant was then treated with 1% (v/v) Triton-X100 for the removal of non-covalent aggregates and was filtered through a 0.22 µm PVDF filter prior to further steps of purification. The glycosylated huIFNα2b was purified by His-tag affinity chromatography followed by Concanavalin A (ConA) lectin chromatography. Whereas non-glycosylated (native) huIFNα2b was purified by His-tag affinity chromatography followed by size exclusion chromatography.

His-tag affinity chromatography was performed for the clarified supernatant, where it was diluted at 1:1 ratio with the binding buffer (20 mM Tris, 500mM NaCl, 20 mM Imidazole, pH 8) and allowed for binding of recombinant huIFNα2b onto His-Trap 5 mL column (GE Healthcare), at a flow rate of 2 mL/min. The binding step was followed by washing with wash buffer (20 mM Tris, 500mM NaCl, 50 mM imidazole, pH 8). Elution of the bound huIFNα2b was carried out using elution buffer (20 mM Tris, 500mM NaCl, 500 mM imidazole, pH 8) with a linear gradient of 50 mM - 500 mM imidazole at a flow rate of 1 mL/min for 60 min. The glycosylated huIFNα2b was further purified by Con A chromatography. The pooled fraction from His-tag chromatography was diluted with Con A binding buffer (20 mM Tris-HCl, 0.5 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 7.4) in 1:1 ratio and applied on a pre-equilibrated Con A column (GE Healthcare). The column was then washed with a 10 column volume of Con A binding buffer. Finally, glycosylated huIFNα2b was eluted from the column with an elution buffer by methyl-α-D-glucopyranoside competition (20 mM Tris-HCl, 0.5 M NaCl, 0.5 M methyl-α-D-glucopyranoside, pH 7.4). The non-glycosylated (native) huIFNα2b was further purified by size exclusion chromatography. The pooled fraction from His-tag chromatography was concentrated using 10 kDa cut-off filter and applied onto pre-equilibrated Hiload 16/60 Superdex 200pg size exclusion column (GE Healthcare) with PBS as elution buffer.