High level production and purification of human interferon α2b in high cell density culture of *Pichia pastoris*

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Abstract

Human interferon α2b gene was cloned in the methylotrophic yeast *Pichia pastoris* under the control of the AOX1 methanol inducible promoter. To optimise the volumetric productivity, we performed different fed-batch studies in a 5-L bioreactor. We demonstrated that hIFNα2b was highly sensitive to proteases activity during high cell density culture. The target protein was totally degraded 20 h after the start of methanol feeding. Replacement of culture medium with fresh medium after glycerol fed-batch culture mode as well as medium enrichment with casamino acids at 0.1% and EDTA at 10 mM, had significantly improved hIFNα2b expression and prevented its proteolysis. Moreover, to further improve hIFNα2b production, three different methanol fed-batch strategies had been assayed in high cell density culture. The optimal strategy resulted in a production level of 600 mg/l while residual methanol level was maintained below 2 g/l.

Clarification of culture supernatant through a 0.1 μm hollow fiber cartridge showed that almost 95% of the target protein was retained within the retentate. Triton X-100 or NaCl addition to the culture harvest before microfiltration had improved the recovery yield of this step. rhIFNα2b was further purified by cation exchange on Sepharose SP resin followed by gel permeation on Sephacryl S-100. The overall yield of the process was equal to 30% (180 mg/l). The biological activity of the purified protein based on the antiviral activity test was 1.5×10^8 IU/mg. The optimised process has a great potential for large scale production of fully functional hIFNα2b.

Keywords: Human interferon α2b; *Pichia pastoris*; High cell density culture; Purification process; Antiviral activity

1. Introduction

Interferons (IFNs) are the first line of defence components of vertebrates that act against infectious agents and tumour development and progression. Recombinant interferon α has been used for almost two decades, as a therapeutic agent in a variety of diseases including viral infections and cancer.

Recombinant human interferons have been expressed in *Escherichia coli* [1–3], yeast [4,5], baculovirus infected insect cells [6,7] and animal cells [8]. The yield of recombinant INFs using *E. coli*, is by far higher than that obtained using other systems. However, IFNα protein expressed in large amount in *E. coli* often precipitates into insoluble, misfolded inclusion bodies that require subsequent solubilization and refolding steps [9,10]. Moreover, refolding from inclusion bodies presents

several drawbacks such as the requirement for optimising the refolding conditions for each target protein. In addition, the resolubilization procedures could affect the integrity of refolded proteins [11]. To overcome the shortcomings of *E. coli* expression system, expression of human interferon alpha in *Pichia pastoris* was recently described [12–14].

P. pastoris is a widely used host for the production of heterologous proteins. Some proteins are produced at gram per liter levels [15–17]. Complex proteins such as human glycoproteins or human collagens, are also successfully processed and secreted by *P. pastoris* [18–20]. Furthermore, this expression system presents many advantages [17,21–22]. The organism grows on defined media to high cell density on either glycerol or methanol as the sole carbon source and heterologous protein production is under the control of a strong but tightly regulated alcohol oxidase (AOX) promoter induced by methanol. *P. pastoris* can be grown to the desired cell density on glycerol as the carbon source and then on methanol for high level heterologous production [23]. In *Pichia* cells fed with methanol at growth limiting rates in biore-

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actor cultures, AOX levels are dramatically induced, constituting 30% of total soluble protein [17].

Besides fermentation process, downstream processing is a key issue that needs to be considered during the design of biotechnological processes. Heterologous expression in *P. pastoris* system can be controlled to direct expression of target proteins to the extracellular medium. Furthermore, *P. pastoris* secretes low levels of its own proteins, therefore secretion mode offers an easier way for isolation and purification of the product compared to other systems which either need complex media or rely on intracellular expression [16,24].

In this work, we optimised the high cell density culture of a recombinant clone of *P. pastoris* KM71H strain that produces hIFNα2b. To reach a high volumetric productivity of the target protein, the effect of various process parameters were investigated. We also developed a down stream process to obtain hIFNα2b at a high degree of purity and assessed the antiviral activity of the purified protein.

2. Material and methods

2.1. Strain and plasmids

The recombinant strain *P. pastoris* KM71H/pPICZα-hIFNα2b used in this study was constructed by Fathallah et al. [25]. The hIFNα2b gene was integrated into the genome of the yeast. The expression of hIFNα2b gene was under the control of alcohol oxidase 1 gene promoter with an N-terminal α peptide sequence for its extracellular production.

2.2. Shake-flask culture

A single colony of *P. pastoris* recombinant clone isolated from 100 μg/ml zeocin-YPD plate, was used to inoculate 50 ml of BMGY medium (buffered complex medium containing glycerol as the sole carbon source and composed of 1% yeast extract, 2% peptone, 100 mM potassium phosphate adjusted to pH 6, 1.34% yeast nitrogen base, 4×10^{-5} biotin, 1% glycerol) in a 500 ml flask and incubated at 30 °C and 250 rpm for 24 h. The cells were harvested by centrifugation at 3500 rpm for 15 min, resuspended in 5 ml BMMY medium (buffered complex medium containing 1% methanol instead of glycerol as the sole carbon source) and incubated at 30 °C and 250 rpm. Methanol was added every 24 h to a final concentration of 1%. After two days of induction, the cells were pelleted by centrifugation at 3500 rpm for 15 min at 4 °C, the supernatant was kept at −20 °C for recombinant protein expression analysis.

2.3. High cell density culture

The recombinant clone of *P. pastoris* isolated from 100 μg/ml zeocin-YPD plate, was grown in 10 ml BMGY medium, in a 125 ml baffled flask for 12 h at 30 °C and 250 rpm.

This culture was then used to inoculate 200 ml of BMGY medium in 2-L baffled flask for 12 h at 30 °C and 250 rpm.

High cell density cultures were carried out in a 5-L bioreactor (Infors, Bottmingen, Switzerland). The batch culture was carried out with an initial culture volume of 2 L inoculated with the 200 ml preculture, the culture was carried out at the following conditions: temperature = 30 °C, stirrer speed = 800 rpm, pH maintained at 5 by addition of 25% ammonia, dissolved oxygen was set at 40% of air saturation by stirrer cascading and enrichment of the inlet air with pure oxygen when required.

The fed batch culture on glycerol was started once the dissolved oxygen increase was observed, corresponding to an optical density at 600 nm (O.D.) around 80. After 24 h of a fed batch culture on glycerol at a constant flow rate, a carbon source starvation period of 2 h was established for total glycerol exhaustion in the culture medium. Upon complete depletion of glycerol indicated

by an increase in dissolved oxygen concentration and reduction in agitation speed, a fed batch culture on methanol was started. The methanol feeding pump was manually modulated to keep methanol residual level under 2 g/l.

The composition of the media used for bioreactor culture were as follow, batch medium: glycerol (40 g/l), K_2SO_4 (18.2 g/l), $MgSO_4$ (7.28 g/l), KOH (4.13 g/l), $CaSO_4 \cdot 2H_2O$ (0.93 g/l) and 85% orthophosphoric acid (26.7 ml/l) dissolved in deionised water and sterilized in the bioreactor. 5 ml/l basal salts of fermentation PTM1 and 2 ml/l of biotin at 0.2 g/l were added to the medium after sterilization, culture medium pH was adjusted to 5 by addition of 25% ammonia (w/v).

The PTM1 solution contains: $CuSO_4 \cdot 5H_2O$ (6 g/l), NaI (0.08 g/l), $MnSO_4 \cdot H_2O$ (3 g/l), $Na_2MoO_4 \cdot 2H_2O$ (0.2 g/l), H_3BO_3 (0.02 g/l), $CoCl_2$ (0.5 g/l), $ZnCl_2$ (20 g/l), $FeSO_4 \cdot 7H_2O$ (65 g/l), biotin (0.2 g/l) and H_2SO_4 98% (5 ml/l).

Fed-batch medium contains glycerol (450 g/l), K_2SO_4 (18.2 g/l), $MgSO_4$ (7.28 g/l), KOH (4.13 g/l), $CaSO_4 \cdot 2H_2O$ (0.93 g/l) and 85% orthophosphoric acid (26.7 ml/l). PTM1 (8 ml/l) and biotin at 0.2 g/l (5 ml/l) were added after heat sterilization.

Methanol fed-batch solution contains methanol (987 ml/l), 500X biotin (5 ml/l) and PTM1 (8 ml/l).

2.4. Down stream processing steps

At the end of the culture, culture medium was centrifuged at 3500 rpm, 4 °C for 15 min. The supernatant was further clarified by a microfiltration cartridge (polysulfone membrane, cut-off 0.1 μm) (Amicon, Massachusetts, USA). The permeate containing rhIFN alpha was desalted on a Sephadex G25 XK 50/60 column (Amersham Biosciences, Uppsala, Sweden) Elution was carried out using 2 column volumes of lactic acid 50 mM pH 4 at a flow rate of 3 cm/min.

Hundred milliliter of desalted permeate containing rhIFNα2b, was loaded on a 1 ml Sepharose SP column (Amersham Biosciences, Uppsala, Sweden). Elution of bound rhIFNα2b was carried out using 25 column volumes of a stepwise gradient of 0.1–1 M NaCl, at a flow rate 2.5 cm/min. rhIFNα2b was eluted at an NaCl concentration ranging from 0.4 to 0.8 M. Fractions containing rhIFNα2b were pooled.

The emergence of proteins in the fractions was monitored by measuring the absorbance at 280 nm. Fractions were analysed by SDS-PAGE, Western blot and ELISA.

rhIFNα2b was then further purified on a Sephacryl-S100 HR XK 16/60 column (Amersham Biosciences, Uppsala, Sweden) using PBS pH 7.2 at a flow rate of 15 cm/h.

2.5. Analytical methods

Total cell concentration was determined by measuring the absorbance at 600 nm (O.D.600) (Jenway, United Kingdom). Samples were diluted if the absorbance was above 0.6. Correlation between O.D.600 and dry cell weight was determined according to standard protocols. One unit of O.D.600 was found to be equivalent to 0.27 g/l dry cell weight (DCW).

Methanol concentration in the culture medium, was estimated by off-line gas chromatography (Shimadzu, Japan) equipped with a flame ionisation detector and a glass column (2 m × 2 mm) packed with Porapak Q80. The temperature of the oven was set at 180 °C, injector and detector temperatures were set at 220 °C.

2.6. rhIFNα2b dosage

The quantity IFNα2b was assessed by ELISA in 96 well plates. Dilution series containing HPLC purified soluble recombinant human INFα2b produced in-house were included in each assay to construct a standard curve. hIFNα2b standard concentrations range from 0.1 to 781 pg/ml. The plates were incubated overnight at 4 °C.

Plates were then washed three times with 0.1% Tween 20-PBS (Phosphate Buffer Saline), and then blocked with 5% fat milk in PBS, at room temperature for 1 h. After washing three times with 0.1% Tween 20-PBS, plates were incubated with anti-human IFNα monoclonal biotin-labeled (Endogen, Rockford, USA) at room temperature for 1 h. After incubation, plates were washed three

times, and then incubated with streptavidine-horseradish peroxidase (Amersham Biosciences, Uppsala, Sweden) at room temperature for 1 h. The plates were finally washed three times and incubated with TMB-substrate solution (Sigma, St Louis, USA) at room temperature for 30 min. The results were quantitated using an ELISA plate reader at 450 nm.

2.7. SDS-PAGE and Western blot analysis

Polyacrylamide gel electrophoresis (PAGE) was performed in 15% polyacrylamide gels under denaturing conditions as described by Laemmli [26].

After separation, the proteins were visualized according to standard procedures by staining with either Coomassie brilliant blue G250 or silver nitrate.

For western blotting, the gels were transferred to nitrocellulose membranes by electroblotting. The membrane was blocked with PBS-5% Fat milk–0.1% Tween 20 (PBS-T-Fat milk) overnight at 4 °C. To detect the target protein in culture supernatant, the membrane was incubated for 1 h with anti-human IFNα polyclonal antibody dilution 1:400 (Endogen, Rockford, USA), followed by the anti-goat/sheep IgG peroxidase conjugated monoclonal antibody (Sigma, St Louis, USA) diluted at 1/10,000. The membrane was finally incubated for one minute with ECL solution (Amersham Biosciences, Uppsala, Sweden).

2.8. Biological activity

The biological activity of the recombinant hIFNα2b preparation was determined by the antiviral assay as described by Meager [27]. The antiviral assay is based on the ability of rhIFNα2b to inhibit the cytopatic effect caused by encephalomyocarditis virus (EMCV) on Human glioblastoma cell line, 2D9. One unit of activity was defined as the amount of recombinant hIFNα2b required to produce antiviral activity equivalent to that expressed by 1IU hIFNα2b international reference standard (code: 95/566; Division of Immunobiology, National Institute for Biological Standards and Control, United Kingdom).

3. Results

3.1. Optimisation of the volumetric productivity of hIFNα2b in high cell density culture of P. pastoris

High cell density fermentation is one of the most important strategies for improving the production level of recombinant proteins. Therefore, *P. pastoris* recombinant clone producing hIFNα2b, was grown at high cell density in a 5-L bioreactor according to the protocol described in Section 2. Glycerol fed-batch culture was started after the initial amount of glycerol had been exhausted as evidenced by the abrupt increase in the level of dissolved oxygen. During this biomass build-up phase (fed-batch growth on glycerol), we tested different glycerol flow rates. The highest biomass level was achieved at a glycerol flow rate of 15 ml/l/h (data not shown). This level was retained for subsequent optimisation studies.

SDS-PAGE analysis of culture supernatants collected at different induction times during high cell density culture of *P. pastoris* clone, shows the presence of rhIFNα2b band several hours after the start of induction (Fig. 1A). However as this band had totally disappeared at 20 h of methanol induction, rhIFNα2b was substantially degraded probably by yeast proteases secreted into the culture medium. Proteolytic degradation has been a perpetual problem when yeasts are employed to express recombinant proteins. Several reports described proteolytic degradation of recombinant proteins produced in *P.*
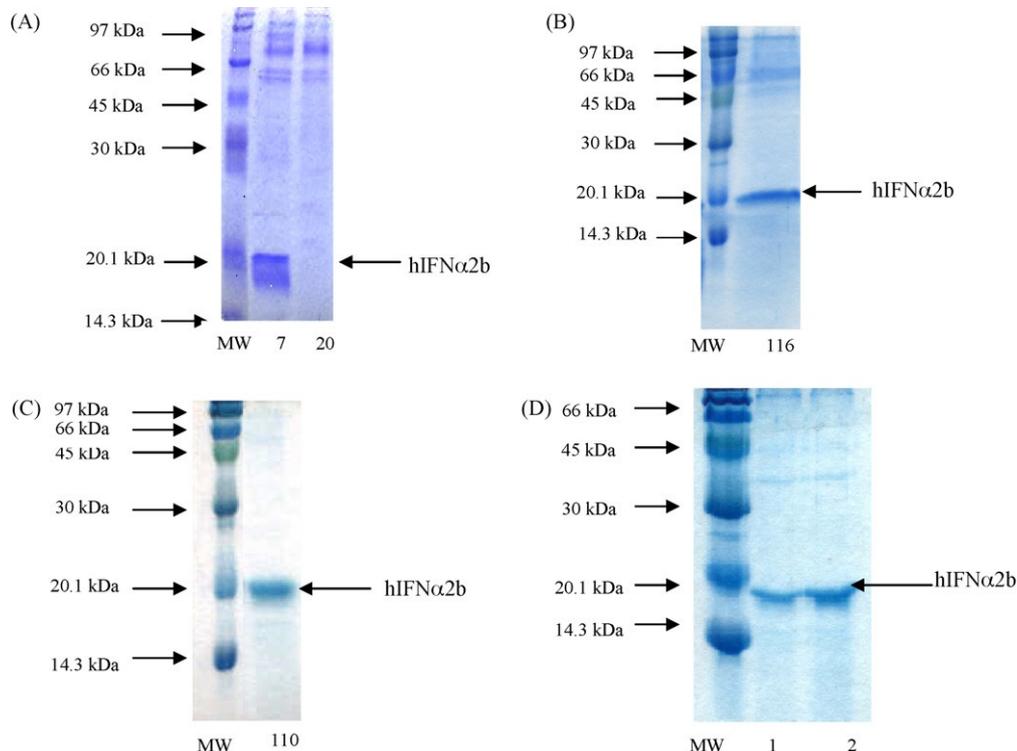
Fig. 1. (A) Coomassie brilliant blue R-250 stained SDS-PAGE (15%) of culture supernatant samples collected during high cell density culture of *P. pastoris* conducted according to the standard procedure, (B) after medium enrichment with casamino acids at 0.1% and (C) after medium replacement and casamino acids addition (0.1%). MW: molecular weight marker. 7, 20, 116, 110 stand for induction time in hours. (D) Effect of EDTA addition on the production level of hIFNα2b by *P. pastoris* grown in shake-flask. MW: molecular weight marker. (1) Induction medium without EDTA. (2) Induction medium containing EDTA at 10 mM (final concentration). For (B)–(D) culture supernatants were previously desalted on PD-10 columns (Amersham Biosciences, Uppsala, Sweden).

pastoris [28,29]. The use of complex nitrogen sources such as casamino acids or peptone is considered as a possible remedial measure to circumvent proteolytic degradation of the recombinant protein. Therefore during induction we added to the culture medium casamino acids at 0.1% every 24 h. Fig. 1B shows that the addition of casamino acids had prevented hIFNα2b degradation and restored the production level. To further improve the expression level of the target protein, we proceeded to a medium change at the end of the glycerol fed-batch phase. The cells were centrifuged and resuspended in the same volume of fresh synthetic medium and returned aseptically to the bioreactor. Fed-batch methanol culture was then immediately initiated at a flow rate of 0.8 ml/l/h. As depicted in Fig. 1C, medium replacement after the glycerol fed-batch phase had further improved the production level of the target protein. Compared to the previous culture (Fig. 1B), the production level was 1.5-fold increased as determined by image J scanning software available at http://rsb.info.nih.gov/ij/, we reached around 250 mg/l as estimated by ELISA.

Metalloproteases secretion during *Pichia pastoris* culture has been reported in several studies [29,30]. Therefore we assessed EDTA capacity to inhibit proteases action in shake flask culture. EDTA was added at 10 mM (final concentration) to the culture medium during the induction phase, cells were grown in synthetic medium and induced by methanol addition at 1% for every 24 h. Fig. 1D indicates that in shake-flask cultures EDTA addition had increased the production level of hIFNα2b by 1.65-fold factor as assessed by image J scanning software.

Another parameter that is likely to influence hIFNα2b production level is methanol feeding profile. It is important to determine a methanol feeding strategy optimal for the needs of a particular production process. The level of transcription initiated from AOX1 promoter can be three to five times greater in cells fed with methanol at growth limiting rates compared to cells grown in excess methanol [17]. Nevertheless other stud-

ies reported different cell behaviour, i.e. keeping a non limiting methanol concentration enhanced protein expression [31,32]. Furthermore, it is crucial to carefully monitor the methanol level to avoid cell toxicity and death. Therefore we tested different feeding profiles denoted 1, 2 and 3 where methanol flow rate and the duration of induction at a specific flow rate were varied. Our goal was to optimise the production level as well as the volumetric productivity and to maintain residual methanol level below 2 g/l. For all profiles and before initiating induction, we proceeded to a medium replacement as described above. Furthermore, we also added to the culture medium casamino acids at 0.1% every 24 h whereas EDTA at 10 mM was added when profiles 2 and 3 were used.

In addition, for all profiles methanol was fed at a flow rate of 0.8 ml/l/h at the beginning of the induction phase to adapt the culture to growth on methanol then different strategies of methanol feeding were adopted (Fig. 2).

Fig. 2A shows that for profile 1 culture, a decrease in cell density was observed when methanol flow rate was increased to 1.6 ml/l/h. Residual methanol was also high and beyond 2 g/l (Fig. 2B). The decrease of the flow rate to 1.2 then to 0.8 ml/l/h did not restore a normal cell behaviour, i.e. cell density level decreased with an accumulation of methanol at 2.8–2.3 g/l.

For profile 2, methanol flow rate was further increased to 2.05 ml/l/h compared to profile 1. Cells showed an active metabolism on methanol till 90 h post induction. Nevertheless the increase of the flow to 2.05 ml/l/h resulted in a decrease of cell density although residual methanol was low. The decrease of methanol feed rate to 1.6 ml/l/h for the last 48 h of induction, had restored cell density level even though at the end of the culture residual methanol was equal to 1.6 g/l (Fig. 2C).

The last feeding strategy that we tested, consisted of a continuous linear step increase (0.4 ml/l/h) of methanol flow rate from 0.8 to 2.45 ml/l/h till the end of the induction phase. Compared to profiles 1 and 2, this strategy had further lowered residual methanol level. Its concentration had not exceeded 1.3 g/l
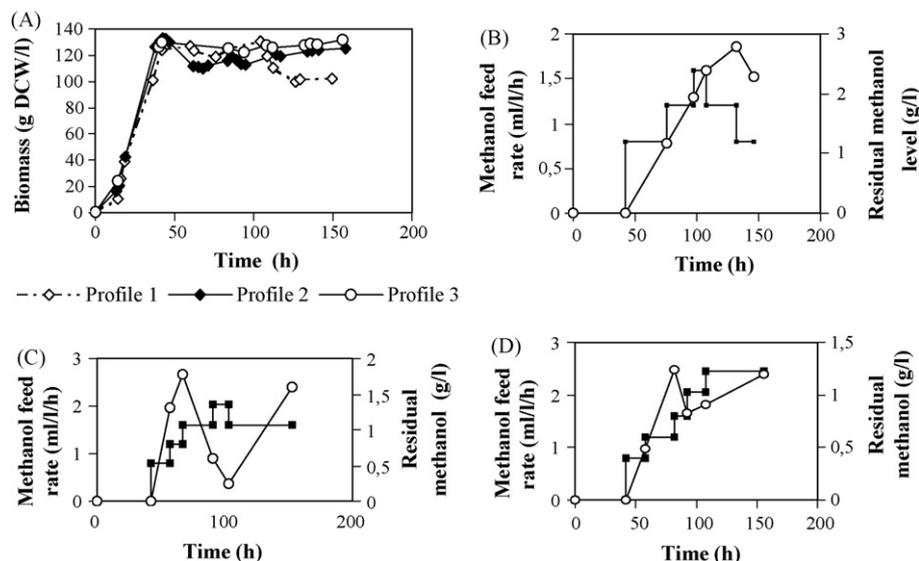
Fig. 2. High cell density culture of *P. pastoris* expressing hIFNα2b using different methanol feeding strategies. Time course of biomass (A), residual methanol level and methanol flow rate during Profile 1 (B), Profile 2 (C) and Profile 3 (D) cultures.
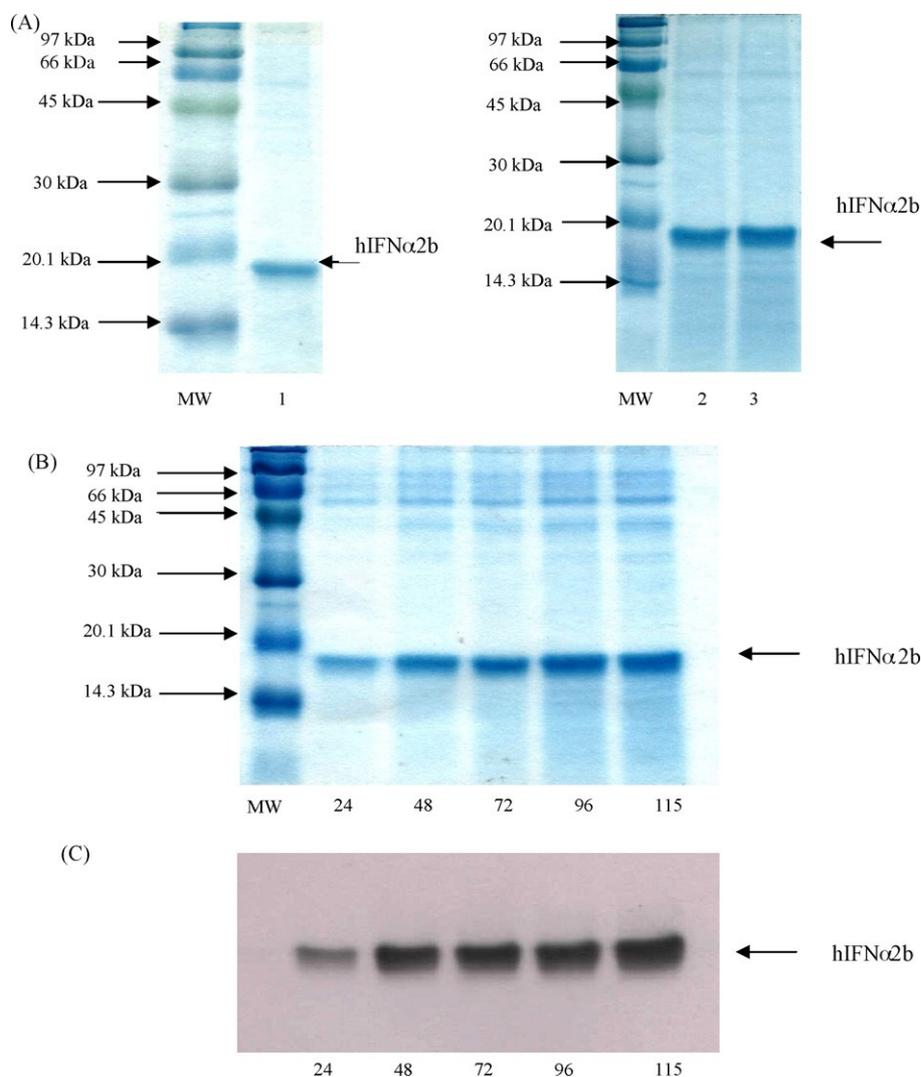
Fig. 3. (A) Coomassie-stained SDS-PAGE (15%) of culture harvest collected during high cell density culture of *P. pastoris* expressing hIFNα2b and conducted using different methanol feeding profiles. MW: molecular weight marker. 1, 2 and 3 stand for methanol profile, respectively. (B) Time course of hIFNα2b accumulation in culture supernatant during high cell density culture of *P. pastoris* where methanol was fed according to Profile 3. MW: molecular weight marker. Each line represents a sample taken at the indicated time of induction in hours. (C) The corresponding immunoblot with polyclonal anti-IFNα antibodies. All supernatants were previously desalted on PD-10 columns.

(Fig. 2D). Cell density level showed a slow increase during the induction phase (Fig. 2A).

Scanning of SDS gels using NIH imageJ showed that the highest level of hIFNα2b was obtained during profile 3 culture (Fig. 3A). In addition, SDS-PAGE analysis of samples collected at different induction times during this culture, shows a continuous increase of the target protein (Fig. 3B). The highest level was achieved at day 5 post induction and was equal to 600 mg/l as assessed by ELISA, the volumetric productivity was 120 mg/l/day. Western blot analysis using anti-IFNα antibodies confirmed this result and shows no proteolysis of the target protein through out the induction period (Fig. 3C).

3.2. Down stream processing design

The supernatant obtained after cell removal by centrifugation was further clarified through a 0.1 μm hollow fiber cartridge. Data shown in Fig. 4A, indicate that most of the rhIFNα2b was

detected in the retentate and not in the permeate. These data were unexpected, as the target protein with a molecular weight of 19.2 kDa, should had been recovered in the filtrate. Therefore it appears that rhIFNα2b is present in culture supernatant either as aggregates or bound to cell membranes.

To improve the recovery yield of the microfiltration step and to prevent rhIFNα2b aggregation, two additives were evaluated NaCl and Triton X-100. Culture supernatant was supplemented with either NaCl at 0.5 M or Triton X-100 at 1% and stirred for 1 h at room temperature. Then, the supernatant was filtrated through the 0.1 μm cartridge. Fig. 4B shows that the amount of rhIFNα2b recovered in the permeate was greatly enhanced. Nevertheless, Triton X-100 appears to be more efficient than NaCl. The recovery yield as estimated by ELISA, was equal to 46 and 35% after Triton X-100 and NaCl addition, respectively.

Two hundred and fifty millilitres of the permeate were desalted using a Sephadex G-25 column, fractions containing rhIFNα2b (4–8) were pooled. The yield of this step was equal
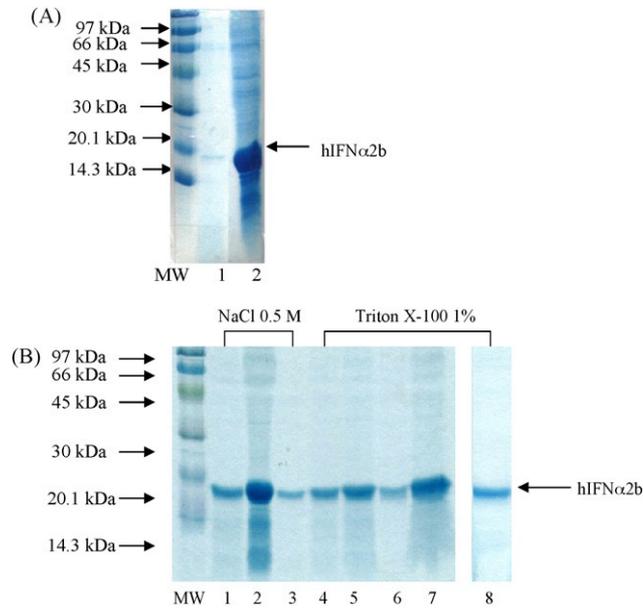
Fig. 4. Determination of protein concentration by SDS-PAGE (15%). Culture harvest supernatant collected in high cell density culture of *P. pastoris* (Profile 3 culture), was clarified on 0.1 µm hollow fiber cartridge. (A) Without any disaggregating agent, permeate (lane 1) and retentate (lane 2). (B) After Triton X-100 or NaCl treatment. MW: molecular weight marker, lane 1: culture supernatant after addition of NaCl at 0.5 M, lane 2: retentate, lane 3: permeate, lane 4: culture supernatant disaggregated with Triton X-100 at 1%, lane 5: retentate recovered after 2 h of microfiltration, lane 6: permeate recovered after 2 h of microfiltration, lane 7: retentate recovered after 4 h of microfiltration and lane 8: permeate recovered after 4 h of microfiltration. All supernatants were previously desalted on PD-10 columns.

to 80% as assessed by ELISA (Fig. 5A). Then 100 ml of the desalted permeate were loaded into a Sepharose SP column as described in Section 2. This cation exchange purification step allowed to eliminate all the contaminating proteins of high molecular weight (Fig. 5B). The recovery yield of this step as assessed by ELISA was equal to 90%.

Twelve millilitres of the pooled fractions (from 12 to 25) containing rhIFNα2b were further purified by size exclusion through a Sephacryl S-100 high resolution column. Fig. 5C shows that rhIFNα2b was correctly separated from the contaminating proteins of low molecular weight. The target protein was also recovered at a high level of purity, no contaminating protein band was observed. The yield of this step as determined by ELISA was equal to 92%. The final recovery yield of the downstream process was equal to 30% (Table 1).

The biological activity of the purified rhIFNα2b was assessed according to antiviral activity. The specific antiviral activity of the purified protein was 1.5×10^8 IU/mg.

Table 1
Purification of rhIFNα2b from 1 L of *Pichia pastoris* high cell density culture

	rhIFNα2b (mg)	Purity (%)	Yield (%)
Harvest	600	69.9	100
0.1 µm filtration	276	83	46
Desalting	221	82.5	37
Cation exchange chromatography	199	91.5	33
Gel filtration	183	100	30
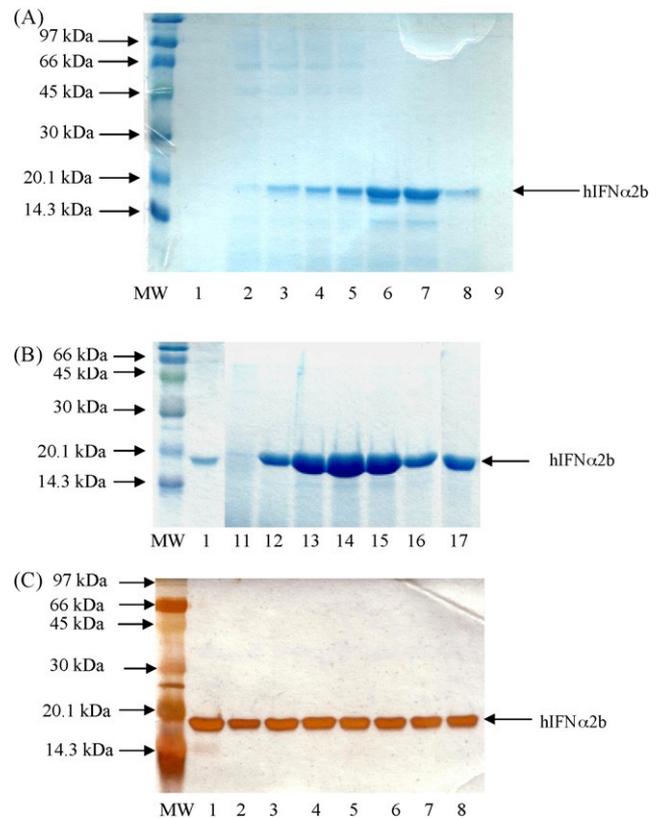
Fig. 5. (A) Desalting of permeate containing hIFNα2b and recovered during 0.1 µm microfiltration after Triton X-100 at 1% treatment. Coomassie blue stained SDS-PAGE analysis of column fractions. MW: molecular weight marker. 2–9 stand for fraction number. (B) Coomassie blue stained SDS-PAGE analysis of fractions collected during cation exchange purification of rhIFNα2b on 1 ml SP Sepharose column. MW: Molecular weight marker. 1: Desalted permeate. Lanes 11–17 correspond to the main fractions containing rhIFNα2b. (C) Silver stained SDS-PAGE (15%) of samples collected through size exclusion purification step. Lane 1: the pooled fractions (from fractions 12–25) obtained during cation exchange purification on SP Sepharose column. Lanes 2–8 correspond to the different fractions. MW: molecular weight marker.

4. Discussion

In this work, we optimised the volumetric productivity of hIFNα2b in high cell density culture of *P. pastoris*. We showed that medium replacement by fresh medium after the glycerol fed-batch phase, had prevented the degradation of the target protein. Similar data were reported by Chen et al. [33]. Such procedure had probably resulted in the removal of the proteases produced during this phase. Furthermore, casamino acids addition to the culture medium during the induction phase had largely contributed to increase the production level of hIFNα2b and to prevent proteases action. Enrichment of culture medium with amino acid rich supplements is a procedure often used to reduce product degradation during biotechnological processes. These supplements can act as alternative and competing substrate for proteases and repress protease induction caused by nitrogen limitation [34].

We also showed that EDTA addition to the culture medium during the induction phase had enhanced the production level of hIFNα2b. Shi et al. [30] reported various types of proteases

during *P. pastoris* growth on methanol; they identified aspartic, cysteine and serine-type proteases and showed that EDTA addition inhibited metalloprotease and cysteine proteases.

Methanol concentration is considered as a another parameter that is likely to influence protein production in *P. pastoris* fed-batch cultures. It is crucial to keep methanol concentration below inhibitory level since *P. pastoris* strains are sensitive to high residual methanol concentration and sudden accumulation of methanol may result in loss of AOX activity and even cell death [34,35]. For a Mut^s strain like *P. pastoris* KM71H in which the chromosomal AOX1 gene is largely deleted, methanol catabolism relies on the much weaker AOX2 gene and therefore this strain grows on methanol at a slow rate [36]. Moreover it is important to determine a methanol feeding strategy optimal for the needs of a particular production process [37,38]. In this study, we demonstrated that a smooth increase of methanol feeding rate represents the best profile, such strategy resulted in the highest level of rhIFNα2b and in a lower level of residual methanol. In addition compared to other profiles, biomass level had slightly increased. Furthermore, under such conditions proteases release due cell to cell lysis was probably lowered, contributing therefore to further enhance the expression level of hIFNα2b.

High cell density culture of *P. pastoris* offers high productivity in the bioreactor but puts high demand on the downstream processing. A feedstock characteristic is the high salt concentration. The cultivation performed with such medium resulted in an accumulation of lipid like substances, which caused major problems in downstream processing and encouraged aggregation of recombinant protein [31,39,40]. This is what probably happened during the clarification step using the hollow fiber cartridge, i.e. the majority of rhIFNα2b was kept within the retentate. Culture supernatant treatment with a protein disaggregating agent such as Triton X-100 or NaCl improved the recovery yield of this step. Triton X-100 is a non-ionic surfactant that was successfully used for the recovery of active proteins from inclusion bodies [41,42]. NaCl can also play a critical role in the inhibition of aggregation of certain proteins such as recombinant factor VIII SQ [43] and α1-antitrypsin [44]. Aggregation of human consensus IFNα in high cell density culture of *P. pastoris* was also described by Hao et al. [13,14]; they showed that either temperature reduction to 20 °C or Tween-20 addition reduced the aggregation of the target protein. They also demonstrated that human consensus IFN-α aggregates were formed dominantly by non-covalent interaction and partially by disulfide bond.

We demonstrated in the present work, that by utilizing two chromatography steps, we recovered from culture supernatant functional and active hIFNα2b at a high degree of purity.

To our knowledge, the recovery yield achieved (∼180 mg/l purified human interferon α2b) in the present study is much higher than that reported in the literature using *P. pastoris* as a host expression system. Liu et al. [12] reported 35 mg/l of purified rhIFNαD in shake flask culture whereas Hao et al. [14] obtained 160 mg of human consensus IFN-α from 1 L of supernatant harvest from high cell density culture of *P. pastoris*.

However improvement of the global yield could be considered, particularly regarding the microfiltration step which is the limiting step where most of the rhIFNα2b was lost. One way will be the use of larger cut-off membranes (0.45 μm or more). Nevertheless the whole process has a great potential for the large scale production of biologically active rhIFNα2b in a cost effective way.

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