Original Article

Cloning and Optimization of Intracellular Expression of Human Interferon β-1a in *Pichia pastoris* GS115

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Abstract

Background: Interferon-beta (IFN-β) is a cytokine with a wide range of biological and pharmaceutical applications, including multiple sclerosis (MS), cancer, some autoimmune disorders, and viral infectious diseases. Thus, many studies have been performed to develop novel strategies for the high-yield production of functional IFN-β in a cost-effective approach. Here, we aimed to improve the intracellular expression of IFN-β-1a in *Pichia pastoris*.

Materials and Methods: The gene of IFN-β-1a was successfully sub-cloned into the pPICZA vector. The recombinant vector was transfected to *P. pastoris* GS115 cells by electroporation. After screening positive *P. pastoris* transformants, the expression of IFN-β-1a was evaluated and the cultivation conditions, including temperature, time of incubation, and methanol concentration, were optimized. The protein expression levels were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Results: The double digestion with *Eco*RI and *Xho*I restriction enzymes and sequence analysis confirmed the correct sub-cloning of the IFN-β-1a gene into pPICZA. SDS-PAGE analysis showed that the highest level of IFN-β-1a (25 mg per 1 L of yeast culture) was produced with 2% methanol at 28°C after 72 h incubation.

Conclusion: Optimization of cultivation conditions for intracellular expression of IFN-β-1a was successfully performed. This approach can be generally applied to improve the production yield and quality of other recombinant proteins in *P. pastoris*.

Keywords: Cytoplasm, gene expression, interferon-betaIFN, *Pichia pastoris*, methanol, temperature

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Introduction

Interferon β-1 (IFN β-1) is a member of the interferon family exhibiting immunomodulatory, antiviral, and anticancer effects. It is mainly secreted by fibroblasts in response to viral infections or double-stranded RNA.[1] Some other cytokines (*e.g.* interleukin-1 and tumor necrosis factor alpha) can also induce the production of IFN β-1.[2]

One of the main therapeutic applications of IFN β-1 is the treatment of multiple sclerosis (MS). MS is a chronic inflammatory demyelinating disease and one of the most common disorders of the central nervous system (CNS).[3] IFN β-1b (produced in bacterial expression system) and IFN β-1a (produced in mammalian expression system) are the two forms that have been approved by the FDA for the treatment of MS.[4]

Although bacterial expression hosts like *Escherichia coli* offer ease of application and low-cost bioprocessing, they have some limitations when it comes to expressing glycosylated proteins. This is due to the accumulation of insoluble and inactive proteins as inclusion bodies.[5] Recombinant protein expression

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in mammalian cells (e.g., Chinese hamster ovary (CHO) cells) offers native-like post-translational modifications and proper protein folding, but these expression hosts also have some drawbacks, primarily high costs and long protein production times.[6] In yeast expression systems, both mammalian and bacterial advantages are combined, including high protein yield, reduced bioprocessing cost and time, and easy scale-up.[7]

Pichia pastoris, a methylotrophic yeast, can be used for intracellular and extracellular production of recombinant proteins.[8] With its ease of genetic manipulation, this strain has many noteworthy features, such as post-translational modifications, proper protein folding, and ease of use.[9] Since *P. pastoris* expresses fewer endogenous proteins than other expression hosts, extracellular secretion of heterologous proteins results in easier downstream processing and purification[9] Nevertheless, it has been observed that some recombinant proteins are unable to secrete efficiently. Furthermore, extracellular secretion can lead to proteolysis and degradation of the target protein.[10] Glycosylation in human cells differs from that in yeast, and secreted proteins are more susceptible to hyper-mannosylation, a factor that could adversely affect their biological activity.[11] Intracellular expression offers several advantages over the extracellular secretion method. By comparison, the product is stable, the expression level is higher, and glycosylation does not occur in the form of hyper-mannosylation.[12]

We have previously described the extracellular expression of IFN β-1a in *P. pastoris*, yielding 15.5 mg/L as a final concentration.[9] In this study, we cloned and expressed IFN β-1a intracellularly in *P. pastoris* GS115 to increase protein expression yield.

Material and Methods

Microorganisms, culture media, and reagents

E. coli DH5α served as a host for the propagation and maintenance of plasmids. Low salt Luria broth (LB) containing 1% tryptone, 0.5% yeast extract, and 0.5% NaCl with 50 μg/mL Zeocin (InvivoGen, USA) was applied for growing *E. coli* DH5α transformants. The host strain *P. pastoris* GS115 (HIS4-) was used for intracellular expression of human IFN β-1a. This strain was grown in YPD medium (10 g of yeast extract, 20 g of peptone, and 10 g of glucose per 1 L, pH 7.5) at 28-30°C. Chemicals used to prepare media and buffers were obtained from Merck (Germany).

The construction of recombinant intracellular expression vectors

To introduce the *Eco*RI and *Xho*I restriction sites, the IFN β-1b gene (541 bp) was amplified from the parental vector (i.e., pPICZαA-IFN-β-1b) developed in our previous study[9] by polymerase chain reaction (PCR) with the following primers: forward 5'-GAA TTC AAA CGA TGT CCT ATA ACT TG-3' (PICZ.IFN-F) and reverse 5'-CTC GAG TTA TCA GTG ATG GTG-3' (PICZ.IFN-R). During the thermal cycler, the following were set: initial DNA denaturation at 5 min at 94°C, 30 amplification cycles (45 s at 94°C, 1 min at 50°C, and 2 min at 72°C), and finally an extension period at 72°C for 5 min. To clean up the PCR, 0.1 volume of sodium acetate 3 M and 2.5 volumes of 96% ethanol were added to the PCR mixture. The mixture was vortexed, incubated on ice, and centrifuged at 14,000 rpm, 4°C for 30 min. The pellet was washed with ice-cold 75% ethanol before air drying and then resuspended in nuclease-free water. In the following step, the sample was digested with *Eco*RI and *Xho*I restriction enzymes) Fermentas, USA), gel-extracted with the QIAquick gel extraction kit (Qiagen, USA), and subcloned into pPICZA vector [Figure 1] using the corresponding restriction sites, resulting in pPICZA-IFN-β-1b plasmid. The resulting plasmid was sequenced to ensure no mutations and confirm the correct expression frame by double digestion experiment and DNA sequence analysis using AOX1 primers (forward: 5′-GAA TTC AAA CGA TGT CCT ATA ACT TG-3′ and reverse: 5 ′-CTC GAG TTA TCA GTG ATG GTG-3′).

Transformation of P. pastoris GS115

pPICZA-IFN-β-1a plasmid was digested with *Sac*I, gel extracted, and transformed to *P. pastoris* GS115 cells by electroporation (1500 V, 150 Ω, 25 μF). Immediately following electroporation, 1 mL of ice-cold 1 M sorbitol was added to the cuvette, and the contents were transferred to a sterile 1.5 mL microtube and incubated for 1 hour at 30°C without shaking. The transformed cells were selected on YPDS (Yeast Extract Peptone Dextrose – Sorbitol) plates containing 1% yeast extract, 2% peptone, 2% glucose, and 1 M sorbitol with varying concentrations of Zeocin (i.e. 50, 100, 200, and 400 μg/mL) for 5 days at 30°C until colonies appear. Twelve positive colonies were isolated, and the IFN-1b gene integration was evaluated by PCR using insert-specific (i.e. PICZ.IFN) primers.

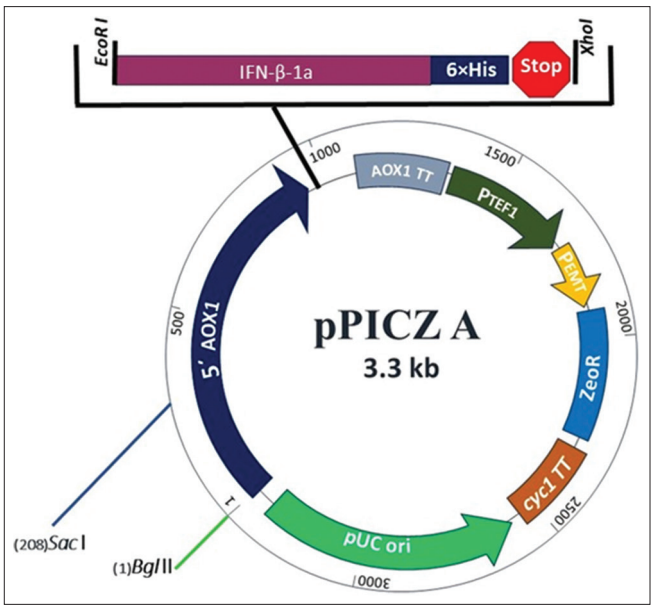

Figure 1: Schematic diagram of the pPICZA-human interferon β-1a expression vector

Intracellular expression IFN-β-1a in P. pastoris

A single colony of *P. pastoris* integrated with pPICZA-IFN-β-1b plasmid was inoculated in 30 mL YPD medium and incubated for 48 hours at 30°C and 200 rpm until reaching the OD_{600} to 2. Then, the cells were centrifuged and resuspended in fresh YPD medium, and 1% (V/V) methanol as an inducer was added every 24 hours for 4 days. For optimization of intracellular expression of IFN-β-1b, cultivations were done at different temperatures (25°C, 28°C, and 30°C) and using different inducer concentrations (0.5%, 1%, 2%, and 3%).

The protein samples were analyzed using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis using anti-His-HRP antibody (Abcam, USA). The expression level of IFN-β-1b was estimated by densitometry analysis of the gels using TL120 software (USA).

Result

The construction of recombinant intracellular expression vectors

Figure 2 shows the successful amplification of the IFN-β-1a gene with PCR [Figure 2a]. The PCR product was sub-cloned in pPICZA vector using *Eco*RI and *Xho*I enzymes. The release of a 535 bp recombinant gene fragment [Figure 2b] and sequences analysis confirmed the correct sub-cloning [Figure 2c].

Transformation of P. pastoris GS115

The recombinant pPICZA-IFN-β-1a plasmid was linearized with *Sac*I, and the *P. pastoris* strain GS115 was transformed using electroporation and screened on YPDS plates containing 50-800 µg/mL zeocin. Then, zeocin-resistant transformants (12 clones) were analyzed by PCR. The expected 541 bp band was found in 10 cases indicating successful integration of the IFN-β-1a gene [Figure 3].

Expression IFN-β-1a in P. pastoris

Seven positive colonies were selected, and their protein expression was analyzed to determine the clone with the highest expression of IFN-β-1a. SDS-PAGE analysis showed that colony 4 expressed the highest level of IFN-β-1a (data were not shown).

Optimization of the intracellular expression IFN-β-1a in P. pastoris

The expression of IFN-β-1 using different concentrations of methanol (0.5%–3%) at 25°C [Figure 4], 28°C [Figure 5], and 30°C [Figure 6] were evaluated. As shown in Figure 5a-c, the maximum protein expression was observed at 28°C. Figure 6a and b showed that the lowest protein production was at 30°C. The methanol concentrations have no significant effect on the expression level of IFN-β-1a. As illustrated in Figure 4a and b, the highest production of protein (25 mg per 1 L of yeast culture) was observed after 48-72 h incubation, whereas a very slightly decreased IFN-β-1a expression took place after 96 h incubation. Figure 7 shows the result of the Western blot analysis of IFN-β-1a expression at 28°C with 2% methanol after 72 h incubation using an anti-His-HRP antibody, confirming the correct and full-size protein expression.

Discussion

IFN-β is a cytokine with a wide range of biological and pharmaceutical applications, including MS, cancer, some autoimmune disorders, and viral infectious diseases (e.g. hepatitis and COVID).[13-15] Thus, many studies have been performed to develop novel strategies for high-yield production of functional IFN in a cost-effective approach.

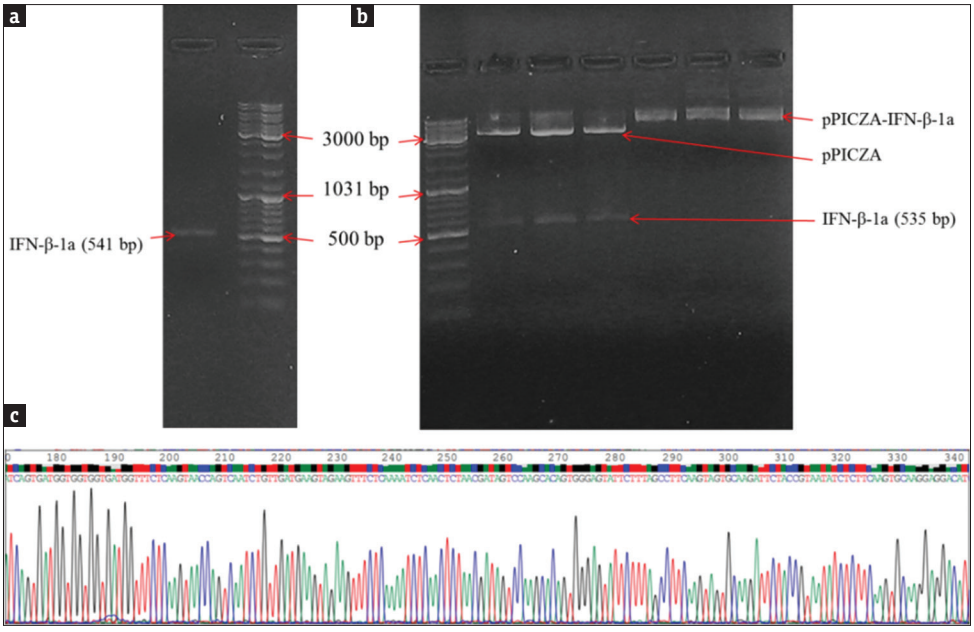

Figure 2: (a) PCR amplification of IFN-β-1a gene. (b) Restriction digestion of recombinant plasmid pPICZA-IFN-β-1a with *Eco*RI and *Xho*I restriction enzymes. (c) Sanger sequencing for pPICZA-IFN-β-1a

Considering the drawbacks of *E. coli* and CHO cell expression systems, some research groups have used *P. pastoris* as an alternative expression host.[16,17] In the present study, we applied *P. pastoris* for intracellular expression of IFN-β-1a due to a higher yield of expression, more stability, and lack of some post-translation modifications (i.e., hyper-mannosylation) compared to extracellular recombinant protein production. We also evaluate the effect of different cultivation factors on the expression of IFN-β-1a.

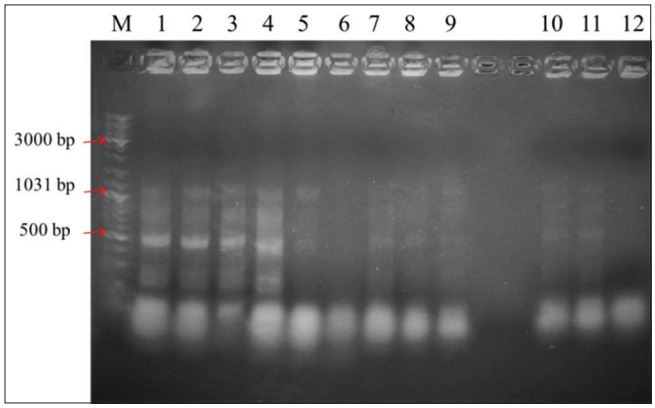

Figure 3: Colony PCR of zeocin-resistant transformants of *P. pastoris*. M: DNA ladder; Lane 1-12: Transformed colonies with pPICZA-IFN-β-1a

Temperature is one of the most critical parameters influencing the expression of recombinant proteins. *P. pastoris* grows at 30°C, above which protein synthesis and cell growth stop. Other studies have shown significant improvement in protein production at lower temperatures as oxygen stress and proteolytic degradation are reduced.[18,19] Another reason might be decreased protease release and an increase in the productivity and stability of the product.[20] Dragosits *et al*.[21] summarized many studies and concluded that other physiological parameters, such as changing expression of glucose transporters and glycolytic enzymes, affect increasing protein yield. In addition, the production of heterologous proteins is increased at 20°C and 25°C, but at 30°C increasing denaturation and aggregation of native proteins. We also observed that 28°C was the optimum temperature for intracellular expression, and 25°C was better than 30°C.

In *P. pastoris* fermentation, methanol plays a crucial role in the growth and productivity of this yeast. *P. pastoris* possesses the unique ability to metabolize methanol as its sole carbon source. As an energy source, *P. pastoris* utilizes methanol through metabolic transformations, like oxidation and assimilation.[22] The growth rate and viability of *P. pastoris* cells are directly influenced by methanol concentration in the culture medium. Higher methanol concentrations can

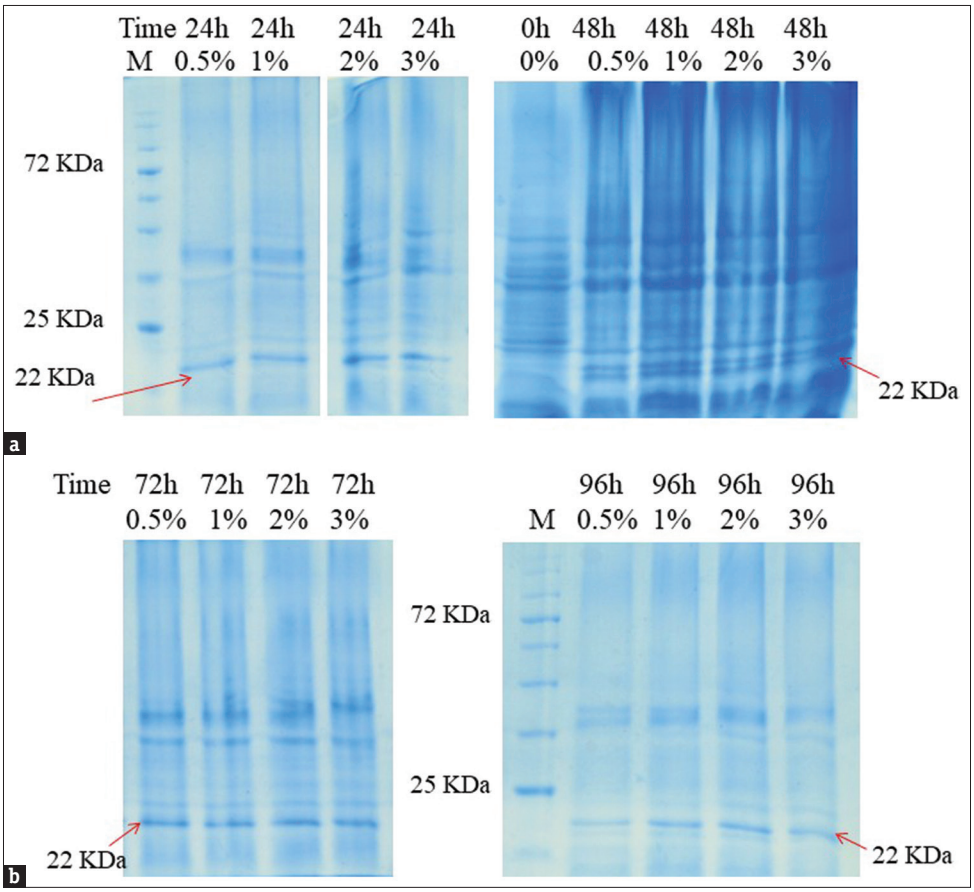

Figure 4: SDS-PAGE analysis of IFN-β-1a expression at 25°C with different methanol concentrations. (a) After 24 and 48 h incubation. (b) After 72 and 96 h incubation. M: Protein marker (SMOBIO PM1500). The arrows indicate the protein of interest

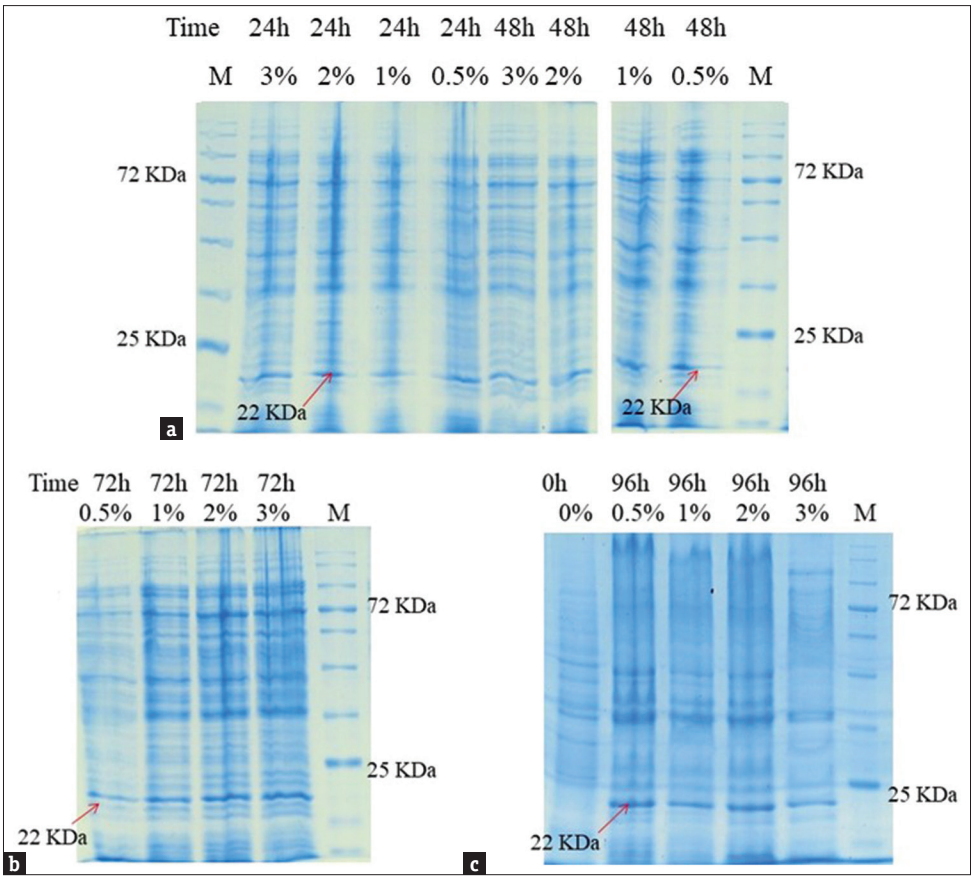

Figure 5: SDS-PAGE analysis of IFN-β-1a expression at 28°C with different methanol concentrations. (a) After 24 and 48 h incubation. (b) After 72 h incubation. (c) After 96 h incubation. M: Protein marker. The arrows indicate the protein of interest

enhance growth, but excessive levels can cause toxicity and inhibit cell proliferation.[19] Karbalaei *et al*.[18] realized that recombinant protein production requires a minimum of 0.5% methanol concentration and a concentration between 2% and 2.5% (W/V) for full expression. Additionally, methanol concentrations above 5% cause formaldehyde and hydrogen peroxide to accumulate, leading to cell death. They also analyzed more than 15 studies over 10 years and summed up the optimum methanol concentration. Methanol concentrations between 0.5% and 3% are considered optimal, while 2% is the most optimal concentration at 33.3% of reviewed articles. They also analyzed more than 15 studies over 10 years and summed up the optimum methanol concentration. Methanol concentrations between 0.5% and 3% are considered optimal, while 2% is the most optimal concentration at 33.33% of reviewed articles. In the current work, we observed that a mild increase in methanol feeding from 0.5% to 1% resulted in a higher level of IFN-β-1a expression. However, increasing the concentration of methanol from 1% to 3% did not exhibit a significant effect on the level of IFN-β-1a production.

Time is considered another factor that might influence protein expression in *P. pastoris*. As mentioned in the study by Skoko *et al*.[23] the incubation time in the *P. pastoris* expression system is relatively long and about 96 h. Adi *et al*.[24] considered that despite the maximum growth of *P. pastoris* cells after 4 days, expressed protein may be digested by proteolytic enzymes. However, our last study about interferon beta incubation admitted the optimum time for protein expression is 72 h and 96 h incubation led to degradation of the secreted protein.[9] Here, we found no evidence of significant proteolysis after 96 h probably due to intracellular expression of IFN-β-1a.

Our study has some limitations that need to be considered for future research. First, other cultivation parameters, including composition and pH of the medium, should be optimized to improve the yield of intracellular expression of IFN-β-1a in *P. pastoris*. Although more yield of IFN-β-1a expression was observed in the intracellular system compared with the extracellular system, we might face with challenges of cell disruption and protein purification. Furthermore, *in vitro* and *in vivo* biological evaluations are still required to confirm the quality of IFN-β-1a produced in this study.

Conclusion

In summary, IFN-β-1a was successfully sub-cloned and intracellularly expressed in *P. pastoris*. Furthermore, the cultivation conditions, including temperature, time of incubation, and methanol concentration, were optimized. The maximum protein expression was observed with 2% methanol at 28°C after 72 h incubation. An intracellular expression

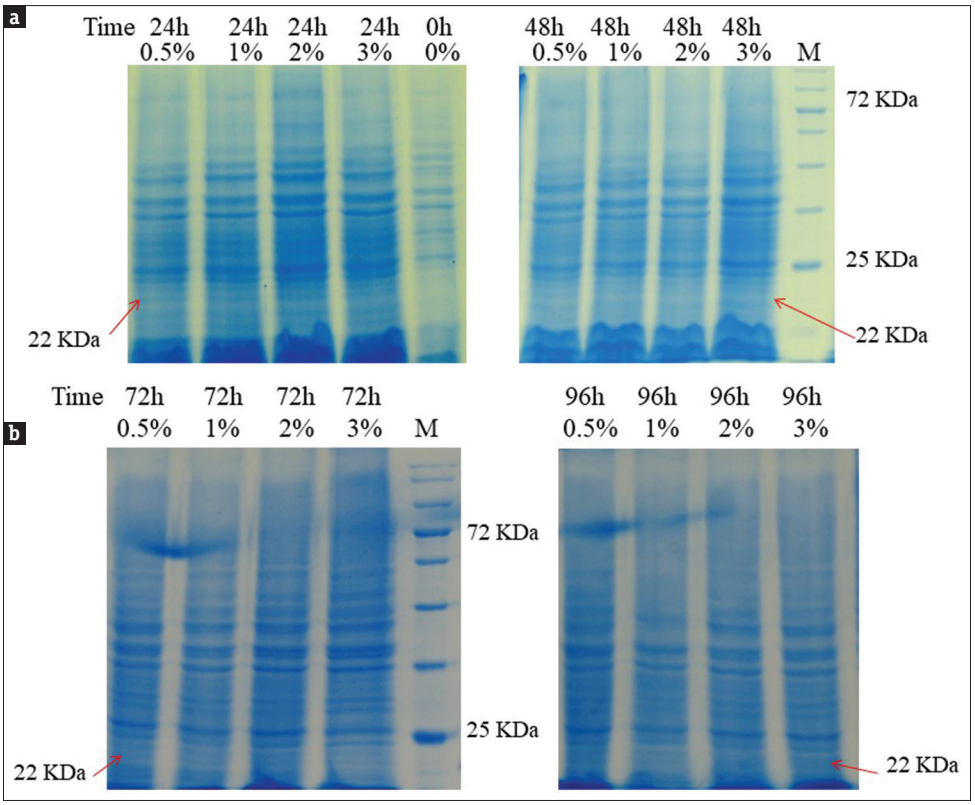

Figure 6: SDS-PAGE analysis of IFN-β-1a expression at 30°C with different methanol concentrations. (a) After 24 and 48 h incubation. (b) After 72 and 96 h incubation. M: Protein marker. The arrows indicate the protein of interest

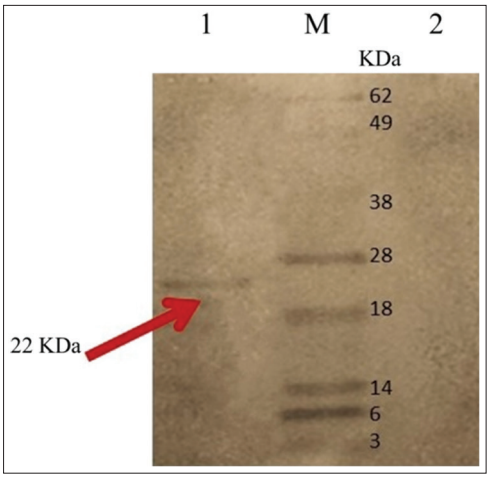

Figure 7: Western blot analysis of IFN-β-1a expression at 28°C with 2% methanol after 72 h incubation using an anti-His-HRP antibody. Lane 1: positive transformant, lane 2: negative transforman, and M: pre-stained protein marker (SeeBlue™, Thermo Fisher Scientific, USA). The arrows indicate the protein of interest

produced approximately two times more IFN-β-1a than our previous study using an extracellular expression system.

Ethics approval and consent to participate

This study was approved by the Research Ethics Committees of the Vice-Chancellor in Research Affairs-Medical University of Isfahan (Ethical approval code: IR.MUI.RESEARCH.REC.1397.423).

Acknowledgment

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Conflicts of interest

There are no conflicts of interest.

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