INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disorder believed to be one of the most common central nervous system (CNS) diseases (Lassmann, 2018). There is a large body of evidence suggesting that autoimmune mechanisms against CNS structures play a pivotal role in the pathogenesis of MS (Kaskow, Baecher-Allan, 2018). Nowadays, interferon-β (INF-β) is generally considered as one of the most commonly used drug for the treatment of MS as characterized by reduced relapse frequency, delayed MS progression and demyelinating formation (Jakimovski et al., 2018).

INF-β, also known as fibroblast interferon, includes a family of cytokines mediating the early innate immune response to viral infections (Hotter, Kirchhoff, 2018). Several studies have demonstrated that INF-β has also antiviral, anti-proliferative and immune-modulatory properties. Therefore, in addition to MS, it can be used for treatment of various human diseases including arthritis, infectious disease, and several types of cancers (Von Kalckreuth, Lohse, Schramm, 2008; Medrano et al., 2017). Up to now, two different types of recombinant INF-β including INF-β-1a (glycosylated form produced by mammalian cells) and INF-β-1b (non-glycosylated form produced by bacterial cells) have been developed and are currently available in the global market. INF-β-1a has 166

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Interferon-β-1a (INF-β-1a) has gained significant attention due to its emerging applications in the treatment of different human diseases. Therefore, many researchers have attempted to produce it in large quantities and also in a biologically active form using different expression systems. In the present study, we aimed to improve the expression level of INF-β-1a by Pichia pastoris using optimization of culture conditions. The codon-optimized INF-β-1a gene was cloned into pPICZαA plasmid under the control of alcohol oxidase I (AOX1) promoter. The protein expression was induced using different concentrations of methanol at different pHs and temperatures. The biological activity of produced protein was evaluated by anti-proliferative assay. The ideal culture conditions for the expression of INF-β-1a by P. pastoris were found to be induction with 2% methanol at pH 7.0 culture medium at 30°C which yielded a concentration of 15.5 mg/L INF-β-1a in a shake flask. Our results indicate that differences in glycosylation pattern could result in different biological activities as INF-β-1a produced by P. pastoris could significantly more reduce the cell viability of HepG-2 cells, a hepatocellular carcinoma cell line, than a commercially available form of this protein produced by CHO.

Keywords: Interferon-β-1a. Pichia pastoris. Expression. Cell survival. Hepatocellular carcinoma.

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residues of amino acids and an identical sequence to its human endogenous form. However, INF-β-1b has 165 amino acids with a mutation in its 17th amino acid (Cys → Ser) (Runkel et al., 1998).

Mammalian expression systems (e.g., CHO) introduce the correct post-translational modifications, the proper protein assembly and folding providing the protein with native structure and full biological activity. However, these expression systems have some disadvantages including expensive and time-consuming bioprocessing, low yields of proteins expression and difficulties to scaling up (Almo, Love, 2014). Bacterial expression systems such as Escherichia coli are usually used as a common host for the expression of non-glycosylated protein. They can provide higher yield of protein expression; however, most of the protein is produced as an insoluble and biologically inactive form due to lack of post-translational modifications. Therefore, the protein must be restored to its active and soluble form through a process called “refolding” which is usually a difficult, inefficient, and expensive procedure (Lilie, Schwarz, Rudolph, 1998; Baneyx, 1999; Schmidt, 2004). Accordingly, the production of proteins at high yield and in a fully bioactive form without requiring further processing would be more attractive.

Yeast expression systems such as Pichia pastoris have some advantages including ease of application, reduced time and cost of bioprocessing and high level of protein expression (Macauley-Patrick, et al., 2005). This strain is as easy to be manipulated as E. coli, while it has many attractive features such as rapid growth at high cell density, proper protein folding and post-translational modifications, tightly regulated promoters and possibility to produce both intracellular and secreted proteins (Damasceno, Huang, Batt, 2012; Ahmad, et al., 2014).

In this study, the codon optimized synthetic INF-β-1a gene was cloned and then expressed in P. pastoris GS115. We also optimized culture conditions to improve the production and secretion of INF-β-1a protein. In order to evaluate the biological activity of INF-β-1a expressed in P. pastoris expression system, its effect on Hep-G2 proliferation was assessed as well.

**MATERIAL AND METHODS**

**Microorganisms and culture media**

*E. coli* Top10 as a host for plasmid propagation was cultured in Low-salt Luria Bertani (LB) medium supplemented with 50µg/mL Zeocin. *P. pastoris* strain GS115 which has a mutant allele of the HIS4 (HIS4-) was used as a host for the expression of recombinant IFβ-1a. This strain was cultured in YPD medium containing 10 g of glucose, 20 g of peptone, and 10 g of yeast extract per 1 L.

**Construction of the INF-β-1a expression vector and transformation**

The INF-β-1b gene was designed based on the amino acid sequences of human interferon beta using the optimum codons for the expression in *P. pastoris. Xho* (5′) and *Xba* (3′) restriction sites, a carboxyl-terminal hexa-His tag and a stop codon were also included into the sequence. The codon-optimized INF-β-1b gene was synthesized and commercially cloned by Biomatik (Canada) into pPICZαA expression vector using *Xho* (5′) and *Xba* (3′) restriction sites. Recombinant pPICZαA–INF-β-1a was propagated in *E. coli* and after isolation, it was linearized by digestion with *Sac* restriction enzyme to facilitate the integration of INF-β-1a into the yeast genome by homologous recombination. Then the linear plasmid was transformed into *P. pastoris* GS115 by electroporation (Bio-Rad, Gene Pulser Xcell at 1500 V, 150 Ω and 25 µF with 0.2 cm cuvette) according to company instruction. The transformed cells were incubated at 30°C on YPDS (1% (w/v) yeast extract, 2% (w/v) peptone and 1% (w/v) dextrose supplemented with 1 M sorbitol) ager plates with 50-400 µg/ml Zeocin for 5 days and then,18 Zeocin-resistant clones were isolated for further study.

**Polymerase Chain Reaction (PCR) analysis of *P. pastoris* integrants**

Integration of the INF-β-1b gene into genome of isolated *P. pastoris* colonies was evaluated by colony PCR
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After cell lysis by heat treatment, the sample was centrifuged and the supernatant containing the genomic DNA was used as a template for PCR, using universal primers for AOX1 (forward: GAC TGG TTC CAA TTG ACA AGC and reverse: GCA AAT GGC ATT CTG ACA TCC). The standard cycling conditions used for PCRs were as follows: five minutes at 95°C, 30 cycles of 45 seconds at 95°C, one minute at 50°C, two minute at 72°C, and one cycle for five minutes at 72°C.

**INF-β-1a expression in *P. pastoris* and optimization of culture conditions**

One hundred milliliters of YPD medium was inoculated with a single positive colony followed by 24 hours incubation at 30°C and 200 rpm until the OD600 reached to 2–5. The cells were then collected by centrifugation followed by induction with methanol for 4 days. At certain time intervals (0, 24, 48, 72 and 96 h), the samples were taken for protein analysis using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

The concentration of secreted recombinant INF-β-1a from *P. pastoris* cells was the main criteria for the optimization. To increase the yield of INF-β-1a production in *P. pastoris*, optimization of growth and induction conditions such as methanol concentrations (0.5, 1, 2, and 3%), pHs (6.0, 7.0, and 8.0) and temperature (20, 25, and 30°C) were performed. *P. pastoris* cell without vector was used as a negative control.

**Purification of INF-β-1a**

The culture supernatant was loaded on a Ni-NTA resin column (Invitrogen, USA) which was pre-equilibrated with 10 column volumes containing 50 mM phosphate, 0.5 M NaCl, and of pH 8.0. The supernatant was then washed with 50 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, and pH of 8.0. Then his-tagged protein was eluted with 50 mM sodium phosphate, 0.5 M NaCl, 250 mM imidazole, and pH of 8.0. The protein concentration in each fraction was evaluated using Bradford method (Kruger, 2002) and equal amount of each sample was subjected to SDS-PAGE.

**SDS-PAGE and immunoblot analysis**

Electrophoresis of the proteins was performed as described previously (Akbari *et al.*, 2014). The proteins were separated using 15% acrylamide gels followed by staining with coomassie brilliant blue. For immunoblotting an equal amount of protein was loaded into a 15% SDS–PAGE gel, and was then transferred to a PVDF membrane. After that, the membrane was then blocked with 3% skimmed milk at 2-8°C overnight. The membrane was then subjected to the Anti-6X His tag® antibody (HRP) (abcam, USA) for 2 h at room temperature. Finally, the protein bands were detected using 3,3’-diaminobenzidine (DAB) solution.

**Cell culture**

The human liver hepatocellular carcinoma cell line HepG2 was obtained from the Pasteur Institute of Iran, Tehran. HepG2 cells were cultured in high glucose Dulbecco’s modified eagle medium (DMEM) supplemented with fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified incubator at 37°C with 5% CO₂. The cells were fed every 2 days and passaged upon reaching a maximum confluence of 80%.

**Cell viability Assay**

The effect of recombinant IFβ-1a on HepG2 cell viability was evaluated by MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay as described previously (Akbari *et al.*, 2015). In brief, the cells (0.6×10⁴ cells per well) were seeded into a 96-well plate followed by an overnight incubation at 37°C to allow cells attachment. Various concentrations of CinnoVex (CinnaGen, Iran) and produced recombinant protein were added to each well and cells were further incubated for 72 h. After that, 20 μl of MTT reagent (5 mg/ml) was added to each well and incubated for 4 h at 37°C. Then the solution was removed and formazan crystals were dissolved by dimethyl sulfoxide (DMSO) and the absorbance was evaluated at 570 nm.
Statistical analysis

Cell viability assay was repeated three times to ensure reproducibility of the results. All data are presented as mean ± standard deviation (SD). Significant differences between positive control (CinnoVex) and the sample were calculated by statistical analysis (Student’s t-test) using SPSS version 16. $P<0.05$ was considered significant.

RESULTS AND DISCUSSION

Synthetic codon optimized INF-β-1a was cloned into pPICZαA plasmid via XhoI and XbaI restriction sites (Figure 1). This recombinant plasmid was linearized by SacI enzyme and was then transformed into the P. pastoris yeast GS115 strain using electroporation. The transformed cells were screened on YPDS agar plates with 50-400 μg/ml Zeocin. Eighteen Zeocin-resistant transformants were evaluated by colony PCR. As shown in Figure 2, six clones were positive recombinants exhibiting a band of 1035 bp. A weak 2070 bp band was also observed indicating the gene amplification in the yeast genome. Six positive yeast colonies, called 1, 2, 5, 11, 16 and 17, were selected based on the colony PCR screening. These recombinants were cultured at 30°C to an OD$_{600}$ of 2-5, and then 1% final concentration of methanol was added every 24 h to induce the expression of INF-β-1a. Every 24 h, samples were collected for further analysis by SDS-PAGE. Protein analysis of the supernatants (Figure 3) showed that the colony 11 secreted the highest level of interferon (a protein band of approximately 22 kDa) among six recombinants. We also found that 72 h fermentation yielded a higher amount of protein.

FIGURE 1 - Schematic diagram of pPICZαA–INF-β-1a expression vector.
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**FIGURE 2** - Colony PCR of *Pichia* transformants with pPICZαA–INF-β-1a. Lane 1-18: eighteen Zeocin-resistant transformants; M: DNA marker; P: The positive control used the expression vector pPICZαA–INF-β-1a.

**FIGURE 3** - SDS-PAGE analysis of INF-β-1a expression at different time intervals (24 h, 48 h, 72 h, 96 h) for culture supernatant of recombinants 1, 2, 5, 11, 16 and 17 (lane 1-6). M: Protein markers (kDa). The expressed INF-β-1a (22 kDa) was indicated by the arrows.
Due to importance of INF-β-1 in the management of MS, many researchers have attempted to produce it in large quantities using different expression systems. For instance, overexpression of INF-β-1-b was reported in *E. coli*; however, most of the proteins were expressed in an insoluble and inactive form (Mobasher et al., 2016). They concluded that low expression of soluble and active from of INF-β-1-b is caused by toxicity of the protein for bacterial cells. In another study, soluble INF-β-1-b was expressed and successfully secreted into the periplasmic space of *E. coli*, but there was no report on its biological activity (Morowvat et al., 2014). In addition to bacterial systems, mammalian cells such as CHO were also used for the expression of INF-β-1-a by many research groups. The major problems that they reported were low level of protein production and instability and aggregation of INF-β-1-a (Rodriguez et al., 2005). However, reducing the culture temperature from 37°C to 30°C significantly increased the yield and stability of the monomeric protein expressed by CHO (Rodriguez et al., 2005). *Saccharomyces cerevisiae* was also used for the secretory expression of INF-β-1-a; however, only 30 µg/L of INF-β-1-a was detected in the culture supernatant due to toxicity of the protein for yeast cells (Demolder, Fiers, Contreras, 1994). Co-expression with chaperone molecules (*e.g.*, HSP70) resulted in a significant improvement in the secretion of INF-β-1-a (150 µg/L) (Demolder, Fiers, Contreras, 1994).

The current study tried to reach the maximum expression of IFN-β-1a by optimization of culture conditions. At first, different concentrations of methanol (0.5-3 %) were used to induce protein expression. As shown in Figure 4.a, a slightly increased INF-β-1a expression was occurred when methanol feeding of 2 % was used. In another study a range of methanol concentrations (0.5-1.5%) was used for the expression of maltooligosyltrehalose synthase by *P. pastoris* (Han et al., 2017). They found that induction with 1% methanol led to higher enzyme activity and biomass production (Han et al., 2017). Feeding the culture with appropriate level of methanol is very important for protein expression using AOX1 promoter. While methanol, as a carbon source, can increase the cell growth, accumulated methanol can lead to cell death (Boettner et al., 2002).

Temperature was another parameter influencing the expression level of IFN-β-1a and the highest protein production was observed at 30°C whereas no significant protein expression was detected at lower temperatures (25 and 20°C) (Figure 4.b). Similarly, many other studies suggested that the induction of protein expression at 28-30°C could result in higher protein production and cell growth. The effect of different temperatures (25, 30, 32 and 35°C) on the production of human epidermal growth factor (hEGF) in *P. pastoris* was investigated and the optimal temperature for protein expression was found to be at be 29°C (Eissazadeh et al., 2017). Finally, to evaluate the effect of pH, the expression of INF-β-1a was induced by 2 % methanol at 30°C, and the pH of medium was adjusted to 6.0, 7.0 and 8.0. The INF-β-1a expression was detected at pH level of 7.0, while no protein expression was observed at pHs of 6.0 and 8.0 after 72 h fermentation (Figure 4.c). However, protein expression at pHs of 6.0 and 8.0 was observed after 24 and 48 h of induction (data were not shown). Lower cell lysis was observed at pH of 7.0 compared with pH of 8.0, suggesting that the expression of protein at pH of 7.0 can improve the yield of protein secretion and stabilize INF-β-1a expression. In addition to temperature, medium pH plays a critical role for high-yield protein production since it can influence both protein expression and stability. *P. pastoris* can grow well between the pHs of 3 to 7. Based on our results, the optimal pH for the expression of IFN-β-1a was 7 as pH value of 8 did not support the cell growth leading to cell lysis; additionally, protein expression was not observed at pH of 6 after 72 h, possibly due to lower stability of the protein and higher activities of the proteolytic enzymes. In agreement with our findings, it was reported that the optimum pH for the protein expression of a human muscarinic acetylcholine receptor by *P. pastoris* was 7.0 (Asada et al., 2011).
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**FIGURE 4** - SDS-PAGE analysis of INF-β-1a expression at different culture conditions for the supernatant of recombinant 11 after 72 h fermentation. **a)** Optimization of inducer concentrations; Lane 1-4: final concentration of methanol 0.5, 1, 2 and 3 %; M: Protein markers (kDa). **b)** Optimization of temperature. Lane 1-3: 20, 25 and 30°C; lane 4: the supernatant of recombinant culture before induction as a negative control; M: Protein markers (kDa). **c)** Optimization of pH. Lane 1-3: pH 8.0, 7.0 and 6.0; non-recombinant *P. pastoris* strain GS115 as a negative control; M: Protein markers (kDa). The expressed protein was indicated by the arrows.

After finding the optimum culture conditions for the expression of INF-β-1-a, the culture supernatant was concentrated (Figure 5.a) using an ultrafiltration membrane (Amicon Ultra-15 centrifugal filter unit, 10K NMWL; Millipore, Germany) and the sample was loaded into Ni-NTA resin column. After washing, the protein was eluted using a buffer containing 250 mM imidazole (Figure 5.b). The purity of INF-β-1a was estimated to be approximately 90%. The concentration of the purified INF-β-1a was measured to be 155 μg/mL by Bradford assay, and 100 ml culture yielded 1.55 mg of purified INF-β-1a. Another study, reported the expression of INF-β-1-a via pPIC9 vector and without codon optimization and the yield was 8 mg/L culture medium (Skoko *et al.*, 2003). In contrast, we used pPICZα A vector and a codon optimized sequence for expression in *P. pastoris*. The lower yield compared to the results of the present study might be due to the effect of codon usage biases on the heterologous expression efficiency of *P. pastoris*. Several studies reported the improvement of recombinant protein expression in host cells via codon optimization. For example, optimization of keratinase gene according to codon biases of *P. pastoris* significantly increased the expression level of the protein (Hu *et al.*, 2013).
FIGURE 5 - a) Western-blot analysis of culture supernatants of GS115 strain of *P. pastoris* expressing INF-β-1a using the Anti-6X His tag® antibody (HRP). Lane 1: Negative control; Lane 2: The non-concentrated sample; Lane 3: The concentrated sample; M: Prestained protein marker (kDa). b) SDS-PAGE analysis of protein purification using Ni-NTA resin column. Lane 1: Flow-through fraction after loading of concentrated protein into the column; Lane 2: Flow-through fraction after washing the column with a buffer containing 20 mM imidazole; Lane 3: Flow-through fraction after elution with a buffer containing 250 mM imidazole; M: protein marker (kDa).

The biological activity of produced INF-β-1a was evaluated based on its effect on proliferation of HepG2 cells. Additionally, we compared the anti-proliferative activity of produced INF-β-1a to a commercially available form of this protein, called as CinnoVex (CinnaGen, Iran). As shown in Figure 6, treatment of cells with 0.18, 0.75 and 3 µg/ml of INF-β-1a for 72 h led to significant reduction of cell viability (*P* value <0.05) to 84.8, 61.3 and 45.1 %, respectively. Similarly, 72 h incubation of cells with the concentration of 0.18, 0.75 and 3 µg/ml of CinnoVex led to reduction of cell viability to 85.5, 74.5 and 64.2 %, respectively. The inhibitory effect of IFN-β on 13 liver cancer cell lines was previously investigated *in vitro* (Ogasawara *et al.*, 2007). The anti-proliferative effect of IFN-β on hepatocellular carcinoma cells might be mediated through IFN-β signal transduction pathway, such as JAK1, Trk2, and STAT1 (Damdinsuren *et al.*, 2007). Here, we also found that IFN-β-1a produced by *P. pastoris* has significantly (*P* value <0.05) more inhibitory effect compared with one produced by CHO (CinnoVex) at concentrations of 0.75 and 3 µg/ml which might be due to differences in glycosylation patterns. It was also reported that recombinant proteins expressed in *P. pastoris* tend to be hyperglycosylated (hypermannosylation) (Dean, 1999). *Similarly, it was showed that* hyperglycosylated IFN-β-1a produced by CHO had better anti-proliferative effect compared to Rebif® (non-glycoengineered one) (Song *et al.*, 2014). Pervious studies also suggested that IFN-β-1a produced in CHO exhibited lower growth inhibition of hepatoblastoma cells compared to the native one produced by fibroblast cells due to its sugar chain structure (higher triantennary sugar chains and more desialylated neutral form) (Utsumi *et al.*, 1995).
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**FIGURE 6** - The effect of INF-β-1a on viability of HepG2 cells as determined by MTT assay. Cells were incubated with various concentrations of INF-β-1a and CinnoVex, as a positive control, for 72 h (mean ± SD, n=8). Asterisk (*) indicates the means which were significantly different (*P*<0.05) from the positive control.

**CONCLUSION**

The present study aimed to improve the expression and secretion of INF-β-1a by *P. pastoris* via optimizing culture conditions. The highest expression (15.5 mg/L) was observed when the expression of protein was induced by 2% methanol, at pH of 7.0 and at temperature of 30°C. The secreted protein was purified up to 90% using Ni-NTA resin column; however, to improve the purity other chromatographic techniques can be applied. The produced INF-β-1a by *P. pastoris* had a significant anti-proliferative activity against HepG2 cell line even more than the commercial form produced by CHO which confirmed by MTT assay. It could be explained based on the difference in the glycosylation patterns of expression hosts.

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**DECLARATION OF INTEREST STATEMENT**

All authors declare that they have no conflict of interest.

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