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Research Article
Expression of Mouse Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) in *Pichia pastoris*

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Abstract

Background and Objectives: Granulocyte-macrophage colony stimulating factor (GM-CSF) is a hematopoietic growth factors involved in proliferation and differentiation of bone marrow precursor cells into granulocytes, monocyte that used in treatment of patient with cancer, therefore, it was decided to express murine GM-CSF as a recombinant protein in *Pichia pastoris* (*P. pastoris*). **Materials and Methods:** The protein sequence of mouse GM-CSF was obtained from Uniprot database and ordered for synthesis after back translation and optimization of coding sequence. It was subsequently cloned into pPICZαA expression vector expressed in *Pichia pastoris*. Expression and activity of GM-CSF was confirmed by SDS-PAGE, dot blot, Western blotting in the culture supernatant. Biological activity of the product was confirmed by promoting FDC-P1 cell growth. Non-linear regression analysis was used for fitting a curve onto the cell proliferation data plotted against the growth factor dilutions and calculation of EC_{50} value. **Results:** Sequence optimization improved CAI from 0.66 in the native to 0.86 in the optimized GM-CSF sequence through replacing 24% of the nucleotides. In dot blot analysis, GM-CSF expression reached its peak on 5 days and started to decline after 7 days. The GM-CSF protein was appeared as bands with apparent molecular mass nearly 16-17 kDa. It was estimated that 18.5 μg mL^{-1} of active murine GM-CSF was secreted into the culture supernatant. **Conclusion:** The recombinant GM-CSF protein was expressed in *Pichia pastoris* yeast and was biologically active in the FDC-P1 cell line proliferation.

Key words: Mouse GM-CSF, *Pichia pastoris*, FDC-P1 cell line, codon optimization

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Granulocyte monocyte colony stimulating factor (GM-CSF) also known as colony stimulating factor 2 (CSF2), is a monomeric glycoprotein. It is secreted by Tcell, endothelial cells, fibroblasts and natural killer cells and stimulates stem cells to produce granulocytes (eosinophil, basophils and neutrophils). Human recombinant GM-CSF can be used to induce the proliferation of neutrophils or monocytes[1-3] in the patients with suppression of bone marrow due to chemotherapy or radiotherapy[4]. Patients treated with GM-CSF showed a significant increase in number of WBCs and thus decrease the use of antibiotics and duration of staying in hospital and mitigating the treatment costs[5]. Host's immunological defect in number and function of lymphocytes, monocytes and neutrophils is crucially associated with opportunistic infections in AIDS patients. A study evaluated the effects of GM-CSF in AIDS patients, a dose-dependent increase in number of neutrophil, monocytes and eosinophils was noted[6,7].

Numerous researches were done on recombinant GM-CSF and its therapeutic applications. The GM-CSF was available in 3 forms of Molgramostim, Sargramostim and Regramostim produced in bacteria, yeast and mammalian cells, respectively[8]. Among eukaryotic systems used for recombinant protein production, *Pichia pastoris* was very popular and generally regarded as a safe microorganism and its recombinant protein products were endotoxin-free with proper folding and post-translational modifications. Additionally, the ability to secret the proteins in culture medium allows collection of the products from the culture supernatant. Therefore, it was used for production of many mammalian recombinant proteins, such as Ecallantide, were produced in large scales[9]. This study reported the production of recombinant murine GM-CSF encoded by an optimized sequence in *Pichia pastoris* expression system.

MATERIALS AND METHODS

Design and analysis of GM-CSF sequence: The GM-CSF peptide sequence (P01587) was obtained from Uniprot website (Fig. 1). The amino acids 18-141, required for its biological activity, were selected for back-translation, codon optimization and synthesis of the coding construct.

Gene optimization and synthesis: Coding sequence for mouse GM-CSF was optimized by GENEray Biotechnology (Shanghai, China). Codon adaptation index (CAI) for the optimized sequence was determined using "rare codon analysis tool" from GenScript which indicated the overall correlation between codon frequencies in a given coding sequence with highly frequent codons in the host. The CAI values above 0.8 were favorable for optimum expression. Additionally, it was believed that codons with less than 30% frequencies in *Pichia pastoris* can hamper the expression. Codon tables for the native and optimized coding sequences for GM-CSF were created by Geneious software (Biomatters, New Zealand) and compared with the codon table for *P. pastoris*. Restriction sites for XhoI and XbaI were also added to the sequence, followed by adjusting the translation frame for correct expression of GM-CSF with α-factor, c-Myc and HIS tag in the expression vector. The final construct was synthesized by Generay Biotech Co, Ltd.

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      10         20         30         40         50
MWLQNLLFLG IVVYSLSAPT RSPITVTRPW KHVEAIKEAL NLLDDMPVTL
      60         70         80         90        100
NEEVEVVSNE FSFKKLTCVQ TRLKIFEQGL RGNFTKLKGA LNMTASYYQT
     110        120        130        140
YCPPTPETDC ETQVTTYADF IDSLKTFLTD IPFECKKPGQ K
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Fig. 1: Murine GM-CSF amino acid sequence. Residues 1-17 (shown in red) act as signal peptide. Residues which are required for its biological activity are shown in black

Expression of GM-CSF: The synthetic DNA encoding GM-CSF was subcloned using XhoI and XbaI restriction sites into pPICZαA vector (Invitrogen, Carlsbad, CA, USA) for expression in *P. pastoris*. The pPICZαA containing GM-CSF fragment (pPICZ-GM-CSF) was propagated and linearized using SacI enzyme before electroporation into competent *P. pastoris* (both KM71H and GS115 strains) as recommended by the supplier (Invitrogen). Transformed KM71H and GS115 cells were selected on YPDS (yeast extract 1%, peptone 2%, dextrose 2%, sorbitol 18.2% and agar 2%) containing 100 μg mL^{-1} of Zeocin (Invitrogen). Single colonies taken from the YPDS plates were inoculated into 2 mL of BMGY (yeast extract 1%, peptone 2%, yeast nitrogen base 1.34%, glycerol 1% and biotin 4×10^{-5}% in 100 mM potassium phosphate buffer (pH 6.0). When the culture's OD_{600} reached 1.5, the BMGY medium was replaced with BMMY (substituting 2% methanol rather than 1% glycerol in BMGY). The cultures were incubated at 25°C with 250 rpm rotation and samples (200 μL) were collected for protein analysis on the following days.

Protein purification and analysis: The samples collected from the culture supernatant, purified GM-CSF and flow-through were analyzed dot blotting, SDS-PAGE and Western blotting. The samples dot blotted on nitrocellulose membrane (Sigma-Aldrich), followed by probing with anti c-Myc mouse monoclonal IgG1 antibody (Santa Cruz Biotech., Dallas, TX) and mouse anti-IgG conjugated with HRP (Sigma-Aldrich, St. Louis, MO). The presence of antibody interaction with the GM-CSF fusion protein was visualized using ECL reagent and the chemiluminescence was recorded by an Alliance Mini instrument (UVItec Limited, Cambridge, UK).

The supernatant samples were also resolved on SDS-PAGE and stained using Coomassie Brilliant Blue R250 (Sigma-Aldrich). Additionally, the proteins transferred onto nitrocellulose membrane detected and recorded as described for dot blot analysis.

Evaluation of the GM-CSF biological activity: Biological activity of the product was confirmed by growth stimulation of murine factor dependent FDC-P1 (ATCC ID: CRL-12103) were used in biological activity assay of the product. Briefly, serial dilutions of the GM-CSF product were prepared in 96-well tissue culture plates in DMEM medium (GIBCO, Invitrogen) with 10% FCS (JRH). The FDC-P1 cells were washed with growth factor-free DMEM medium and 4×10^4 cells were added to each well and the plates were incubated at 37°C in a humidified 5% CO_2 in air atmosphere for 48 h. Then 20 µL of resazurin reagent[10] were added into each well and incubated for 4 h. The relative number of viable cells per well was determined based on measuring fluorescence intensity of resorufin produced by reduction of resazurin using a Victor X5 plate reader (Perkins Elmer, Waltham, MA). The results were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA).

RESULTS

Optimization of coding sequence for GM-CSF: Results depicted in Fig . 1, the residues 1-17 of murine GM-CSF act as signal peptide and are not necessary for biological activity. Therefore, only coding sequence for residues 18-124 were included in the synthetic construct. The coding sequence was optimized for expression or in *P. pastoris*. Sequence alignment revealed that 24% of nucleotides changed upon optimization of native GM-CSF. These nucleotide substitutions resulted in CAI improvement from 0.66 in the native to 0.86 in the optimized GM-CSF sequence. The GC content however was not significantly changed (39.65 vs. 39.61%) upon optimization. In the original GM-CSF sequence 42% of codons were the codons with frequency range of 91-100 codons which increased up to 61% in the optimized sequence. Additionally, all the codons with less than 50% frequency were replaced with more frequent ones in the optimized sequence (Fig. 2). Cloning simulation of optimized sequence of GM-CSF into pPICZαA plasmid demonstrated that the GM-CSF was in the correct frame to be expressed as a fusion protein with c-Myc and 6xHIS tag (Fig. 3).

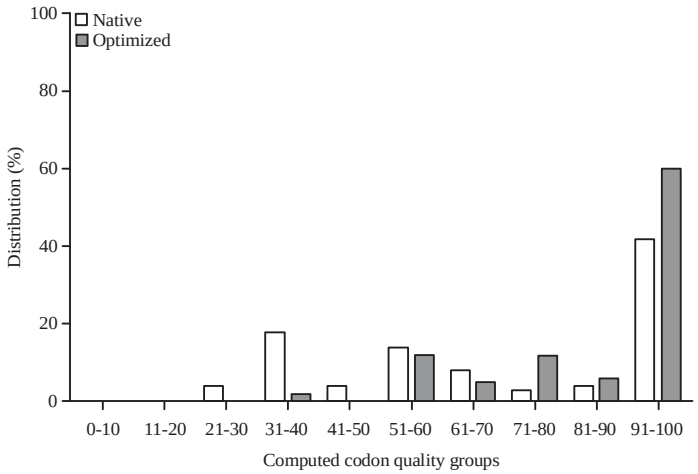

Fig. 2: Percentage of codons grouped based on their quality in the native and optimized GM-CSF. The group 91-100 indicates codons with highest frequency for a given amino acid in the organism. Codons with numbers lower than 30% can interrupt translation

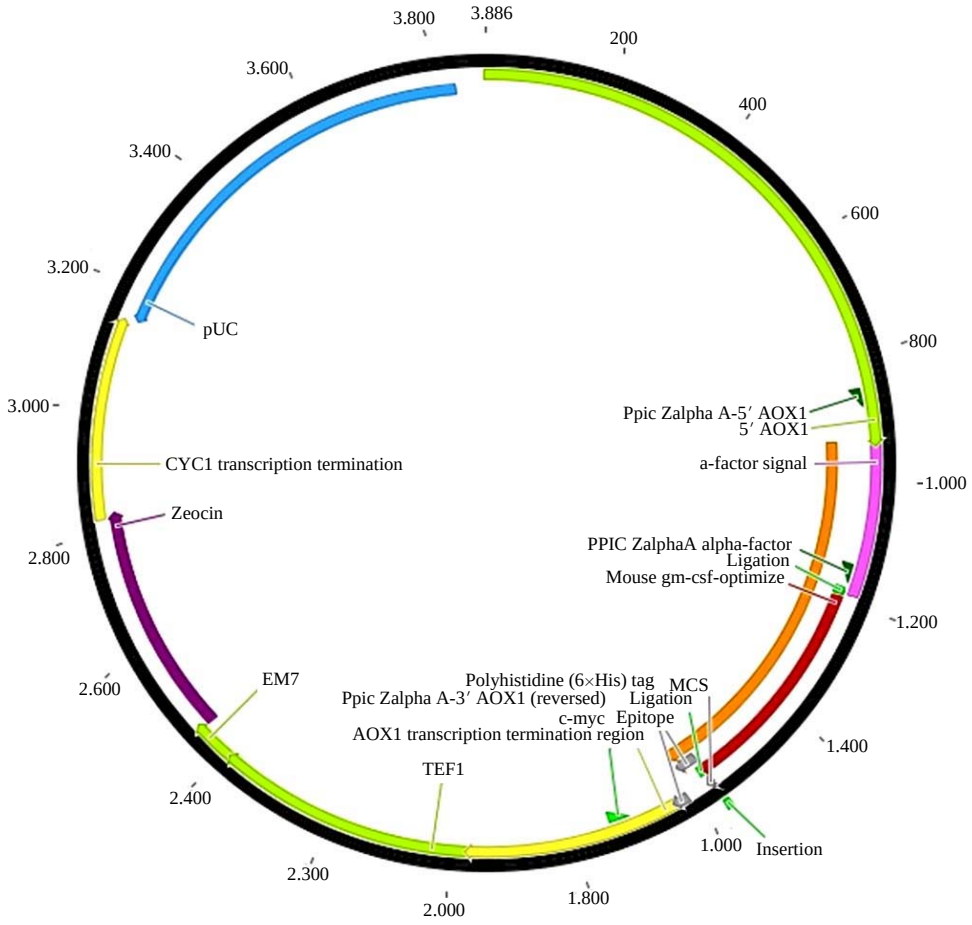

Fig. 3: Mapping GM-CSF in pPICZαA plasmid. Optimized GM-CSF segment (green) is cloned downstream to α-factor (pink) and upstream to c-Myc and 6xHIS taq (grey) coding sequence. Primer binding sites are indicated with green arrows. Ligation sites for GM-CSF are also shown with green rectangles

Subcloning GM-CSF fragment from pGH into pPICZαA vector: Double digestion of the pGH vector resulted in extraction of GM-CSF (a384-bp fragment) (Fig. 4), which was subsequently ligated into digested pPICZαA vector (Fig. 5). The products of ligation was transformed into and propagated in JM109. Presence of a 384 bp fragment in the double digested plasmid confirmed the insertion of GM-CSF fragment in the pPICZαA vector (Fig. 6). Sequencing the pPICZαA-GM-CSF plasmid demonstrated the presence of accurate GM-CSF coding sequence and translational frame in the pPICZαA-GM-CSF plasmid.

GM-CSF expression in *P. pastoris* (KM71H strain): In dot blot analysis, the supernatant of the KM71H culture transformed using pPICZ-GM-CSF (KM-GM-CSF) reacted strongly with c-Myc antibody (Fig. 7) which indicated that GM-CSF expression reached its maximum on 5 days and remained constantly high up to 7 days. However, after 7 days the GM-CSF degradation overtook its production rate.

In SDS-PAGE analysis, the GM-CSF protein was appeared as two bands with apparent molecular mass about 16-18 kDa in the supernatant of the KM-GM-CSF (Fig. 8). This was consistent with the prediction that the GM-CSF fusion protein c-Myc epitope and 6xHIS taq to have 147 amino acids and molecular weight of 16.8 kDa. Such a protein band did not existed in the supernatants of *P. pastoris* which was not transformed (lane 2) and the yeast transformed with only pPICZαA empty vector. Additionally, GM-CSF seems to be the major protein bands in the supernatant of KM-GM-CSF.

Results showed in Fig. 9 predict that the Western blotting results of the 3 different clones of KM-GM-CSF. This demonstrates that both protein bands with molecular mass between 15 and 25 kDa were indeed GM-CSF fusion protein. Differences in the molecular weight may resulted from variations in the glycosylation.

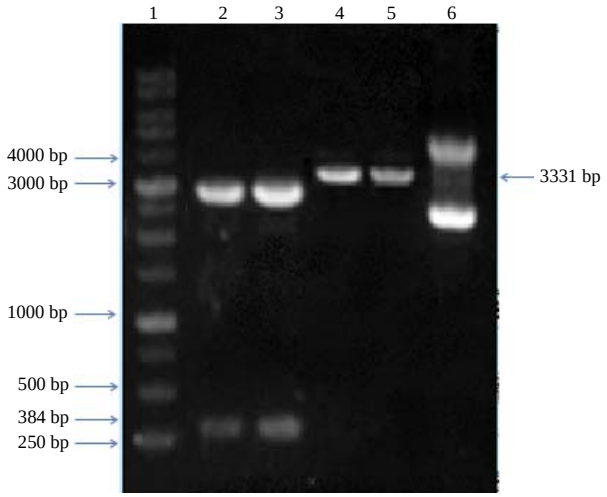

Fig. 4: Electrophoresis of pGH-GM-CSF plasmid extracted from JM109 bacteria and its enzymatic digestion. 1 DNA marker. 2, 3 Double digestion of plasmid with XbaI/XhoI enzymes. 4 and 5 Single digestion of plasmid with Xbaland XhoI, respectively. 6 Undigested plasmid

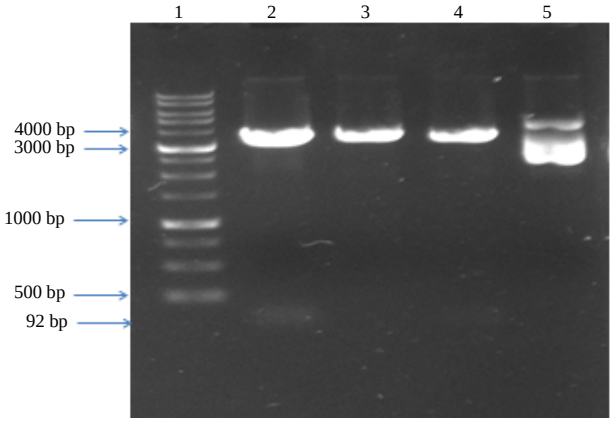

Fig. 5: Electrophoresis of extracted pPICZαA plasmid enzymatic digestion. 1 DNA marker, 2 Double digestion of plasmid with XbaI/XhoI enzymes, 3 and 4 Digestion of plasmid with XbaIandXhoI enzymes, respectively, 5 Undigested plasmid

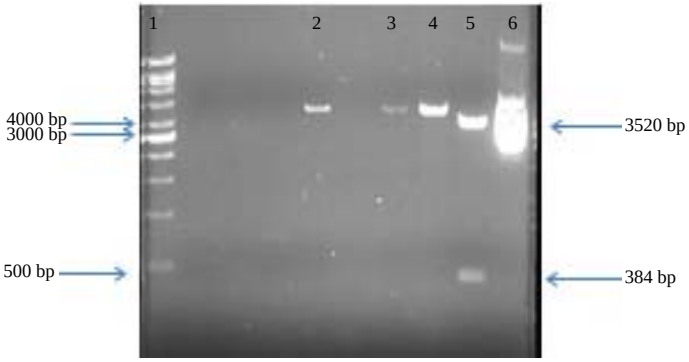

Fig. 6: Digestion of pPICZαA-GM-CSF plasmid with XhoI and XbaI enzymes. 1 Kb1 marker, 2 Enzymatic digestion with SacI, 3 and 4 Enzymatic digestion with XbaI and XhoI, respectively, 5 Double digestion with XhoI/XbaI, two DNA fragments of 3520 and 384 bps are indicated with arrows in the right-side, 6 Undigested recombinant plasmid

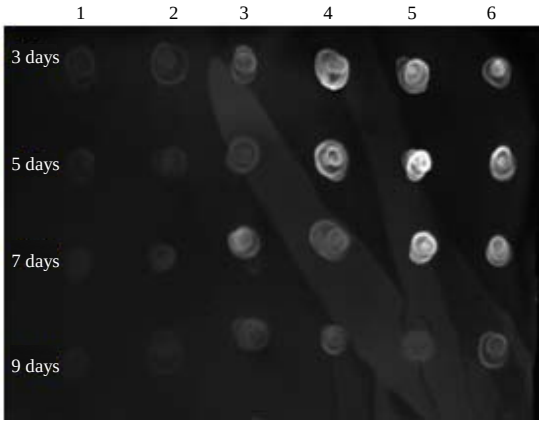

Fig. 7: Dot-blot analysis for GM-CSF fusion protein expression. Columns 1 and 2: Negative controls (untransformed KM71H strain and KM71H transformed with pPICZαA empty vector), Columns 3-6: KM71H transformed with pPICZ-GM-CSF vector. The product probed using anti c-Myc epitope

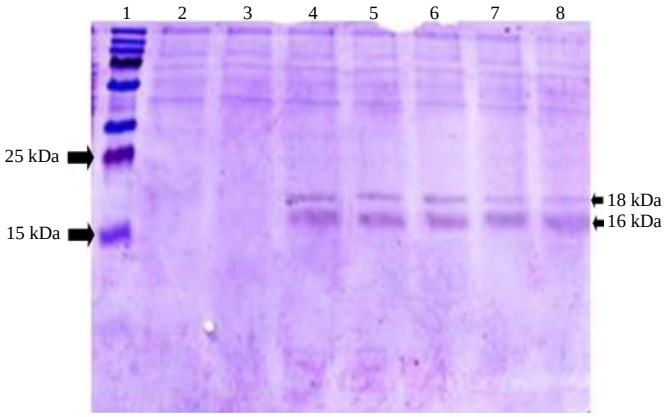

Fig. 8: SDS-PAGE analysis of GM-CSF expression in the culture supernatants. Lane 1: Protein marker, lane 2: Untransformed KM71H), lane 3: KM71H transformed with pPICZαA empty vector, lanes 4: 5 days from KM71H transformed with pPICZ-GM-CSF (clone 1). Lanes 5 and 6: 4 and 5 days from KM71H transformed with pPICZ-GM-CSF (clone 2). Lanes 7 and 8: 4 and 5 days from KM71H transformed with pPICZ-GM-CSF (clone 3). The protein bands corresponding to the molecular mass of GM-CSF are indicated by arrows on the right side

Evaluation of recombinant GM-CSF biological activity: Biological activity of the recombinant GM-CSF in stimulating proliferation of FDC-P1 cells was showed in Fig. 10 which indicate that the supernatant of the KM-GM-CSF could support 50% of the maximum growth when diluted 371200 times.

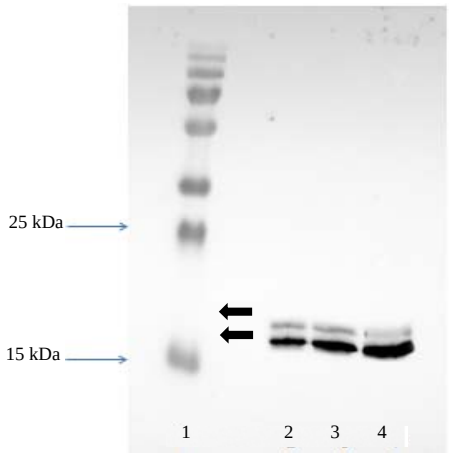

Fig. 9: Western blot analysis of the culture supernatants. Lane 1: Protein marker. Lanes 2-4: KM71H transformed with pPICZ-GM-CSF (clones 1-3)

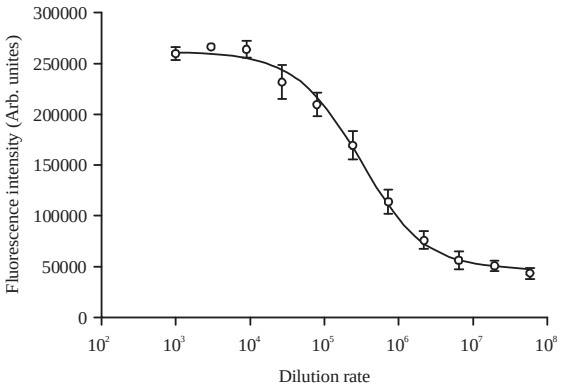

Fig. 10: Biological Activity of the recombinant GM-CSF protein. The FDC-P1 cell growth was determined in serial dilutions of the culture supernatant using resazurin assay

Comparing to the commercial product (Peprotech) with the ED_{50} value of 0.05 ng mL^{-1}, it was concluded that there was 18.5 μg mL^{-1} of active murine GM-CSF in the KM-GM-CSF culture supernatant.

DISCUSSION

Prokaryotic systems such as *E. coli* were routinely used to express recombinant proteins, however, their protein product was not glycosylated and had low stability and half-life in the laboratory settings. Additionally, these products might be contaminated to bacterial lipopolysaccharide which can induce fever and influence accuracy of laboratory results[11]. Yeasts as a eukaryote, were more sophisticated expression system with capacity for

processing, folding and post-translation modifications of heterologous proteins.

In this study, GM-CSF sequence was optimized for *P. pastoris* by replacing uncommon codons and removing negative cis-elements interfering with optimal expression. The CAI of the native GM-CSF sequence was 0.66 which increased to 0.86 after optimization which was above the 0.8 cut-off for optimal expression.

According to the dot-blot results, it seems that the GM-CSF expression was detectable on 3rd day and reached its maximum on 5th day. In SDS-PAGE analysis, GM-CSF appeared as two bands between 15 and 25 kDa which may resulted from various types of glycosylation. The GM-CSF sequence analysis revealed two N-glycosylation sites on amino acids 83 and 92. This heterogeneity in the molecular mass of the product was previously reported for CSF-1[12], FLT3L[13] and Staphylokinase[14]. During expression of staphylokinase, the protein band with higher molecular mass, which assumed to be more glycosylated, was disappeared upon adding tunicamycin to the culture media[14].

Donahue and colleagues compared GM-CSF expression in *E. coli* bacteria and *S. cerevisiae*. The recombinant GM-CSF from *E. coli* and *S. cerevisiae* were active in dilution rates up 1:1000 and 1:100000[15]. In present study, the supernatant of KM-GM-CSF culture was able to induce maximum cell growth up to a dilution rate of 1:100000, indicating that the yield was comparable with the other studies.

Based on its biological activity, the yield was estimated to be about 18.5 μg mL^{-1} of active murine GM-CSF. However, Asn83 is in the vicinity of GM-CSF interaction site with the receptor and its glycosylation may interrupt the ligand's function. Therefore, further investigation using glycosidase enzymes and tunicamycin (a glycosylation inhibitor) is required to confirm glycosylation effects on GM-CSF stability and function.

Using *P. pastoris* as the host for expression of GM-CSF has some advantages over *E. coli* and *S. cerevisiae*. This product not contain lipopolysaccharide which was pyrogenic with adverse effects on accuracy of the research experiments[16]. Additionally, *P. pastoris* unlike *S. cerevisiae* does not hyperglycosylate proteins[17,18], O-glycosylation rarely occurred and oligosaccharide chains composed of 8-14 mannose chain which were shorter than those produced by *S. cerevisiae* (50-150 mannose chain)[19]. Oligosaccharides with α 1, 3 bond which were highly antigenic, making the products more appropriate for therapeutic applications were not produced. Recently, GlycoS with technology was used for genetic engineering of *Pichia* for production of recombinant proteins with humanized glycosylation[20].

CONCLUSION

Recombinant mouse GM-CSF was expressed as a biologically active product in *P. pastoris* with high efficiency (18.5 μg mL^{-1} of the culture supernatant). The product seems to have some heterogeneity in glycosylation level demonstrated with two distinct bands of nearly 16 and 18 kDa. These results show that the optimized sequence used in this study can be used for production of GM-CSF to produce endotoxin-free product for various applications. Additionally, this procedure can be applied to produce other growth factors for research and potentially for therapeutic applications.

SIGNIFICANCE STATEMENT

This study discovers an optimized method for preparation of recombinant murine GM-CSF in *Pichia pastoris*, which resulted in production of 18.5 μg mL^{-1} of active, endotoxin-free GM-CSF in the culture supernatant. It was in secretary form which facilitates its homogeneous production and reduces the cost of further purification. This product can be used for *in vitro* and *in vivo* research involving culture of the hematopoietic cells.

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REFERENCES

1. Srour, E.F., J.E. Brandt, R.A. Briddell, S. Grigsby, T. Leemhuis and R. Hoffman, 1993. Long-term generation and expansion of human primitive hematopoietic progenitor cells *in vitro*. Blood, 81: 661-669.
2. Foss, D.L., A.M. Bennaars, C.A. Pennell, M.D. Moody and M.P. Murtaugh, 2003. Differentiation of porcine dendritic cells by granulocyte-macrophage colony-stimulating factor expressed in *Pichia pastoris*. Vet. Immunol. Immunopathol., 91: 205-215.

3. Qiu, J.T., T.C. Chang, C.T. Lin, Y.M. Chen, F.Q. Li, Y.K. Soong and C.H. Lai, 2007. Novel codon-optimized GM-CSF gene as an adjuvant to enhance the immunity of a DNA vaccine against HIV-1 Gag. Vaccine, 25: 253-263.
4. Mitsuyasu, R.T. and D.W. Golde, 1989. Clinical role of granulocyte-macrophage colony-stimulating factor. Hematol. Oncol. Clin. North Am., 3: 411-425.
5. Ehsani, M.A., M. Ordouee, P. Salamati, E. Shahgholi and K. Sotoudeh *et al*., 2006. Evaluation of efficacy and side effects of FILGRASTIM versus PD-Grastim in prevention of neutropenia in patients with neuroblastoma under treatment with OPEC chemotherapy protocol-A comparative study. Iran. J. Pediatr., 16: 319-324.
6. Groopman, J.E., R.T. Mitsuyasu, M.J. DeLeo, D.H. Oette and D.W. Golde, 1987. Effect of recombinant human granulocyte-macrophage colony-stimulating factor on myelopoiesis in the acquired immunodeficiency syndrome. N. Engl. J. Med., 317: 593-598.
7. Baldwin, G.C., J.C. Gasson, S.G. Quan, J. Fleischmann and R. Weisbart *et al*., 1988. Granulocyte-macrophage colony-stimulating factor enhances neutrophil function in acquired immunodeficiency syndrome patients. Proc. Natl. Acad. Sci. USA., 85: 2763-2766.
8. Yamauchi, T., K. Yada, A. Umemura, E. Asakura, T. Hanamura and T. Tanabe, 1996. Effect of recombinant human macrophage-colony stimulating factor on marrow, splenic and peripheral hematopoietic progenitor cells in mice. J. Leukocyte Biol., 59: 296-301.
9. Cregg, J.M., I. Tolstorukov, A. Kusari, J. Sunga, K. Madden and T. Chappell, 2009. Expression in the yeast *Pichia pastoris*. Methods Enzymol., 463: 169-189.
10. Mashkani, B., M.H. Tanipour, M. Saadatmandzadeh, L.K. Ashman and R. Griffith, 2016. FMS-like tyrosine kinase 3 (FLT3) inhibitors: Molecular docking and experimental studies. Eur. J. Pharmacol., 776: 156-166.
11. Nuc, P. and K. Nuc, 2006. Recombinant protein production in *Escherichia coli*. Postepy Biochem., 52: 448-456, (In Polish).
12. Mashkani, B., A.F. Odell, E.M. Byrnes, R. Griffith and L.K. Ashman, 2013. Expression of biologically active human colony stimulating factor-1 in *Pichia pastoris*. Protein Expression Purif., 88: 93-97.
13. Karimi, E., H. Faraji, D.H. Alamdari, M. Souktanloo, M. Mojarrad, L.K. Ashman and B. Mashkani, 2017. Over expression of functional human FLT3 ligand in *Pichia pastoris*. Applied Biochem. Microbiol., 53: 421-428.
14. Faraji, H., M. Ramezani, H.R. Sadeghnia, K. Abnous, F. Soltani and B. Mashkani, 2017. High-level expression of a biologically active staphylokinase in *Pichia pastoris*. Prep. Biochem. Biotechnol., 47: 379-387.
15. Donahue, R.E., M.M. Johnson, L.I. Zon, S.C. Clark and J.E. Groopman, 1987. Suppression of *in vitro* haematopoiesis following human immuno deficiency virus infection. Nature, 326: 200-203.
16. Wakelin, S.J., I. Sabroe, C.D. Gregory, I.R. Poxton, J.L. Forsythe, O.J. Garden and S.E. Howie, 2006. Immunol. Lett., 106: 1-7.
17. Daly, R. and M.T.W. Hearn, 2005. Expression of heterologous proteins in *Pichia pastoris*: A useful experimental tool in protein engineering and production. J. Mol. Recognit., 18: 119-138.
18. Tang, H., S. Wang, J. Wang, M. Song and M. Xu *et al*., 2016. N-hypermannose glycosylation disruption enhances recombinant protein production by regulating secretory pathway and cell wall integrity in *Saccharomyces cerevisiae*. Scient. Rep., Vol. 6. 10.1038/srep25654.
19. Brondyk, W.H., 2009. Selecting an appropriate method for expressing a recombinant protein. Methods Enzymol., 463: 131-147.
20. Laukens, B., C. de Wachter and N. Callewaert, 2015. Engineering the *Pichia pastoris* N-glycosylation pathway using the glycoswitch technology. Methods Mol. Biol., 1321: 103-122.