

Expression of granulocyte colony stimulating factor (GCSF) in *Hansenula polymorpha*

Yeganeh Talebkhan^{1*}, Tannaz Samadi¹, Armin Samie^{1,2}, Farzaneh Barkhordari¹, Mohammad Azizi¹,
Vahid Khalaj¹, Esmat Mirabzadeh¹

¹Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran

²Department of Biology, Azad University of Damghan, Iran

Received: October 2015, Accepted: December 2015

ABSTRACT

Background and Objectives: During past decades *Hansenula polymorpha* has attracted global attention for the expression of recombinant proteins due to its high growth rate, minimal nutritional requirements and use of methanol as a low cost inducer.

Materials and Methods: The corresponding nucleotide sequences for the expression of heterologous genes in *Hansenula polymorpha* were extracted and assembled in an *E. coli* vector. The constructed expression cassette included formate dehydrogenase promoter (pFMD), a secretory signal sequence, a multiple cloning site (MCS) and methanol oxidase (MOX) terminator. Zeocin resistance gene fragment and complete cDNA encoding granulocyte colony stimulating factor (GCSF) were cloned downstream of the expression cassette in-frame with signal sequence. Restriction mapping and sequence analysis confirmed the correct cloning procedures. Final vector was transformed into *Hansenula* and recombinant host was induced for the expression of GCSF protein by adding methanol. SDS-PAGE and immuno-blotting were performed to confirm the identity of r-GCSF.

Results: The expression cassette containing *gcsf* gene (615bp) and zeocin resistance marker (*sh-ble*, 1200bp) was prepared and successfully transformed into competent *Hansenula polymorpha* cells via electroporation. Zeocin resistant colonies were selected and GCSF expression was induced in recombinant *Hansenula* transformants using 0.5% methanol and an approximately 19kDa protein was observed on SDS-PAGE. Western blot analysis using serum isolated from GCSF-treated rabbit confirmed the identity of the protein.

Conclusions: Molecular studies confirmed the designed expression cassette containing *gcsf* gene along with pFMD and signal sequence. The expressed 19kDa protein also confirmed the ability of designed vector in expressing heterologous genes in *Hansenula* cells.

Keywords: *Hansenula polymorpha*, Expression cassette, GCSF.

INTRODUCTION

Expression of heterologous genes is usually per-

formed in prokaryotic host cells especially *E. coli* which have high growth rates and simple nutritional requirements. On the other hand, the mammalian expression systems attracted much attention for the production of those pharmaceuticals with high level of post-translational modifications. However statistical analyses show that in the worldwide biopharmaceutical marketing, beside *E. coli* and mammalian host cells, low eukaryotic microbial cells including yeasts

*Corresponding author: Yeganeh Talebkhan Garoosi, Biotechnology Research Center, Pasteur Institute of Iran, No. 69, Pasteur Ave. 13164, Tehran, Iran
Tel/Fax: + 98-21-66480780
E-mail: talebkhan@pasteur.ac.ir; ytgaroosi@gmail.com

offer important roles in manufacturing pharmaceuticals with low to moderate post-translational modifications such as glycosylation (1). Although the use of the first and the most characterized yeast cell, *Saccharomyces cerevisiae*, has been FDA approved for the production of recombinant insulin and Hepatitis B vaccines (2) but its drawbacks and limitations such as hyper-glycosylation of the proteins through α 1,3 bond of mannose chains to the N terminus of the produced protein has been confirmed to be allergenic.

Hansenula polymorpha (*Pichia angusta* or *Ogataea angusta*) is one of the substitutes for the baker's yeast which can grow in the presence of methanol (MeOH) as the only source of carbon and energy (3). In the presence of glucose, methanol oxidase (MOX) and catalase will be completely repressed whereas shifting to methanol induces their expression (4). *Hansenula's* whole genome sequence (5) has provided an excellent opportunity for molecular investigation of this yeast towards efficient expression of biopharmaceutical proteins. The key important features include the presence of inducible promoters in methanol metabolism and thermo-tolerance behavior of *Hansenula* cells which is critical for large scale fermentation process. Furthermore, it has been shown that the amount of secreted proteins in *Hansenula* medium is very low which facilitates the purification downstream processing. All mentioned features have made *Hansenula* as a suitable host for the expression of biopharmaceutical proteins such as interferon alpha-2a and hepatitis B vaccine (6).

The basic elements in *Hansenula* specific transcriptional unit are: a strong inducible promoter related to the MeOH metabolism, a suitable transcriptional terminator and a selection marker for screening the transformant cells. The latter can be an auxotrophic marker involved in pyrimidine or amino acid biosynthesis such as URA3, LEU2 (7) or a dominant resistance marker. For effective integration of the expression unit into the genome, *H. polymorpha*-derived autonomously replication sequence (e.g. *HARS1*), as a replication signal, will be added. Signal sequences can also be fused to the target protein for the secretion in to the medium.

In the present study, the construction of a *H. polymorpha* expression vector possessing zeocin resistance gene as the screening marker has been described and GCSF as a single chain peptide with minimum glycosylation pattern was expressed to examine the efficiency of the designed expression

cassette.

MATERIALS AND METHODS

Construction of *Hansenula* Expression cassette. A home made expression cassette in an *E. coli* cloning vector (pGH, 2900bp) was designed (pYT) containing the following transcriptional elements: pFMD (promoter of formate dehydrogenase) and MOX gene terminator (methanol oxidase, GenBank No. X02425.1). Nucleotide sequence of these elements was extracted from the published genome sequence of *Ogataeaapara polymorpha* (Strain CBS 4732) (5). A 25bp fragment containing restriction sites of unique enzymes including *Bam*HI, *Sac*I, *Hpa*I and *Bcl*I was also inserted into the downstream of selected promoter in the synthetic vector in an engineered design to be in one open reading frame with any gene of interest as a cloned gene fragment. In order to construct a secretory expression vector, *S. cerevisiae* alpha-mating factor (*alpha*-MFI) leader/signal sequence (267bp) was also inserted at the upstream of the multiple cloning site adjacent to the promoter region. *HARS1* (*H. polymorpha*-derived autonomously replication sequence-1) gene sequence (GeneBank No. X03540.1) was also inserted at 5' end of the expression cassette which is necessary for insertion of cloned gene into the *Hansenula* genome. Schematic view of the designed vector is shown in Fig. 1.

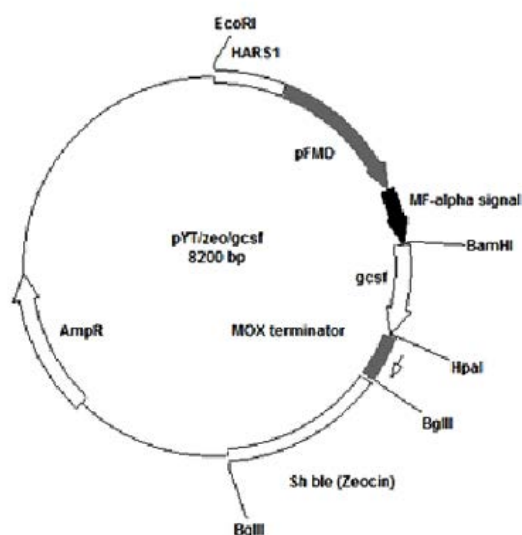


Fig. 1. Schematic view of constructed vector

Cloning of zeocin resistance selection marker.

Due to the prototrophic characteristics of available *Hansenula* host cell, a selection marker was required to identify recombinant clones. Zeocin resistance (*sh-ble*, *Streptoalloteichus hindustanus bleomycin*) gene was amplified through PCR with specific primers (F: 5'-gtagatcttgcggatcccc-3' and R: 5'- gtagatcttg-gtctccagcttgc-3') using *Pichia* pPICZ A vector (Invitrogen, USA) as a template DNA. Both sense and antisense primers contained 5' *BglIII* restriction site to facilitate the cloning procedure. PCR cycles were as follows: Initial denaturation at 94°C (5min), 30 cycles of 94 °C (1min), 60 °C (30sec), 72 °C (30sec) which followed with 3min extra extension at 72 °C. The resulting PCR product (approximately 1200bp in length) was cloned into *BglIII* site of final expression vector (Fig. 1). The recombinant plasmid was isolated from zeocin resistant *E. coli* clones grown on agar plates supplemented with zeocin (25µg/ml) and was verified through restriction analysis and PCR.

GCSF encoding sequence. A 615bp cDNA fragment encoding full length GCSF protein was extracted from Genbank (NM_172219.1) and was optimized for a variety of parameters including codon usage bias, GC percentage, mRNA secondary structures and restriction sites which may interfere with cloning procedures. The optimized DNA fragment harboring *BamHI* and *HpaI* restriction sites at 5' and 3' ends was chemically synthesized. This fragment was sub-cloned into the *BamHI/HpaI* linearized constructed *Hansenula* expression vector (pYT/zeo).

Verification of recombinant pYT/zeo/gcsf vector.

Cloned *gcsf* and zeocin genes were confirmed with designed primers (for *gcsf* gene: F-primer: 5'-gtagatcttgcggatcccc-3'; R-primer: 5'-gtagatcttg-gtctccagcttgc-3'; for zeocin gene: F-primer: 5'-gtagatcttgcggatcccc-3' and R-primer: 5'-gtagatcttggtctccagcttgc-3'). Restriction digestion analysis using *BamHI*, *EcoRI*, *HpaI* and *BglIII* and final sequencing analysis also confirmed the accurate reading frame in the synthetic expression vector.

Transformation of *Hansenula polymorpha*. Prototroph *Hansenula polymorpha* RB11 strain (efficient in orotidine-5'-phosphate decarboxylase; *ura3* positive) was inoculated into non selective YPD broth medium and incubated on a shaker at 37 °C overnight. The culture was diluted with fresh YPD

medium to the optical density of 0.8-1.2 at 600nm. Cellular pellet was harvested at 2000 g (5min) and re-suspended in 50mM potassium phosphate buffer (pH 7.5) with a final concentration of 25mM DTT. After 15min incubation at 37 °C, cells were washed twice in STM buffer (270mM Sucrose, 10mM Tris-HCl, 1mM Magnesium Chloride, pH 8.5) and resuspended in STM buffer (0.005 initial volume) and kept in ice. The recombinant vector (pYT/zeo/gcsf) was digested at unique *EcoRI* site located at the most 5' end of the expression cassette, extracted from agarose gel and transformed into the prepared competent *Hansenula* cells via electroporation. Transformation was carried out according to the previously published protocol. In brief, 5-10µg linearized pYT/zeo/gcsf expression vector and the backbone vector alone (pYT/zeo) were added to 60µl (approximately 6 X 10⁹ cells/ml) prepared competent cells in prechilled 0.2cm cuvettes. Electric pulses (200Ω, 2 kV and 25µF) were given using a BioRad GENE Pulser II and 1ml YPD broth was immediately added to the cell-DNA mixture and incubated at 37 °C for 1 hour. Cells were centrifuged and spread on zeocin free YPD agar plates and incubated at 37 °C for 3-5 days. The grown colonies were selected on YPD plates supplemented with different zeocin concentrations ranging from 400 to 1600µg/ml during several passages and zeocin specific PCR was performed to screen the positive clones for GCSF protein analysis.

GCSF expression induction in *Hansenula polymorpha*. Selected recombinant *Hansenula* clones were screened for the expression of recombinant GCSF. In brief, colonies were inoculated into BMGY (Buffered Glycerol-complex medium) and incubated in a high speed shaker (300rpm) at 30 °C until OD_{600nm} reached to 2-6 (16hrs). After centrifugation (2000 g for 5min), cellular pellet was resuspended in BMMY (Buffered Methanol-complex medium) expression medium and incubated for more growth. To induce protein expression, methanol with the final concentration of 0.5% was added every 24 hours to the medium and incubation at 30 °C extended till 96 hours (4 days). The broth medium was centrifuged at 2000 g (5min) and the supernatant was collected for further analysis.

SDS-PAGE analysis of expressed protein. To prepare more homogenous supernatant, samples were passed through Centriprep YT50 filters (Mil-

lipore, USA) and final samples were run on a 15% SDS-PAGE. The gel was stained with silver nitrate and the expression of GCSF protein was investigated in the samples representing supernatant before filtration (named as crude supernatant or total proteins), filtrate (named as <50kDa) and retentate (named as >50kDa) samples of each control and recombinant *Hansenula* clones.

Identity confirmation of recombinant protein by western blotting. Protein samples were run on a 15% SDS-PAGE and transferred to the nitrocellulose membrane. Rabbit anti-GCSF polyclonal serum and HRP conjugated goat anti-Rabbit IgG monoclonal antibody were used as the primary and secondary antibodies in the blotting test and recombinant bands were developed using DAB as the substrate. Commercially available recombinant GCSF produced in *E. coli* was used as positive control in this test.

RESULTS AND DISCUSSION

Construction of *Hansenula* expression cassette. Molecular studies have conducted to develop a wide range expression system applicable for different yeast species including *H. polymorpha*. The established platform, CoMed™ system, has been applied for comparative expression of several proteins in different yeast host cells (8). Due to the limitations in purchasing native *Hansenula* strain and its commercial expression vectors, we aimed to construct an appropriate vector for expressing different heterologous genes in the available *H. polymorpha* cell which was prototroph. Designed expression cassette was constructed in pGH backbone cloning vector, mainly harboring ampicillin resistance gene and ColE1 origin of replication. FMD promoter and MOX terminator fragments (approximately 1000 and 200bp in length, respectively) were integrated in the expression cassette with a multiple cloning site in the middle for cloning of any heterologous gene fragment. *Alpha-MF1* signal sequence was also inserted between pFMD and MOX terminator in frame with any cloned target gene in the designed MCS (Fig. 1). A 500bp fragment of *HARS1* was also designed at 5' region of the cassette in order to get more genome integrations and stable transformants. The cassette (*HARS1*-FMD promoter-*MFa1* signal sequence-MCS-MOX terminator) was confirmed by restriction

digestion through designed sites at MCS of the constructed vector (Fig. 1).

Previous studies have suggested a variety of selection markers in screening recombinant *Hansenula* cells. Beside auxotrophic characteristics of the host strains such as URA3, LEU1 and TRP3 markers, use of dominant markers such as kanamycin and phleomycin resistance genes have also been recommended. Zeocin has been categorized as a member of phleomycin/bleomycin antibiotic family and has anti-bacterial as well as anti-fungal activity (9, 10). Previous studies revealed a zeocin sensitive behavior in *Hansenula polymorpha* in which its 100µg/ml concentration was successfully used in screening *Hansenula* transformants. An approximately 1200bp *sh-ble* gene fragment consisting all essential gene fragments encoding zeocin resistance protein, was amplified and the *BglIII* ended fragment was cloned into the same digested expression vector, at the downstream region of the expression cassette. The above cloned fragment contained Transcription Elongation Factor 1 (TEF1) promoter that initiates expression of zeocin resistance gene in yeast cell, EM7 promoter, a prokaryotic promoter expressing zeocin resistance protein in *E. coli*, *sh-ble* ORF and 3' end of *Saccharomyces cerevisiae* CYC1 gene which is involved in efficient processing and stability of 3' end of zeocin mRNA (pPICZ A, B and C expression vectors, Invitrogen, USA). This cloning procedure was also confirmed through zeocin specific PCR and *BglIII* digestion (Fig. 2A). The encoded protein binds to the supplemented zeocin in the agar medium and inhibits its DNA cleavage activity and results in zeocin resistance phenomenon.

Mature human GCSF is an 18.8kDa protein as a single chain polypeptide consisting 174 amino acids (11). The codon optimized full length GCSF encoding fragment was synthesized and inserted into the final expression vector. Cloning procedure was confirmed through the *gcsf* specific PCR and restriction digestions representing a 615bp fragment of *gcsf* gene on agarose gel (Fig. 2B). Sequencing analysis also revealed its correct fusion to the *MFa1* signal sequence to secrete the protein into the medium under the control of pFMD promoter.

Recombinant *Hansenula* transformants. The constructed plasmid, pYT/zeo/*gcsf* carrying *HARS1* telomeric region and zeocin as a selection marker, was transformed into available *Hansenula polymor-*

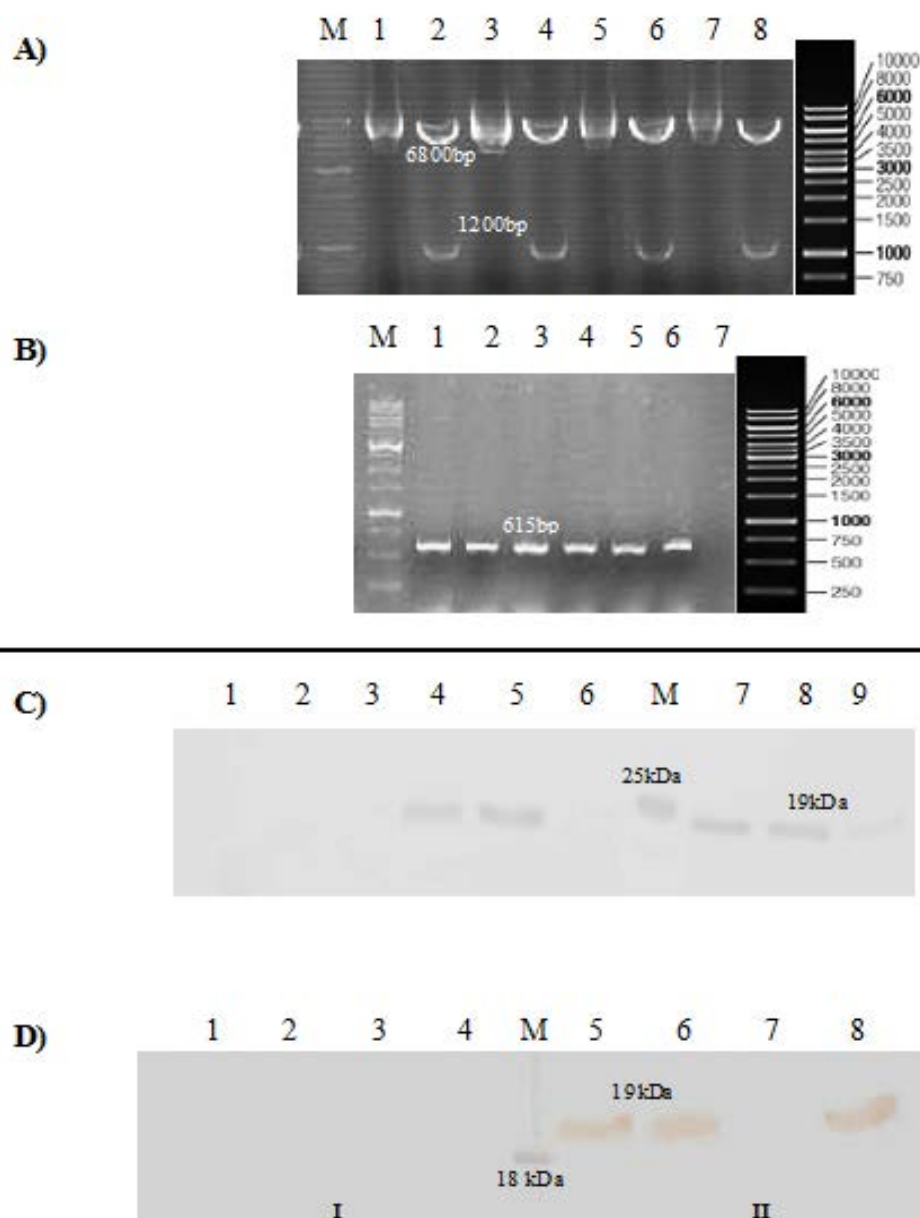


Fig. 2. Molecular confirmation of constructed expression vector.

(A) *BglIII* digestion analysis of final vector: #1, 3, 5, 7: undigested expression vectors; #2, 4, 6, 8: *BglIII* digested vectors; M: DNA 1kb-ladder (Thermo Scientific, Cat No. SM0313).

(B) *gcsf*/PCR confirmation of expression vector: #1-6: *gcsf* positive recombinant expression vectors; #7: PCR negative control; M: DNA 1kb ladder.

(C) SDS-PAGE analysis of GCSF expression: #1-3: Background *Hansenula* clone (crude supernatant, <50kDa sample and >50kDa sample), respectively; #4-6: Clone #2 (crude supernatant, <50kDa sample and >50kDa sample), respectively; #7-9: Clone#19 (crude supernatant, <50kDa sample and >50kDa sample), respectively; M: Protein size marker (Thermo Scientific, Cat No. 26610).

(D) Immunoblotting confirmation of r-GCSF: #1, 8: Commercial r-GCSF (positive control); #2, 7: Background *Hansenula* clone (<50kDa sample); #3, 6: Clone#2 (<50kDa sample); #4, 5: Clone#19 (<50kDa sample); M: Protein size marker (Thermo Scientific, Cat No. 26610)(I, II: Rabbit serum samples before and after immunization with r-GCSF protein).

pha strain. The grown colonies on initial YPD agar plate (in the absence of zeocin) were spread on plates supplemented with initial 100µg/ml concentration of zeocin according to the previous studies (12). Zeocin concentration gradually increased ranging from 100 to 1600µg/ml and final zeocin resistant colonies (on the plate containing 1600µg/ml of zeocin) were stabilized through several passages and genetically confirmed by the presence of approximately 1200bp zeocin resistance gene fragment through specific designed PCR on *Hansenula* genomic DNA. Our molecular investigation confirmed that the expression cassette and all necessary elements are theoretically located in their correct positions in the designed constructed vector (Fig. 1).

Expression of recombinant GCSF in *Hansenula polymorpha*. Methanol utilization as the only carbon source has been described for several yeast strains (13) including *Hansenula polymorpha* (3) and key enzymes involved in MeOH metabolism have been discovered through extensive molecular studies. In *Hansenula polymorpha*, MOX and FMD promoters are repeatedly used for the expression of heterologous genes (14-16). These promoters are repressed in the presence of glucose, ethanol or glycerol and adding methanol to the culture medium can induce their activity (17, 18). BMGY and BMMY media used in this study are optimized for the expression of secretory proteins via using phosphate buffer considering a range of pH in which yeast extract and peptone may decrease the proteolysis activity against secreted proteins and allow better biomass production. The expression of recombinant GCSF was induced by adding methanol to the liquid medium. Methanol was added to the medium every 24 hours to control the concentration of the inducer in the culture during 96 hours incubation period.

Previous studies have confirmed that the presence of α -MF1 signal sequence originated from *S. cerevisiae* resulted in the secretion of the recombinant proteins in the culture medium. Culture supernatants were passed through centriprep YM50 filters (with 50kDa cutoff) to collect proteins below 50kDa (mentioned as <50kDa sample in Fig. 2C) including r-GCSF (approximately 19kDa) and collected samples were separately run on 15% SDS-PAGE. A protein of approximately 19kDa was detected on the gel stained with silver nitrate in below 50kDa samples corresponding to *Hansenula* clones numbered 2 and

19 which was absent in the negative control cell (harboring empty vector) and retentate samples (supernatants above 50kDa named as >50kDa sample) (Fig. 2C).

Identity confirmation of recombinant GCSF protein by immuno-blotting. Western blotting was performed to confirm the expressed protein as GCSF using a rabbit polyclonal antibody produced against commercially available recombinant GCSF (Filgrastim; Pooyesh Darou Inc., Iran). Membrane was firstly incubated with 1:500 dilution of rabbit polyclonal serum against r-GCSF and then incubated with 1:1000 dilution of the secondary antibody. The result indicated the presence of expected band of 19kDa in the supernatants of confirmed clones in comparison with negative control which was the supernatant of *Hansenula* cell culture harboring the expression vector (empty vector) (Fig. 2D).

The application of yeast cells in biotechnology refers to many years ago when they were applied in production of breads and alcoholic drinks. Increasing knowledge in their fermentation process extended their application into the production of biopharmaceuticals. These GRAS (generally regarded as safe) organisms and *S. cerevisiae* in particular, have been well-characterized and several engineered strains have been developed in recent years (19). In the high growing pharmaceutical market *E. coli*, *S. cerevisiae* and mammalian cells are the main host cells. Beside high growth rate of *E. coli* and its simple medium requirements, mammalian cells are the main hosts in producing proteins with huge post-translational modifications (20, 21). On the other hand, yeast cells can serve as the simplest eukaryotic microbes in order to provide high growth rate on simple media as well as specific limited post-translational modifications of specific desired proteins. *S. cerevisiae* has been served as one of the main yeast cells in biopharmaceutical industry. Its application began with production of recombinant insulin in 1987 and continued by several vaccines and blood factors (20, 22).

Among other yeast strains, methylotrophic yeast *Hansenula polymorpha* and *Pichia pastoris* have attracted scientific interests as suitable hosts for the expression of several heterologous genes such as IFN alpha-2a, insulin and hepatitis B vaccines and a few of them have been approved by the FDA (6, 22, 23). This attraction can be due to their advantages in comparison with *S. cerevisiae* which include the ability

to grow in high cell density in simple media and high rate of plasmid integration into the genome (24, 25). On the other hand, the most frequent promoters in *S. cerevisiae* vectors, GAL1 or GAL10, will be induced by galactose which is an expensive carbon source molecule while methanol acts as a suitable economic carbon source for inducing corresponding strong promoters in other two mentioned yeast cells. In *S. cerevisiae*, hyper glycosylation through α -1,3-mannose linkages (50-150 residues per protein) will cause shorter serum half life of produced proteins and immunogenic responses in the body. To overcome this issue, several genetic engineering approaches have been conducted in other yeast strains including *H. polymorpha* to minimize the level of α -1,3-mannose residues link to the expressed proteins between 8 and 14 (26, 27).

Cloning of *gcsf* gene was selected to examine the efficiency of constructed vector in expressing heterologous genes. Mature human GCSF protein has two disulfide bonds and a free cysteine in position#17. Without any N-glycosylation site, it has just one O-glycosylation site at Thr-133 position (11) assuming as a GCSF stabilizing moiety (28) via supportive activity for cysteine group (29). This study confirmed the integrity of the expression cassette for the expression of heterologous genes which can be examined for more complicated pharmaceutical proteins.

ACKNOWLEDGEMENTS

This study was funded and supported by a grant (#673) from Pasteur Institute of Iran.

REFERENCES

- Kamionka M. Engineering of therapeutic proteins production in *Escherichia coli*. *Curr Pharm Biotechnol* 2011; 12: 268-274.
- Melmer G. (2005). Biopharmaceuticals and the industrial environment. In: Production of recombinant proteins: novel microbial and eukaryotic expression systems. Ed, G Gellissen. Wiley-VCH, Weinheim. pp. 361-383.
- Sudbery PE. The non-*Saccharomyces* yeasts. *Yeast* 1994; 10: 1707-1726.
- van der Klei IJ, Yurimoto H, Sakai Y, Veenhuis M. The significance of peroxisomes in methanol metabolism in methylotrophic yeast. *Biochim Biophys Acta* 2006; 1763: 1453-1462.
- Ramezani-Rad M, Hollenberg C, Lauber J, Wedler H, Griess E, Wagner C, et al. The *Hansenula polymorpha* (strain CBS4732) genome sequencing and analysis. *FEMS Yeast Res* 2003; 4: 207-215.
- Seo HS, Park JS, Han KY, Bae KD, Ahn SJ, Kang HA, et al. Analysis and characterization of hepatitis B vaccine particles synthesized from *Hansenula polymorpha*. *Vaccine* 2008; 26: 4138-4144.
- Gietl C, Faber KN, van der Klei IJ, Veenhuis M. Mutational analysis of the N-terminal topogenic signal of watermelon glyoxysomal malate dehydrogenase using the heterologous host *Hansenula polymorpha*. *Proc Natl Acad Sci USA* 1994; 91: 3151-3155.
- Steinborn G, Böer E, Scholz A, Tag K, Kunze G, Gellissen G. Application of a wide-range yeast vector (CoMed™) system to recombinant protein production in dimorphic *Arxula adenivorans*, methylotrophic *Hansenula polymorpha* and other yeasts. *Microbial Cell Fact* 2006; 5: 33.
- Drocourt D, Calmels TPG, Reynes JP, Baron M, Tiraby G. Cassettes of the *Streptoalloteichus hindustanus ble* gene for transformation of lower and higher eukaryotes to phleomycin resistance. *Nucleic Acids Res* 1990; 18: 4009.
- Baron M, Reynes JP, Stassi D, Tiraby G. A Selectable bifunctional β -galactosidase: phleomycin resistance fusion protein as a potential marker for eukaryotic cells. *Gene* 1992; 114: 239-243.
- Kubota N, Orita T, Hattori K, Oh-eda M, Ochi N, Yamazaki T. Structural characterization of natural and recombinant human granulocyte colony-stimulating factors. *J Biochem* 1990; 107: 486-492.
- vanDijk R, Faber KN, Hammond AT, Glick BS, Veenhuis M, Kiel JAKW. Tagging *Hansenula polymorpha* genes by random integration of linear DNA fragments (RALF). *Mol Genet Genomics* 2001; 266: 646-656.
- Waterham HR, Digan ME, Koutz PJ, Lair SV, Cregg JM. Isolation of the *Pichia pastoris* glyceraldehyde-3-phosphate gene and regulation and use of its promoter. *Gene* 1997; 186: 37-44.
- Yurimoto H, Sakai Y, Kato N (2002) Methanol metabolism. In: *Hansenula polymorpha*- Biology and Applications. Ed, G Gellissen. Wiley-VCH, Weinheim, pp. 61-75.
- Barnes CS, Krafft B, Freeh M, Hofmann UR, Pappendieck A, Dahlems U, et al. Production and characterization of saratin, an inhibitor of von Willebrand's factor-dependent platelet adhesion to collagen. *Semin Thromb Hemost* 2001; 27: 337-347.
- Kang HA, Gellissen G (2005) *Hansenula polymorpha*. In: Production of Recombinant Proteins – Novel Microbial and Eukaryotic Expression Systems. Ed, G

- Gellissen. Wiley-VCH, Weinheim, pp. 111-142.
17. Eggeling L, Sahm H. Derepression and partial insensitivity to carbon catabolite repression of the methanol dissimilating enzymes in *Hansenula polymorpha*. *Eur J Appl Microbiol Biotechnol* 1978; 5:197-202.
 18. Gellissen G, Melber K. Methylotrophic yeast *Hansenula polymorpha* as production organism for recombinant pharmaceuticals. *Arzneimittelforschung* 1996; 46: 943-948.
 19. Martinez JL, Liu L, Petranovic D, Nielsen J. Pharmaceutical protein production by yeast: towards production of human blood proteins by microbial fermentation. *Curr Opin Biotechnol* 2012; 23: 965-971.
 20. Walsh G. Post-translational modifications of protein biopharmaceuticals. *Drug Discov Today* 2010; 15: 773-780.
 21. Berlec A, Strukelj B. Current state and recent advances in biopharmaceutical production in *Escherichia coli*, yeasts and mammalian cells. *J Ind Microbiol Biotechnol* 2013; 40: 257-274.
 22. Walsh G. Biopharmaceutical benchmarks 2010. *Nat Biotechnol* 2010; 28: 917-924.
 23. Janowicz ZA, Melber K, Merckelbach A, Jacobs E, Harford N, Comberbach M, et al. Simultaneous expression of the S and L surface antigens of hepatitis B, and formation of mixed particles in the methylotrophic yeast, *Hansenula polymorpha*. *Yeast* 1991; 7: 431-443.
 24. Gellissen G, Strasser AWM, Suckow M (2005). Key and criteria to the selection of an expression platform. In: Production of Recombinant Proteins – Novel Microbial and Eukaryotic Expression Systems. Ed, G Gellissen. Wiley-VCH, Weinheim, pp. 1-5.
 25. Deml L, Schirmbeck R, Reimann J, Wolf H, Wagner R. Purification and characterization of hepatitis B virus surface antigen particles produced in *Drosophila Schneider-2* cells. *J Virol Methods* 1999; 79: 205-217.
 26. Hamilton SR, Gerngross TU. Glycosylation engineering in yeast: the advent of fully humanized yeast. *Curr Opin Biotechnol* 2007; 18: 387-392.
 27. Cheon SA, Kim H, Oh DB, Kwon O, Kang HA. Remodeling of the glycosylation pathway in the methylotrophic yeast *Hansenula polymorpha* to produce human hybrid-type N-glycans. *J Microbiol* 2012; 50: 341-348.
 28. Wang C, Eufemi M, Turano C, Giartosio A. Influence of the carbohydrate moiety on the stability of glycoproteins. *Biochemistry* 1996; 35: 7299-7307.
 29. Arakawa T, Prestrelski S, Narhi L, Boone T, Kenney W. Cysteine 17 of recombinant human granulocyte-colony stimulating factor is partially solvent-exposed. *J Protein Chem* 1993; 12: 525-531