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***RESEARCH ARTICLE***

# Cloning, Transformation and Expression of Human IFN-γ by Genetic Engineering in *Saccharomyces cerevisiae*

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## ABSTRACT:
Human interferon-gamma (hIFN-γ) is a Glycoprotein pertinence to a distinct group of interferon, called type II interferons, which have an immunological function to respond to antigenic stimuli such as bacteria, viruses, fungi, and any infections by a microorganism. The hIFN-γ is produced by natural killer T (NKT) and natural killer (NK) cells during the immune response as part of the innate immune response and by Th1 CD4 and CD8 cytotoxic T lymphocytes (CTL) effector T cells upon the development of antigen-specific immunity. Recombinant hIFN-γ has been produced in different expression systems comprising prokaryotic, insect, fungal (yeasts), protozoan, mammalian cells, and plant. In the present study, pET28a plasmid was used in this research for preparation to insert and pYES2 plasmid for cloning and expression of human IFN-γ gene in yeast. The trademark of hIFN-γ has been produced in Escherichia coli is termed ACTIMMUNE®, but the human interferon-gamma was produced in the prokaryotic expression system is unglycosylated form. This result increases in cost due to increased purification, as well as short-life in the bloodstream, but it is biologically active. This study aimed to use Saccharomyces cerevisiae 4741 strain as a eukaryotic system for expression of hIFN-γ cDNA instead of a prokaryotic system in glycosylation patterns as modified translation. But the results are not satisfactory as the produce of yields.

**KEYWORDS:** *S. cerevisiae*, Recombinant Human Interferon-Gamma, Glycocytokine, Expression vector.

## INTRODUCTION:
The recombinant proteins therapeutic are exogenous proteins that are produced in a production organism and utilized for the prevention of disease or treatment in humans or animals[1-3].

Some human diseases are caused by a deficiency of proteins and their inefficient function and most of them can be relieved with suitable protein drugs. So, they must be available at a higher amount as pharmaceutical usage of these proteins. Two strategies are utilized for the production of human proteins: extraction of protein from blood and using recombinant protein technology, if the protein manufacture in the body is low[4,5].

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Yeasts are eukaryotic microbial host cells are producing biopharmaceutical proteins and have unique advantages for this purpose; yeasts can easily accommodate genetic modifications, growth on simple media, post-translational modifications. *Saccharomyces cerevisiae* has been utilized as a major host for the expression of biopharmaceuticals[6].

Interferons (IFN) are cytokine, a set of signaling proteins which have an important role against the multiplication of viruses, infections of intracellular bacteria, fungi, parasites, foreign macromolecules, foreign cells, or many other compounds and involves in the inflammatory process and regulation of a cellular cycle[7-12] which plays a role in the immune response in host cells, and produce defense mechanism made and released by host cells, and synthesized and secreted[13]. Depending on their functional activity, structure, and receptor specificity, interferons consist of three groups: type I (IFN-α (12 subtypes), β, ω), type II involves only (IFN-γ) and type III (IFN-λ) (IFN-λ1, IFN-λ2, IFN-λ3 and IFN-λ4[14] But the interferons have fundamental three distinct groups:

IFN-α, -β, and IFN-γ, which are produced by leukocytes, fibroblasts, and lymphocytes respectively. The action-type of this cytokine is comprised of 143-amino acid polypeptides and 23 amino acids as a signal sequence at first of the sequence[15,16]. The human gene of IFN-γ is 6 kb in size and contains four exons and three introns. The gene for human IFN-γ has been localized to human chromosome 12 (I2 q24.1)[17-20] or (12q14)[19]. It is secreted by natural killer (NK) cells as part of the innate immune response, and by CD4+ and CD8+ T cells when they are activated upon the evolution of antigen-specific immunity[8,16,22]. The IFN-γ has a conclusive role in the immune system to resist pathogens. However, the presence of this cytokine alone is not sufficient to protect against diseases[23]. hIFN-γ containing 30% carbohydrates [24]. It has been shown that the carbohydrate of this molecule is not necessary for its biological activity[25-29].

When the IFN-γ attached to specific receptors on the target cells. It has been several biologically activates such as antiviral activities, preventing the growth of macrophages and neoplastic cells as well as induce the antiviral activities of IFN-α and–β. IFN-γ is unrelated to the Type I interferons at both the genetic and the protein levels[25-31].

Recombinant technology is the process of choosing among all recombinant expression hosts using various expression hosts and *Escherichia coli* (*E. coli*) is the most conventional organism[32-34]. Because IFN-γ is a cytokine that is used for clinical application, it's required for production in large amounts for the final goal[35]. Human IFN-γ has been approved by USFDA for the treatment of many diseases such as severe malignant osteopetrosis and chronic granulomatous disease because has a highly potent biotherapeutic. Furthermore, approximately 350 clinical experiments of Human IFN-γ are either ongoing or perfected for the treatment of different diseases ranging from tuberculosis to cancer [36]. A few human sicknesses emerged from defects in useful protein creation in various tissues.

Scientists are using special hosts for recombinant protein drug expression to solve this issue. Recombinant medication makes the process of the proficient isolation and purification of recombinant protein from the other proteins is the essential stage[37,38].

Since 1982 the human interferon-gamma has been produced in vitro by different expression systems including *E. coli*[6,36-42] culture monkey cells[30], yeast[43], parasite (*Leishmania tarentolae*) [44], Chinese hamster ovary cells[45], and plant[12,46].

The aim of this study was the production of IFN-γ by genetic engineering utilizing *Saccharomyces cerevisiae* 4741 strains for its advantages over other host systems for foreign gene expression. It is a safe host, can be cultivated on simple media and produces no endotoxins.

## MATERIALS AND METHODS:

The IFN-γ gene was synthesis by the Genescrpit Company (Denmark) and cloned into the $pYES_2$ expression vector. The recombinant DNA was expressed in *Saccharomyces cerevisiae* BY4741 strain and verified by cultured plates with ampicillin and gentamycin (G-418) then purified by affinity $Ni^{++}$ and SDS analysis.

### Materials:

### Strains, Plasmids, and gene:

*Saccharomyces cerevisiae* BY4741 (MATa his3Δ1 leu2Δ0 met15A0 ura3Δ0)[47] was utilized for the building of IFN-γ -pYES2 cassette (Figure 1). It was used in transformation and expression experiments as a control strain provided by Prof. Dr. Khalaj (The Pasteur Institute, Iran). Strains of *Escherichia coli*; DH5α (Invitrogen) were used as a host for plasmid which was used in this study were purchased from Genescrpit company, Denmark. The pYES2 plasmid (Invitrogen Corp (USA), is resistant to ampicillin for selection in the *E. coli* DH5α strain. This plasmid contains a GAL1 promoter sequence as well as an aURA3 gene for high-copy-number maintenance and selection in a Ura mutant of *S. cerevisiae*. Yeast strains were grown and cultured in The YPD medium (2% polypeptone, 1% yeast extract, and 2% dextrose) was used for cultured yeast strains[32]. Yeast nitrogen base medium (YNB; Sigma-Aldrich, U.S.) was prepared 0.67% and complemented with 2% glucose. For the selection of IFN-γ–pYES2 strains, the YPD medium was complemented with ampicillin and gentamycin (G-418). The pYES2 cloning vector was utilized as a middle vector for cloning of different DNA fragments. Restriction endonucleases (BamHI and XhoI) and ligase of T4 DNA were obtained from Fermentas (Waltham, United States). Primers were synthesized by Genscrpit, Denmark (Table 1). The DNA extraction kit and purification kit, which were from Roche Applied Science Kit (Roche).

**Table 1: The primers used for the amplification of PCR amplicons for IFN-γ expression**

| Primers Sequence |
|---|
| pESC-HIS FW 5'-CGGATCCGATGAAGTACACCAGTTA-3' |
| pESC-HIS RV 5'-CCGCTCGAGTTAGTGGTGGTGATGGTGGTG 3' |
| pYES2 Fw 5'-TAATACGACTCACTATAGGG-3' |
| pYES2 Rv 5'-CTACCTCTGAATAAC ATTTG-3' |

### Design of the synthetic gene (hIFN-γ):

Designing the synthetic of the hIFN-γ gene was done with the (Genescrpit, Denmark) and cloning into pYES2 vector (Invitrogen) by using BamHI and XhoI site containing GAL1 promoter (Figure 1).

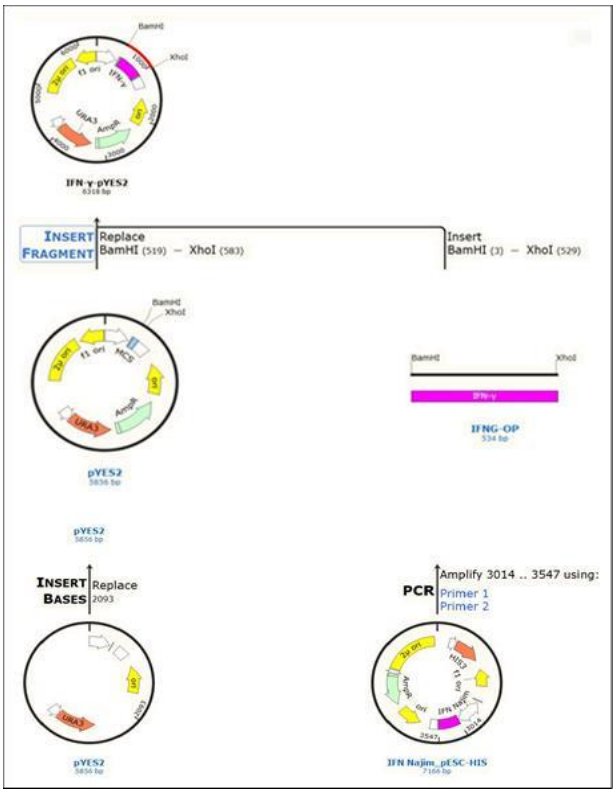



**Figure 1: Schematic representation of the pYES2-IFNG. To construct IFNG from pESC-HIS was used pair of primers, the obtained amplicon (534 bp) and was cloned in pYES2 plasmid. *E. coli* BL21 was preparation for transformed with these plasmids. *Saccharomyces cerevisiae* (4741) strains were transformed with pYES2 plasmid and induced by galactose for expression of interferon-gamma gene.**

**Preparation of competent cell and transformation:**

*Escherichia coli* (DH5α strain) was prepared as competent cells by chemical method ($CaCl_2$) and was transformed with the hIFN-γ–pYES2plasmid. Bacterial clones including the recombinant plasmid were incubated on ampicillin Luria-Bertani (LB) agar. The plasmids were isolated and the presence of the desired fragment was examined by digestion.

**Transformation of bacteria (*E. coli*) and the Plasmids Propagation:**

Reactions of ligation for the transformation of *E. coli* DH5α strains and transformants were plated out on Luria Bertani plates (LB) containing the antibiotic (ampicillin). Raise colonies and incubated overnight in Luria Bertani medium and let them grow to utilize the same antibiotic as before purification of plasmids.

**Transformation of *S. cerevisiae*:**

Transformed utilizing a standard electroporation protocol as described before with slight modification[48]. Briefly, Incubated YPD broth medium with a colony of *S. cerevisiae* for overnight in 30°C and 200rpm shaking to reach an OD600 of 1.5. The OD 600 of culture Cells was diluted to 0.4 in 100ml of (YPD) medium and grown for an additional 3-4 h and OD600 reach 1.6 and collected by centrifugation. The cell pellet was washed twice with 50ml of cold water, and then with 50ml buffer of electroporation (1M sorbitol/1mM $CaCl_2$). Before electroporation, the cells were conditioned in 20 mM LiOAc/10mM DTT solution and cultured for 30 min at 30°C with shaking 200rpm. Wash more than time by electroporation buffer, then suspended in electroporation buffer (1M sorbitol/1mM $CaCl_2$)[48], then resuspended in 200µl of the same buffer to reach 1ml volume (corresponds to ~$1.6x10^9$ cells/ml). Electroporation was performed with plasmid DNA+insert (5µg) for each volume of 100µl mixture of cells suspension utilizing Gene Pulser XcellTM electroporation system (Bio-Rad Laboratories, United States), the Cells were electroporated at 2.4 kV, 25 mF, 34.5 ms in a BioRad GenePulser cuvette. The cells were suspended in 10ml of 1:1 mix of 1 M sorbitol: YPD media after electroporation then incubated at 30°C for 1 h. The transformation mixture was then spread on the selective medium agar after collected by centrifuge, the positive transformants cells were recovered after 3days incubation at 30°C.

**Induce of Protein Synthesis:**

A single colony of transformed S. cerevisiae 4741 was cultured into 100ml of Selective medium include 2% glucose and incubated at 30°C with shaking (200rpm) for 12-14h. The $OD_{600}$ was estimated and diluted the culture for reaching an $OD_{600}$ of 0.4 in an induction medium (YNB 1x, Gal 2% and 0.1 g/lit Amino acid mix) was prepared, after precipitating by centrifuge, suspended the pellet in induction medium and incubated at 30°C with shaking (200 rpm) for intervals from 0- 10 h. For each time, takeout 5ml from culture and centrifuge, then the pellet was resuspended in 500µl sterile water. To induce the expression of hIFN-γ in our recombinant *S. cerevisiae* 4741, 2% galactose was added onto the culture medium[49]. For later studies, stored at -80°C.

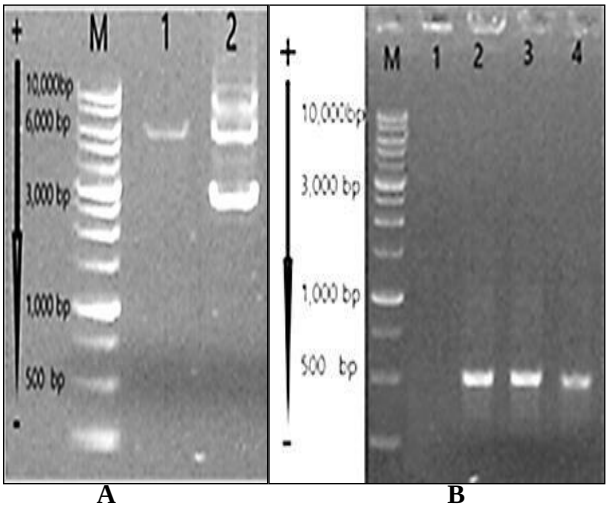
**Protein Extraction and Quantification:**

The cell pellets were suspended in 500µl breaking buffer (1mM EDTA, 5% glycerol, 1mM phenylmethylsulfonyl fluoride, and 50mM sodium phosphate, pH:7.4,) centrifuged at 2500rpm for 5 min at 4°C. Discarded the supernatant and the cells were suspended to obtain an OD 500 of 50-100 in the same breaking buffer. To lyse the cells were added an equivalent volume of glass beads and vortexed for 30 min. Centrifugation of the lysate at maximum speed for 10 min. The supernatant was transferred to a fresh tube for protein concentration following the application. The final concentration of the lysate was 1× with SDS-PAGE sample buffer and was boiled the mixture for 5-10 min. later, 20µg of the

sample was loaded on an SDS-PAGE gel with 10µl of loading buffer (5X solution of 250mM Tris· HCl, pH 6.8, 10% SDS, 30% (v/v) Glycerol, 10mM DTT, 0.05% (w/v) Bromophenol Blue) and electrophoresed.

**SDS-PAGE**:
The YPD selective medium was utilized for the grown of yeast clones to optical density $OD_{600}$ of 2 and the yeast clones were grown increase for intervals from 0-10 h after the replacement of glucose by galactose. Then, cells were discarded and the supernatants were concentrated by two techniques; Millipore Ultra Centrifugal Filter Unit or lyophilization (Freeze-drying). The loading buffer (2×) was utilized for dissolution of the lyophilized yeasts (1:1) then undergo to (12.5% w/v) SDS-PAGE gel electrophoresis.

**Analytical procedures:**
The strategy for cell culture turbidity was utilized as a method for estimating cell growth and optical density (OD) was determined at a wavelength of 600nm. The proportion of 12.5% polyacrylamide gel in Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was utilized for the estimation of expression levels for hIFN-γ and the gels were stained with Coomassie brilliant blue R250. The Coomassie brilliant blue R250 stained gels were scanned using a densitometer for estimated the purity of the expressed hIFN-γ for the screen of plasmid stability in the recombinant strain of *S. cerevisiae* 4741were utilized Colony PCR and SDS PAGE techniques. The efficiency of cell lysis by sonication was quantitatively estimated by culturing the resulting disrupted cell suspension on NYB agar culture plates and counting subsequent colonies.



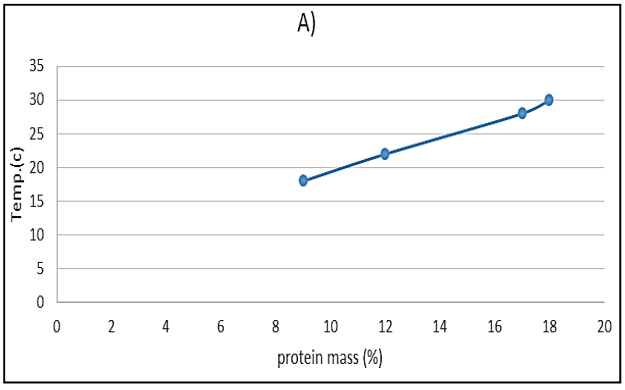

A B

**Figure 2: A) gel electrophoresis picture on 1% agarose of the undigested and digested pYES2 plasmid vector. M: Ladder, 1: digested vector, 2: undigested vector. B) Amplified Human Interferon γ gene fragment. M: ladder, 1: negative, 2-4: cDNA fragment.**

## RESULTS:

**Cloning into pYES2:**
The hIFN-γ gene syntheses by Genescrpit Company and cloning into plasmid vector pYES2 were enzymatically digested using the BamHI and XhoI restriction enzymes. Figure 2 represents a gel electrophoresis image of the digested and undigested vector alongside the amplified hIFN-γ gene fragments. The digested vector and the hIFN-γ were successfully ligated and the results were confirmed by PCR on gel electrophoresis.

**Expression of hIFN-γ:**
The different mass of the production of rhIFN-γ protein in different conditions to an estimated mass of the yeast proteins after expression induction with galactose is represented in Figure 3. The final specific yield was estimated by scanning of SDS-PAGE gels.



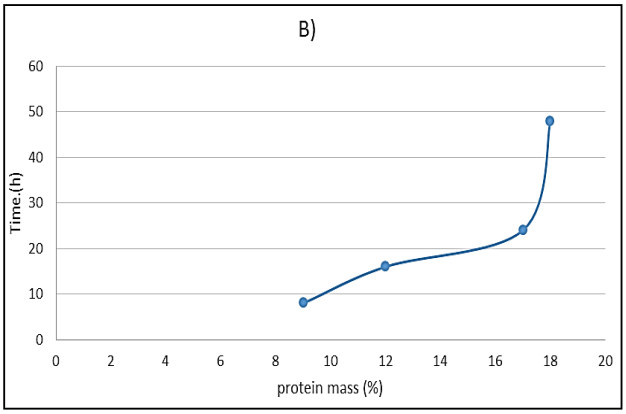




**Figure 3: These figures are the presentation of the effects of different conditions for the expression of hIFN-γ. A) effect of different temperatures of incubation, B) effects of different culture times**

**Analytical procedures:**
The method of cell culture turbidity was utilized as a manner of measuring cell growth as well as optical density (OD) was estimated at a wavelength of 600nm.

The SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis) with 12.5% polyacrylamide gel was utilized for the estimation of hIFN-γ expression levels with Coomassie brilliant blue

R250 was utilized to stain the gels (Figure 4). In an attempt to determine the purity of the expressed hIFN-γ, the Coomassie brilliant blue R250 stained gels were scanned using a densitometer. SDS PAGE and Colony PCR techniques were utilized to screen for the stability of plasmid in the recombinant *S. cerevisiae* 4741 strain. The efficiency of cell lysis by sonication was quantitatively measured by culturing the resulting disrupted cell suspension on YPD agar culture plates and counting subsequent colonies.

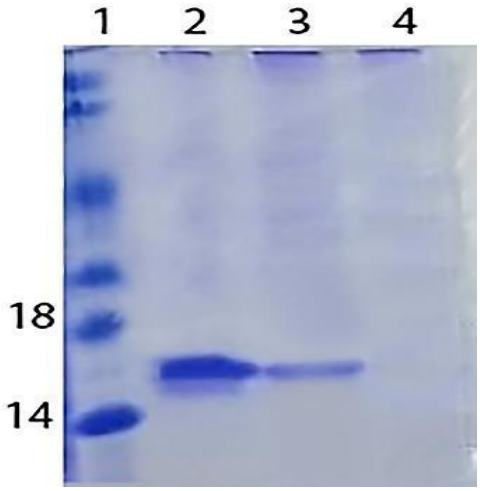



**Figure 4: SDS-PAGE of hIFN-γ proteins; 1: ladder, 2: The results of purified rhIFN-γ which concentrated by Millipore filter, 3: purification of rhIFN-γ eluted with 500mM imidazole.**

## DISCUSSION:

Interferons have a crucial role for active unlimited host defense against numerous viruses, and antitumor effects which make them successful therapeutics for clinical use. Until this point in time, the most utilized and proficient articulation framework for the expression of recombinant hIFNγ is *E. coli* because of the simplicity of recombination procedures and high yield, nevertheless, bacterial production systems have numerous drawbacks. To control the inefficiency of the bacterial expression systems, the yeast *S. cerevisiae* has been vastly utilized as a host for the production of foreign proteins because it has the availableness of powerful controllable promoters, its loss of pathogenicity, the simplicity of handling it, the capacity of posttranslational modifications, ability to secreted proteins into medium and not toxins.

The human interferon-gamma has a potentiating impact of cytotoxic and antiviral activity and Participates in these activities with α- and β-IFNs as well as has effects of antiproliferative in vitro on malignant cells is expected to be 10- to 100-fold further than the other types of interferon.

Interferon-gamma (IFN-γ) is one of the immune-significant cytokines in the innate and adaptive immune responses[12,50] which makes it an especially attractive protein from a manufactural and pharmaceutical perspective, so there had been a lot of specialists and genetically engineered drugs organizations efforts to expression it in huge industrial scales utilizing various sorts of the biological host including *E. coli*, yeast, and CHO cells. In this present study, the *Saccharomyces cerevisiae* has been used in the production of IFN-γ proteins because it is considered a safe host of biotechnical processes, which makes it a good host for the production of foreign proteins, able to perform most posttranslational modifications, and can be cultured in sample YPD media. All of the reasons that led to choosing *S. cerevisiae* as our protein expression host in this study.

In this study, the biologically active hIFN-γ, which is a homodimer that has interactions with IFN-γ receptor[14,51], was successfully expressed in *S. cerevisiae* 4741 with a reasonable production yield and biological functionality in comparison to various other related research papers published in this field using the commercially inducible pYES2 inserted into the BamHI and XhoI restriction sites. In the current study, we performed a purification of rhIFN-γ and the results from the method were favorable. Besides the final production yield of the hIFN-γ protein, its functionality as a cytokine is an important factor throughout the process of its production. Although some studies have reported lower biological activity for recombinant hIFN-γ in comparison to the standard which could potentially result from the formation of complex structures or monomers instead of homodisdzaz Setmers [22].

There are different methods utilized to assess the bio-functionality of the recombinant hIFN-γ produced by different expression systems. As we mentioned earlier, it has been proven that IFN-γ induces an up-regulation in the expression level of PDL-1 in several mouse and human cell lines through the activation of downstream signaling cascades (Mouse myeloma cell line B16-F10 and human myeloma cell line SK-MEL-37). On the other hand, similar studies have also used a method named “Antiviral assay” to evaluate the bio-functionality attributed to the recombinant hIFN-γ.

## CONCLUSION:

The biologically active hIFN-γ, which is a homodimer that has interactions with IFN-γ was successfully expressed in *S. cerevisiae* 4741 with a reasonable production yield and biological functionality. Further scientific investigations need to be conducted to improve the productivity of the recombinant hIFN-γ as well as increasing their biological functionality alongside other multimeric complexes which are considered detrimental to the bioactivity of hIFN-γ.

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