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Production of recombinant human epidermal growth factor in *Pichia pastoris*

Samira Eissazadeh[a], *Hassan Moeini*[b,*], *Maryam Ghandizadeh Dezfouli*[c], *Somayyeh Heidary*[a], *Rubina Nelofer*[d], *Mohd Puad Abdullah*[a]

[a] *Universiti Putra Malaysia, Faculty of Biotechnology and Molecular Science, Department of Cellular and Molecular Biology, Malaysia*
[b] *Universiti Putra Malaysia, Institute of Bioscience, Malaysia*
[c] *Arak University, Faculty of Science, Department of Biology, Iran*
[d] *Pakistan Council of Scientific and Industrial Research, Laboratories Complex, Lahore, Pakistan*

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ABSTRACT

This study was carried out to express human epidermal growth factor (hEGF) in *Pichia pastoris* GS115. For this aim, the hEGF gene was cloned into the pPIC9K expression vector, and then integrated into *P. pastoris* by electroporation. ELISA-based assay showed that the amount of hEGF secreted into the medium can be affected by the fermentation conditions especially by culture medium, pH and temperature. The best medium for the optimal hEGF production was BMMY buffered at a pH range of 6.0 and 7.0. The highest amount of hEGF with an average yield of 2.27 μg/mL was obtained through an induction of the culture with 0.5% (v/v) methanol for 60 h. The artificial neural network (ANN) analysis revealed that changes in both pH and temperature significantly affected the hEGF production with the pH change had slightly higher impact on hEGF production than variations in the temperature.

Introduction

Human epidermal growth factor (hEGF) is a 6.2 kDa polypeptide composed of 53 amino acid residues with three intramolecular disulfide bonds.[1] One of its major biological functions is to promote the generation of new epithelial and endothelial cells, and to stimulate tissue repairs.[2] As such, it has a powerful mitogenic activity, which can speed up the healing process of damaged tissues due to, among other things, ulcers and wounds. hEGF was also found to be effective in the treatments of wrinkles, age spots, freckles and acnes.[3,4] Traditionally, hEGF is purified from animal urine through a complicated purification step.[5] However, the yield and purity of the hEGF from this process are low and could not meet the demand from the industries. Genetic engineering is one approach in which pure hEGF can be potentially produced in a large scale. Over the last two decades, hEGF had been produced in various host systems including *Escherichia coli*,[6] *Bacillus brevis*,[7] *Saccahromyces cerevisiae*[8] and *baculovirus*.[9] In *E. coli*, the yield is not up to the level appropriate for the industrial needs, as cytoplasmic hEGF tends to form inclusion bodies, which can be rapidly degraded by proteases, leading to the formation of misfolded hEGF molecules. As a result, the overall production cost is increased due to the extra downstream processing steps required to release the hEGF from the

* *Corresponding author at*: Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Malaysia.
E-mail: moeini82@yahoo.com (H. Moeini).
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inclusion bodies. In addition, the hEGF produced by prokaryotic system exhibits lower biological activities compared to those produced by eukaryotic systems. Therefore, eukaryotic expression systems may hold a promising host for the production of active hEGF if post-translational processing is really needed especially in a large-scale production.

Among the eukaryotic expression systems, *Pichia* may offer a cheaper alternative host for hEGF production. *Pichia pastoris* is widely applied as host systems for the expressions of many heterologous proteins. As a methylotrophic yeast, *P. pastoris* is able to grow in the presence of methanol; therefore, methanol can be used an inducer without any toxic effects. In the present study, the secretary expression vector (pPIC9K) equipped with a strong methanol inducible promoter (AOX1) was used to express and secrete hEGF extracellularly.[10,11] Pichia has both the advantages of *E. coli* and eukaryotic expression systems for being an inexpensive host system, able to highly express heterologous proteins, and able to modify proteins post-transnationally.

Materials and methods

Microorganisms and plasmids

E. coli DH5α, as a host for construction of DNA plasmids, was cultured in Luria Bertani (LB) medium supplemented with 50 μg/mL ampicillin. *P. pastoris* strain GS115, as a host for the production of hEGF, cultured in YPD medium [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose (glucose)] at 30 °C. DNA plasmid pFLAG-ATS-hEGF, obtained from the Faculty of Biotechnology and Biomolecular Sciences, UPM, Malaysia, was used a template for the amplification of hEGF gene. The pPIC9K expression plasmid (Invirtrogen, USA) was used for the expression of hEGF in *P. pastoris*.

Construction of pPIC9K-hEGF DNA plasmid

The *hEGF* gene (GeneBank Accession No. M15672) was amplified from the previously constructed plasmid pFLAG-ATS-hEGF[12] using the specific primers 5′-GAATTCATGAACTCAGATAG-3′ and 5′-CCTAGGTCAACGTAATTCC-3′. Amplification was carried out in a thermocycler (Gene Amp PCR system, BioRad, USA); after denaturation at 95 °C for 2 min, the sample was subjected to 35 cycle of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 20 s followed by final extension at 72 °C for 5 min. The amplified fragment was then inserted into the *Eco*RI and *Avr*II cloning sites of the yeast expressing plasmid pPIC9K. After transformation of the recombinant plasmids into *E. coli* DH5α cells, gene insertion was checked by restriction enzyme digestion and PCR. The integrity of the insert was checked by double-stranded sequencing (1st BASE DNA Sequencing Services, Malaysia).

Expression of hEGF in P. pastoris

Electro-transformation of pPIC9K-hEGF into P. pastoris

A single colony of *P. pastoris* GS115 was cultured in 5 mL YPD medium in a 50 mL conical flask overnight in a shaker incubator at 30 °C with 250 rpm agitation rate. The culture was transferred into fresh YPD medium (250 mL) and incubated under the same condition until the $OD_{600\,nm}$ reached 1.3–1.5. The cells were then harvested at 1500 × *g* for 5 min at 4 °C; following 2 times washing with 500 and 250 mL ice-cold sterile dH_2O, the cells were resuspended in 20 mL ice-cold 1 M sorbitol. Finally, the cells were centrifuged and resuspended in 1 mL ice-cold 1 M sorbitol. For electro-transformation, in a 0.2 cm cuvette (BioRad, USA), the *P. pastoris* competent cells were mixed with 0.5 μg of the recombinant pPIC9K-hEGF plasmid; after 5 min incubation on ice, electroporation was carried out at 1.5 kV. Immediately after electroporation, 1 mL of ice-cold 1 M sorbitol was added to the cells; in a sterile microtube, the cells were mixed with 1 mL YPD medium and incubated as at 30 °C for 1 h. Around 200 μL of the mixture was spread on MD agar plates [1.34% yeast nitrogen base with ammonium sulfate without amino acids (YNB), 4×10^{-5}% biotin and 2% dextrose]. The plates were incubated at 30 °C until recombinant colonies were appeared (2–5 days).

Direct screening of multiple inserts

Positive transformants were screened on the YPD agar supplemented with geneticin at the final concentrations of 0.25, 0.5, 0.75, 1.0, 1.5, 1.75, 2.0, 3.0 and 4 mg/mL. Positive clones were screened by direct colony PCR as previously described by Linder et al.[13] with some modifications. Briefly, a single colony of the transformants was picked and resuspended in 10 μL of sterile dH_2O. Then, 5 μL of 5 U/μL solution of lyticase was added to the cells; after 10 min incubation at −80 °C, the extract was subjected to PCR using the 5′ and 3′ AOX1 primers according to the following cycles: 95 °C for 5 min for pre-denaturation, 30 cycles of amplification at 95 °C for 1 min, 54 °C for 1 min (annealing) and 72 °C for 1 min followed by a final extension step at 72 °C for 5 min.

In vivo screening of multiple insertions

His^+ clones were resuspended in dH_2O. Then, the suspensions were transferred to 50 mL Falcon tube, vortexed for 8 s and cell density was determined at 600 nm using a spectrophotometer. The cells were placed on YPD containing 0.25, 0.5, 0.75, 1.0, 1.5, 1.75, 2.0, 3.0 and 4 mg/mL geneticin, separately. The plates were incubated at 30 °C and monitored for survivals every day.

Production of recombinant hEGF

In a 250 mL baffled flask, a single colony of the recombinant *P. pastoris* was inoculated in 25 mL BMGY [buffered glycerol-complex medium: 1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate, pH 6.0, 1.34% (w/v) yeast nitrogen base (YNB), 4×10^{-5}% (w/v) biotin] containing 1% glycerol (v/v) followed by overnight incubation at 30 °C in a shaking incubator (250 rpm) until the optical density at 600 nm reached 2 (log-phase growth). The cells were then harvested at 2500 × *g* for 5 min at RT; the pellet was resuspended in 150 mL of BMMY medium supplemented with 0.5% methanol and incubated until the optical density at 600 nm reached 1.0. In a 1 L baffled flask, the culture was further incubated at 29 °C with agitation at 270 rpm for 96 h. In order to maintain the induction, methanol was added to a final concentration of 0.5% every 24 h. The culture was checked for hEGF production at 0, 6, 12, 24, 36, 48, 60, 72, 84 and 96 h of incubation.