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



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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 $\mu\text{g}/\text{mL}$ 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.

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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 acne.^{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 *Saccharomyces 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

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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 DH5 α , as a host for construction of DNA plasmids, was cultured in Luria Bertani (LB) medium supplemented with 50 μ g/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 (Invitrogen, 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-hEGF¹² using the specific primers 5'-GAATTCATGAACCTCAGATAG-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* DH5 α 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 \times g for 5 min at 4 °C; following 2 times washing with 500 and 250 mL ice-cold sterile dH₂O, 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 μ g 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 μ L of the mixture was spread on MD agar plates [1.34% yeast nitrogen base with ammonium sulfate without amino acids (YNB), 4 \times 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 μ L of sterile dH₂O. Then, 5 μ L of 5 U/ μ L 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 dH₂O. 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 \times 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 \times 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.

SDS-PAGE analysis

Secreted proteins were separated on a 12% SDS-polyacrylamide gel. Eighty micro-liter of each sample was boiled in a 20 μ L sample buffer (312 mM Tris-HCL pH 6.8, glycerol 50% (v/v), bromophenol blue 0.05% (w/v) and dH_2O) for 10 min before the sample mixture were being loaded onto the gel. Standard hEGF protein (65.0 kDa) (R&D SYSTEM) was used as a positive control. hEGF protein was purified directly from the SDS gel using a commercially available polyacrylamide gel protein purification kit (Thermo Scientific).

Quantikine hEGF immunoassay

For hEGF expression assay, the Quantikine Human EGF immunoassay kit (R&D system, USA) was used according to the manufacture's instruction. A standard curve was created by plotting the mean absorbance at 570 nm for each standard on the y-axis against the concentration on the x-axis. The amount of hEGF was determined by referring the absorbance value of the samples at 570 nm to the hEGF standard curve. The data were linearized by plotting the EGF concentrations versus the log of the absorbance and the best-fit line was determined by regression analysis.

Effect of culture conditions on growth and hEGF production

Culture medium

In 1-L baffled flask, the cells were grown on 150 mL of YPD, BMMY, MM or BMGY. Cell biomass and hEGF production were determined at 0, 12, 24, 36, 48, 60, 72 and 84 h after culturing. The growth condition used in this experiment was similar with the one described earlier. Methanol was used at a final concentration of 0.5% (MM and BMMY) every 24 h in order to maintain a continual induction and its final concentration. The best medium was selected for further optimization as below.

Methanol concentration

The recombinants were grown in the same condition as previously described. Different concentrations of methanol [0, 0.25, 0.5, 1, 1.5 and 2% (v/v)] were evaluated. The respective final concentrations of methanol were maintained by adding the appropriate amounts of methanol to the cultures for every 24 h. Samplings of the cultures were done every 12 h as described above.

pH

Optimization was done in two steps; in the first step, a large range of pH from 4 to 7 was used; in the second step, the pH range was further fine-tuned based on the two best pH obtained from the first step. The cultures were incubated using the same culture condition that was described before. The samples were taken every 12 h for analysis.

Combination of pH and temperature

The range of pH used in this study was between 4 and 7 comprising four different levels of pHs i.e. 4.0, 5.0, 6 and 7; whereas the effect of temperature was evaluated at 25 °C, 30 °C, 32 °C and 35 °C. A total of six combinations were performed and the growth condition used was the same with the one mentioned

before. The artificial neural network (ANN) was used to optimize pH and temperature for hEGF production. We used ANN with three layers: (1) an input layer with 2 neurons, (2) a hidden layer with 7 neurons, and (3) an output layer with 1 neuron. The topology of the network contained three layers (2:7:1): one input layer for two fermentation variables, pH and temperature; middle hidden layer of 7 neurons, and one output layer for hEGF production.

Statistical analysis

The data were analyzed by using one way analysis of variance followed by liner correlation tests where a *p* value of less than 0.05 was considered as significant. The statistical analysis of the ANN data and plots were performed using the Statistical software version 7.

Results

Construction of pPIC9K-hEGF expression plasmid

The hEGF gene was amplified from the pFLAG-ATS-hEGF plasmid and inserted into the expression vector pPIC9K. After transformation into *E. coli* DH5 α , positive clones were selected with direct PCR screening and restriction enzyme digestion. Finally, the identity of the inserts were checked by double-stranded sequencing pPIC9K-hEGF plasmid was sequenced and compared to the original hEGF sequence. The comparison analysis showed the cloned hEGF was 100% similar to the original hEGF (GeneBank Accession No. M15672).

Generation of recombinant *P. pastoris*

The DNA plasmid construct pPIC9K-hEGF was linearized by *Sac*I and electro-transformed into *P. pastoris*. The recombinant clones were screened on YPD supplemented with different concentrations of geneticin from 0.25 to 4 mg/mL. According to the results (data not shown), most of the transformants were found to be resistant to the low concentration of Geneticin (0.25, 0.5 and 0.75 mg/mL) and just a few were resistant to the high concentrations. Direct PCR proved successful insertion of the hEGF gene into *P. pastoris* genome (Data not shown).

Production of recombinant hEGF

Five recombinant clones selected from the hyper-resistant clones were tested for the production of hEGF by ELISA at ten different time points after induction. As shown in Table 1, the highest level of hEGF production was obtained 60 h post-induction. Very weak absorbance readings considered as background reading of the ELISA assay were observed in the control sample (GS115). Both B1 and D7 clones showed the highest hEGF production, 60 h post-induction, however, in D7, the level of hEGF was found to be decreased rapidly. Therefore, the B1 clone was selected for the optimization of fermentation conditions and medium compositions.

Based on the highest level of hEGF protein production, the presence of hEGF in the culture filtrate was further analyzed by SDS-PAGE, as shown in Fig. 1. The hEGF protein band

Table 1 – hEGF production in different clones of *P. pastoris*.

Time (h)	hEGF ($\mu\text{g/mL}$)					
	GS115 (control)	Recombinant <i>P. pastoris</i> strains				
		D7	A5	A8	B1	C2
0	0	0	0	0	0	0
6	0	0	0	0	0	0
12	0	0	0	0	0	0
24	0	0.44 \pm 0.26	0.20 \pm 0.1	0.27 \pm 0.40	0.63 \pm 0.60	0.05 \pm 0.30
36	0.01 \pm 0.25	0.76 \pm 0.15	0.42 \pm 0.21	0.44 \pm 0.25	0.89 \pm 0.15	0.08 \pm 0.11
48	0.03 \pm 0.31	1.31 \pm 0.21	0.62 \pm 0.17	0.64 \pm 0.21	1.31 \pm 0.15	0.18 \pm 0.21
60	0.06 \pm 0.28	2.25 \pm 0.15	0.89 \pm 0.21	0.89 \pm 0.40	2.27 \pm 0.21	0.4 \pm 0.25
72	0.08 \pm 0.41	2.1 \pm 0.10	0.86 \pm 0.23	0.83 \pm 0.36	2.20 \pm 0.10	0.36 \pm 0.2
84	0.05 \pm 0.28	1.85 \pm 0.05	0.84 \pm 0.21	0.77 \pm 0.30	2.11 \pm 0.17	0.31 \pm 0.17
96	0.04 \pm 0.45	1.61 \pm 0.25	0.79 \pm 0.25	0.67 \pm 0.25	2.00 \pm 0.25	0.26 \pm 0.25

represented the expected size of the protein (6.2 kDa) as compared to the standard hEGF and the marker. The total amount of secreted proteins in the medium was also determined by the Bradford assay, where 10.4 mg/mL protein was detected for the B1 strain. The hEGF protein was then purified.

Growth curve and hEGF production

The growth curve of the recombinant *P. pastoris* showed a short lag phase, lasting for a few hours, followed by the exponential growth for 50 h, before reaching the stationary phase 10 h later (Fig. 2). The first appearance of hEGF was observed after 24 h of culture i.e. shortly after being induced at 20 h. A rapid hEGF production occurred concomitantly with an increase in the growth. Under this condition, the hEGF was continually produced even after the culture reached its stationary phase.

Optimization of culture condition for hEGF production

Baffled flask was used for optimization of the culture condition for growth and hEGF production in *P. pastoris*.

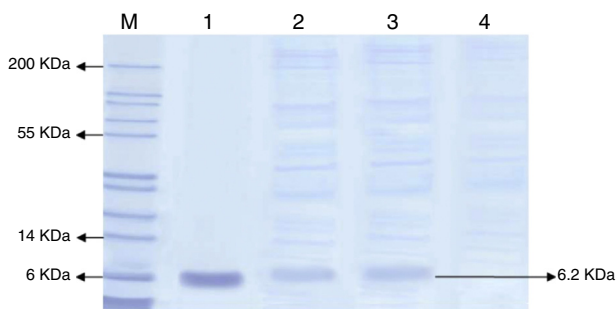


Fig. 1 – SDS-PAGE of total protein secreted from the wild and recombinant strain of *P. pastoris*. The SDS gel was stained by Coomassine blue. M: stained protein ladder; lane 1: standard hEGF protein; lanes 2 and 3: samples of recombinant *P. pastoris* B1 clone; lane 4: sample of wild type *P. pastoris* GS115. Each well was loaded with 100 μL of culture filtrate from recombinant *P. pastoris* B1 and wild type *P. pastoris*.

Culture medium

A time course investigation of hEGF production and biomass accumulation in different media in shake flasks was carried out where the highest hEGF production was obtained in BMMY and MM medium, as shown in Fig. 3. In term of biomass production, all of the media were found to support good growth of the culture. Although, the best cell biomass production was observed in the YPD medium, this medium failed to promote good production of hEGF.

Methanol concentration

The effect of different concentrations of methanol (0.0%, 0.25%, 0.5%, 1.0%, 1.5% and 2%) on growth of the recombinant *P. pastoris* and hEGF production are presented in Fig. 4A and B. A decrease in the production of hEGF and cell biomass was detected, when the concentration of methanol was either too low or too high, although the growth rate was found to be higher only at relatively low concentration (0.25% of the final concentration). The highest level of hEGF production was observed when the concentration of methanol was 0.5% (v/v).

pH and temperature

As shown in Fig. 5, higher yield of hEGF was detected at a pH range of 6–7. Even though *Pichia* can grow on a pH range of 4 and 5, which is slightly acidic, the amount of hEGF was low. Fig. 6 shows the effect of a narrow pH range on hEGF production. The result also showed that the recombinants could grow

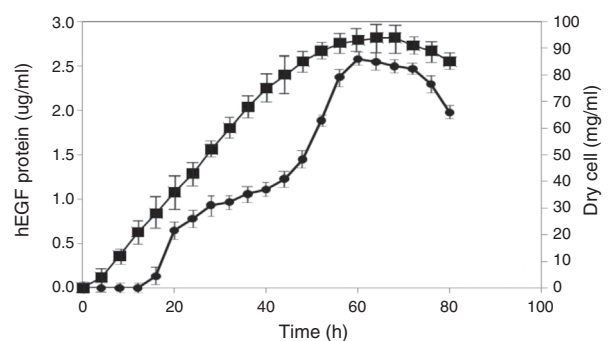


Fig. 2 – A time course of growth and hEGF production by the recombinant *P. pastoris*. Data are means of biological triplicate readings. Dry cell (—■—), hEGF protein (—●—).

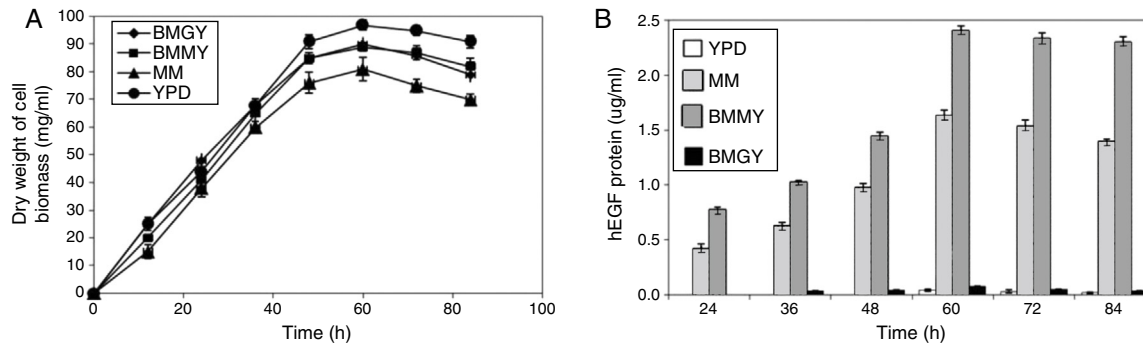


Fig. 3 – A time-course growth and hEGF production by the recombinant *P. pastoris* on different media. (A) Growth curves of the recombinant *P. pastoris* on different media. (B) hEGF production on different media. Data are means of biological triplicate readings.

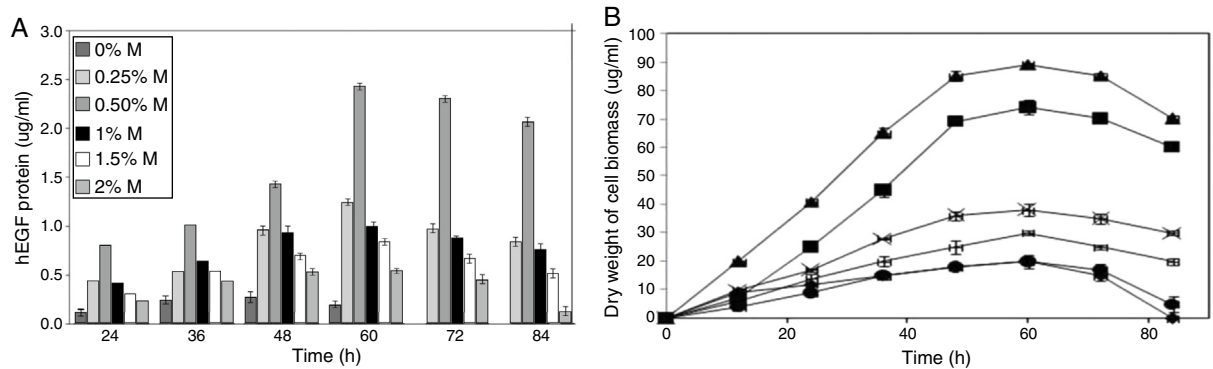


Fig. 4 – A time-course hEGF production and cell biomass of recombinant *P. pastoris* after being induced by different concentrations of methanol. (A) Effects of different methanol concentrations on hEGF production. (B) Effects of methanol concentrations on growth of the recombinant yeast. Data are means of biological triplicates. 0% methanol (—◆—), 0.25% methanol (—■—), 0.5% methanol (—▲—), 1% methanol (—×—), 1.5% methanol (—○—) and 2% methanol (—●—).

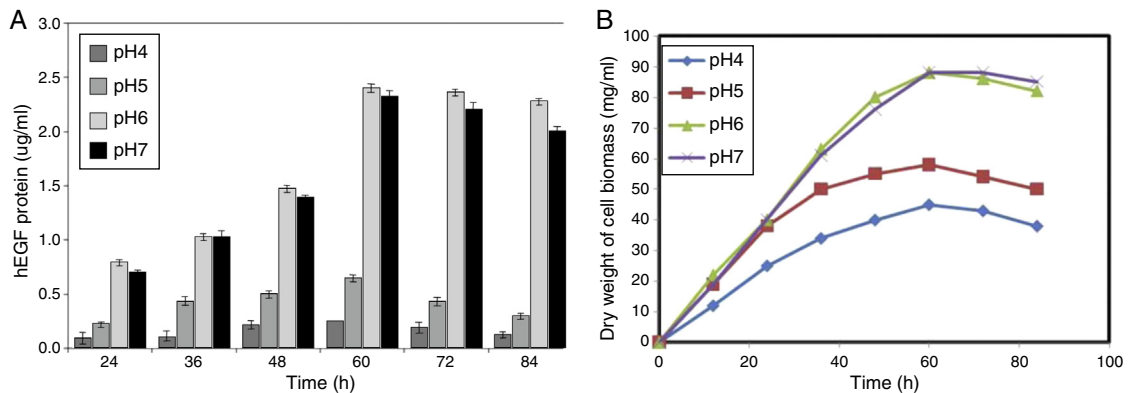


Fig. 5 – Effects of pH on cell biomass and hEGF production by the recombinant *P. pastoris*. (A) Effect of pH on hEGF production. (B) Effect of pH on growth of the recombinant culture. Data are means of biological triplicates.

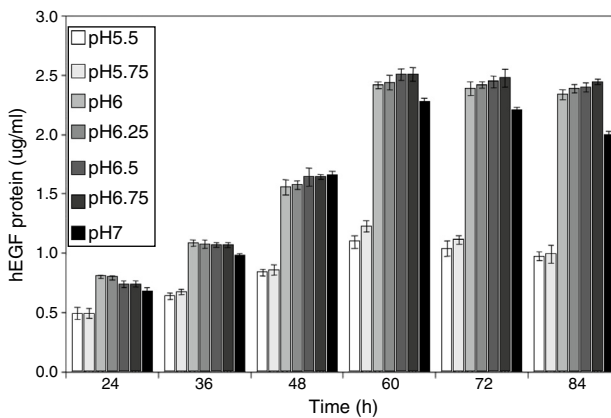
in a wide pH range from 3.0 to 7.0, however the growth rate was found to be lower at pH below 6.0.

Data of the combination effects of pH and temperature analyzed by ANN, as shown in Table 2, revealed significant effects of both pH and temperature on hEGF production. The optimum pH and temperature calculated by the ANN were found to be 7 and 29°C, respectively. The predicted amount of hEGF production at these levels was 2.371 µg/mL. The combinatorial effects of pH and temperature were

represented by the surface plot where changes in the culture media pH showed more impact on the hEGF production than temperature (Fig. 7A). The analysis of ANN sensitivity was carried out to analyze the ratio and ranking of the variables where variables with a ratio higher than one were considered as significant.¹⁴ Based on this, both parameters significantly influenced the production of hEGF (Table 3). The effect of individual factor on hEGF production is shown in Fig. 7B and 7C. The production of hEGF was increased as the pH

Table 2 – Effect of different pH and temperature on hEGF production.

Time (h)	hEGF ($\mu\text{g/mL}$)					
	25 °C			30 °C		
	pH					
	4	5	6.5	4	5	6.5
0	0	0	0	0	0	0
12	0	0	0	0	0	0
24	0	0	0.45	0.09	0.25	0.27
36	0	0.21	0.78	0.11	0.45	1.05
48	0.07	0.25	1.35	0.22	0.49	1.62
60	0.11	0.40	2.21	0.26	0.65	2.48
72	0	0.19	2.17	0.18	0.44	2.44
84	0	0.05	2.10	0.12	0.30	2.37

**Fig. 6 – A time-course of hEGF production by the recombinant *P. pastoris* in a narrow range of culture media pH. Data are means of biological triplicates.****Table 3 – Sensitivity analysis by ANN.**

	pH	Temperature
Ration	1.994860	1.696055
Rank	1.000000	2.000000

increased higher than 4.0. The optimal temperature for hEGF production was 29 °C and the levels of hEGF was expected to be decreased when the temperature was higher than 31 °C.

Discussion

This study was carried out to produce recombinant hEGF in *P. pastoris* GS115 using a commercially available expression vector equipped with a specific signal peptide that directs the transportation of the hEGF protein into the culture medium. The ability of *P. pastoris* to use methanol as a sole carbon and energy source allows the use of methanol alcohol oxidase gene (AOX1) promoter for the expression of the desire gene.^{15,16} Glycerol was used as a sole carbon source until they reached the log-phase growth where methanol was supplemented in the medium for hEGF induction as suggested.¹⁷

The hEGF gene was inserted into the pPIC9K expression vector, and then integrated into the yeast by electroporation.

The clones were screened on YPD supplemented with different concentrations of geneticin from 0.25 to 4 mg/mL. The level of geneticin resistance depends on the copy number of the kanamycin gene integrated into the *Pichia* genome and it seems that multiple integrations of the recombinant gene in the *Pichia* genome increase the expression of the desired protein.^{18–21} Therefore, clones resistant to the high concentration of geneticin were selected for the production of recombinant hEGF.

ELISA-based assay confirmed successful secretion of hEGF in the BMMY medium. Highest level of hEGF was detected in the hyper-resistant clone B1, 60 h post-induction.

The amount of hEGF secreted into the medium was shown to be affected by the fermentation condition. Optimization of culture conditions is one of the determining factors to the success of recombinant protein production in yeast strains. Shaken flask, small-scale expression methods are often the first step in order to optimize the protein expression levels and selecting the best culture conditions,²² although the level of protein production from shake flask cultures is 10-fold lower than the level that would be achieved using a fermenter because of its lower cell density²³ and limited aeration.^{24,25}

Highest level of hEGF was detected in BMMY and MM medium. It can be due to the presence of methanol in these media which acts a sole source of carbon and energy as well as an inducer for the gene expression system. It is a common practice for high production of recombinant protein, high densities culture is used to promote high yield protein production based on the fact that the level of recombinant protein production is proportional to cell density.¹¹ However, this was not happened in this study, as YPD medium which promoted good growth of the yeast culture failed to induce hEGF production. BMMY which support good growth and highest level of hEGF production was selected as the cultivating medium for the recombinant B1 strain. This medium has been shown to be also the best medium for the expression of SAG2 and bikunin.^{26,27}

Optimization of methanol concentration in *Pichia* fermentation system is extremely important because a high level of methanol can be toxic to the yeast.²⁸ In some cases, increasing methanol concentration causes increasing in the expression level such as expression of human β -2-glycoprotein I.²⁹ However the accumulation of methanol has a negative result of cell growth and can lead to a decrease in the expression.^{25,30} On

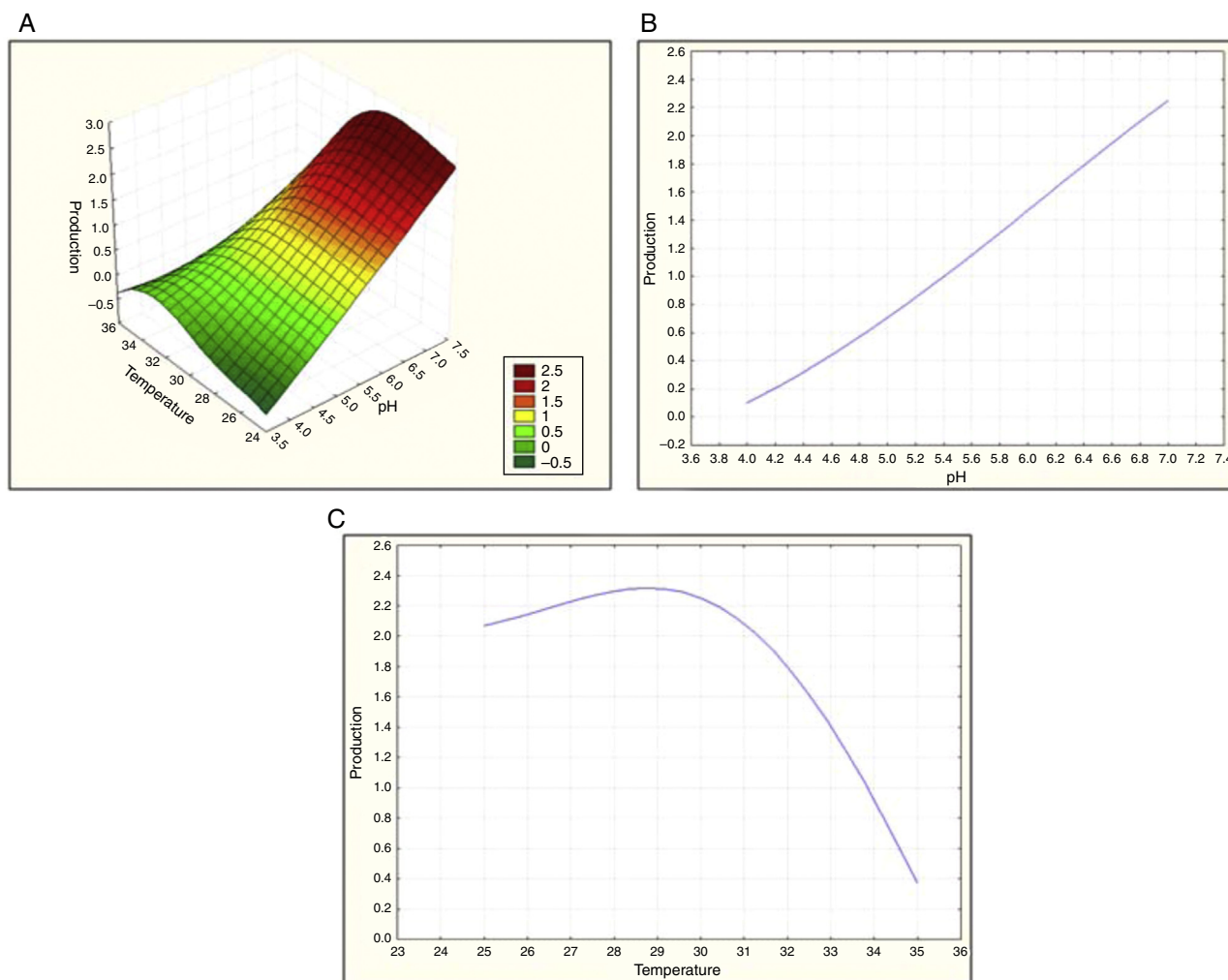


Fig. 7 – (A) Three-dimensional (3D) surface plot showing the effect of pH and temperature on hEGF production. (B) One-factor contour plot showing the effect of pH on hEGF production. (C) One-factor contour plot showing the effect of temperature on hEGF production.

the other hand, a low level of methanol may not be enough to initiate the transcription of the recombinant gene.¹⁰ Methanol concentration of 0.5% (v/v) was considered as the optimal concentration for the production of hEGF which is similar to what has been reported for laccase production in *P. pastoris* host system.³¹ It was shown that methanol with concentration higher than 0.5% (v/v) might inhibit growth whereas very low concentration of methanol might not be enough to support energy metabolism leading to poor growth of cultures. In fact, methanol was not only used to provide a sole carbon source for *Pichia* but also to induce hEGF production. However, the levels of methanol should be kept low to avoid the toxicity effect of methanol,³² which would inhibit cell growth and protein production. The toxicity effect of methanol was evident at 1% (v/v) with the poor growth rate of the culture, which in turn reduced the hEGF production.

Like temperature, pH of culture media plays an important role for optimized heterologous protein production as it is not only affect the yield of heterologous protein production but also affect the integration of the protein structure.³³ In addition, the pH of culture media would also affect the activities

of various proteases that may degrade heterologous proteins. Our results showed that the recombinant *Pichia* could grow in a wide pH range from 3.0 to 7.0, although highest yield of hEGF was obtained at the pH range of 6–7. This feature is an extra advantage of the yeast for recombinant protein production where a wide range of pH can be used for the optimization of the recombinant protein production as demonstrated in this study. Data analysis by ANN showed the significant effects of both pH and temperature on hEGF production, although changes in pH were found to be more effective. ANN analysis recommended 29°C as the optimal temperature for hEGF production in *P. pastoris* and the yield of hEGF was expected to be decrease at temperatures higher than 31°C, possibly due to poor stability of the protein at higher temperatures, releasing of proteases from dead cells, and misfolding of the protein.^{34,35}

In conclusion, this study supports the use of *P. pastoris* as a host for hEGF production, specifically when the overall hEGF production is compared with the production in *E. coli*. The BMMY medium with pH 7 supplemented with 0.5% (v/v) methanol is recommended as the best induction medium for hEGF production in *P. pastoris*.

Conflicts of interest

The authors declare no conflicts of interest.

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REFERENCES

- Carpenter G, Cohen S. Epidermal growth factor. *J Biol Chem.* 1990;265:7709–7712.
- Schultz G, Clark W, Rotatori DS. EGF and TGF- α in wound healing and repair. *J Cell Biochem.* 1991;45:346–352.
- Ojo-Amaize E, Nchekwube E, Cottam H, Oyemade O. Hypoestoxides, derivatives, agonists and crude extracts thereof for use in cosmetic compositions. Google Patents; 2004.
- Orvar BL, Mantyla E. Use of plant-derived recombinant growth factors in skin care. Google Patents; 2009.
- Wang C, Davis L JR, Wu C, Japar S, Niki H, Weinstock B. Epidermal growth factor: identification of a new hormone in human urine. *Appl Phys Lett.* 1972;21:209.
- Ptitsyn L, Al'tman I. Extracellular production of recombinant human epidermal growth factor (hEGF) in *Escherichia coli* cells. *Bioorg Khim.* 1999;25:923–929.
- Ebisu S, Takagi H, Kadowaki K, Yamagata H, Udaka S. The efficient production of human epidermal growth factor by *Bacillus brevis*. *Ann N Y Acad Sci.* 1996;782:115–122.
- Topczewska J, Bolewska K. Cloning and expression of the hEGF gene in *Saccharomyces*. *Acta Biochim Pol.* 1993;40:4–7.
- Yu W, Chen J, Zhao X, Lv Z, Nie Z, Zhang Y. Expression of polyhedrin-hEGF fusion protein in cultured cells and larvae of *Bombyx mori*. *Afr J Biotechnol.* 2006;5.
- Cereghino JL, Cregg JM. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS Microbiol Rev.* 2000;24:45–66.
- Cregg JM, Cereghino JL, Shi J, Higgins DR. Recombinant protein expression in *Pichia pastoris*. *Mol Biotechnol.* 2000;16:23–52.
- Razis AFA, Ismail EN, Hambali Z, Abdullah MNH, Ali AM, Lila MAM. The periplasmic expression of recombinant human epidermal growth factor (hEGF) in *Escherichia coli*. *Asia-Pac J Mol Biol Biotechnol.* 2006;14:41–45.
- Linder S, Schliwa M, Kube-Granderath E. Direct PCR screening of *Pichia pastoris* clones. *Biotechniques.* 1996;980–982.
- Lou W, Nakai S. Application of artificial neural networks for predicting the thermal inactivation of bacteria: a combined effect of temperature, pH and water activity. *Food Res Int.* 2001;34:573–579.
- Ellis SB, Brust PF, Koutz PJ, Waters A, Harpold MM, Gingeras TR. Isolation of alcohol oxidase and two other methanol regulatable genes from the yeast *Pichia pastoris*. *Mol Cell Biol.* 1985;5:1111–1121.
- Tschopp JF, Brust PF, Cregg JM, Stillman CA, Gingeras TR. Expression of the lacZ gene from two methanol-regulated promoters in *Pichia pastoris*. *Nucleic Acids Res.* 1987;15:3859–3876.
- Clare JJ, Romanes MA, Rayment FB, et al. Production of mouse epidermal growth factor in yeast: high-level secretion using *Pichia pastoris* strains containing multiple gene copies. *Gene.* 1991;105:205–212.
- Vedvick T, Buckholz RG, Engel M, et al. High-level secretion of biologically active aprotinin from the yeast *Pichia pastoris*. *J Ind Microbiol Biotechnol.* 1991;7:197–201.
- Cregg JM, Vedvick TS, Raschke WC. Recent advances in the expression of foreign genes in *Pichia pastoris*. *Nat Biotechnol.* 1993;11:905–910.
- Scorer CA, Clare JJ, McCombie WR, Romanos MA, Sreekrishna K. Rapid selection using G418 of high copy number transformants of *Pichia pastoris* for high-level foreign gene expression. *Nat Biotechnol.* 1994;12:181–184.
- Vassileva A, Arora Chugh D, Swaminathan S, Khanna N. Effect of copy number on the expression levels of hepatitis B surface antigen in the methylotrophic yeast *Pichia pastoris*. *Protein Expr Purif.* 2001;21:71–80.
- Daly R, Hearn MTW. Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production. *J Mol Recognit.* 2005;18:119–138.
- White C, Kemp N, Komives E. Expression of highly disulfide-bonded proteins in *Pichia pastoris*. *Structure.* 1994;2:1003–1005.
- Barr K, Hopkins S, Sreekrishna K. Protocol for efficient secretion of HSA developed from *Pichia pastoris*. *Pharm Eng.* 1992;12:48–51.
- Romanos M. Advances in the use of *Pichia pastoris* for high-level gene expression. *Curr Opin Biotechnol.* 1995;6:527–533.
- Thiruvengadam G, Init I, Fong M, Lau Y. Optimization of the expression of surface antigen SAG1/2 of *Toxoplasma gondii* in the yeast *Pichia pastoris*. *Trop Biomed.* 2011;28:506–513.
- Gou X-H, Liu Y-Y, Chen Q-L, et al. High level expression of bikunin in *Pichia pastoris* by fusion of human serum albumin. *AMB Express.* 2012;2:1–7.
- Zhang W, Bevins MA, Plantz BA, Smith LA, Meagher MM. Modeling *Pichia pastoris* growth on methanol and optimizing the production of a recombinant protein, the heavy-chain fragment C of botulinum neurotoxin, serotype A. *Pap Biotechnol.* 2000:1–8.
- Katakura Y, Zhang W, Zhuang G, et al. Effect of methanol concentration on the production of human [beta] 2-glycoprotein I domain V by a recombinant *Pichia pastoris*: a simple system for the control of methanol concentration using a semiconductor gas sensor. *J Ferment Bioeng.* 1998;86:482–487.
- Sarramegna V, Demange P, Milon A, Talmont F. Optimizing functional versus total expression of the human [mu]-opioid receptor in *Pichia pastoris*. *Protein Expr Purif.* 2002;24:212–220.
- Hong F, Meinander NQ, Jönsson LJ. Fermentation strategies for improved heterologous expression of laccase in *Pichia pastoris*. *Biotechnol Bioeng.* 2002;79:438–449.
- Katakura Y, Zhang W, Zhuang G, et al. Effect of methanol concentration on the production of human β 2-glycoprotein I domain V by a recombinant *Pichia pastoris*: a simple system for the control of methanol concentration using a semiconductor gas sensor. *J Ferment Bioeng.* 1998;86:482–487.
- Fullbrook PD, Godfrey T, West S. *Industrial Enzymology*. London: MacMillan Press; 1996.
- Hong F, Meinander NQ, Jonsson LJ. Fermentation strategies for improved heterologous expression of laccase in *Pichia pastoris*. *Biotechnol Bioeng.* 2002;79:438–449.
- Jahic M, Wallberg F, Bollok M, Garcia P, Enfors SO. Temperature limited fed-batch technique for control of proteolysis in *Pichia pastoris* bioreactor cultures. *Microb Cell Fact.* 2003;2.