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Enhanced production of human epidermal growth factor (EGF) in *Escherichia coli*

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Abstract

Epidermal Growth Factor (EGF) is a monomer polypeptide consists of 53 amino acids with three intra-molecular disulfide bridges bonds. EGF is produced from different tissues in human and possesses valuable therapeutic effects. In the present study, we reported the high throughput expression of hEGF in *Escherichia coli* BL21 (DE3). For achieving this purpose, the designed vector was constructed based upon pET-28a (+) with T7 promoter. The synthetic hEGF gene was cloned in pET28a using *NcoI* and *HindIII* sites. Recombinant vector, pET28-hEGF, was transferred into *E. coli* BL21 (DE3) and induced for expression in a lab and bench scale. For this reason, at first effects of medium, temperature, and induction time in three-level were investigated on production enhancement of hEGF by using Taguchi experimental design in shake flask. Data processing by Qualitek-4 software was shown that maximum production acquires from TB medium at 28°C with IPTG concentration of 0.1mM. Under these conditions the final cell dry weight and the ultimate of hEGF concentration were 5.61g/l and 1.094 g/l respectively. Then, effects of induction time and glycerol concentration were examined at three levels in a bench bioreactor with full factorial approach. Under optimized conditions glycerol 15 g/l and induction time at OD_{600}=5 with medium TB obtained final cell dry weight and the last of hEGF concentration were 10.58g/l and 2.28 g/l respectively. This amount of protein is one of the highest values which have been reported in non-continuous system.

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Introduction

Epidermal Growth Factor (EGF) is a 53 amino acid protein hormone with three intra-molecular disulfide bridges bonds (Harris RC *et al.*, 2003; Lu HS., 2001). Disulfide bonds divided EGF structure to three loops which are called from A-C.A loop contains helical bundle while B loop has β-sheet structure (Ogiso H *et al*,. 2002). The residue of Gln^{43}, Arg^{41}, Tyr^{37}, His^{16}, Leu^{15}, and Leu^{47} are essential for EGF function (Groenen LC., 1994; Van Zoelen *et al.*, 2000; Dreux AC *et al.*, 2006).

EGF has a lot of applications in medicine and Pharmaceutical Science. Mitosis induction, Implantation, Morphogenesis, Cytoprotection, tissue Trophism, and epithelial regeneration are critical roles of EGF protein (Venturi S and Venturi M, 2009; Chia CM *et al.*, 1995; Thesleff I *et al.*, 1995; Kato M *et al.*, 1995; Gupta C, 1996; Goldman B *et al.*, 1996;). The main source of EGF is Parotid gland. Production of EGF from animal's source has many disadvantages and it is not suitable for human applications (Barrow RE *et al.*, 1993; Liu A *et al.*, 1992; Stahlman MT *et al.*, 1989; Thesleff I *et al.*, 1988). The greatest way for production of this protein is recombinant DNA technology, batch fermentation and High cell density culture for *E. coli* (Choi JH *et al.*, 2006; Lee S.Y, 1996). *Escherichia coli* have been used as a heterologous expression host for both industrial and academic research applications. *E. coli* can grow rapidly and at high density on economical substrates, and the large number of compatible tools available for biotechnology particularly cloning vectors and host strains and the simple process scale up (Baneyx F, 1999; Babaeipour V *et al.*, 2013). Using *BL21* derived strain of *E. coli* can reduce proteolyses of heterologus protein (Chem R, 2011). The most favorable expression of recombinant protein in *E. coli* can be effortlessly achieved when the growth conditions are controlled.

Medium components, growth temperature, and induction time have insightful effects on the way in which recombinant protein is produced. The aim of the present study was to investigate the expression efficiency of hEGF in E. coli expression system in shake flasks and bench top bioreactor**.** To achieve this goal, we design a synthetic gene and clone in pET28a using *Hind III* and *NcoI* sites. Recombinant vector, pET28a-hEGF, transferred into *E. coli* BL21 (DE3). So as to reach maximum expression different medium like LB, TB and 32Y with different temperatures such as 24, 28, 32 and 37°C was applied. The results of experiments were analyzed by SDS-PAGE, cell growth and recombinant protein production kinetics. The major objective of this study is over- production of recombinant human insulin-like growth factor I(rhIGF-I) through a developed process by recruiting effective factors in order to achieve the most recombinant protein. In this study we investigated the effect of culture medium,induction temperature and amount of inducer on cell growth and EGF production.

Materials and methods

All chemicals were procured from Sigma, Merck and promega. *Escherichia coli* strain BL21 (DE3) was used as the expression host for hEGF. For gene cloning and expression pET28a was used. *E. coli* BL21 (DE3) were grown in Luria-Bertani medium (LB) 1 (w/v) % peptone, 0.5 (w/v) % yeast extract and 1(w/v) % NaCl. The bacterial cells were grown in TB medium (1.2 (w/v)% peptone, 2.4 (w/v)% yeast extract, 4ml Glycerol, 0.17M KH_2PO_4, 0.72 M K_2HPO_4) and 32Y (0.8 (w/v)% peptone, 3.2 (w/v)% yeast extract, 500mM NaCl). The medium were supplemented with 50µg/mL Kanamaycin (Sigma) after autoclaving.

Cloning and expression

Synthetic hEGF gene was cloned in pET28a (+) (Novagen) via *NcoI* and *HindIII* sites. Plasmids containing hEGF gene were transformed in to *E. coil* BL21 (DE3) competent cells using CaCl2 method, plated on LB agar contains 30µg/ml Kanamaycin and incubated at 37°C over night. Freshly transformed *E. coil* BL21 (DE3) cells were grown in LB broth to an approximate optical density OD_{600} of 0.7 in a shaker (200rpm) at 37°C in total volume of 50ml contains 40µg/ml Kanamaycin. Plasmids were purified using plasmid kits from Vivantis (Selangor, Malaysia).

Protein expression was induced by the addition of an IPTG with final concentration of 0.1mM. After 4h of growth, cells were harvested by centrifugation at 6000g for 5 min at 4°C, resuspended in 100µl of sample buffer and 5 µl was loaded on to 17.5% sodium dodecyl sulfate-poly acryl amide gel electrophoresis (SDS-PAGE). The bacterial stocks were kept at −70 °C in 20 % (v/v) glycerol for long-term usage.

Small scale expression cultures

Optimization of culture conditions were performed for maximum expression of hEGF in *E. coil* BL21 (DE3). Protein expression was tested in TB, LB and 32 Y medium at 24°C, 28°C and 32°C with three IPTG concentration (0.05, 0.1and 0.15 mM).these experiment was done according to taguchi methods.

Inoculum preparation for fermentation

An individual colony was selected from LB agar plate and inoculated into 5 mL of terrific broth (TB) medium containing 50µg/mL kanamaycin. The 5 mL *E. coli* culture was incubated in shaking incubator overnight at 37 °C. 100 µL of the overnight culture was inoculated into 200 mL TB medium containing 50µg/mL kanamaycin. It was grown in shaking incubator at 37 °C and after reaching to OD600nm=0.7, then it was transferred to the bioreactor as seed culture.

Batch Fermentation

Bacteria were grown in a 3.5L Fermenter (Infors) containing 1.3L medium which was supplemented with 40 µg/ml kanamaycin and 0.1% (v/v) silicone antifoam. The medium was inoculated with 200ml of overnight culture and then incubated at 37°C by constant stirring (800 -900 rpm) with 1 vvm air supply. The pH was controlled at 7±0.05 with the addition of HCl 1N or NaOH 1M. Dissolved oxygen was controlled at 30-40% of air saturation by the control of both the inlet air and agitation rate. When cell density of culture reach 2.3and 4.6 g/L the temperature was reduced to 28°C and protein expression induced by IPTG to final concentration of 0.1 mM. In batch fermentation protein expression was tested in TB medium which carbon source was substitute with glucose and glycerol. At first experiment, carbon source was 10g glucose and when cell density of culture reach 4.6 g/L inductions was done. In the second and third experiment carbon source was 10 and 20 g/L and induction was done at2.3 and 4.6 g/L respectively. The cells were harvested by centrifugation at 6000 g for 5 min at 4°C.

SDS-PAGE and Bradford analysis

Electrophoresis was carried out in 0.7mm thick gels, using a BioRad setup followed by coomassie staining. Gels quantified by gel densitometer and Total protein were analyzed by bradford assay with bovine serum albumin as standard.

Results and discussion

Cloning and expression of hEGF

For an efficient expression of recombinant hEGF in *E. coli*, Construction of hEGF DNA encoding sequence was designed and Codon optimization was performed. Open reading frame (ORF) was inserted between *NcoI* and *HindIII* in pET28a restriction site. Figure 1 also describes the schematic diagram of the protein which was used in this work. The designed construct was analyzed using Vector NTI 11.0 software to ensure that the restriction sites cannot interfere with cloning.

The prepared vector encoding was transformed into the host cells for recombinant protein production. The expression of the recombinant protein was induced using IPTG at final concentrations of 0.1 mM at 37 °C. IPTG addition induces the expression of T7 RNA polymerase resulting in transcription of the hEGF gene under the control of the T7 promoter in the recombinant cells harboring the constructed plasmid. The theoretical molecular mass of hEGF is 6.2 kDa. SDS-PAGE analysis showed an outstanding polypeptide band in conformity with the expected molecular mass (Fig. 2).

Effects of different parameters on protein expression in shake flask batch cultivation

Different factors such as Culture conditions,

induction time, growth temperature, and IPTG concentration have a profound effect on protein expression. The conditions that decrease the rate of protein expression, such as induction at low temperatures may increase target protein expressio. According to our studies, the expression of recombinant hEGF was examined in three medium, three different temperatures, and three IPTG concentrations according to taguchi methods. There is a comparison between final cell density and recombinant protein expression level in Table-1 and fig. 3.

Table 1. The effects of medium type, IPTG concentration, and temperature on protein expression and final cell density.

hEGF(g/L)	Percentage of expression	Total protein (g/L)	Dry weight g/L	OD_{600} Final	medium	Inducer concentration	temperatures	Test number
0.09	13٪	0.70	1.47	3.2	LB	0. 05	24	1
0.53	29٪	1.86	3.58	7.8	TB	0.1	24	2
0.14	15٪	0.99	2.07	4.5	32Y	0.15	24	3
0.25	23٪	1.09	2.39	5.2	LB	0.1	28	4
0.89	32٪	2.80	5.61	12.2	TB	0.15	28	5
0.40	27٪	1.49	3.12	6.8	32Y	0.05	28	6
0.16	19٪	0.88	1.93	4.2	LB	0. 15	32	7
0.56	30٪	1.87	3.91	8.5	TB	0.05	32	8
0.48	31٪	1.58	3.31	7.2	32Y	0.1	32	9

Table 2. Effect of induction time and medium component on hEGF production and final cell density.

Test number	Cell density at induction time OD_{600}	Glycerol concentration (g/L)	Total protein (g/L)	OD_{600} Final	Dry Cell weight g/L	Percentage of expression	hEGF(g/L)
1	1	10	3.31	13.1	6.02	23	0.76 ± 0.03
2	5	10	3.86	15	6.9	30	1.16 ± 0.04
3	10	10	4.42	16	7.36	35	1.94 ± 0.05
4	1	15	3.52	14	6.44	20	0.704 ± 0.03
5	5	15	6.35	22	10.58	36	2.28 ± 0.05
6	10	15	4.9	18	8.28	35	1.175 ± 0.04
7	1	20	3.95	15	6.9	33	1.3 ± 0.04
8	5	20	5.45	21	9.66	30	1.635 ± 0.04
9	10	20	4.95	20	9.2	25	1.24 ± 0.04

Effects of medium type

To study the effect of medium types on cell growth and recombinant protein expression three various medium including LB, TB, and 32Y was selected. As fig. 4 shows Level 2 (TB medium) is the most appropriate for hEGF expression. The TB medium increased the final cell density and recombinant protein expression. Also, the LB produced the lowest final cell density.

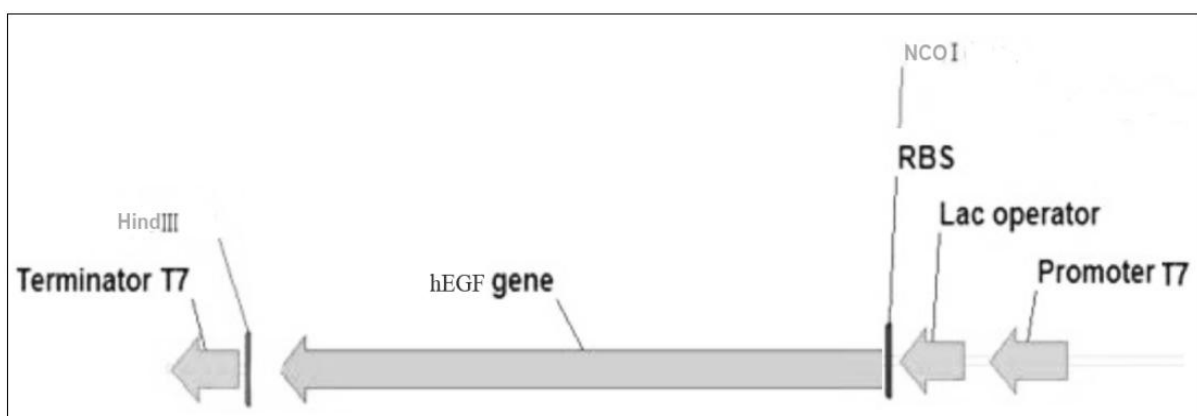

Fig. 1. The schematic diagram of the protein which was used in this work The expression vector was constructed on pET28a under the control of T7 promoter.

Accordingly, 32Y medium resulted in greater cell mass and protein expression than LB, but no rising was seen in recombinant protein expression level than TB. Translation of the recombinant protein in host cells causes metabolic demands which lead to slow the growth rates of host (Singh AB and Mukherjee K, 2013). Gordon and coworkers showed that decreasing the level of peptone leads to increase growth rate and the maintenance of cells in stationary phase. When the concentration of peptone in medium is low, the levels of yeast extract can determine the cell growth (Gordon E *et al.*, 2008).

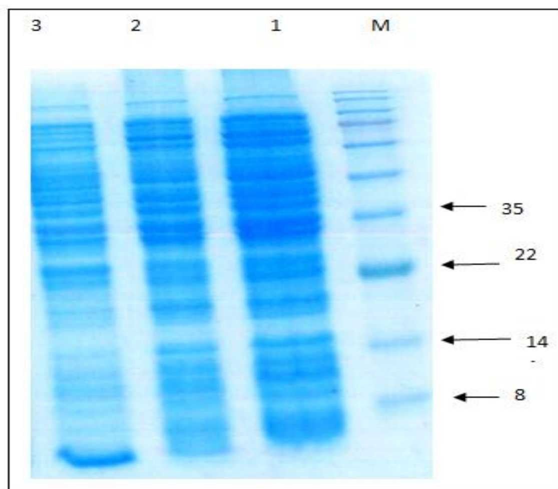

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis for overexpression of hEGF in E. coli BL21. Lane M; Molecular weight marker, lane 1; total protein before addition of IPTG , lane 2; total protein 1h after addition of IPTG in BL21 (DE3) lane 3; total protein 4h after addition of IPTG in BL21 (DE3).

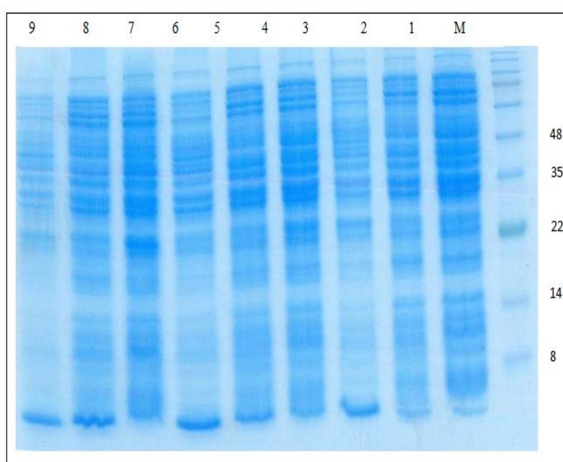

Fig. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis for overexpression of hEGF in E. coli BL21. Lane M; Molecular weight marker, lane 1; LB medium at 24°C, lane 2; 32 Y medium at 24°C, lane 3; TB medium at 24°C, lane 4; LB medium at 28°C, lane 5; 32Y medium at 28°C, lane 6; TB medium at 28°C, lane 7; LB medium at 32°C, lane 8; 32Y medium at 32°C, lane 9; TB medium at 32°C.

The yeast extract contains nitrogen source and different growth factors in contrast with peptone. this may lead to protein expression in TB medium is more than LB medium .TB medium have glycerol as carbon source and phosphate-buffer which can reduces the production of acetate an drop in pH respectively. Presence of nitrogen-containing complex compounds in LB and 32Y medium results in ammonium production and increasing pH. In contrast to LB and 32 Y medium, TB is a phosphate-buffered medium. Presence of k2HPO4 and kH2PO4 as phosphate buffer can retain the pH of the medium during the culture growth. This prevents cell death of bacteria due to a fall in pH (Kahaki AF *et al.*, 2014). Therefore, TB medium was chosen as suitable medium for following steps. As it shows is suitable for.

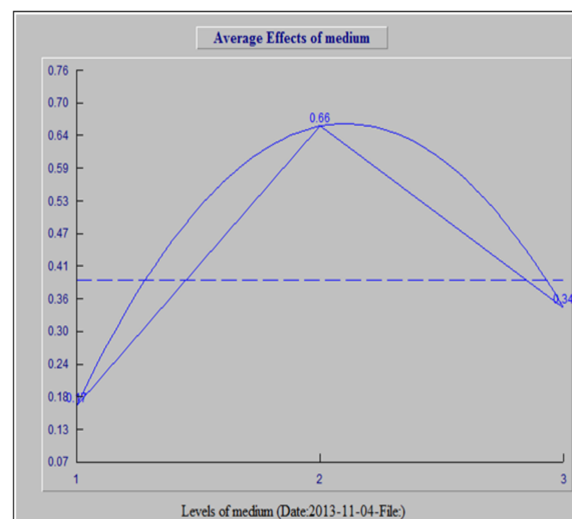

Fig. 4. The average effect of medium on hEGF expression. Level 2 (TB medium) is the most suitable for hEGF expression.

Growth temperature

The effects of growth temperature on recombinant protein expression were examined in three values of 24 °C, 28°C, and 32°C. Decreasing temperature from 32°C to 28°C increased hEGF expression. Heat can cause stress for cell which induce heat shock protease and hindering over-expression of target protein

(Cunningham F and Deber CM, 2007).According to this, increasing hEGF expression may be due to decreasing in proteolysis degradation. Fig.5 shows the average effect of growth temperature on protein expression. The results were showed that combination of TB medium with an IPTG concentration of 0.1mM and 28°C are the best condition for hEGF expression. The last OD_{600} and dry cell weight at this condition was 12.2 and 5.61g/L respectively. The final biomass in TB medium with an IPTG concentration of 0.1mM and 28°C was 5.61 g/L which is noticeable for the production of membrane proteins in *E. coli* in shake flask batch cultivation.

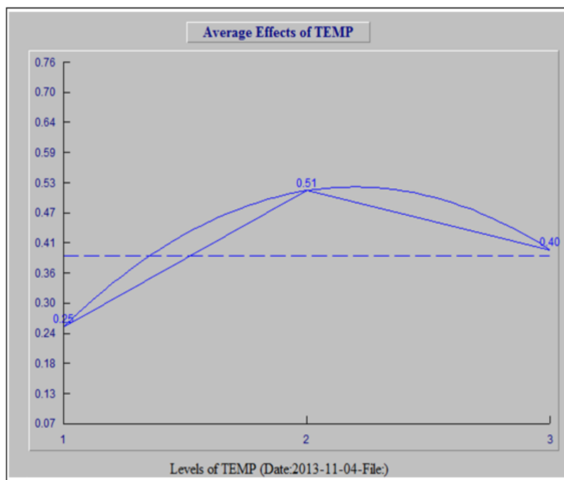

Fig. 5. the average effect of growth temperature on protein expression, 28°C is the best temperature for recombinant hEGF expression.

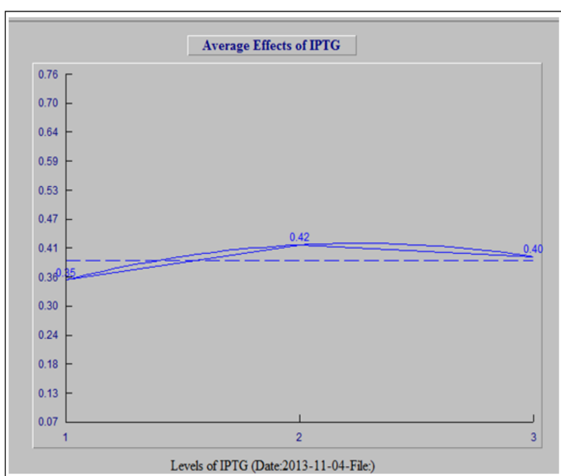

Fig. 6. The average effect of IPTG on hEGF expression. Level 2 (0.1 mM) is the most suitable for hEGF expression.

IPTG concentration

IPTG addition induces the expression of T7 RNA polymerase resulting in transcription of the hEGF gene under the control of the T7 promoter in the recombinant cells harboring the constructed plasmid (Klepsch MM, 2011). Three concentrations of IPTG (0.05, 0.1 and 1.5mM) were used for increasing expression level. Increasing the concentration of IPTG from 0.05 to 0.1 increased expression level but more IPTG did not affect the level of protein expression and increased the cost of production process. Therefore; an IPTG concentration of 0.1mM was chosen as proper value for recombinant fusion protein expression at subsequent steps of this study. Fig. 6 indicates that 0.1 mM IPTG concentration is the most suitable for hEGF expression.

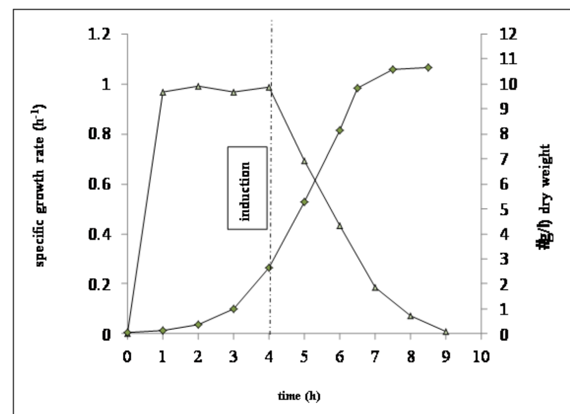

Fig. 7. specific growth rate in TB+15gr glycerol medium and Induction at OD_{600}=5 Dry weight.

Effects of induction time and medium component on expression in batch fermentation

In addition to medium and temperature the yield of recombinant protein expression will also depend on the point in the growth phase at which expression induced (Babaeipour V *et al.*, 2013; Kahaki AF *et al.*, 2014; Choi JH *et al.*, 2004). For this reason, the effect of induction time on hEGF expression in batch fermentation was studied after flask scale expression. Induction was done at three induction time ($OD_{600}$of 1.0, 5.0 and 10.0) and three glycerol concentration (10,15and 20 g/L). The induction time was chosen according to the growth curve of *E. coli* BL21 (DE3) in TB medium and 28°C for non-induction situation (Fig. 7). Table 2 indicates the effects of induction time and medium component on hEGF production and final cell density. The final cell density was enhanced

to 10.58 g/L by induction at OD_{600}=5 and adding 15g/L glycerol to medium. It seemed that induction at cell density of OD_{600}=1 in comparison with the induction at OD_{600}=5 cause cell growth to end by over expression of hEFG before cell density reaches to highest level. Induction time at OD_{600}=10 decreases productivity of hEGF. This may be due to the bacterial cells that enter the stationary phase. The final concentration of hEGF increased to 0.05 g/l, which showed valuable amount of hEGF production of in *E. coli*.

Conclusion

Here, we introduce the over expression of recombinant hEGF in *E.coli* expression system. This study focused on optimization of hEGF expression in different medium, temperature, induction time, and IPTG concentration. This enhancement of production yield suggests that this plan may be successfully applied to practical large-scale fermentations of E. coli expression systems.

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