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Optimization extracellular secretion of Recombinant Human Epidermal Growth Factor (hEGF) in *Escherichia coli* BL21 (DE3) pD881-OmpA-hEGF by using Response Surface Method (RSM)

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ABSTRACT

Human Epidermal Growth Factor is techno-economically protein that has the function for stimulates a process of proliferation and cell differentiation. Because of that function, it can be a candidate for wound healing in ulcer diabetic. In the previous study, hEGF can extracellular secreted by OmpA signal peptide using recombinant DNA. The production method of recombinant hEGF will be extracellular secreted by *Escherichia coli*. However, the optimum secretion of Outer Membrane Protein A (OmpA) has not been studied. Therefore the purpose of this study is to determine the optimum conditions using the RSM method. This research begins with a rejuvenation of culture, extracellular secretion, and optimization with the RSM method, followed by protein production at optimum condition. Secreted recombinant protein measured by Sandwich ELISA method and LOWRY. The result showed that the optimum condition was found in the medium concentration of 1.5x and induction time at 2 hours 10 minutes and the total protein yield of expression was 21.247 mg/mL with optimization percentage 2.96% and efficiency 50.56 % and recombinant protein hEGF concentration in fermentation media 416 μg/mL.

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INTRODUCTION

Diabetes mellitus (DM) is one of the major health problems in Indonesia. Data from a global study show that the number of people with Diabetes Mellitus in 2011 has reached 366 million people. If there is no action taken, the patient number is expected increased until 552 million in 2030 century. Diabetes mellitus has been caused of 4.6 million people deaths. Diabetes Mellitus is a metabolic disorder disease and characterized by the increase of blood sugar due to decreasing insulin secretion by pancreatic beta cells or insulin resistance (Setyorogo and Trisnawati, 2013). Ulcer or diabetic gangrene is a chronic complication that affects many diabetic patients (Ruan *et al.*, 2013).

Human Epidermal Growth Factor is 6.2 kDa protein consisted of 53 amino acid and single chain polypeptide. It has three disulfide bridges for have biological activity. Protein isolated from the submandibular cell of male rate, and known had activity for stimulates migration and proliferation of epithelial

cells (Chen *et al.*, 2014). Human EGF is had potential as effective wound healing and had function as problems of premature ageing (Maksum *et al.*, 2017a). Therefore, the method for produce large and high efficiency of Human EGF needed. One of the most method used is by recombinant DNA technology, which is by transforming human EGF (hEGF) gene coding from human into other organisms (Calnan *et al.*, 2000).

In the previous study, hEGF protein have been extracellular secreted using *Sec-Dependent* signal peptide role by Outer Membrane Protein A (OmpA) protein with hEGF protein level 71.25 μg/mL into culture media (Fahmi, 2016). In another our previous study hEGF protein has been optimize using Response Surface Method for Pectate Lyase B (PelB) signal peptide and have been optimize into 185.7% using fermentor and get hEGF protein for about 121,76 g/mL (Maksum *et al.*, 2017b). Therefore, in this study, we will determine the optimum conditions for expression hEGF by using OmpA signal peptide with Response Surface Method and determines the levels of rhEGF that secreted into the fermentation media through extracellular secretion under optimum conditions.

MATERIALS AND METHODS

Reagents and Chemical

Material used in this study were bacto agar (Oxoid), agarose (Genetika Sains), ammonium persulfate (Bio Basic INC), Bromphenol Blue (Merck), EDTA (Merck), *E. coli* BL21 (DE3) (Novagen), Gel red (Biotium), hEGF synthetic gene inserted to pD881-OmpA plasmid (synthesized by DNA 2.0, California, USA), DNA 1 kb marker (Thermo Fischer Scientific), Precision Plus Protein™ Dual Color Standards (Biorad), Sodium Deodecyl Sulphate (Merck), TEMED (Sigma), Tris Base (Merck), Tricine (Sigma), Tryptone (Sigma), Yeast Extract (Merck).

Statistical Analisis Software

Statistical analysis will show by Minitab[17] Software from Minitab™.

Regeneration of *E. coli* BL21 (DE3) [pD881-OmpA-hEGF]

500 μL of glycerol stock of *E. coli* BL21 (DE3) [pD881-OmpA-hEGF] spread into solid LB media with kanamycin total concentration 4mM. Then it was incubated about 16-18 hour in 37ºC. Single clone adds into 5 mL liquid LB media with 5μL kanamycin antibiotic 4mM, 16-18 hour in temperature 37ºC with shaking speed of 200 rpm. *E. coli* BL21 (DE3) [pD881-OmpA-hEGF] culture added 20 μL into a microtube which has been contained 180 μL glycerol 10% and then saved in temperature -20ºC.

Expression Test of *E. Coli* BL21 (DE3) [pD881-OmpA-hEGF]

E. coli BL21 (DE3) [pD881-OmpA-hEGF] that was characterized, grown in 5 mL of liquid LB media which contained kanamycin 4mM for about 16-18 hours, in temperature 37° C and 200 rpm shaking speed. Cell culture of *E. coli* BL21 (DE3) [pD881-OmpA-hEGF] was added 1000 μL into Erlenmeyer flask containing 100 mL liquid LB and kanamycin 4 mM. *E. coli* BL21 (DE3) native bacteria also grown in 5 mL of liquid LB media for 16-18 hours, 37ºC and 200 rpm shaking speed, then cultured in 1000 of LB media.

Then all cultures in the Erlenmeyer flask incubated at 37ºC and shaking speed 200 rpm until OD_{600} reached at 0.6. Then 1 mL culture of *E. coli* BL21 (DE3) [pD881-OmpA-hEGF] taken as t_0 (before induction of L-rhamnose) and collected into a 1.5 mL microtube, then cell pellet and it's supernatant separated by using centrifugation with velocity 3000 *g*, 4ºC for 20 minutes. Then the culture of *E. coli* BL21 (DE3) [pD881-OmpA-hEGF] and *E. coli* BL21 (DE3) native in Erlenmeyer flasks added by L-rhamnose until had the total concentration of 4 mM in culture, then incubated at 37ºC, shaking speed 200 rpm for 20 hours. For about 2 mL of *E. coli* BL21 (DE3) [pD881-OmpA-hEGF], culture was taken as t_i (after induction of L-rhamnose) 1 mL, separated the cell pellet and its supernatant by centrifugation with a velocity of 3000 *g*, 4ºC for 20 minutes.

Making the Growth Curve of *E. coli* BL21 (DE3) [pD881-OmpA-hEGF]

E. coli BL21 (DE3) [pD881-OmpA-hEGF] then grown in 5 mL liquid LB media which has contained kanamycin 4 Mm until 16-18 hours, in temperature 37ºC and 200 rpm shaking speed. Cell culture of *E. coli* BL21 (DE3) [pD881-OmpA-hEGF] was added 1000 μL into an Erlenmeyer flask containing 100 mL liquid LB and kanamycin antibiotic 4 mM. Culture grown in shaking incubator for about 12 hours, temperature 37º C and 200 rpm shacking speed and culture measured in spectrophotometer OD_{600} every hour.

Optimization of rhEGF Secretion By Response Surface (RSM) Experimental Design

Response Surface Method experimental design and processing of experimental we used software Minitab 17 to get the experimental optimum condition. Factors that test at least in three levels, that is minimum, medium, and maximum or equivalent to -1, 0, 1 that called as unit code. These three levels

are within the range of the upper and lower limits of a variable that input in this experimental design. In this research, we used two factors namely induction time and media concentration. The upper and lower limits for the concentration factor of the media are concentrations 1 until 2x media concentration, and for the upper and lower limits for induction time variable determined from the growth curve of *E. coli*. Then, the upper and lower limit variables entered into Minitab 17 software and this software has given the experimental design. The design used as expression optimization strategy and perform running following experimental design. The result of each variable on the media measured by LOWRY method for getting total protein yield.

After total protein measured by using Lowry, the data of each run obtained in Minitab 17 software and the equation was used to determine the prediction of total protein concentration in optimum condition. After getting optimum condition we determine the validation of the method. Then the optimum condition did in fermentor scale.

SDS-PAGE analysis

Secreted proteins were separated on a 15% Tricine SDS-polyacrylamide gel. 10 μL of each sample was boiled in a 5 μL sample buffer (312 mM Tris–HCl pH 6.8,glycerol 50% (v/v), bromophenol blue 0.05% (w/v) and dH2) for10 min before the sample mixture was being loaded onto the gel by using (Haider *et al.*, 2012) protocol.

Measurement of rhEGF content with ELISA Method

Measurement of hEGF concentration by using the ELISA method with protocols and kit from Qiayee-Science.

Production of recombinant hEGF on the fermentor 2L

The fermentation process begins with the preparation of inoculum, a single colony on a solid LB is taken and fed into 20 mL liquid LB media which has been added kanamycin 4mM, then incubated at 37ºC, shacking speed 200 rpm for 18 hours. Then inoculum added into fermentor that was contained 2L LB media and kanamycin 4mM with fermentation conditions in pH 7, temperature 37 º C, 250 rpm shacking speed, and dissolved oxygen rate 30-40%. The fermentation process is carried out with two stages, the HCDC phase in the first 4 hours, then after 4 hours followed by a feed-batch phase carried out by given feeding with speed rate 3 mL / hour. Induction of L-rhamnose 4mM is in optimum condition. Then the fermentation did in 18-20 hours after induction.

RESULTS AND DISCUSSION

Human Epidermal Growth Factor is protein has activity for cell differentiation and proliferation cell. EGF acting as an extracellular signal that can transmission signal into the nucleus cell and activated regulation of gene expression for certain proteins (Carpenter and Cohen, 1990). Due to it benefit, hEGF can be used as a candidate for wound healing of diabetic ulcers, by trigger the proliferation and expression of growth genes in epithelial cell. Therefore, hEGF should produce in large by using Recombinant DNA technology with various microorganisms. The commonly used microorganism to express hEGF recombinant is using *E.coli. E. coli* is a bacteria gram negative that mostly used for expression recombinant protein because *E. coli* is able to secrete recombinant protein into media culture. This extracellular secretion is essential in the production of recombinant proteins because the product obtained is easier to purify than in intracellular secretion. Therefore, a lot of research is done to determine the optimum operation condition, in order to obtain hEGF protein in large quantity with high efficiency.

Rejuvenation Stock Glycerol *E. coli* BL21 (DE3) [pD881-OmpA-rhEGF]

The mechanism of storage is very important in expression because it was one of the factors that influence the quality and performance of the microorganisms as the host of expression. The short-term microbial storage for laboratory purposes is removing the microbes from the old medium and added cryoprotectant agent as a protective cell during storage in -20 ° C temperature. Cryoprotectant agents are added to cell culture to keep the cells from storage in extreme temperatures (very cold) and minimize the damage of cell during clotting (Susilawati and Purnomo, 2016). Some of the cryprototective agents that can be used include dimethylsulfoxide (Me_2SO), glycerol, DMSO and polyvinylpyrrolidone (PVP).

Cryoprotectant agent which used to store the preparation of *E. coli* transformant is 10% glycerol or stock glycerol. This method is a type of short-term storage in cell preparations, so that should be done rejuvenation in period maximum for three months. Glycerol can be used as a storage media because glycerol can improve the stability of protein structures and can reduce the risk of degradation from thermal processes and protein aggregation. In addition, glycerol can inhibit cell metabolism. Glycerol can be used as a cell storage media because glycerol can enhance the stability of protein structure by binding water to the surface protein in the cell. Glycerol

also inhibits cell metabolism by altering free energy and reaction equilibrium in biochemical reactions in order to extend cell storage time.

In the previous study, it was seen that the unrefined *E. coli* glycerol stock for about 6 months effected decreasing 50% of expression (Calnan *et al.*, 2000). This is possible because the cryoprotectant agent is unable to stabilize culture and make the transformers cell died. So the number of cells in the culture decreases and the product expression decrease. Therefore in this study rejuvenation of transformers cell culture repeated in every 1 month.

Growth Curve of *E. coli* BL21 (DE3) [pD881-OmpA-rhEGF]

Prokaryotic organisms growth is defined as the increased density of cell it also means the increasing number of cells in media (Kaitu *et al.*, 2013). Log or exponential phase in bacteria Growth curve is the phase which the cell in the active state divides by a constant velocity. Induction performed in this phase will increased target protein concentration. Therefore, the growth curve in this study aims can be the best range as the upper and lower limits of induction time.

Many another study said that expression product increased in double strength media condition or double the initial concentration (Maksum *et al.*, 2017a). It was clear that we make double strength media concentration was one factor that will optimize in this study. Our range of media concentration is just until double strength because if the medium concentration is too high, it will inhibit the cell growth rate and the production cost much higher. So in this research, Range of Log phase Growth curve as a determinant of production factor is done in normal media concentration until double strength media concentration. The result of the production curve is shown In Figure 1.

In Figure 1 was seen that the increase of media concentration didn't make differences of the curve but in the rate of growing bacteria. Higher media make cell growth faster. Although the different cell growth rates, the growth of *E. coli* in the three variations of concentrations media show similar patterns of log phases occurring from the 3^{rd}hour until the 7^{th} hour. It shows that the change in media concentration has an effect on the microbial growth rate but does not affect the microbial growth characteristic.

Therefore we can conclude that production factor variable that will optimize is for lower limit induction time is at in 3^{rd} hour and the upper limit in the 7^{th} hour after cell growing, and the media concentration variable was in the upper limit of 2x and the lower limit of 1x media. Upper and lower limits variable are used in RSM experimental design.

Experimental Design

After obtaining the upper and lower limits of each factor, the data is entered into the software Minitab 17 for created the RSM experimental design. RSM experimental design is more effective than looking for optimum factors one by one because not only for saving time and materials for research, but also data can be processed statistically so the determination of optimum conditions is more accurate (Dutka *et al.*, 2015). The resulting experimental design is shown In Table 1.

Table 1: RSM Experimental Design

Running Number	Induction Times (h)	Medium Concentration
1	5	1.5
2	5	1.5
3	7	1
4	5	2.2
5	7.5	1.5
6	3	2
7	2.5	1.5
8	5	1.5
9	5	0.8
10	5	1.5
11	7	2
12	5	1.5
13	3	1

After obtaining optimum conditions total protein concentration. The condition is then used on the production of fermentor scale.

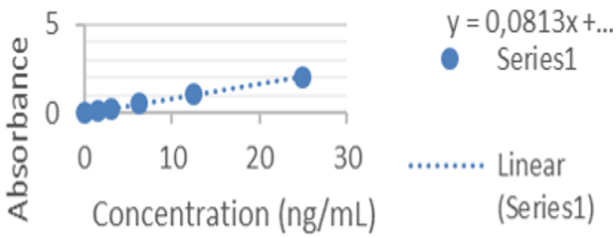

Figure 3: Standard Curve of ELISA hEGF

In RSM experimental design, the data of each factor is divided into three regions namely -1, 0, 1 and from those three areas will be varied. Because the type of RSM used is CCD, a lot of repetition done on the 0.0 variation of the two factors or also called a center point. In this experimental design it is shown that the center point is in the running number 1, 2, 8, 10 and 12. The center point function is to improve the

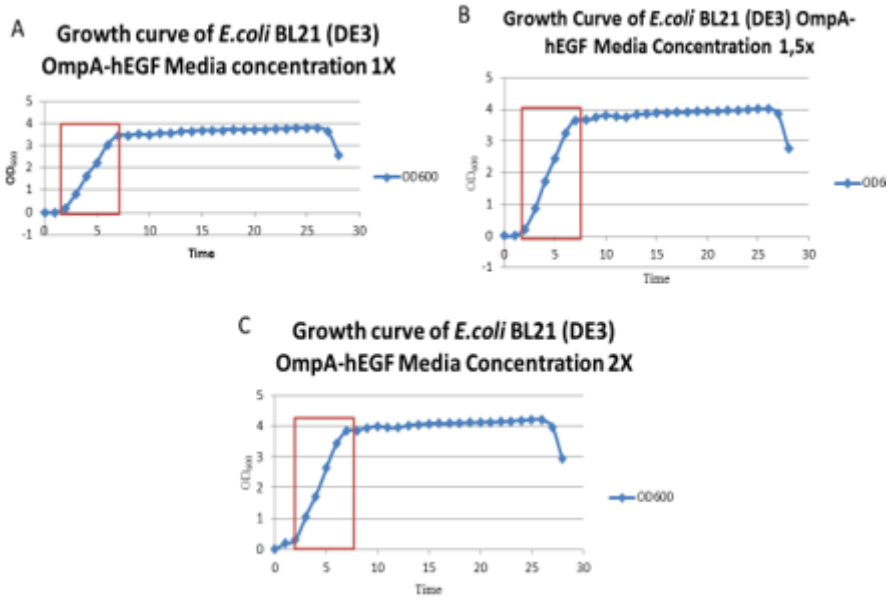

Figure 1: Growth Curve of E.coli BL21 (DE3) [pD881-OmpA-hEGF]

Table 2: Result of characterization of total protein level at every running

Run	Induction Time (h)	Medium Concentration	Total Protein Levels (mg/mL)
1	5	1.5	13.58
2	5	1.5	18.49
3	7	1	13.84
4	5	2.2	9.62
5	7.5	1.5	12.27
6	3	2	20.88
7	2.5	1.5	17.21
8	5	1.5	14.04
9	5	0.8	1.40
10	5	1.5	16.05
11	7	2	19.05
12	5	1.5	16.49
13	3	1	22.19

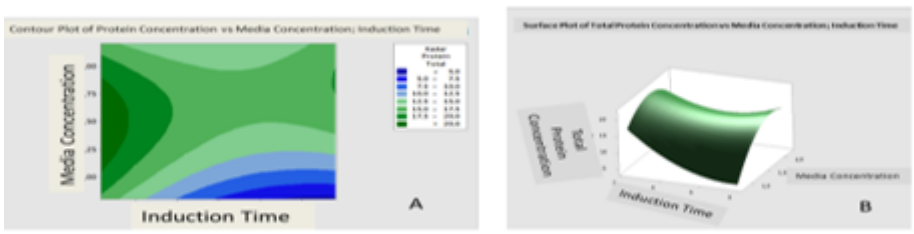

Figure 2: (a) Contour plot optimization of total protein production conditions. (b) Surface plot optimization of total protein production conditions

Table 3: Results of statistical analysis ofoptimization of recombinant hEGF production conditions

Run	Induction Time (h)	Medium Concentration	Total Protein (mg/mL)	Total Protein Prediction (mg/mL)	Eror Rate
1	5	1.5	13.58	20.82	34.8
2	5	1.5	18.49	20.82	11.2
3	7	1	13.84	13.12	5.5
4	5	2.5	9.62	17.40	44.7
5	7.5	1,5	12.27	21.60	43.2
6	3	2	20.88	22.22	6.0
7	2.5	1.5	17.21	27.62	37.7
8	5	1.5	14.04	20.82	32.5
9	5	0.8	1.40	10.56	86.8
10	5	1.5	16.05	20.82	22.9
11	7	2	19.05	21.22	10.2
12	5	1.5	16.49	20.82	20.8
13	3	1	22.19	20.64	7.5

Table 4: Data on optimization of recombinant hEGF production

Repetition	Induction Time(h)	Concentration	Protein Levels (mg/mL)
Center	5	1,5	20,8175
Optimal	2	1,5	21,115
	2	1,5	21,556
	2	1,5	21,070
Average			21,247

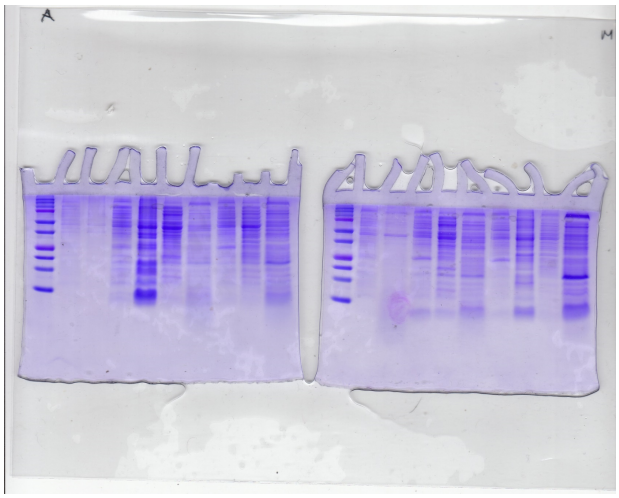

Figure 4: SDS-PAGE analysis of hEGF protein

accuracy of the experimental design with the repetition of data so the resulting data can represent all points of variation. Center point is also used as a base line in calculating the percentage of optimization (Dutka *et al.*, 2015). The resulting experimental design is shown in Table 1. In RSM experimental design used in this research to get the optimum condition. All experiments were performed in an incubator at 37^{o}C with a shacking speed 200 rpm and followed by this experimental design.

Recombinant hEGF Secretion

From the experimental design that has been obtained, then performed running based on the conditions in the experimental design. Total protein levels are make as a response for determine the best condition. Running of all experiment must be done simultaneously agar conditions other than variables varied remain the same. The total protein concentration of each run at the 20th hour after induction characterized by Lowry. The characterization of Lowry results are shown In Table 2.

From the data in Table 2, it was shown that the ratio of total protein content to the variation of induction time and the concentration of the medium components gave different patterns. This is influenced by different cell growth at each concentration of the medium component but it is also influenced by the number of cells that have been secreted. As shown In Figure 1, the number of cells in the third to the fourth hour is a high rate of growth. It is cleared because at normal cell media concentration gives significant cell growth rate occurs at the fourth hour until the seventh hour, therefore if at the

fifth time induction is done a competition of energy usages between recombinant hEGF expression and cell growth unavoidable so can make expression hEGF recombinant protein won't be maximal. If induction is done in log phase the rate of growth cell is not too rapid and cell energy can be focused on the expression of the recombinant protein (Choi and Lee, 2004).

Whereas at double strength media, cell growth significant rate occurs at the 7^{th} hour to the 9^{th} hour. Consequently, the highest total protein content is present at the time of the 3^{rd} and the smallest induction at the time of 5^{th} hour after induction. Meanwhile, at 1.5x medium concentration, the total protein content of each induction isn't much different because the rate of cell growth at one 1.5x media concentration tends to be constant or doesn't have a significant difference from the beginning to the end of the exponential phase.

Optimum production condition is determined by data processing of total protein content as a result from significant factor response. After the data is processed statistically, we get an equation that can be used to determine the total predictive protein concentration, the equation is,

Total Protein Concentration = 12.2 - 7.82 Induction Time + 30.5 Medium Concentration + 0.430 Induction Time*Induction Time - 11.58 Medium Concentration*Medium Concentration.

The comparison of total protein levels of the characterization results with predictive total protein levels of prediction is shown In Table 3. The results showed an average accuracy of the experimental design approached 50.66%. This figure indicates that the experimental design is reliable enough to be used in determining the optimum conditions of recombinant hEGF production. From that total protein concentration, we analyze design experiment and it gives contour plot and surface plot diagram can be drawn up in Figure 2 which can be used to look at the conditions that produce the higher Total Protein Concentration in the statistical equation. Figure 2 shows that the Total Protein coentration are optimum at the media concentration 2x and induction time between the 5^{th} and 6^{th} hours. Data optimization is shown In Figure 2.

After the optimum condition data is obtained, the validation process must be ensured to see whether the statistical equation matches the experiment. The results of the run were then compared with the data center point and hEGF prediction levels in the optimum conditions shown In Table 4. The table shows that the protein levels produced under optimum conditions are much greater than the predicted level of 14.22 μg / mL and approaching the target to be achieved is 30μg/mL. This shows that the optimization of recombinant hEGF production has been done with the optimization percentage of 185.7% compared to the center point value.

Production of hEGF Recombinant Fermentor Scale

The HCDC stage was performed in the 1^{st} until 4^{th} hours which serves to increase cell concentration in culture on carbon-rich media, among them sucrose, in this way it will increase the cell growth rate significantly. Then after the 4^{th} hour fed-batch stage is performed to maintain the cell growth rate and recombinant hEGF expression rate in culture.

The yield of hEGF protein the fermentor scale is characterized by ELISA and Tricine SDS-PAGE.

Standard curve of ELISA method showed in Figure 3. From the calculation results obtained ELISA levels of 416 $\mu g/mL$ and 6x fold compared to the results of expression in previous studies (Fahmi, 2016). And the total protein content of expression was 21,247 mg / mL with optimization percentage 2.96% and efficiency 50.56%.

In SDS PAGE there was protein band under protein 10 kDa that indicated hEGF. Ethanol precipitation makes band strong because it can decrease dissociation constant of protein. The optimum conditions for recombinant hEGF production using *E. coli* BL21 (DE3) [pD881-OmpA-hEGF] are the concentrations of medium components including tripton 20 mg / mL, yeast extract 10 mg / mL, sodium chloride 20 mg / mL and induction time 2 hours 10 minutes with 2.96% optimization percentage and efficiency of 50.56%. hEGF protein levels secreted by to medium fermentor were 416 μg / mL.

CONCLUSION

The optimum conditions for recombinant hEGF production using *E. coli* BL21 (DE3) [pD881-OmpA-hEGF] are the concentrations of medium components including trypton 20 mg / mL, yeast extract 10 mg / mL, sodium chloride 20 mg / mL and induction time 2 hours 10 minutes with 2.96% optimization percentage and efficiency of 50.56%. hEGF protein levels secreted by medium fermentor were 416 μg / mL.

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