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Optimization of The Expression of Recombinant Universal Infleunzavaccine Candidatein Escherichia Coli Using Response Surface Methodology

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Abstract: Vaccinations are considered among the effective biosecurity programs in preventing the bird flu virus. Avian influenza virus mutations are able to induce Hemaglutinin (HA) and Neuraminidase (NA) variants. This can in turn cause a pandemic that causes new viruses to make the existing vaccines to become ineffective. Recombinant protein M2e (2-16-k-25) has been successfully designed and expressed on E.coli ER2556 as a fusion protein (M2e (216) -K-P25 intein CBD), and used as a vaccine candidate base on epitope M2e has the potential as a universal influenza vaccine. The optimum determination of expressed fusion proteins is necessary for efficient variables that are important in the design of expression systems. This study aims to determine the optimum fusion protein expression variables. The overexpression fusion protein in E.coli ER2566 was optimized in shake flasks using design experiments Surface Methodology of Box-Behnken. Variables such as IPTG concentration, incubation temperature, and post induction time were applied to determine the optimum expression of fusion proteins. The statistical approach used in this study employed Minitab software 17. The characterization of fusion proteins was performed using gel electrophoresis dodecyl dodecylsulfate-polyacrylamide, followed by the quantification by densitometry using the ImageJ program. The results of this research demonstrate that the most influencing variable is induction temperature (p = 0,002), along with induction time (p = 0,173) and IPTG concentration (p = 0,243). The optimization of fusion protein expression at 0.33 mM IPTG concentration for 18 h at 18 ° C, yields 0.15 ± 0.013 mg / mL.

Keywords: Avian Influenza Vaccine, IPTG concentration, temperature and post-induction time, Box-Behnken.

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I. INTRODUCTION

Until March 2017, Indonesia has recorded 199 positive cases infected with the Avian Influenza virus, 167 of which died (WHO, 2017). Vaccination is among the most effective and economical biosecurity programs for the prevention and control of the spread of the Avian Influenza virus (Subbarao and Luke, 2007). The Avian Influenza vaccine component currently consists of Hemagglutinin (HA) and Neuraminidase (NA) (Palese, 2006). The virus is very fast to have mutations (antigenic drift) on the antigenic HA and NA. Therefore, the vaccine used today is only effective for influenza seasons. In order to compensate for the antigenic drift of the Avian Influenza virus, a new vaccine strain must be selected, manufactured, processed and commercialized on an annual basis, causing vaccine accumulation. To reduce the deficiency then develop the virus-based antigenic Avian Influenzathat is highly conserved, such a universal vaccine is based on the M2 ectodomain (M2e) protein (De Filettea et al., 2008).

M2e is highly conservedin the human Avian Influenza virus,as well as in poultry (Rudolph and Ben-Yedidia, 2011). M2e is the 23th amino acid sequence (SLLTEVETPIRNEWGCRCNDSSD), which is highly conserved throughout the strain of the Avian Influenza virus. The N-terminal SLLTEVET (residue 2-9) is on a 100% sustained M2e epitope in human Avian Influenza virus and over 99% in all subtypes of the Avian Influenza virus (Kesik-Brodacka and Plucienniczak, 2014). The M2e epitope is recognized by anti-M2 monoclonal antibodies (MAb) capable of inhibiting Avian Influenza virus replication. Therefore, a sustainable epitope-based vaccine using M2e epitope is an alternative to stop the spread of Avian Influenza (Tompkins et al., 2007).

Previous research by Subroto*et al.*(2012) on gene fragment M2e (2-16) -K-P25 has been successfully designed, cloned and expressed in *Escherichia coli (E.coli)* ER2566 as fusion protein M2e(2-16)-K-P25-intein-

CBD (MeKPC). The expression of fusion proteinMeKPC was carried out under conditions of 18˚C for E.coliER2566, with 1mM IPTG concentration, and 5 hours of induction time. The success of this expression is not only seen from the success or failure of recombinant proteins expressed. The results of expressed proteins can be increased by optimizing the parameters that affect the expression (Shafiee et al., 2017).

In order to obtain the production of high-yield recombinant proteins from *E. coli,* it is crucial to efficiently perform the critical stages in the design of recombinant expression systems,including strain development, expansion optimization, and bioprocess optimization. The resulting protein concentrations and cell growth are strongly influenced by medium compositions such as carbon, nitrogen, and isopropyl β-D-1-thiogalactopyranoside (IPTG) concentrations (Khalili et al.,2017). Low concentrations of IPTG inducers can lead to inefficient induction (low recombinant protein yields), whereas excessive addition can result in economic losses.In addition to the toxic effects, it can reduce cell growth and the concentration of recombinant proteins produced (Papaneophytou et al., 2014). Other factors that affect protein expression and solubility are temperature and induction time. High temperatures may increase cell growth, but are detrimental to protein expression, because higher growth rates lead to the formation of inclusion bodies (Papaneophytou et al., 2014). The formation of inclusion bodies should be avoided because they must be refolded,which requires optimization of refolding conditions (Majerle et al., 1999). The formation of inclusion bodies in the recombinant protein in *E.coli* can be minimized by lowering the induction temperature. This approach has proven to be effective in increasing the solubility of recombinant proteins (De Groot and Ventura, 2006).

Larentis *et al.* (2011) successfully evaluated induction conditions (IPTG concentration, temperature and induction time) on *E. coli* performed by Response Surface Methodology (RSM) technique, in order to increase the expression level of recombinant protein. RSM is a collection of statistical techniques for designing experiments, evaluating the effect of process factors, and searching for optimum conditions, and has been successfully applied in the optimizing bioprocess. RSM can potentially reduce the number of experimental experiments (Singh and Sharma, 2012).

Therefore, assessing the effects of expression protein variables on recombinants is necessary to determine the optimum conditions. This study aims to determine expression variable (optimum IPTG concentration, temperature and induction time) on expression of MeKPCfusion protein expressedin *E.coli* ER2566 using the RSM Box-Behnken method.

II. EXPERIMENTS

Chemicals

Isopropyl β-D-1-thiogalactopyranoside (IPTG), ampicilin from Sigma, Luria Bertani (yeast extract 0.5%, trypton 1% and Sodium chloride 1%) were used as a growth medium.

Strain

The expression host used was the *E.coli* ER2566 strain of the RNA polymerase T7 gene inserted after the lacZ promoter. The expression vector used was pTyB21, which carries the MeKPC gene derived from previous research (New England Biolabs).

Screening of expression condition

In 100 µL of transformant glycerol stock,E.coli ER2566 was grown in 5 mL of Luria Bertani medium containing the ampicillin 100 µg / mL antibiotic, for 16 h at 37 ºC.It was shakenat a rate of 150 rpm. A total of 100 µL of these cultures were grown into a 100 mL Luria Bertani liquid medium containing ampicillin 100 µg / mL, and then incubated for 3.5 h at 37 ºC, at a shake rate of 150 rpm. Furthermore, the measurement of the OD600 nm value on the UV-Vis spectrophotometer was 0.6-0.7. A 200 rpm shake speed was applied, with variationsin the IPTG concentration, temperature and induction time, according to RSM experimental design. The expression results were harvested and centrifuged at a speed of 8,000 g, a temperature of 4 ºC, for a period of 10 min. The expression pellet was added with 54 mL of lysis buffer.Sonication was performed for 3x10 min (2 sec on / 2 second off), and then centrifuged at 15,000 g, at 4 ºC for 20 min. The supernatant as a dissolved fraction is characterized by SDS-PAGE.

SDS-PAGE

Supernatant results from the expression were characterized further by SDS-PAGE (Laemmli, 970), and a gel with a composition of 12% separating gel and 4% stacking gel was used. Visualization was performed using Coomassie Briliant Blue.

Quantification of fusion proteins

Fusion Me2KPC proteins characterized by SPS-PAGEwere then quantified using the densitometric method with the ImageJ program. The fusion protein concentration was calculated by linear regression equation y = a + bx, with Bovine Serum Albumin (BSA) as standard. Standard BSA is made with concentration series 0, 0.01, 0.05, 0.1, 0.15, and 0.2 mg / mL.

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Experimental design and statistical analysis

The experimental design for determining IPTG concentration, optimum temperature and induction time used RSM Box-Behnken.A statistical method was used with the help of Minitab 17 software. In this study 15 run experiments were applied. Statistical analysis of the model was done in the form of variance analysis (ANOVA) for the determination of the significant variables. The variables and expression levels are shown in Table 1.

Table 1 Variables and levels of Box-Behnken Design Experiments

Parameter	Code	Levels		
		-1	**0**	**+1**
IPTG concentration (mM)	x	0.010	0.505	1.000
Induction temperature (°C)	y	18	27	37
Induction time (hour)	z	2	9	16

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III. RESULTS AND DISCUSSION

The determination of the concentration of IPTG, temperature, and optimum induction time of expression of the M2eKPC fusion protein in *E.coli* ER2566 strain used the RSM Box-Behnken design. The quantification of the M2e KPC used the ImageJ program's densitometry analysis. The Box-Behnken design allows the calculation of responses to be performed with minimal experimental count compared to other designs (Shafiee et al., 2017). This research uses three factorial Box-Behnken designs (-1, 0, +1) with three variables (namely, IPTG concentration, temperature, and induction time), using the Minitab 17 program.A total of 15 experimental designs were obtained, and the expression and quantification M2eKPC fusion protein was prformed. The measurement of rendement of the M2eKPC fusion proteinused a densitometry method withthe ImageJ program on the result of characterization of the M2eKPC fusion protein with SDS-PAGE (Figure 1). The quantification of the fusion proteins was calculated from the area of M2eKPC fusion band with the BSA standard linear regression equation, y = 130709 x, with regression coefficient 0.978, from the standard BSA solution series (0.01; 0.05; 0.10; 0.15 and 0.2%). The mathematical equation for the determination of fusion protein yield prediction based on the analysis results, obtained the following model:

$$Rendement = 0{,}1851 + 0{,}0125x - 0{,}00827y + 0{,}00928\,z - 0{,}0442x^2 + 0{,}000102y^2 - 0{,}000578z^2 + 0{,}0000xy + 0{,}00216xz - 0{,}000038yz$$

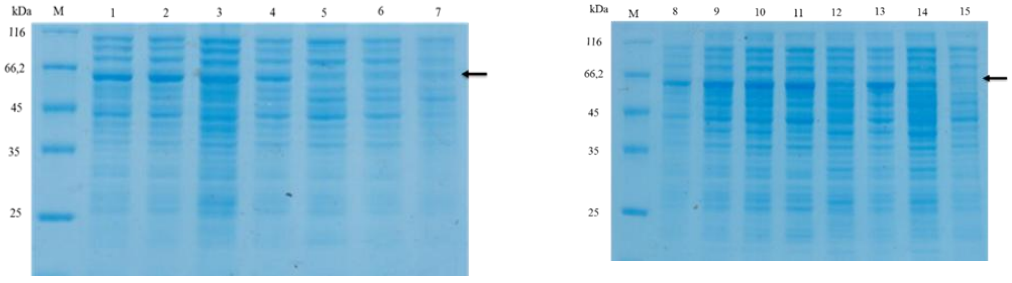

Figure 1 Characterization of the M2eKPC fusion protein with SDS-PAGE, experimental results with the Box-Behnken design. M: Marker; 1-15: experiment run. (←): M2eKPC [~ 65kDa].

Table 2 Experimental Results of RSM Box-Behnken

Run	Design Box-Behnken			x (mM)	y (˚C)	Z (hr)	*Actual* (mg/mL)	*Predicted*(mg/mL)
1	-1	-1	0	0.010	18	9	0.10	0.11
2	+1	-1	0	1.000	18	9	0.10	0.06
3	0	-1	+1	0.505	18	16	0.07	0.06
4	0	-1	-1	0.505	18	2	0.07	0.08
5	-1	+1	0	0.010	37	9	0.03	0.07
6	0	+1	-1	0.505	37	2	0.03	0.03
7	0	+1	+1	0.505	37	16	0.02	0.02
8	+1	+1	0	1.000	37	9	0.03	0.02
9	0	0	0	0.505	27	9	0.06	0.07
10	-1	0	-1	0.010	27	2	0.06	0.05
11	0	0	0	0.505	27	9	0.07	0.07
12	+1	0	-1	1.000	27	2	0.02	0.02
13	0	0	0	0.505	27	9	0.07	0.07
14	-1	0	+1	0.010	27	16	0.02	0.05
15	+1	0	+1	1.000	27	16	0.01	0.01

The statistical significance of this design was evaluated using variance analysis (ANOVA), and data processing was performed with Minitab 17. The ANOVA results show that the right model for this case is a second-order model (Square). This is seen from the p-value of the second-order model (Square),which is less than α 5%. The lack of Fit test of the model obtained a P-value = 0.110,which is greater than the degree of significance α = 0.05.It can be concluded that the regression model is appropriate. To check the significance of the second-order model, we can check the p-value of the model. The value of p-value = 0.022, which is smaller than the degree of significance α = 5%.This means that free variables such as IPTG concentration, temperature and induction time have a significant effect on the yield of the M2e fusion protein (2-16) -K-P25- intein-CBD. The most influential variable on expression of M2e fusion protein (2-16) -K-P25-intein-CBD is induction temperature (p-value = 0.002), followed by induction time (p-value = 0.173) and IPTG concentration (p-value = 0.243).

The resulting 3D Surface for the recovery of the yield of the M2e fusion protein (2-16) -K-P25-intein-CBD is shown in Figure 3. The effect of the induced temperature reduction can increase the yield of the M2e fusion protein (2-16) -K-P25-intein -CBD (Figures 3A and 3C). This is because lowering the induction temperature can decrease the formation of inclusion bodies, so that the soluble protein increases. While high temperatures may increase cell growth, it is detrimental to protein expression, because high growth rates lead to the formation of inclusion bodies (Papaneophytou et al., 2014). The formation of inclusion bodies occurs due to the chemical denaturation and proteolysis process as the temperature increases (Sánchezde and Ventura, 2006). Additionally, as the rate of the expression increases, it does not increase the solubility of proteins. The faster the expression rate increases the likelihood of folding errors, because in this condition the protein does not have sufficient time to perform the folding process correctly (Hartinger et al., 2012). Recombinant proteins in the form of inclusion bodies expressed by E. coli should be re-folded (refolding),which requires optimization of refolding conditions (Majerle et al., 1999).

While the effects of IPTG concentrations are too low (0.010 mM) and too high (1 mM),this leads to a decrease in the yield of fusion protein (Fig. 3B and 3C). Concentrationsof 0.010 mM and 1.000 mM IPTG decrease expression levels, where significantly low IPTG concentrations lead to low recombinant protein yields, and significantly high IPTG concentrations can potentially have a toxic effect of reducing cell growth and the concentration of recombinant protein expressed. In addition, high concentrations of IPTG can increase the fusion protein in the non-soluble fraction (Papaneophytou*et al.,* 2014). Optimum IPTG concentrations are reported to increase dissolved proteins due to the accumulation of low target proteins that can avoid crowding effects (Jhamb and Sahoo, 2012). Increased concentrations of IPTG may increase the rate of expression of *E.coli* fusion protein, but this increase in expression rate is not accompanied by the correct folding of the target protein, so the target protein is generated as inclusion bodies (Muntari et al., 2012).

The effect of induction time can be observed in Figs 3A and 3B. An induction time that is too short elongates results in a slight recovery yield.This may be due to the solubility and refolded recombinant fusion protein increasing at a given time to obtain a mature fusion protein. Optimum induction time is important for

controlling the extent of the plasmid gene expression as a recombinant protein that can effectively inhibit host cell growth, possibly due to its toxicity. In addition, the metabolic burden given to host cells through heterologous gene expression causes growth delay (Muntari et al., 2012).

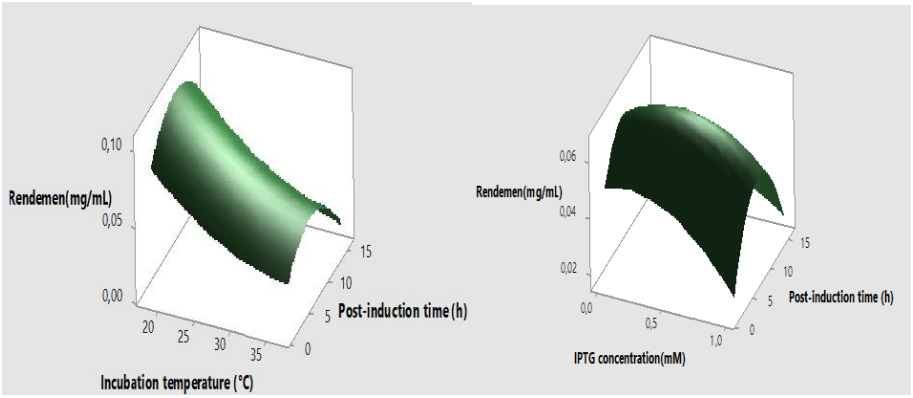

Figure 2*3D Surface* 3D Surface Interaction Variable Expression Design RSM *Box-Behnken*. A: Post-Induction time vs incubation temperature; B:Post-induction time vs IPTG consentration; C:Post-induction time vs IPTG concentration

Based on the results of the analysis using Mini Tab 17, the optimum value of expression of the M2eKPC fusion protein was at the concentration of IPTG 0.33 mM, and temperature of 18˚C for 8 hours, with prediction yield of 0.1 mg / mL (Figure 4). Furthermore, the testing of laboratory expression on the optimum conditions, the test was performed for five replications (Table 4). Quantification of the results of the characterization of fusion proteins (Figure 5) by the densitometric method using the ImageJ program obtained the rendement of the M2e fusion protein (2-16) -K-P25-intein-CBD by 0.15 ± 0.013 mg / mL.

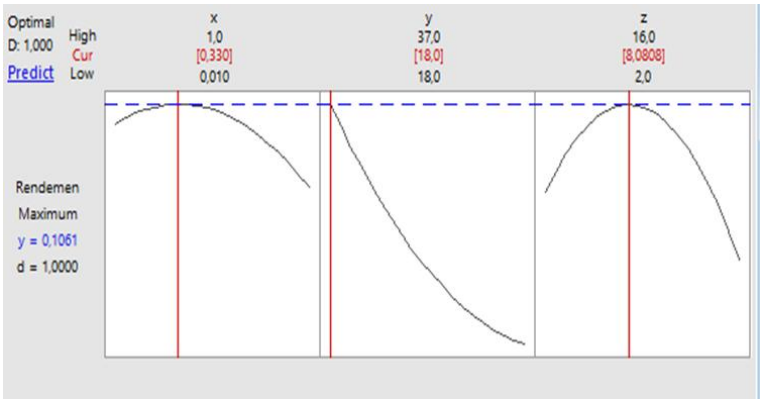

Figure 3 Prediction of the optimum value of expression of the M2eKPC fusion protein. x: concentration of IPTG; y: induction temperature; z: induction time

Table 3 Quantification of optimization conditions of fusion protein expression-M2eKPC

Run	Rendement (mg/mL)
1	0,14
2	0,16
3	0,15
4	0,14
5	0,17
Rerata	0,15
SD	0,013

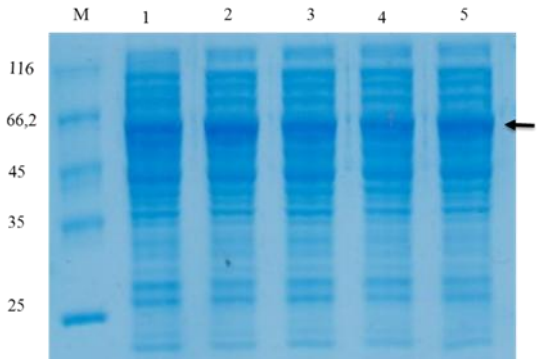

Figure 4 Characterization of the optimum conditions of fusion protein expression. 1-5: Test replication.(←): M2eKPC [~ 65kDa].

IV. CONCLUSIONS

The optimum conditions obtained through the experimental design of Response Surface Methodology Box-Behenkenfor the expression of M2eKPC fusion protein is a concentration of IPTG 0.33 mM at 18˚C for 8 hours, with a yield of M2e fusion protein (2-16) -K-P25-intein-CBD of 0.15 ± 0.013 mg / mL.

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REFERENCES

[1]. WHO, Cumulative Number of Confirmed Human Cases of Avian Influenza A (H5N1) Reported to WHO, 16 March 2017, Available http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/. [20/03/2017].
[2]. Subbarao, K and Luke, C. 2007. H5N1 viruses and vaccines. PLoS Pathog. 3(3).e.40
[3]. Palese, P. 2006. Making better influenza virus vaccines?. Emerging Infectious Diseases. 12(1).61-65
[4]. De Filettea, M., Martensa, W., Smet, A., Schotsaert, M., Birkett, A., Londo˜no-Arcila, P., Fiers, W., and Saelens, X. 2008. Universal influenza A M2e-HBc vaccine protects against disease even in the presence of pre-existing anti-HBc antibodies, Vaccine. 26: 6503–6507.
[5]. Rudolph, W., and Ben-Yedidia, T. 2011. A universal influenza vaccine. Human Vaccines. 7(1): 1-2.
[6]. Kesik-Brodacka, M and Plucienniczak, G. 2014. A universal flu vaccine. Acta APB Biochmica Polonica. 61: 523-530.
[7]. Tompkins S.M., Zhao, Z.S., Lo, C.Y., Misplon, J.A., Liu, T., Ye, Z., Hogan, J.S., Wu, Z., Benton, K.A., Tumpey, T.M., and Epstein, S.L. 2007. Matrix protein 2 vaccination and protection against influenza viruses, including subtype H5N1. Emerging Infectious Diseases. 13(3): 426-435.
[8]. Subroto, T., Gaffar S and Hidayat, A.D. 2012. Pengembangan vaksin influenza universal berbasis epitop. Prosiding InSINas.
[9]. Shafiee,F., Rabbani, M, and Najafabadi, A.J. 2017. Optimization of the Expression of DT386-BR2 Fusion Protein in Escherichia coli using Response Surface Methodology. Adv Biomed. 6 (22).
[10]. Khalili, E., Khoshayand, M.R., Abbasi, E., Lakzaee, M., Parvizi, M.R., Faramarzi, M.A., Aminian , M., and Nojoomi, F. 2017. Medium optimization for synaptobrevin production using statistical methods. Archives of Medical Laboratory Sciences. 3(1): 7-14.
[11]. Papaneophytou, C.P., and Kontopidis, G. 2014. Statistical approaches to maximize recombinant protein expression in Escherichia coli: A general review. Protein Expression and Purification. 94:22–32.
[12]. Majerle, A., Kidric, J., and Jerala, R. 1999. Expression and refolding of functional fragments of the human lipopolysaccharide receptor CD14 in Escherichia coli and Pichia pastoris. Protein Expression and Purification. 17: 96-104.
[13]. De Groot, N.S., and Ventura, S. 2006. Effect of temperature on protein quality in bacterial inclusion bodies. FEBS Letters. 580 : 6471–6476.
[14]. Larentis, A.L., Argondizzo, A.P.C., Esteves, G.D., Jessouron, E., Galler, R., Medeiros, M.A. (2011). Cloning and optimization of induction conditions for mature PsaA (pneumococcal surface adhesin A) expression in Escherichia coli and recombinant protein stability during long-term storage. Protein Expr. Purif. 78 38–47.
[15]. Singh J and Sharma A. 2012. Application of response surface methodology to the modeling of cellulase purification by solvent extraction. Advances in Bioscience and Biotechnology. 3: 408-416.
[16]. Sánchezde, N and Ventura, G.S. 2006. Effect of temperature on protein quality in bacterial inclusion bodies. FEBS Letters. 580(27): 6471-6476.
[17]. Hartinger, D., Heinl, S., Schwartz, H.E., Grabherr, R., Schatmayr, G., Haltrich, D., and Moll W.D. 2010. Enhacement of solublitiy in E.coli amd purification of aminotransferase from Ssphingoyxis sp. MTA144 for deamination of hydrolized fumonisin B1. Microbial Cell Factories. 9:62.
[18]. Jhamb, K., and Sahoo, D.K. 2012. Production of soluble recombinant proteins in Escherichia coli: Effects of process conditions and chaperone co-expression on cell growth and production of xylanase. Bioresource Technology. 123: 135–143.
[19]. Muntari, B., Amid, A., Mel, M., Jami. M.S, Salleh, M.H. 2012. Recombinant bromelain production in Escherichia coli: process optimization in shake flask culture by response surface methodology. AMB Express. 2(12).
[20]. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227(5259): 680-685.

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