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PHYSICAL OPTIMIZATION OF THERMOSTABLE ALKALINE PROTEASE BY *E. COLI* BL21 (DE3) PLYSS HARBORING 50A PROTEASE GENE USING RESPONSE SURFACE METHODOLOGY

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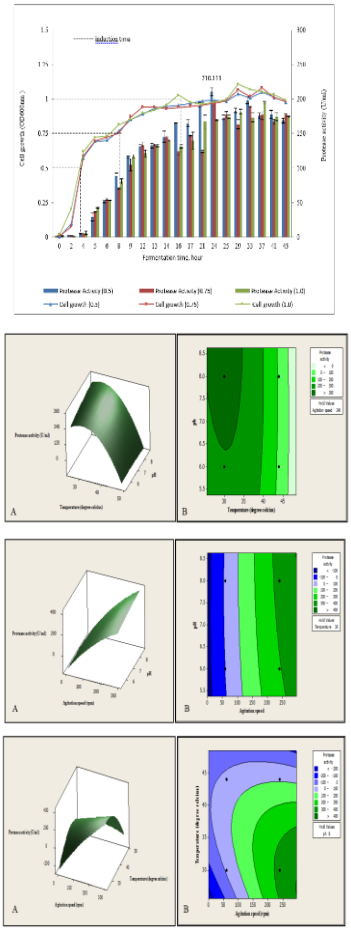
Graphical abstract



Abstract

Physical optimization is important for enzyme production by fermentation process. In general, fermentation process at optimal condition increases the expression and production level of enzyme to many times in comparison with their natural production. This study was focused on the optimization of the physical factors that influenced the thermostable alkaline protease production. The induction and incubation time were studied using conventional method while the other three factors which are incubation temperature, initial pH of medium and agitation speed were optimized by response surface methodology (RSM). The interaction effects among these factors were explained using response plot and the model adequacy was satisfactory as the coefficient of determination (R^2) was 96.48%. The enhancement of thermostable protease from 197.83 U/ml to 325.89 U/ml was achieved using both conventional and statistical approach of response surface methodology (RSM). This present study proved that physical optimization significantly affects the protease production and the optimum physical condition obtained may applied in large scale process.

Keywords: Optimization, Response Surface Methodology, thermostable protease

Abstrak

Pengoptimuman fizikal adalah penting bagi penghasilan enzim melalui proses fermentasi. Secara umumnya, proses fermentasi pada kondisi optima dapat meningkatkan perembesan dan tahap penghasilan enzim beberapa kali ganda berbanding penghasilan secara semulajadi. Kajian ini tertumpu pada proses pengoptimuman fizikal yang mempengaruhi tahap penghasilan termostabil alkali protease. Masa induksi dan inkubasi telah dikaji menggunakan kaedah konvensional manakala tiga faktor lain iaitu suhu inkubasi, pH awal medium dan kelajuan goncangan telah dipertingkatkan lagi melalui aplikasi metodologi tindak balas permukaan (RSM). Kesan hubungkait antara faktor-faktor ini telah dijelaskan melalui plot tindak balas dan model ini juga menunjukkan keputusan yang memuaskan kerana mendapat pekali penentu (R^2) sebanyak 96.48%. Peningkatan penghasilan termostabil protease daripada 197.83 U/ml kepada 325.89 U/ml telah dicapai menggunakan kedua-dua pendekatan secara konvensional dan statistik oleh metodologi tindak balas permukaan. Kajian ini membuktikan bahawa pengoptimuman fizikal memainkan peranan penting dalam mempengaruhi penghasilan protease dan keadaan optima yang diperolehi dapat diaplikasikan dalam proses berskala besar.

Kata kunci: Pengoptimuman, metodologi tindak balas permukaan, termostabil protease

1.0 INTRODUCTION

Proteases are enzymes that catalyze hydrolytic reactions in which protein molecules are degraded to peptides and amino acids. These enzymes have been employed in a wide array of applications for many years with satisfactory result. Because of their wide application in industries, this enzyme has accounted for 60 percent of worldwide sales of industrial enzymes [1-3]. Among various types of microbial protease, alkaline serine proteases are preferred because they are generally active from neutral to alkaline pH [4]. Alkaline proteases also had an ability to digest proteinaceous stains such as keratin, blood, milk and gravy on fabrics [5].

Referring to biotechnological process and bioprocessing technology, fermentation process is the familiar way used for optimization technique in order to increase the production yield as well as to fill the increasing demand of protease in industry. Besides, to meet the growing demands in the industry, it is necessary to improve the performance of the system and thus increase the yield without increasing the cost of production [6]. The optimization approach usually divided into two parts which are nutritional optimization and physical optimization.

This present study was aim for physical optimization of the fermentation culture by a newly cloned bacteria of *E.coli* BL21(DE3) pLysS harboring 50a protease gene. Four important factors that influenced the protease production were selected in this study which is induction and incubation time, incubation temperature, initial pH of medium and agitation speed. All these factors were investigated using both conventional and modern statistical approach of Response Surface Methodology (RSM).

In conventional approach, one-factor-at-a-time strategy was used and this approach provided a simple way of optimization [7]. However, nowadays, statistical approach are preferred due to utilization of fundamental principles of statistics, randomization, replication and duplication [8]. Response surface methodology (RSM) through central composite design (CCD) is one of the popularly used optimization procedures which is useful and suitable for developing, improving and optimizing processes. This design also provide an efficient strategy in the determination of optimal conditions for multivariable system [9].

2.0 METHODOLOGY

2.1 Bacterial Strain and Inoculums Preparation

E.coli BL21(DE3) pLysS harboring 50a protease gene was grown on the Luria Bertani-Skim Milk agar (LB-SMA) plates containing ampicilin (50 ug/ml) and chloroamphenicol (34 ug/ml). The plates were incubated overnight at 37°C. A single colony of the growth from the agar plate was inoculated into 50 ml Luria Bertani (LB) broth supplemented with 50 µg/ml ampicillin and 34 µg/ml chloroamphenicol and incubated 24 hour at 37°C, 150 rpm in incubator shaker. The cells were harvested for inoculum preparation by centrifugation at 10,000rpm, 4˚C for 10 min. The bacteria pellet was dissolved in saline (0.85% NaCl) to give an absorbance reading of 0.5 at 540 nm [10-11]. Inoculum was then inoculated into the fermentation medium.

2.2 Fermentation Medium in Shake Flask Conditions

Inoculum (2.5%) was inoculated in 250 ml Erlenmeyer flask containing 150 ml of basal medium with the following composition (g/l): Tryptone, 20; Sorbitol, 5; $CaCl_2.H_2O$, 2 and the initial pH of basal medium was uncontrolled. The fermentation cultures was incubated at 37°C and induced with 0.5 mM isopropyl-β-D-thiogalactopyronoside (IPTG). The induction time was varied according to the cell growth of the cultures as described in section 2.5.1.

2.3 Culture Recovery

The induced cultures were harvested by centrifugation at 12,000 x *g* for 10 min at 4°C. The supernatant were discarded and the cell pellet was resuspended in 0.1M Tris-HCl, 2 mM CaCl2 buffer, pH 9 and further sonicated for 2min to lyses the cell. Then, the cell suspension was centrifuged at 12,000 x *g* for 10 min at 4°C and the supernatant was collected as a crude enzyme solution. The crude enzyme solution was purified using heat treatment method by incubated the crude enzyme in hot water bath at 70°C for 3 hour to precipitate the mesophilic *E.coli* proteins [12]. The purified enzyme was centrifuged again to remove the denatured contaminated proteins. The supernatant obtained was assayed for protease activity.

2.4 Proteolytic Assay

Protease activity was measured using sulphanilamide azocasein substrate according to the modification method [10]. One unit (U) of azocaseinase activity was defined as the amount of enzyme activity that produces a change of absorbance (0.001 per min) at 450 nm at 80°C under standard assay conditions.

2.5 Physical Optimization

2.5.1 Determination of Suitable Induction and Incubation Time

The induction time was determined based on the cell growth which measured by the optical density (OD) at 600 nm. The fermentation broths were induced when the cell growth reached OD at 0.5, 0.75 and 1.0 respectively. The protease production profile at three different induction times were monitored and recorded. The culture samples were withdraw at 2 hours interval and monitored the cell growth and protease activity closely.

2.5.2 Optimization of Physical Condition Using RSM

After identifying the suitable induction cell growth and optimal incubation time, the second step in the optimization of physical condition was to determine the optimum levels of initial culture of pH, incubation temperature and agitation speed for protease production. Each factors was studied at two different level (-1, +1) and centre point (0), with all factors taken at a central coded value of zero (Table 1). Total number of 20 experiments, with an axial point (a=1.682) and six replicates at the centre points was employed (Table 2). The protease yield for each run was used as the response variable. The data were also subjected for analysis of variance (ANOVA). Multiple regression analysis, response surface plot and statistical analyses were performed using Minitab V.16 statistical software®.

2.6 Verification of Central Composite Design

The validation of the proposed experimental methodology was verified with respect to all the three variables within the design space. A random set of five experimental combinations were performed to study the protease production in 250 ml shake flask.

Table 1 Experimental factor at different levels using Central Composite Design (CCD)

Factors	Units	Symbol code	Actual factors at coded variable levels				
			-α (-1.682)	Low (-1)	Centre (0)	High (+1)	+α (+1.682)
Agitation rate	rpm	Y_1	0	60	150	240	296
Temperature	^{0}C	Y_2	25.57	30	37	44	48.43
pH		Y_3	5.37	6	7	8	8.63

3.0 RESULTS AND DISCUSSION

3.1 Suitable Induction and Incubation Time

The time of induction with IPTG was important for the cell growth and recombinant protein synthesis [13]. The induction time was studied at three different cell growths (OD_{600nm}) which are 0.5, 0.75 and 1.0 for 45 hours of incubation time. The fermentation culture was induced with constant IPTG concentration (0.5mM). The growth profiles for protease production by *E.coli* BL21 (DE3) pLysS harbouring 50a induced at different cell growth: 0.5 (middle exponential growth), 0.75 (late exponential growth) and 1.0 (stationary growth) were illustrated in Figure 1.

However, the different induction times showed only small difference and produced almost same growth curve. This result suggesting that induction time was not an important factor for expression of the protease 50a in *E.coli*. The recombinant 50a strain exhibited sigmoidal growth curve in optimized basal medium. At 0 to 2 hour of incubation, the strains were adjusting to the lag phase and enter the exponential (log) phase form 2 hour to 12 hours of incubation. After that, the bacterial growth comes to the stationary phase. At this stationary phase, the cells being produced become equal to the dying cell. The cultures start to enter the death phase when incubated more than 37 hour.

It is important to detect the optimum incubation time at which an organism exhibits maximum enzyme activity since organisms show considerable variation at different incubation periods [14]. Therefore, in order to determine the optimum protease activity and growth rate, it is essential to find the optimum fermentation time. Referring to Figure 1, the protease activity was higher at stationary phase of 24 hours incubation with optimum value of 210.111 U/ml by the culture induced at 0.5 (OD_{600nm}). Similar to previous research, the protease production by *Bacillus licheniformis* ATCC 12759 also exhibits maximum protease synthesis at 24 hours of incubation [6]. In another study, the recombinant protein production by *E.coli* BL21(DE3) also obtained maximum production of extracellular invertase at the beginning of stationary phase [13]. As the protease activity does not depend on the cell growth, it can be concluded that the protease activity was not directly proportional to the cell growth and the optimum fermentation time for the recombinant 50a strain was 24 hours after IPTG induction at 0.5 (OD_{600nm}).

3.2 Optimization of Physical Conditions by CCD

The second part of process optimization was focused on incubation temperature, pH and agitation speed were carried out using central composite design. The optimum medium composition and incubation time achieved from previous optimization phase were applied in this part. Three variables of agitation rate (Y_1), incubation temperature (Y_2) and initial pH medium (Y_3) were studied based on the design decided in Table 1.

This last part of optimization studies was necessary to be carried out in order to identify optimum process condition which resulted in maximum protease production. The overall experimental design developed by CCD together with the predicted and experimental response was tabulated in Table 2. The highest protease production was recorded in the last run order of 20 with protease activity of 325.889 U/ml. The experimental data were analyzed using analysis of variance (ANOVA) and the regression coefficients

were also estimated by the statistical software. Both of the analysis of variance and estimated coefficients were presented in Table 3 and Table 4.

According to the total number of run developed by CCD (Table 2), it was possible to complete all the run at one time. Blocks were chosen in this design as the experimental work and data were run and collected on different time. The blocks (Table 3 and Table 4) obtained p-value more than 0.05 which means that the data collected on different time does not give significant effect on the protease activity. The coefficient of determination R^2 of the model was calculated to be 96.48 % indicated that the model was statistically significant. The variation in the responses was adequately explained by the model since the lack of fit was insignificant (p-value equal to 0.133).

Table 2 Experimental design of CCD for process condition optimization together with predicted and experimental responses

Std order	Run order	Blocks	Uncoded variable level			Protease activity (U/ml)	
			Y_1:Agitation rate	Y_2:Temperature	Y_3:pH	Predicted value	Experimental value
16	1	3	150.00	48.43	7.00	-52.990	2.000
15	2	3	150.00	25.56	7.00	107.413	78.667
19	3	3	150.00	37.00	7.00	180.021	199.000
18	4	3	150.00	37.00	8.63	178.101	180.222
17	5	3	150.00	37.00	5.37	166.223	190.333
20	6	3	150.00	37.00	7.00	180.021	199.000
14	7	3	296.00	37.00	7.00	274.681	273.889
13	8	3	0.00	37.00	7.00	-13.136	7.889
8	9	2	60.00	44.00	6.00	36.414	10.778
12	10	2	150.00	37.00	7.00	180.021	156.222
7	11	2	240.00	30.00	6.00	284.556	304.000
9	12	2	60.00	30.00	8.00	4.136	21.667
11	13	2	150.00	37.00	7.00	180.021	199.000
10	14	2	240.00	44.00	8.00	80.106	74.333
4	15	1	60.00	44.00	8.00	17.354	7.000
1	16	1	60.00	30.00	6.00	-5.192	9.667
2	17	1	240.00	44.00	6.00	74.886	66.444
5	18	1	150.00	37.00	7.00	180.021	180.000
6	19	1	150.00	37.00	7.00	180.021	192.000
3	20	1	240.00	30.00	8.00	318.164	325.889

Coefficients of the model were evaluated by regression analysis and tested for their significance summarized in Table 4. For linear regression terms, the agitation speed and incubation temperature have highly significant effect on the protease production by recombinant 50a strain. Only initial pH of medium showed no significant value. The non-significant value of the initial pH may represent the possibility that the initial pH of medium within the design range was not significantly affect the protease production or the design level (range) selected was not suitable.

According to Table 4, only interaction between agitation and temperature terms (Y_1Y_2) gave a significant effect as the p-value less than 0.05. This result showed that the effect of agitation speed on protease production depends on the incubation temperature. The other interaction between agitation and pH (Y_1Y_3) and temperature and pH (Y_2Y_3) were not significant which means that the effect of each terms on protease production were not depending on each other. For the squared (quadratic) effects, only the p-value for temperature*temperature (Y_2Y_2) are significant indicated that the relationship between temperature and protease activity follow a curved line. While the other term agitation*agitation (Y_1Y_1) and pH*pH (Y_3Y_3) were not significant indicated that there was no curvature in the response surface.

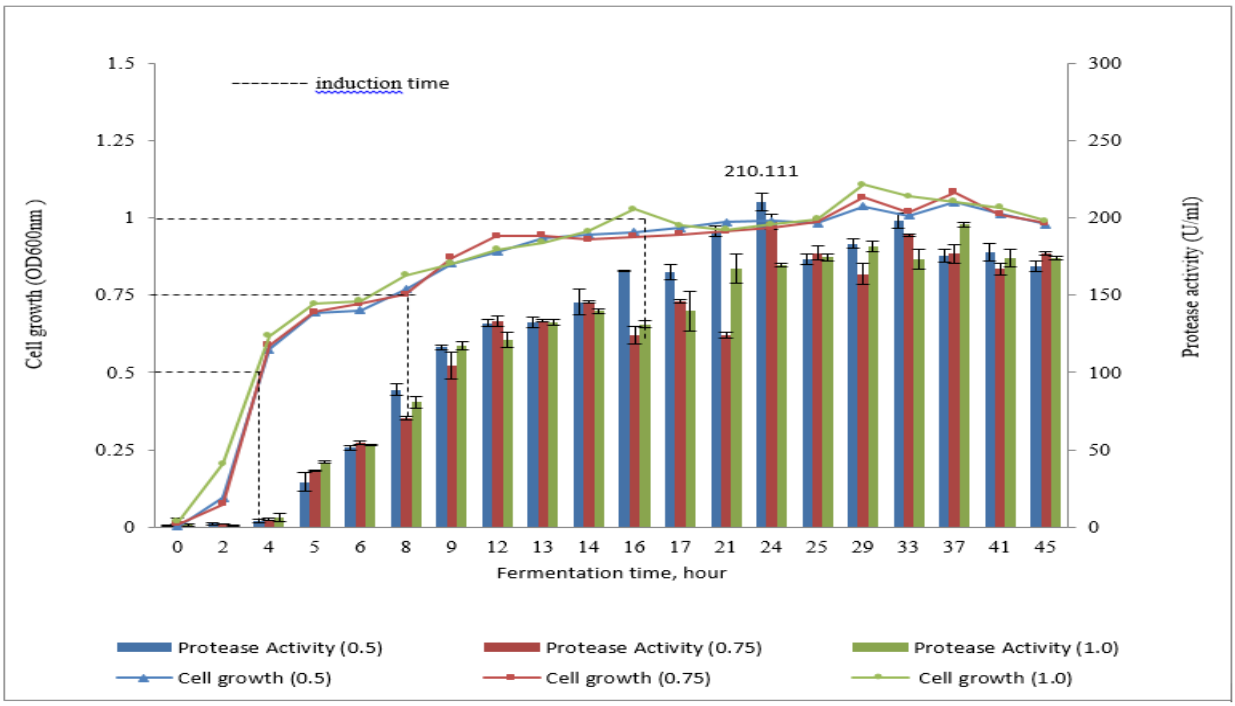


Figure 1 The growth profiles by recombinant 50a strain induced at different cell growth in optimized medium (uncontrolled pH) at 37°C and 150 rpm

Table 3 Analysis of variance (ANOVA) for central composite design

Source	Degree of freedom	Sum of square	Mean square	F-value	p-value
Blocks	2	647	357	0.36	0.707
Regression	9	215664	23963	24.26	0.000
Linear	3	137240	45470	46.04	0.000
Square	3	46156	15385	15.58	0.001
Interaction	3	32268	10756	10.89	0.003
Residual Error	8	7902	988	-	-
Lack of Fit	5	6915	1383	4.20	0.133
Pure error	3	987	329	-	-
Total	19	224213	-	-	-

The coefficients data (Table 4) obtained from the regression analysis calculated by statistical software were fitted into second-order polynomial equation. The final predictive equation can be written as:

Predicted protease activity (U/ml) =

$$187.267 - 0.124 - 7.124 + (88.125\ Y_1) - (49.113\ Y_2) + (3.637\ Y_3) - (18.468\ Y_1^2) - (57.303\ Y_2^2) - (2.947\ Y_3^2) - (62.819\ Y_1Y_2) + (6.070\ Y_1Y_3) - (7.097\ Y_2Y_3) \quad (1)$$

Y_1, Y_2 and Y_3 represent variables as in the Table 2.

The theoretically calculated response using Equation 1 was found to be good agreement with the experimental responses (Table 2). The 2D (contour) and 3D (surface) of the response plot were then plotted (Figure 2 to 4) to explain the interaction among process conditions and the optimum condition of each variable required for the protease overproduction. In response plot, two variables varied at a time when the other variable maintained at fixed level.

Table 4 Model coefficients estimated by multiple linear regressions

Term	Coefficient	Standard error	t-value	p-value
Constant	187.269	12.817	14.611	0.000
Block 1	-0.124	10.255	-0.012	0.991
Block 2	-7.124	10.255	-0.695	0.507
Y_1- Agitation rate	88.125	8.585	10.265	0.000
Y_2-Temperature	-49.113	8.607	-5.706	0.000
Y_3- pH	3.637	8.607	0.423	0.684
Y_1Y_1	-18.468	8.553	-2.159	0.063
Y_2Y_2	-57.303	8.651	-6.624	0.000
Y_3Y_3	-2.947	8.650	-0.341	0.742
Y_1Y_2	-62.819	11.111	-5.654	0.000
Y_1Y_3	6.070	11.111	0.546	0.600
Y_2Y_3	-7.097	11.111	-0.639	0.541

3.2.1 Interaction Effect of Incubation Temperature and pH on Protease Production

Incubation temperature was one of the important variables that greatly influenced the protease activity [15]. According to Figure 2, the surface plot represents a simple maximum response and the elliptical contour plot. The optimum protease production by *E.coli* BL21 (DE3) pLysS harboring 50a protease gene was observed in the temperature range of 25 to 35°C with the pH value of 6.5 to 8.63.

The incubation temperature was significantly affected the protease production as the linear and quadratic effect calculated by software obtained p-value more than 0.05. However, the protease activity was reduced at the incubation temperature below than 25°C or higher than 35°C. Almost similar range of temperature (below 35°C) also obtained for higher protease activity by the interaction of temperature and agitation speed showed in Figure 4. Thus, by combining both of the responses obtained from Figure 2 and Figure 4, the optimum growth temperature for overproduction of protease were between 25 to 35°C.

Specific temperature requirement are important as the growth of microorganisms can be inhibited at unsuitable temperature and activated at another temperature [14]. Besides, different temperatures also have different effects on the survival and growth of microbes [16]. The gram negative bacteria, *Escherichia coli* was categorized as mesophilic bacteria as their ability to grow in temperature ranging from 20°C to about 40°C with optimum growth temperature was 37°C [16].

However, comparing with the optimization response, *E.coli* BL21 (DE3) pLysS harboring 50a protease obtained optimum growth temperature between 30°C to 36°C. Previous study has shown that optimal temperatures for maximum neutral protease production by *E.coli* BL21 (DE3) pLysS harboring NPRC10 gene were found at 20°C [17].

3.2.2 Interaction Effect of Initial pH of Medium and Agitation Speed on Protease Production

Basically, pH also becomes one of the important physical requirements for microbial growth. pH can be defined as the measurement of hydrogen ions concentration in a solution [16]. In this optimization study, the pH of the medium only focused on the initial pH of the medium not for the overall fermentation process due to the limitation of using shake flask conditions. But, for the large scale process using fermenter system, the pH of the culture can be observed and maintained for overall fermentation process. The pH 7 was set as a centre level in the design range (Table 1) of the pH variable regarding the instruction manual of the competent cell of *E.coli* BL21 (DE3) pLysS medium preparation.

Figure 3 shows the response of the interactive variables of agitation speed and initial pH of medium. It was evident that increasing the shaker speed had a positive influence on protease production by recombinant 50a strain. However, increasing the initial pH of medium was not significantly affect the protease production as the effect remain the same for the range of pH 5.5 to 8.5. Considering other interaction effect between temperature and pH illustrated in Figure 2, the optimum protease activity was achieved at pH range 6.5 to 8.63. The linear, interaction and quadratic effect estimated by regression analysis in Table 4 already stated that the initial pH of medium were not significant on protease production.

The insignificant value of initial pH on protease overproduction indicated that either the initial pH within the design range selected was negligible or the design range was unsuitable and should be extended in order to determine more significant pH value that affected the protease production. According to previous study by Romsomsa *et al*., 2010 [18], the initial pH of the medium also showed insignificant effect on the protease production by *Bacillus subtilis* C4 using Plackett-Burman design as the p-value more than 0.05

(p value = 0.20). This negligible effect of pH on protease production also agreed by Calik *et al*., 2002 [19] which reported that uncontrolled pH was more favourable than controlled pH operation for serine alkaline production by *B. licheniformis*.

3.2.3 Interaction Effect of Agitation Speed and Temperature on Protease Production

The selection of suitable agitation rate is important because too high agitation rate led to cell lysis and excessive cell permeability due to abrasion by shear forces that finally caused reduction in protease yield [20]. However, low agitation rate also caused poor bacteria growth due to insufficient aeration supply and nutrient transfer [21]. In this line, suitable agitation speed should be used when culturing the strain in order to achieve maximum cell growth and protease production. A maximum protease activity of more than 300 U/ml was observed, corresponding to a high level of agitation speed (240 to 296 rpm) at a low temperature setting (25 to 35°C). This was evident that by increasing the agitation speed along with maintaining the incubation temperature at low level had a positive influence on protease overproduction. The surface and contour plots in Figure 4 represent a rising ridge surface.

Both surface and contour plots are based on a regression model. The linear and interaction effect of these variables (agitation speed*temperature) estimated by multiple regression were significant with p-value less than 0.05 (Table 4). In view of the other response plot between agitation speed and pH in Figure 3, the high protease activity also observed in the high level of agitation speed between 240 to 296 rpm. Both of the response in Figure 3 and 4 showed that the high level of agitation speed (240 to 296 rpm) resulted in higher protease production. Similar to previous study, the shaker speed showed significant value on protease production [18] while lowering the agitation speed drastically inhibited the protease activity [22].

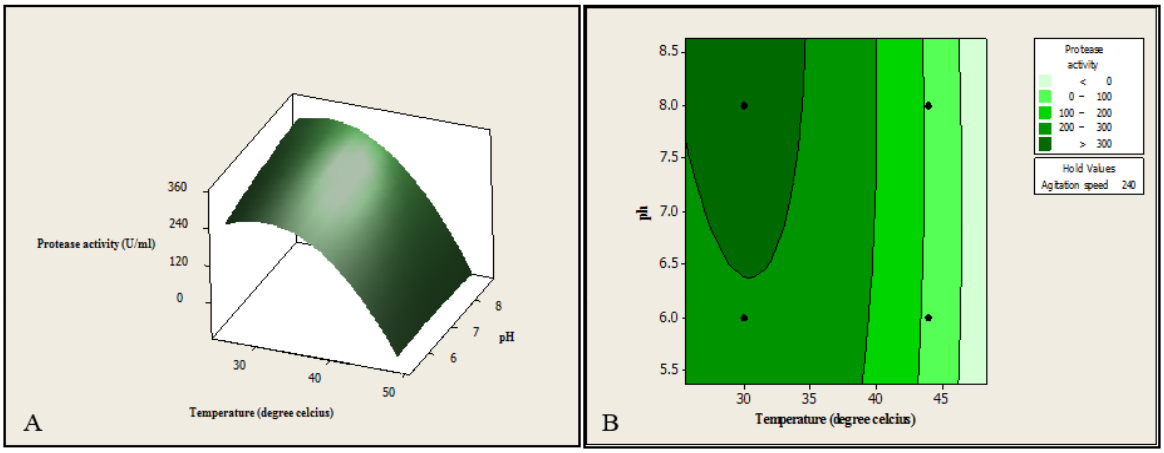


Figure 2 Surface plot (A) and contour plot (B) of protease production by recombinant 50a strain; the effect of temperature and initial pH of medium

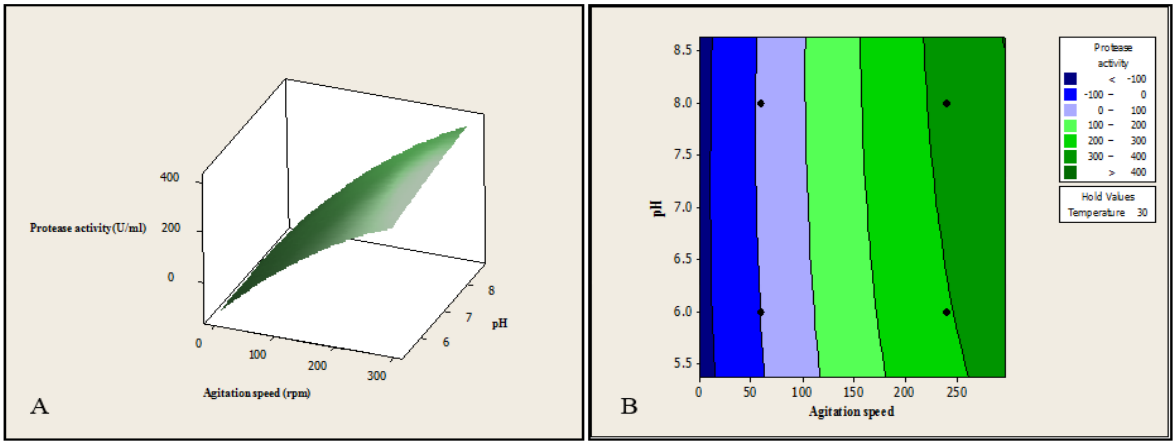


Figure 3 Surface plot (A) and contour plot (B) of protease production by recombinant 50a strain; the effect of agitation speed and initial pH of medium

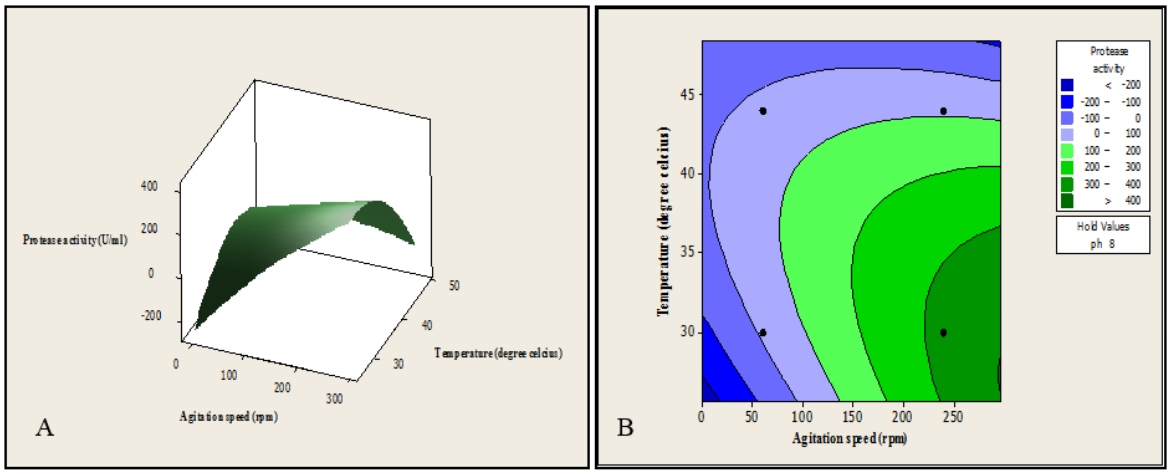


Figure 4 Surface plot (A) and contour plot (B) of protease production by recombinant 50a strain; the effect of agitation speed and incubation temperature

3.3 Experimental Verification of Culture Conditions Optimization using CCD

The validity of the model was done to verify the results obtained from the statistical analysis, experimental runs were developed using random set of four conditions combinations (Table 5) according to the overall higher response of protease activity from surface and contour plot (Figure 2 to 4). Only pH variable were varied as the optimum value were not obtained from the response plot. The control condition was performed using fixed agitation speed, temperature and uncontrolled initial pH of production medium. A high degree of similarity was observed between predicted and observed response. The highest production of 327.456 U/ml was achieved with the corresponding conditions of agitation speed (240), incubation temperature (30) and initial pH of production medium (8.63) compared to control conditions. The alkaline pH optimum at 8.63 reveals the alkaliphilic nature of the recombinant 50a strain.

Table 5 Validation of quadratic model within design space

Run order	Uncoded variable level			Protease activity (U/ml)	
	Y_1:Agitation rate	Y_2:Temperature	Y_3:pH	Predicted value	Experimental value
Control	150	37	Uncontrol	-	210.000
1	240	30	6	284.556	250.667
2	240	30	7	304.307	301.552
3	240	30	8	318.164	320.111
4	240	30	8.63	323.889	327.456

4.0 CONCLUSION

The used of both conventional and statistical design for physical optimization of thermostable alkaline protease by *E.coli* BL21 (DE3) pLysS harboring 50a protease were successful and significant. The optimum physical condition obtained with incubation time at 24 hours after induction process at cell growth of 0.5 (OD_{600nm}), initial pH medium of 8.63, temperature of 30°C and 240 rpm of agitation speed. The optimum condition provided an increment in protease production approximately 164.73% from the control physical condition.

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