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Response Surface Methodology for Optimization Membrane Disruption Using Thermolysis in Lipase *Lk2* and *Lk3*

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Abstract

***Lk2* and *Lk3* were thermostable recombinant lipase and highly expressed in *Escherichia coli* BL21 (DE3). However, *Lk2* and *Lk3* accumulated as an inclusion body. To further characterize both recombinant lipases, the soluble enzyme must be obtained first. This study aimed to optimize the disruption of the cell membrane in order to obtain soluble and active lipases. The effects of temperature lysis, pH, and SDS concentration on lipolytic activity *Lk2* and *Lk3* were investigated using a three-factor Box-Behnken design response surface methods. The optimum condition for the temperature variables at 50°C, pH 8, and 0.34% SDS which gave a lipolytic activity of 0.9 U for *Lk2*. Meanwhile, *Lk3* lipolytic activity of 0.9 U obtained at the temperature of 50°C, pH 8, and 0.1% SDS. This result showed efficient one-step membrane disruption methods using thermolysis with addition of a low concentration of detergent at pH 8. The methods used were effective and applicable in the production of active and soluble thermostable recombinant lipase.**

Keywords: Lipase, thermostable, recombinant, inclusion body, thermolysis

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INTRODUCTION

Lipases or triacylglycerol acylhydrolases catalyze carboxyl ester bond synthesis and hydrolysis. Various types of synthetic reaction catalysed by lipase, i.e.: esterification, alcoholysis, acidolysis, aminolysis, and interesterification.[1,2] Through these widely reaction that could be catalysed by lipase, these enzymes were categorized as important industrial enzymes Lipase is utilized extensively in the dairy, food, flavor, biofuels, pharmaceutical, cosmetics, detergents, leather, and chemical sectors.[3,4] Different sources of lipase include animals, vegetables and microbes.[5-7] Another important source of lipase is from metagenome. In a metagenomics study, the lipase gene inserts into the vector plasmid and typically transforms into an *Escherichia coli* host. The source of the lipase gene could originate from any environment such as wastewater, soil, sea sediment, and compost.[8]

Lk2 and *Lk3* are recombinant lipases obtained through metagenome study from domestic compost. These lipases are highly similar to the lipase of *Pseudomonas stutzeri* categorized as true lipase.[9] *Lk2* and *Lk3* are strongly over expressed on *Escherichia coli* BL21 (DE3), were thermostable and showed hydrolytic activity on para-nitrophenyl dodecanoate.[10] However, high expression level of these recombinant lipase resulting inclusion body (IBs). IBs are misfolded proteins formed in the cytosol bacterial environment. There are two types of IBs which are classic and not classic. Conventional term for recovering active proteins from IBs are made up of four steps: 1. Isolation inclusion body, 2. Solubilization through detergent or other agents, 3. Refolding and 4. Purification of the refolded proteins.[11,12]

Solubilization and refolding of inclusion body are crucial steps to recover functional recombinant lipase. A variety of recombinant protein solubilized using two step denaturation and refolding.[13] Solubilisation using denaturant agent in various pH and activation of certained recombinant lipase by lif protein (lipase specific foldase) or chaperone discussed detailed in the literature.[14,15] All of these strategies are based on the OFAT (one-factor-at-a-time) method of optimization, which keeping the other variable constant and changing one variable at a time.[16] These conventional methods were time-consuming, therefore require other more efficient methods.

Optimisation employing response surface methodology (RSM) could be used to select effective methods. RSM is a set of mathematical and statistical tools for process optimization. Parameters that affect the process are called independent variables and the dependent variables are called response.[17,18] For example, RSM was used to optimize the refolding of recombinant lipase from the inclusion body *Escherichia coli*.[19] The optimization cell disruption methods using the RSM-based Box-Behnken design were done by variable temperature lysis, pH and time. Temperature at 77°C, pH 7.71 with incubation duration in 20-minute were the best values for the variables.[20] However, this previous study used the thermolysis technique to disturb the *Escherichia coli* membrane cell.

The thermolysis method was done by incubating crude lysate of recombinant protein at heat temperature.[21] Two previous studies successfully using thermolysis methods for partial purification of recombinant lipase from Geobacillus stearothermophilus strain AH22 and Bacillus pumilus.[22,23] Thermolysis is suitable to be used in thermostable protein that can retain activity on high temperature where the other protein can denature. The present study aimed to optimizing cell disruption by using RSM methods based on three factorial models (Box-Behnken design) to recover the active soluble and thermostable recombinant lipase *Lk2* and *Lk3*.

MATERIALS AND METHODS

Materials

The *LK2* and *LK3* lipase genes were previously cloned in the expression vector pET-30a (+) and introduced into the host *E. coli* BL21(DE3).[10] 4-nitrophenyl dodecanoate, 2,4-nitrophenol, sodium dodecyl sulphate, Triton™ X-100, and sodium deoxycholate monohydrate were purchased from Sigma (Sigma, Chemicals, USA.). Disodium hydrogen phosphate anhydrous, NaOH, and sodium hydrogen phosphate monohydrate were pure analysis grade from Merck (Merck, Germany). All other chemicals were of the highest commercially available reagent grade.

Methods

Heterologous Expression

Each clone with kanamycin sulfate (50 g/mL) was grown in LB medium. Overexpression was achieved by inducing the cells with 1 mM IPTG and then incubating at 37°C and 150 rpm for 4 hours. Cell pellets collected by centrifugation and kept in -20°C for further methods.

Cell disruption using thermolysis with detergent added

In a 50 mM, pH 8.0 sodium phosphate buffer, the cells were resuspended. Cells expressing recombinant *Lk2* and *Lk3* were subsequently subjected to membrane cell solubilization by thermolysis with detergent added. This was performed by cells incubated at 50°C in 30 minutes by adding 0.1% detergents. Different detergents were used, ie. Sodium dodecyl sulfate, sodium cholate hydrate and Triton™X-100. After incubation, the cells were centrifuged for 20 minutes at 11,000 g to separate protein supernatant and cellular debris. SDS PAGE was used to examine the protein profile of the supernatant.

SDS PAGE

SDS-PAGE was done at 110 V on a 12% running gel with SDS Running Buffer.

Protein bands were visualized by using 0.1 percent Commasie Brilliant Blue to stain the gel. The purified enzyme's molecular mass was estimated using the peqGOLD Protein Marker III (Peqlab) molecular mass standard.

Optimisation of membrane disruption using the response surface methodology.

Optimization of membrane disruption has been determined as recovery of soluble recombinant lipase expressed as lipolytic activity (U/mL). This was performed using the surface response method based on Box Benhken (BBD) design. BBD were generated by MINITAB (Trial version 19). Three independent variables included temperature, pH, and SDS concentration (Table 1.). Each experiment's response value (lypolitics activity) is the average of three triplicates. The F-test score verified the importance of the response surface model. The coefficient of adjustment R2, the determination R2, and the lack of fit were used to assess the quadratic model's quality. For a better understanding of the correlation between independent variables and responses, the response surface and contour plots were determined.

Table 1. Variable levels to optimise thermolysis

Independent	Variable levels		
	-1	0	1
A) Temp. (°C)	50	70	90
B) pH	8	9	10
C) SDS (%)	0.1	0.55	1

Lypolitic activity assay

In a 0.9 mL acetonitrile:ethanol:buffer 1:4:95 substrate solution (v:v:v), added 0.3 mL crude enzyme and incubated at 50°C for 15 minutes. Lipolytic activity was observed by hydrolysis of 4-nitrophenyldodecanoate providing 2,4-nitrophenol which is detectable by spectrophotometry at 405 nm. The quantity of mol pNP released in one minute is one unit of enzyme activity.[24]

RESULTS AND DISCUSSION

Heterologous Expression and Membrane disruption Optimization

Heterologous expression of *LK2* and *LK3* lipase genes in *Escherichia coli* BL21 (DE3) produces a large amount of recombinant lipase (Fig. 1).

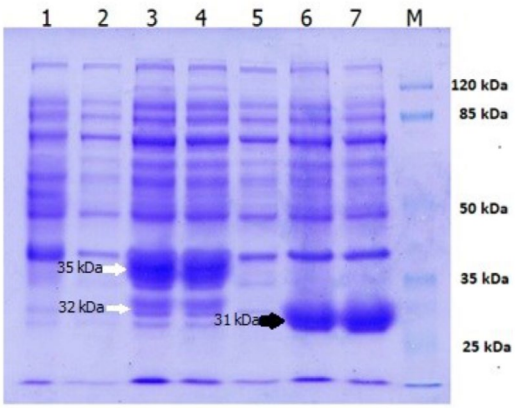

Fig. 1. SDS-PAGE of over expressed Lk2 and Lk3 in *E. coli* BL21(DE3): [1] pET-30a vector; [2] Lk2 without IPTG induction; [3]&[4] Lk2 with IPTG induction; [5] Lk3 without IPTG induction; [6]&[7] Lk3 with IPTG induction. Lk2 protein bands are indicated by white arrows. Lk3 protein bands are indicated by a black arrow.

The *LK2* and *LK3* lipase genes have a high degree of homology with the *Pseudomonas stutzeri* gene according to family 1.1, ranging from 96 to100% 10. The presence of peptide signals in the *LK2* gene sequence results in two proteins (Fig. 1, line 3 and 4) that are overexpressed. Based on the *in silico* analysis, the approximate weight of the fusion protein is 35 kDa that still contains a putative signal peptide, when the region was cleaved resulting 277 amino acid residues corresponded to the protein with molecular weight of 32 kDa. The *LK3* gene sequence produces a single protein band of 31 kDa in size.

Isolation of recombinant lipase *Lk2* or *Lk3* by routine ultrasonication method could not overcome the problem of inclusion bodies, this showed by large amount of protein bands in the cell debris (Fig. 2, line 1 and 4). So we did thermolysis with detergent added to get soluble recombinant lipase.

Thermolysis with a 0.1% added detergent was carried out with varies; sodium cholate hydrate, triton X-100 or SDS. The result was only thermolyzing by adding SDS can be used to obtain soluble *Lk2* and *Lk3* (Fig. 3). Based on this, thermolysis optimization was done using SDS added. SDS classified as strong ionic detergents are capable of lysing the cell within a second.[25] However, after the soluble recombinant lipase was obtained, SDS binding protein was precipitated using 30 mM K_2HPO_4 to prevent denaturation effect.[26]

Thermolysis has been classified as a nonmechanical (physical) cell lysis method.[27] This method was first performed by[28] in which, at 80°C, *Escherichia coli* would release recombinant thermostable esterase. At elevated temperatures, the membrane did not explode completely, but became readily permeable. In this situation, the detergent easily attaches itself with a membrane component like lipopolysaccharides and proteins. The disruption of the cell membrane is therefore efficient.

Membrane disruption optimization based on response surface methodology

To study the optimization variable within the recovery of recombinant lipases *Lk2* and *Lk3*, temperature, pH and SDS concentration were studied to assess their effect on the recovery of soluble recombinant lipase using rsm based on a three level BBD factorial design. A total 15 run experiments and the resulting enzyme activity (U/mL) are presented in Table 2.

Multiple regression was used to evaluate the data in Table 2 and was modified to the second order (quadratic) regression model for enzymatic activity, the following equation:

Uncoded Units Regression Equation for *Lk2*

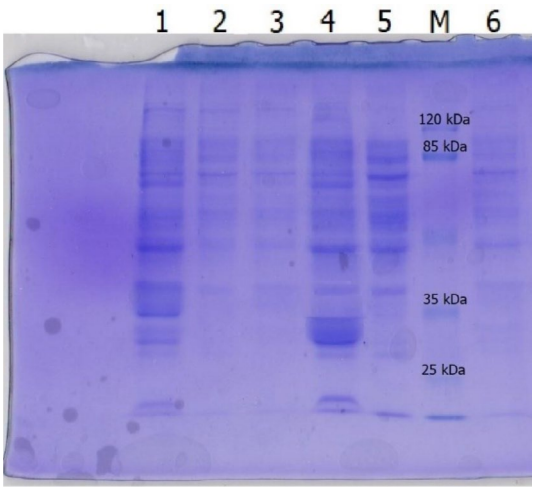

Fig. 2. SDS-PAGE of lysis cell using ultrasonication method; [1] Lk2 cell debris, [2]&[3] Lk2 cell supernatant; [4] Lk3 cell debris, [5]&[6] Lk3 cell supernatant.

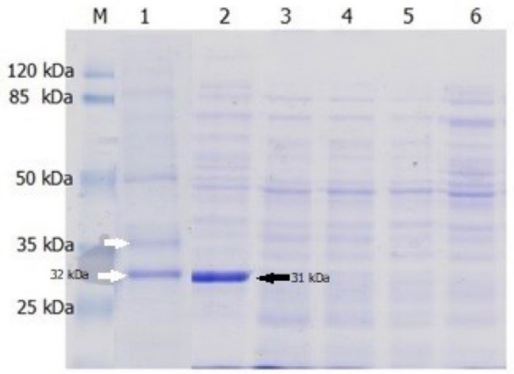

Fig. 3. The lysate's profile on SDS-PAGE: [M] Protein Ladder, [1] LK2 from thermolysis+0.1% SDS at 50 °C, [2] LK3 from thermolysis+0.1% SDS at 50 °C, [3] LK2 from thermolysis+0.1% sodium cholate hydrate at 50 °C, [4] LK3 from thermolysis+0.1% sodium cholate hydrate at 50 °C, [5] LK2 from thermolysis+0.1% triton x-100 at 50 °C, [6] LK3 from thermolysis+0.1% triton x-100 at 50 °C.

Table 2. Independent variables and response values in a Box-Behnken design

Std Order	Run Order	PtType	Blocks	Temp. (°C)	pH	SDS (%)	Activity (U/mL)	
							Lk2	Lk3
1	1	2	1	50	8	0,55	0.90	0.89
2	2	2	1	90	8	0.55	0.54	0.63
3	3	2	1	50	10	0.55	0.67	0.80
4	4	2	1	90	10	0.55	0.30	0.53
5	5	2	1	50	9	0.1	0.82	0.86
6	6	2	1	90	9	0.1	0.49	0.59
7	7	2	1	50	9	1	0.69	0.84
8	8	2	1	90	9	1	0.39	0.57
9	9	2	1	70	8	01	0.57	0.76
10	10	2	1	70	10	0.1	0.38	0.69
11	11	2	1	70	8	1	0.55	0.73
12	12	2	1	70	10	1	0.35	0.66
13	13	0	1	70	9	0.55	0.52	0.70
14	14	0	1	70	9	0.55	0.55	0.70
15	15	0	1	70	9	0.55	0.52	0.65

Table 3. ANOVA for quadratic model of *Lk2*

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	6	0.383704	0.063951	72.78	0.000
Linear	3	0.333816	0.111272	126.63	0.000
Temp.	1	0.232721	0.232721	264.84	0.000
pH	1	0.090488	0.090488	102.98	0.000
SDS	1	0.010608	0.010608	12.07	0.008
Square	3	0.049888	0.016629	18.92	0.001
Temp.*Temp.	1	0.037568	0.037568	42.75	0.000
pH*pH	1	0.003409	0.003409	3.88	0.084
SDS*SDS	1	0.005333	0.005333	6.07	0.039
Error	8	0.007030	0.000879		
Lack-of-Fit	6	0.006607	0.001101	5.21	0.170
Pure Error	2	0.000422	0.000211		
Total	14	0.390734			

S=0.0296430, R2=98.20%, adjusted R2=96.85%, predicted R2=92.99%.

Activity = 0,85 – 0.04383 Temperature + 0.441 pH + 0.1255 SDS + 0.000252 Temperature*Temperature – 0.0304 pH*pH – 0.1877 SDS*SDS

Uncoded Units Regression Equation for *Lk3*

Activity = 1,6945 - 0,01067 Temperature - 0,04098 pH - 0,02800 SDS + 0,000029 Temperature*Temperature

The significance of the model for thermolysis optimization *Lk2* and *Lk3* was confirmed by an analysis of variance (ANOVA) as shown in Tables 3 and 4.

The high F-value of *Lk2* (72.78) and *Lk3* (821.06) and the small P-value (0.000) confirmed that both models were significant. Table 3 and Table 4 indicated that the linear terms (temperature, pH and SDS) were important for the activity response. This means that each of the independent variables gives effect to the cell disruption and can be observed based on recombinant lipase activity. The terms cross-interaction between Temperature*pH, Temperature*SDS and pH*SDS have no significant effects and are therefore not included in the final equation of the model. For square interaction in the *Lk3* quadratic regression model, the terms

pH*pH and SDS*SDS were eliminated as they have no significant effect.

The coefficient of determination (R2) is a goodness-of-fit test used in ANOVA to show how well a statistical model fits a set of data. The range value between 0-1 and the best model closest to 1. According to R2 value for each quadratic model, 98.20% of the total of variance can be explained by the model for *Lk2* and 99.70% for *Lk3*, respectively. The high adjusted R2 also confirm significance of the model.[29] The *Lk2* and *Lk3* regression models have a non-significant lack of fit when compared to the pure error, according to the ANOVA table. This indicates that the model adequately describes the experimental data.[30]

A final test of model significance was obtained by comparing the experimental response to the predicted response (Fig. 4).

Fig. 4, shows less difference between the clustering of the experimental run and the predicted values. These plots show the validation of the models.

The effect of factors on membrane disruption was studied using response surface analysis

To visualize the interaction effects of the covariates on the response, the amended regression equation was shown as a three-dimensional response area and two-dimensional contour plot. Fig. 5 and 6 indicate the impact of temperature, pH, and SDS concentrations on enzymatic activity response, as well as their reciprocal effects.

Fig. 5 demonstrates how raising the temperature range from 50°C to 90°C and the pH from 8 to 10 reduces *Lk2* activity. Within the SDS range of 0.1 to 0.6 percent and pH range of 8 to 9 at 50°C, high activity (>0.8 U) is visible.

An *Lk3* regression model produced the response surface and contour plot for the

Table 4. ANOVA for quadratic model of *Lk3*

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	4	0.157647	0.039412	821.06	0.000
Linear	3	0.157160	0.052387	1091.37	0.000
Temp.	1	0.142453	0.142453	2967.72	0.000
pH	1	0.013438	0.013438	279.95	0.000
SDS	1	0.001270	0.001270	26.45	0.000
Square	1	0.000486	0.000486	10.13	0.010
Temp.*Temp.	1	0.000486	0.000486	10.13	0.010
Error	10	0.000480	0.000048		
Lack-of-Fit	8	0.000454	0.000057	4.45	0.196
Pure Error	2	0.000026	0.000013		
Total	14	0.158127			

S=0.0069283, R2=99.70%, adjusted R2=99.58%, predicted R2=99.27%

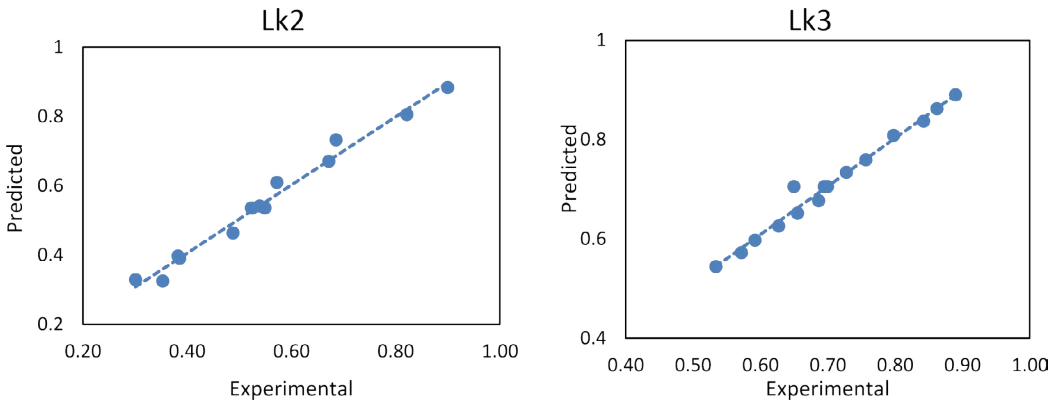

Fig. 4. Predicted vs experimental plot for Thermolysis optimization.

interaction variable and activity illustrated in Fig. 4.

The surface and contour graphs in Fig. 4 demonstrate this, the higher the temperature, pH and SDS concentration, the lower the activity of Lipase *Lk3*. High activity >0.85 U observed at SDS concentrations from 0.1 to 0.2%, pH 8 at 50°C.

The predicted optimal conditions can be calculated statistically from the regression model which was performed. The response optimizer predictions were calculated using Minitab based on the *Lk2* and *Lk3* regression models. This can be done once we know that the model was valid and significant according to the above result. Response optimization was set to be maximized to achieve optimum activity. *Lk2* optimum activity predicted at 0.90 U with independent variable as follows; temperature at 50°C, pH 8 and 0.34% SDS. Meanwhile, for *Lk3*, the optimum activity was expected to be 0.90 U with temperature variables at 50°C, pH 8 and 0.1% SDS.

The two recombinant lipase *Lk2* and *Lk3* showed the best lysis condition (lipase activity) at medium temperature at 50°C. Optimization

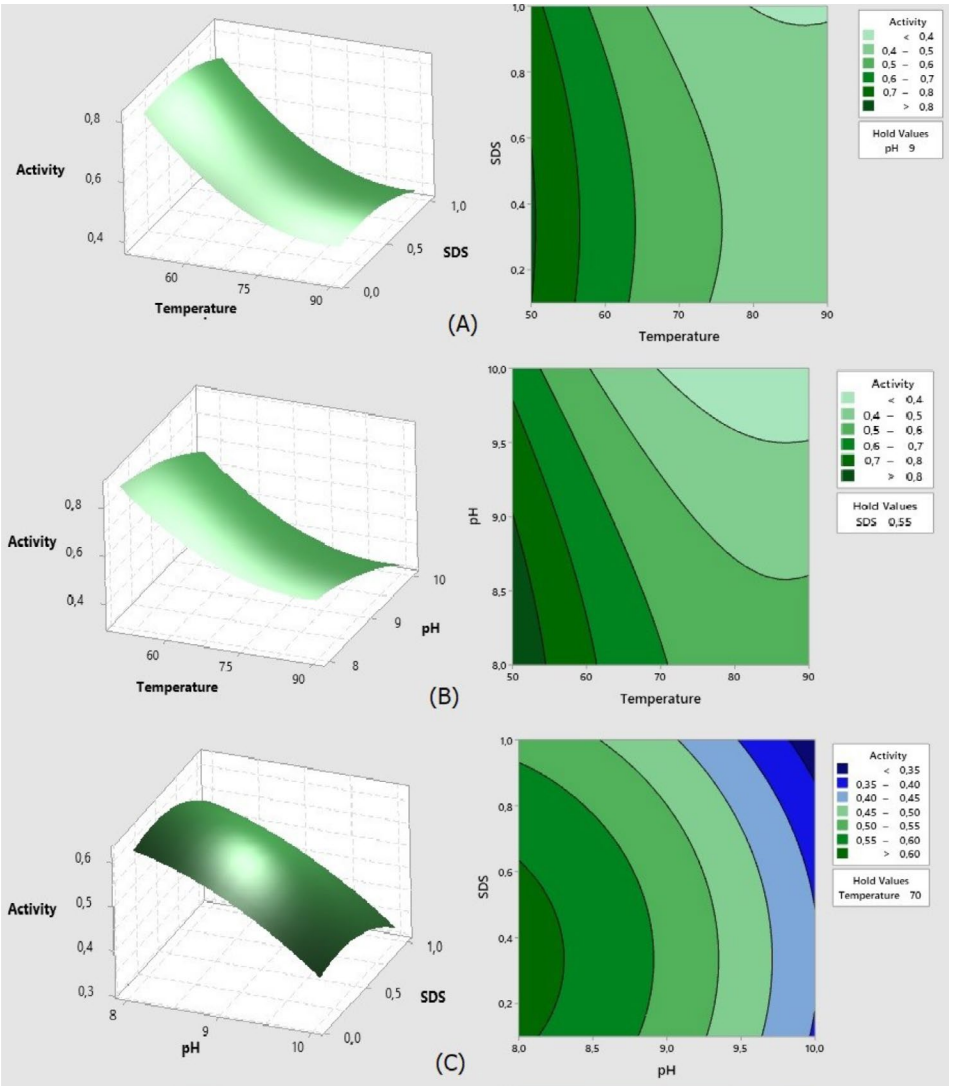

Fig. 5. Surface and contour graphs of the response Lk2; (A) At pH 9, the effect of temperature and SDS content on lipase activity was investigated.; (B) At a concentration of 0.55 percent SDS, the effect of temperature and pH on lipase activity was investigated.; (C) Effect of pH and SDS concentration at temperature 70°C.

of thermolysis as well as activity measurements were also performed from thermostable cellulase FnCe15A,[20] thermostable laccase,[31] hyperthermophilic esterase were expressed in *E. coli*.[21] The temperature of these thermolysis studies varied from 60 to 96°C. In the previous reported methods, thermolysis with addition of 0.2% triton-X detergent successfully isolating recombinant steryl glucosidase (SGs) from *E. coli* host.[32] The detergent may bind the hydrophobic and hydrophilic site of the bacterial cell membrane. Detergent can disrupting the lipid-lipid, lipid-protein, and also protein-protein interaction.[33] Combination of low concentration of detergent (0.05–1%) with a 0.5–1 mol urea effectively solubilized inclusion body of recombinant enzyme and enhanced the activity.[34]

This study demonstrated that the optimal pH for lysis cells was at pH 8. In the previous thermolysis study, pH 9 showed an optimal condition for release of recombinant and indigenous *Escherichia coli* proteins. The pH has affected the charged bacterial cell wall molecule as well as the inclusion of body proteins.[35] When the charged species is established, anionic detergent such as SDS attaches easily. This pH and detergent collaborate to permeate cell membrane and solubilized inclusion body.

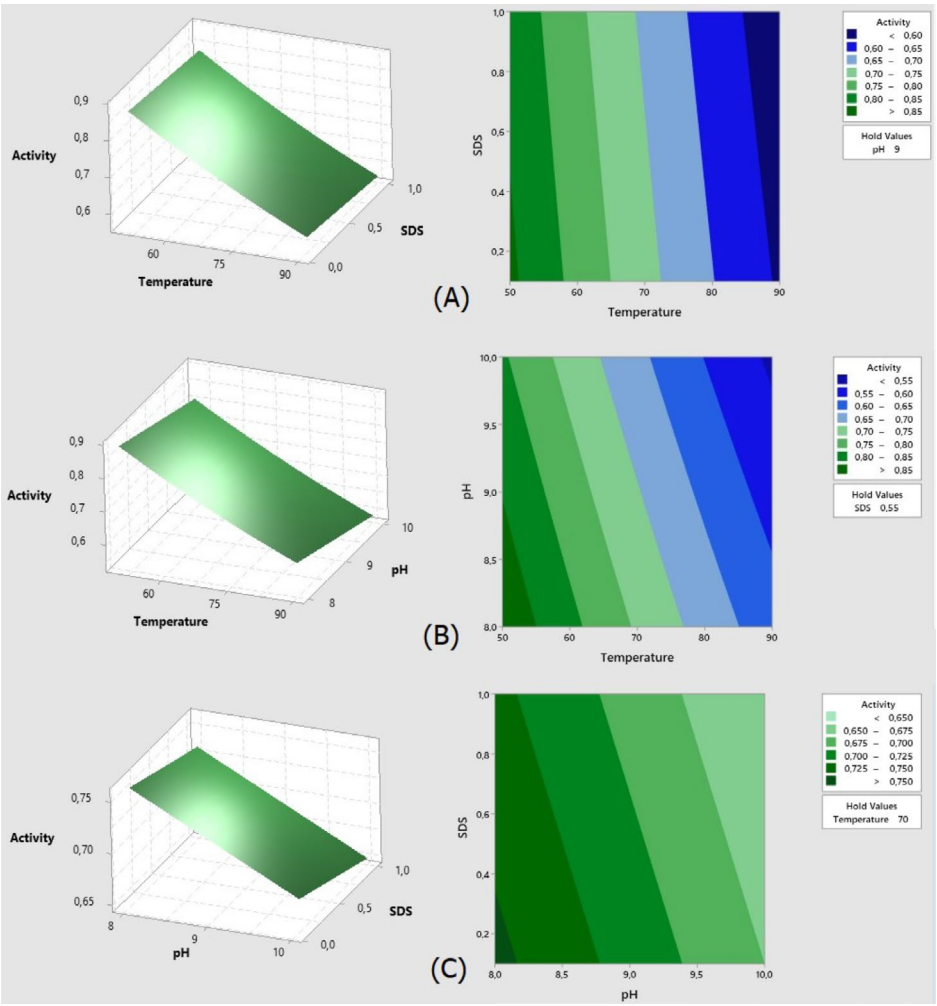

Fig. 6. Surface and contour graphs of the response Lk3; (A) At pH 9, the effect of temperature and SDS content on lipase activity was investigated.; (B) At a concentration of 0.55 percent SDS, the effect of temperature and pH on lipase activity was investigated.; (C) Effect of pH and SDS concentration at temperature 70°C.

These results demonstrate the importance of optimizing the thermolysis method with activity determination to achieve soluble and active recombinant thermostable lipase.

CONCLUSION

In summary, two recombinant thermostable lipases *Lk2* and *Lk3* can be easily isolated by thermolysis using low concentration detergent. The present study is based on a three-factor Box-Behnken factorial design-response surface methodology to find the best thermolysis condition or maximal activity. By statistical analysis, the optimal conditions of temperature and pH were determined at 50°C and pH 8, where SDS concentration 0.34% for *Lk2* and 0.1% for *Lk3*, respectively. The maximum lipolytic activity of 0.9 U for *Lk2* and *Lk3* can be achieved under the above mentioned conditions. Isolation and solubilization of recombinant thermostable lipase as an inclusion body could easily be achieved by thermolysis with the addition of a low concentration of SDS, which could be directly applied in biocatalytic or purified first.

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None.

CONFLICT OF INTEREST

All authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication. All authors read and approved the final manuscript for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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