Optimization of Trypsin-like Protease Production by *Lactobacillus plantarum* FNCC 0270 using Response Surface Methodology

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Abstract

The purpose of this study was to get optimum medium composition and agitation to trypsin-like protease production by *Lactobacillus plantarum* FNCC 0270. The medium composition and agitation for enzyme production was optimized using Central Composite Design and Response Surface Method with Design Expert software version 7.1.5. Fermentation was carried out in erlenmeyer flask at initial pH 8, 37 °C, with shaker incubator at 87.5 rpm. The results of the best of enzyme activity 1.0 mU/mL, protein levels of 0.557 mg/mL and desirability value of 0.740. Numerical optimization was performed to approach the ideal state of the fermentation or desirability value of 1. The medium composition of fermentation used was: 3.64% baker's yeast, 1.21% glucose, and 0.13% skim milk. The enzyme activity reached was 1.51 mU/mL and protein levels of 0.205 mg/mL. After numerical optimization, the fermentation process was verified using 125 mL Erlenmeyer in shaking incubator at 77 rpm, initial pH 8, 37 °C, 15 h of fermentation. The verification results showed that the enzyme activity and protein levels was 1.273 ± 0.227 mU/mL and 0.248 ± 0.012 mg/mL, respectively.

Abstrak

Optimization of Trypsin-like Protease Production by *Lactobacillus plantarum* FNCC 0270 using Response Surface Methodology. Tujuan penelitian ini adalah memperoleh komposisi medium dan agitasi optimum untuk produksi protease serupa tripsin oleh *Lactobacillus plantarum* FNCC 0270. Protease serupa tripsin (PST) dihasilkan oleh *L. plantarum* FNCC 0270 melalui proses fermentasi dengan optimasi komposisi medium dan agitasi menggunakan *Central Composite Design* dan *Response Surface Methode,* dengan *software Design Expert* versi 7.1.5. Fermentasi dilakukan dalam erlenmeyer pH awal 8 dan suhu 37 °C menggunakan shaker inkubator pada 87,5 rpm. Hasil eksperimen terbaik menunjukkan aktivitas enzim 1 mU/mL, kadar protein 0.557 mg/mL dan diperoleh nilai *desirability* 0,740. Untuk mendekati keadaan ideal atau nilai *desirability* 1 dilakukan optimasi melalui simulasi numerik, yaitu fermentasi dengan komposisi *baker yeast* 3,64%, kadar glukosa 1,21%, konsentrasi *skim milk* 0,13%, dan agitasi 77 rpm, sehingga diperoleh aktivitas enzim 1,51 mU/mL dan kadar protein 0,205 mg/mL. Setelah optimasi numerik kemudian dilakukan verifikasi fermentasi di laboratorium, dalam erlenmeyer menggunakan *shaker* inkubator, agitasi 77 rpm, pH awal 8, suhu 37 °C, selama 15 jam. Hasil verifikasi menunjukkan aktivitas enzim dan kadar protein masing-masing $1,273 \pm 0,227$ mU/mL dan $0,248 \pm 0,012$ mg/mL.

Keywords: central composite design enzyme activity, Lactobacillus plantarum, protein content, response surface methodology

Introduction

The value of pharmaceutical enzyme imported in 2007 was $ 2.988 billion and the estimated value in 2008 and 2011 were $3.91 billion and $ 4.55 billion, respectively [1], approximately 90% of the demand, while the world enzyme demand also continued to increase by 6.5% per year, up to $ 5.1 billion in the year 2009 [2]. Trypsin is a protease enzyme produced by the pancreas and secreted to the duodenum, where it hydrolyses proteins into peptides during the digestion of food. Trypsin is memberof the serine protease family, which digest protein from the carboxyl terminal (C-terminal) of the amino acid lysine (Lys) and arginine (Arg) [3].

Trypsin deficiency can also cause a variety of problems in the physiology of the body as impaired amino acid absorption in case of inflammation of the pancreas. One effort to address them is by giving intake digestive enzyme (trypsin) from outside the body [4]. Until now a lot of research has been done on the isolation of trypsin from various species of fish including pomfret fish, bigeye snapper, red snapper, chinook salmon, sardine monterey, mandarin fish and skipack [5].

Trypsin can also be isolated from pork and beef. It can be a bit problematic because there is a fear the spread of bovine spongiform enchephalopathy (mad cow disease). This led to increased interest in the proteases of microbial because microorganisms can be produced on a large scale, relatively short production time, and production can be run continuously.

Lactic acid bacteria (LAB) is one among microbes received GRAS status (Generally Recognized As Safe) from the FDA and safe for food products. One source of microbes potential as trypsin–like protease producer is lactic acid bacteria (LAB). LAB is commonly used in food productions, thus has GRAS status (Generally Recognized as Safe). This is, of course, important if the enzyme is intended for human consumption.

Media composition and growth condition play important roles in providing the culture's growth rate and productivity, as well as the activity of the product. To obtain optimum media composition and growth conditions, such as temperature, pH, or agitation, requires good experimental design and reproducible empirical data. To design more focused and well targeted experiments, statistical methods are often used for example, the Response Surface Methodology (RSM). RSM is a statistical method used to explore the relationships between several explanatory variables and one or more response variables. Response surface methodology is a collection of mathematical and statistical techniques that are useful for the modeling and analysis of problems, in which a response of interest is influenced by several variables. Using RSM, we can determine which explanatory variables (e.g. medium components or culture conditions) have impacts to the response variables of interests (e.g. growth rate, protein level or productivity) [6]. CCD technique contains an imbedded factorial or fractional factorial design with 'center points' that is augmented with a group of `star points' allowing estimation of curvature [7]. Response Surface Methodology (RSM) and Central Composite Design (CCD) with four numerical factors (temperature, intensity, pH, aeration) and one response (μmax) was performed to optimize the growth of *Nannochloropsis oculata* [8]. Response surface methodology (RSM) was applied to optimize the medium constituents. A 2^4 full-factorial central composite design (CCD) was chosen to explain the combined effects of the four medium

constituents, viz. moisture content, particle size of the substrate, *di*-potassium hydrogen phosphate and trace ion solution concentration, for the production of lovastatin by using strain of *Aspergillus terreus* UV 1718 [9]. In this study, we performed medium optimization for the production of trypsin-like protease using the RSM and 2^4 full-factorial CCD with 4 numerical factors (concentration of baker's yeast, glucose, skim milk and the agitation speed) with two responses, trypsin activity and protein contents.

The best medium composition and agitation will be used to scale up and purification as well as characterization of trypsin-like protease.

Materials and Methods

Microorganism. Lactic Acid Bacteria used as producer of a trypsin-like protease (TLP) is *Lactobacillus plantarum* FNCC 0270, collection of Laboratories for Technology Development of Agro-Biomedical Industries (LAPTIAB)–Badan Pengkajian dan Penerapan Teknologi (BPPT) [10].

Growth medium. The growth medium used in this study was 5.2% de Man Rogosa Sharpe (MRS) medium, sterilized by autoclave at 121 °C for 15 min. All fermentations were performed at 37 °C, pH 8.

Production medium and fermentation. Baker's yeast was used instead of yeast extract because it was a cheap and edible alternative of nitrogen source. Each medium composition was prepared separately. Glucose of carbon source was dissolved in water and was adjusted to pH 8 using HCl 0.1M. Baker's yeast was dissolved in water and then centrifuged for 15 min at 25 °C 3000 rpm to obtain the supernatant. Glucose solution and baker's yeast supernatant were mixed and homogenized, then autoclaved at 121 °C and 2 atm for 15 min. Skim milk of inducer was suspended in water and then pasteurized at 110 °C and 2 atm for 15 min. Prior to use, glucose – baker's yeast solution and skim milk suspension were mixed aseptically. Fermentation was carried out in erlenmeyer flask 125 mL at initial pH 8, 37 °C, with shaker incubator. The results of a preliminary study to determine the number of cells is recommended in this study $\geq 10^8$ cells/mL.

To prepare starter culture, glycerol stock of lactic acid bacteria isolates FNCC 0270 was inoculated into 40μl growth medium and incubated at 37 °C 150 rpm for 5 h. 100μL starter culture was used to be inoculate in 5mL growth medium and then incubated at 37 °C 150 rpm. After 5 h, cells were harvested by centrifugation at 4 °C 6000 rpm for 5 min, and then used to inoculate the production medium. Production culture was then incubated for 24 h at 37 °C with agitation. After 24 h,

Table 1. Midpoint Value of the Four Factors on Media Optimization and Agitation with RSM Method

Factor	Name	Coded Level				
		$-\alpha$	-1	0	1	α
A	Baker's yeast	1	1.5	2	2.5	3
B	Glucose	0.5	0.75	1	1.25	1.5
C	Skim milk	0.05	0.15	0.25	0.35	0.45
D	Agitation	12.5	50	87.5	125	162.5

the cell number was determined by OD 560 and enzyme was harvested by centrifuging the culture for 15 minutes at 4 °C 6000 rpm using *High Speed Refrigerated Centrifuge Himac* CR21G and rotor R10A2, supernatant was taken as crude enzyme.

The preliminary experiments to define the glucose and skim milk concentration range for the production of trypsin-like protease from *L. plantarum* FNCC 0270 was performed using medium with the following compositions; 2% w/v baker's yeast, 0.5% and 1% w/v glucose, and 0.25, 0.5, 0.75, and 1.0% w/v skim milk. Agitation speed used was 87.5 rpm. Observation of trypsin-like activities and protein levels expressed at various concentrations was performed every 6 hours for 24 hours. From eight combination experiments obtained by a five combination consisting of medium composition baker's yeast 2% w/v, glucose 1% w/v, skim milk 0, 25% w/v and 87.5 rpm agitation the best enzyme activity was obtained so based on a five combination determined value range of media composition and agitation baker's yeast is 1.5-2.5% (w / v), glucose from 0.75 to 1.25% (w/v), skim milk from 0.15 to 0.35% (w/v) and agitation varies between 50-125 rpm, to put the software Design Expert 7.1.5. (Stat Ease Inc., USA). The determination of the value of the middle point can be seen in Table 1, used in this experimental plan.

Analitycal methods. The number of cells used as starter of fermentation was estimated using spectroscopy at 560 nm (OD$_{560}$). Trypsin – like protease activity and protein content from crude enzyme fraction were analyzed using Khantaphant and Benjakul method [5] and Bradford method [11], respectively. Preliminary studies for analysis of proteins using Bovine Serum Albumin, the protein content 0.293 mg/mL using centrifuge and 0.326 mg/mL without centrifugation, whereas the trypsin-like proteases production of *L plantarum* FNCC 0270, the protein content 0.167 mg/mL at fermented 7 hours.

Experimental design and optimization. The present study used program Design Expert version 7.1.5., with study type: Response Surface, using Central Composite Design (CCD) as the initial design and Quadratic design model, by four numerical factors or independent variable, which were A: baker's yeast (%w/v), B: glucose (%w/v), C: skim milk (%w/v), D: agitation (rpm)

Table 2. The Central Composite Design (CCD) Matrix Employed for Four Numeric Factors

Design	Coded of level factor				Response	
	A	B	C	D	Enzyme activity	Protein content
Factorial	-1	-1	-1	-1	Y_1	Y'_1
	1	-1	-1	-1	Y_2	Y'_2
	-1	1	-1	-1	Y_3	Y'_3
	1	1	-1	-1	Y_4	Y'_4
	-1	-1	1	-1	Y_5	Y'_5
	1	-1	1	-1	Y_6	Y'_6
	-1	1	1	-1	Y_7	Y'_7
	1	1	1	-1	Y_8	Y'_8
	-1	-1	-1	1	Y_9	Y'_9
	1	-1	-1	1	Y_{10}	Y'_{10}
	-1	1	-1	1	Y_{11}	Y'_{11}
	1	1	-1	1	Y_{12}	Y'_{12}
	-1	-1	1	1	Y_{13}	Y'_{13}
	1	-1	1	1	Y_{14}	Y'_{14}
	1	-1	-1	-1	Y_{15}	Y'_{15}
	1	1	1	1	Y_{16}	Y'_{16}
Star po-int	-2	0	0	0	Y_{17}	Y'_{17}
	2	0	0	0	Y_{18}	Y'_{18}
	0	-2	0	0	Y_{19}	Y'_{19}
	0	2	0	0	Y_{20}	Y'_{20}
	0	0	-2	0	Y_{21}	Y'_{21}
	0	0	2	0	Y_{22}	Y'_{22}
	0	0	0	-2	Y_{23}	Y'_{23}
	0	0	0	2	Y_{24}	Y'_{24}
Cen-ter po-int	0	0	0	0	Y_{25}	Y'_{25}
	0	0	0	0	Y_{26}	Y'_{26}
	0	0	0	0	Y_{27}	Y'_{27}
	0	0	0	0	Y_{28}	Y'_{28}
	0	0	0	0	Y_{29}	Y'_{29}
	0	0	0	0	Y_{30}	Y'_{30}

and two responses, which were enzyme activity $(Y_1,$ U/mL) and protein contents $(Y_2,$ mg/mL).

The CCD matrix employed for four numerical factors is presented in Table 2, consisting of: 2^4 (16) factorial points with eight (8) star points and six (6) replications at the center points leading to a total number of 30 experiments. Activity and protein content were determined and assigned as responses 1 and 2, respectively.

Experiments on each condition expressed in factorial and star points were performed in duplicates. A total of 54 runs were performed.

The experimental results were plotted on a second-order polynomial equation and processed with the program Design Expert version 7.1.5. The mathematical model is as expressed in equation (1) .

$$Y = a_0 + a_1A + a_2B_+ a_3C_+ a_4D_+ a_{12}AB + a_{13}AC + a_{14}AD + a_{23}BC + a_{24}BD + a_{34}CD + a_{11}A^2 + a_{22}B^2 + a_{33}C^2 + a_{44}D^2 \quad (1)$$

where, Y : the response that comes from each treatment, in this case is the enzyme activity (Y_1) and protein content (Y_2).

A, B, C, D, : numeric factors
a_0 : intercept
a_1, a_2, a_3, a_4 : coefficients estimate of linear effect of factor
a_{12}, a_{13}, a_{14}, a_{23}, a_{24}, a_{34} : coefficients estimates of interaction terms
a_{11}, a_{22}, a_{33}, a_{44} : coefficients for quadratic terms

Results and Discussion

The results of preliminary studies of trypsin-like proteases production of *L. plantarum* FNCC 0270 baker's yeast as a source of N and replacement of yeast extract gives almost the same specific activity 0.68 mU/mg and 0.75 mU/mg respectively.

Design Expert 7.1.5 provide the three models (Sequential Model Sum of Squares, Lack of Fit Test and Model Summary Statistics) were presented influence of different experimental variables, i.e. the percentage of baker's yeast, glucose, skim milk and agitation towards response of enzyme activities. The Model Summary Statistics towards response of enzyme activities at Table 3.

The Model Summary Statistics indicated that amongst the available models, *i.e.* linier, 2FI, quadratic and cubical, the Qudratic Model was the one most suitable representing the relationship between the experimental variables and enzyme activities.

The Analysis of variance (ANOVA). The Analysis of Variance (ANOVA) results of the Response Surface Quadratic Model of enzyme activityare presented in Tabel 4. The F value of 5.39 demonstrated that noise or error do not play significant role. There is 0.01% chance that model with F value higher that 5.39 demonstrated

error. Less than 0.05 probability (prob>F) value indicated that the model component plays significant role. In this case BD and were the significant components. The F value obtained from the Lack of Fit Test was 2.04, indicating the model was not significant to response. When the model was not significant to response, it means that the model had been selected correctly. A second equation was obtained from Design Expert 7.1.5 (Equation 2), which explains the data response on enzyme activity

$$Y_1 = +2.086E\text{-}004 + 1.21E\text{-}005A - 1.6820E\text{-}005B + 1.172E\text{-}005 C + 1.262E\text{-}005\ D\ -1.732E\text{-}006\ \ AB + 2.636E\text{-}005\ AC - 5.947E\text{-}005\ AD + 3.665E\text{-}005\ BC - 1.929E\text{-}004\ BD - 4.331E\text{-}005\ CD + 1.208E\text{-}004\ A^2 - 1.637E\text{-}005\ B^2 - 2.173E\text{-}005\ C^2 + 2.743E\text{-}005D^2 \quad (2)$$

Model accuracy was predicted by comparing the actual experimental results and model prediction using paired Student's t test using online GraphPad software (http://www.graphpad.com/quickcalcs/ttest2.cfm). The two – tailed p value of the t-test was 1, indicating that at 95% confidence any difference between the predicted and actual enzyme acivities occured by chance (not repoted).

From Design Expert version 7.1.5, there are six pictures of three-dimensional interaction between media composition baker's yeast, glucose, skim milk and agitation on the response of the enzyme activity, only four pictures are presented in Figure 1. Figures 1 (a, b, c, d) show that the enzyme activity increases with increasing concentrations of baker's yeast as a source of N, the concentration of glucose as a source of C and a reduction in the concentration of skim milk as an inducer while agitation speed do not play a significant role in the increase enzyme activity.

The Analysis of Variance (ANOVA) results for the response protein content are presented in Tabel 5. The F value of 2.03 demonstrated that noise or error do not significantly influence protein level. There is only 4.15% chance that models with F value higher than 2.03 could have occured by error. Less than 0.05 probability (prob>F) value, indicates that the model component plays significant role. In this case C and D^2 were the significant components.

Table 3. Model Summary Statistics towards Response of Enzyme Activities

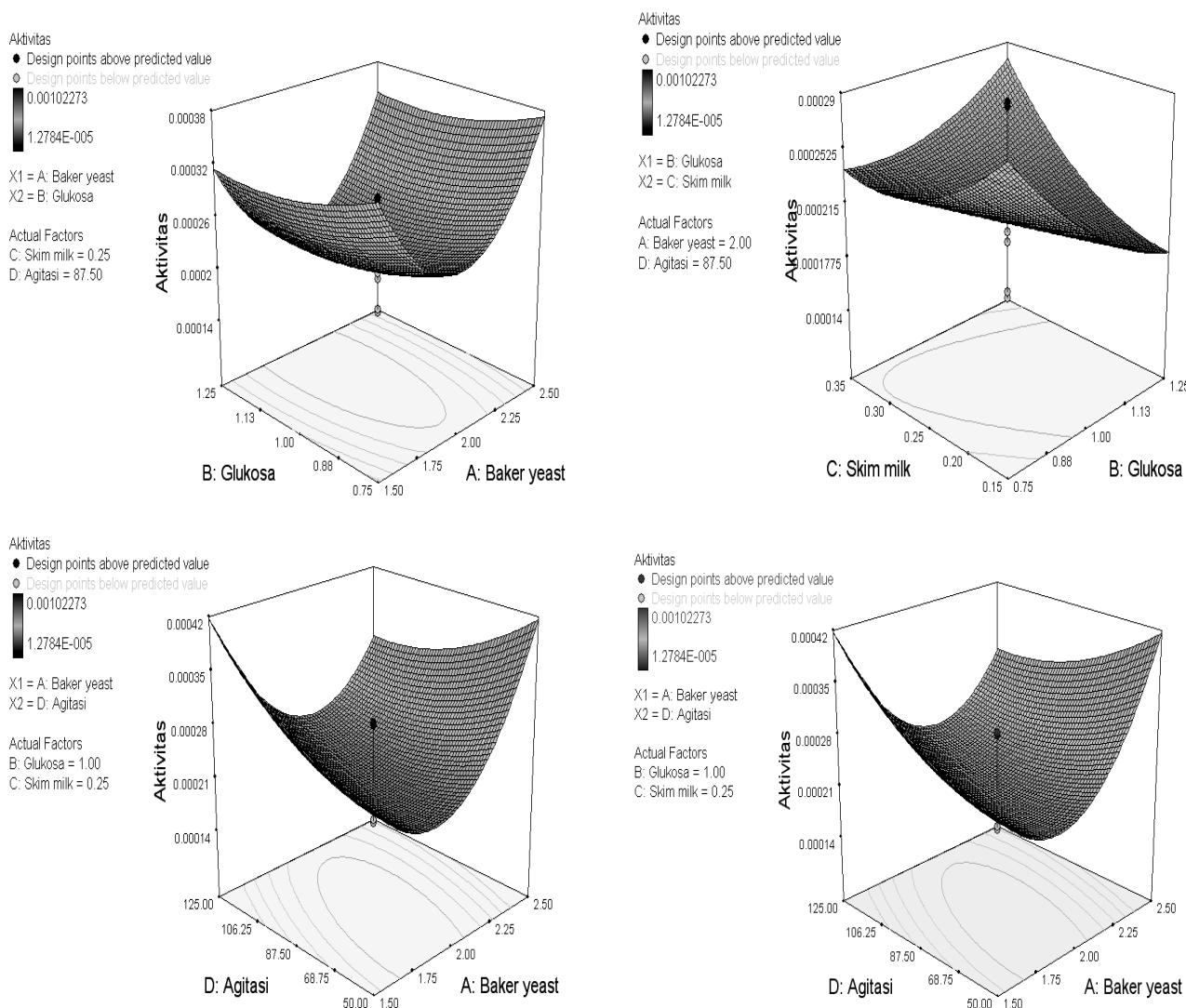| Source	Std. Dev	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS	
Linear	2.550E-004	0.0108	-0.0699	-0.2148	3.913E-006	
2FI	2.021E-004	0.4546	0.3277	0.1340	2.790E-006	
Quadratic	1.678E-004	0.6592	0.5369	0.3238	2.178E-006	Suggested
Cubic	1.537E-004	0.7726	0.6112	0.2359	2.461E-006	Aliased

Figure 1. Interaction Variables baker's Yeast, Glucose, Skim Milk and Agitation on the Response of the Activity of Trypsin-like Proteases

The F value obtained from the Lack of Fit Test was 1.86, indicating the model was not significant to response. When the model was not significant to response, it means that the model had been selected correctly. A second equation was obtained from Design Expert 7.1.5 (Equation 3), which explains the data response on protein level:

$$Y = 0.44 - 0.023A - 4.278E\text{-}003B + 0.045C - 7.445E\text{-}003D - 5.129E\text{-}003\ AB - 0.016\ AC + 0.015AD - 6.506E\text{-}003AC - 7.494E\text{-}003BC + 0.022CD - 0.013A^2 - 0.011B^2 + 0.011C^2 - 0.033D^2 \quad (3)$$

Figure 2 demonstrates the distribution of actual and predicted protein contents. The predicted values were expressed as straight line and the actual values were expressed in squares. Model accuracy was predicted by comparing the actual experimental results and model prediction using paired Student's t test using online GraphPad software (http://www.graphpad.com/quick calcs/ttest2.cfm). The two – tailed p value of the t-test was 1, indicating that at 95% confidence, any difference between the predicted and actual protein contents was not significant and occured only by chance.

Figure 2 shows that the actual and predicted values are spread away from the straight line, indicating the low standard deviation (0.091) as calculated based on ANO-VA. The low standard deviation indicated that the model has good accuracy and the model is not suitable. From Design Expert version 7.1.5, there are six pictures of three-dimensional interaction between media composition baker's yeast, glucose, skim milk and agitation on the response of the protein content, only four pictures are presented in Figure 3.

Table 4. Analysis of Variance for Enzyme Activity Respons

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	2.1E-006	14	1.5E-007	5.39	<0.0001	significant
A-Baker's yeast	7.1E-009	1	7.0E-009	0.25	0.6198	
B-Glukosa	1.4E-008	1	1.4E-008	0.48	0.4914	
C-Skim milk	6.6E-009	1	6.6E-009	0.23	0.6312	
D-Agitasi	7.6E-009	1	7.7E-009	0.27	0.6051	
AB	9.6E-010	1	9.6E-010	0.003	0.9537	
AC	2.2E-012	1	2.2E-012	0.79	0.3796	
AD	1.1E-007	1	1.1E-007	4.02	0.0519	
BC	4.3E-008	1	4.3E-008	1.53	0.2239	
BD	1.2E-006	1	1.2E-006	42.31	<0.0001	
CD	6.0E-008	1	6.0E-008	2.13	0.1522	
A^2	6.2E-007	1	6.2E-007	22.12	<0.0001	
B^2	1.1E-008	1	1.1E-011	0.41	0.5277	
C^2	2.0E-008	1	2.0E-010	0.072	0.4027	
D^2	3.2E-008	1	3.2E-008	1.14	0.2922	
Residual	1.1E-006	39	2.8E-008			
Lack of Fit	4.5E-007	10	4.5E-007	2.04	0.0662	Not significant
Pure Error	6.5E-007	29	2.2E-007			
Cor Total	3.2E-006	53				

Std. Dev. = 1.678 E-004   R Squared = 0.6592
Mean = 3.742E-004   Adj R-Squared = 0.5369
C.V. % = 44.84   Pred R-Squared = 0.3238
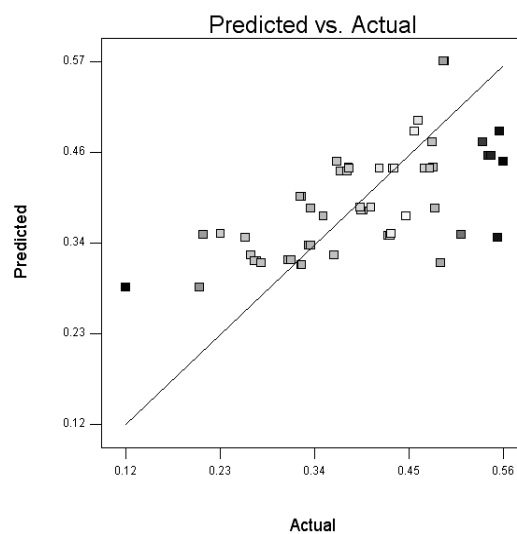PRESS = 2.176E-006   Adeq Precision = 7.240

Figure 2. Distribution of Actual and Predicted Protein Contents

Figure 3 indicated that protein contents will decrease with the increase of baker's yeast and skim milk concentrations as well as the increase of agitation speed, while glucose cocentration did not significantly influence the decrease of protein level

Numerical optimation. Expert version 7.5.1 demonstrated numerical optimation program. The experimental data indicated that the best solution was obtained when using baker's yeast = 2.5 %, glucose = 1.25% or skim milk = 0.35 % and 50 rpm agitation speed. The ratio of C/N 0.064 in the fermentation substrate, baker yeast and glucose as a source of N and C respectively, while skim milk as an inducer. At this condition the followings were obtained; enzyme activity 0.76 mU/mL, protein level 0.328 mg/mL and desirability value = 0.74. To get the optimum enzyme activity and minimum protein level, ideally the

desirability value should be close to 1. Therefore, varying the variables within the experimental range and adjusting the activity at maximum and protein level at in range that the numerical solution closest to ideal could be achieved.

The ranges selected for the concentrations of baker's yeast, glucose and skim milk, as well as agitation speed were 2-4%, 0.05-0.15%, 0.75-1.25 %, and 50-100 rpm, respectively and goal maximize for enzyme activity. Amongst the solutions offered by Design Expert version 7.1.5, the eight solution was selected. The following condition was used: baker's yeast concentration = 3.64 %, glucose concentration = 1.21 %, skim milk concentration= 0.13% and agitation speed 76.79 rpm. At this condition 1.51 mU/mL enzyme activity and 0.20474 mg/mL protein level were obtained.
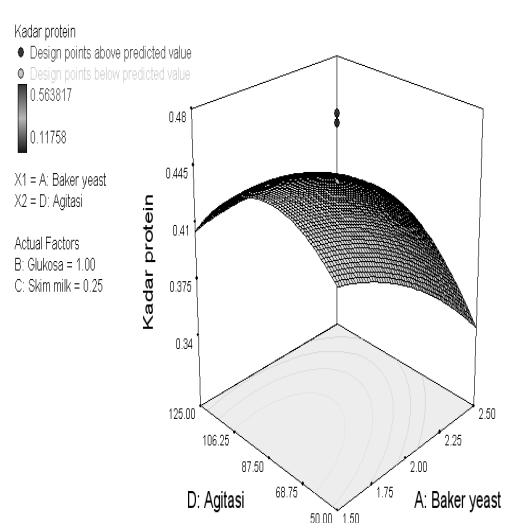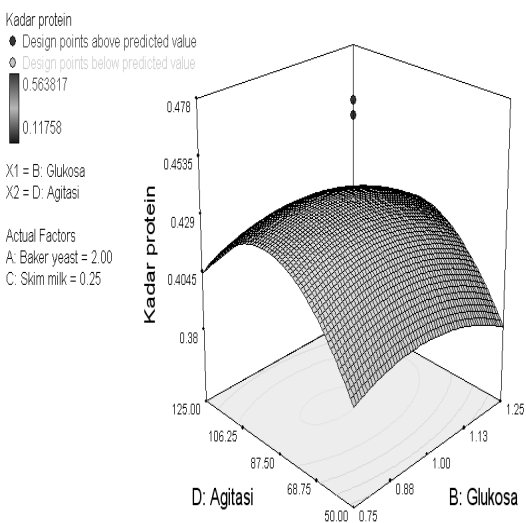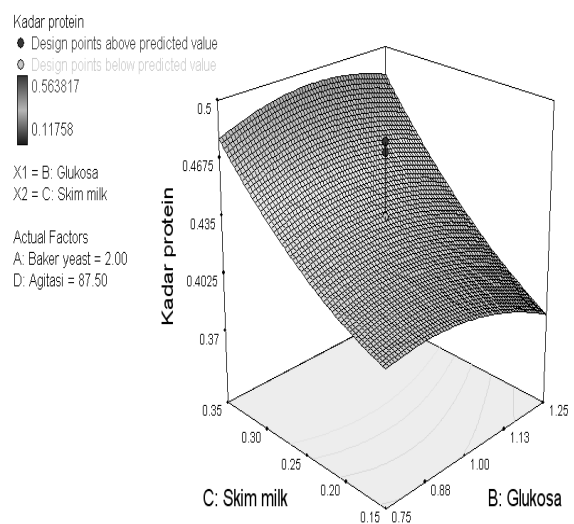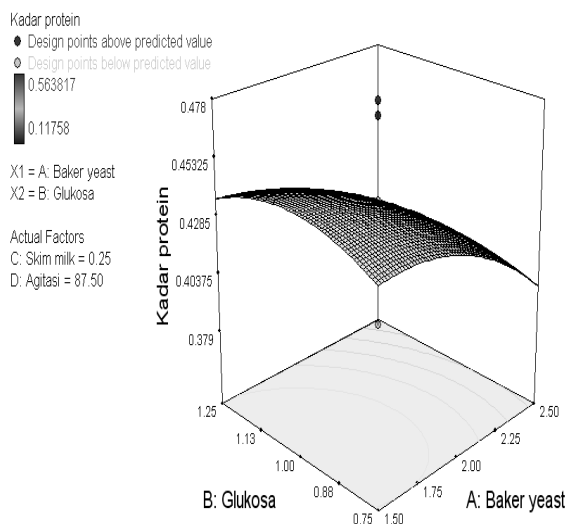

Figure 3. Interaction Variables Baker's Yeast, Glucose, Skim Milk and Agitation on the Response of the Protein Contents of Trypsin-like Proteases

Table 5. Analysis of Variance for Protein Content Respons

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	Source
Model	0.23	14	0.017	2.03	0.0415	significant
A-Baker's yeast	0.026	1	0.026	3.09	0.0864	
B-Glucosa	8.8E-004	1	8.8E-004	0.11	0.7458	
C-Skim milk	0.099	1	0.099	12.01	0.0013	
D-Agitation	2.7E-003	1	2.7E-003	0.32	0.5731	
AB	8.4E-004	1	8.4E-004	0.10	0.7510	
AC	8.1E-003	1	8.1E-003	0.99	0.3209	
AD	7.4E-003	1	7.4E-003	0.89	0.2048	
BC	1.4E-003	1	1.4E-003	0.16	0.6874	
BD	1.8E-005	1	1.8E-005	0.0022	0.9630	
CD	0.015	1	0.015	1.86	0.1799	
A^2	6.7E-003	1	6.7E-003	0.81	0.3735	
B^2	5.2E-003	1	5.2E-003	0.63	0.4316	
C^2	4.8E-003	1	4.8E-003	0.58	0.4496	
D^2	0.047	1	0.047	5.69	0.0220	
Residual	0.32	39	8.2E-003			
Lack of Fit	0.13	10	0.013	1.86	0.0942	Not significant
Pure Error	0.20	29	6.8E-003			
Cor Total	0.54	53				

Std. Dev. = 0.091   R Squared = 0.4211
Mean = 0.40   Adj R-Squared = 0.2133
C.V. % = 22.90   Pred R-Squared = -0.1475
PRESS = 0.64   Adeq Precision = 5.868

To verify the selected condition, fermentation was performed in 125 mL erlenmeyer flask using incubator shaker and medium composition and growth conditions as follows; baker's yeast, glucose and skim milk concentrations 3.64%, 1.21%, and 0.13%, respectively, agitation speed 77 rpm, initial pH 8. The results of the verification of enzyme activity and protein content 0.001273 ± 0.000227 U / mL and 0.248167 ± 0.011805 mg /mL, respectively. The ratio of C/N 0.043 in the fermentation substrate, baker yeast and glucose as a source of N and C respectively, while skim milk as an inducer. This value of C/N ratio then be used for larger-scale fermentation substrates in future studies.

In particular the response surface method (RSM) is a sequential procedure with an initial objective of leading the experimenter rapidly and efficiently to the general vicinity of the optimum. RSM has already been successfully employed for the optimization of microalgae cultures [6]. Central composite designs which maximize both the precision and the accuracy of estimates of the external point of a second-order response surface for fixed values of the model parameters are constructed [12].

The results of this study indicated that numerical factors or variables; baker yeast, glucose and skim milk and agitation may not be the only factors affecting the enzyme activity and protein levels. This was indicated

by the R squared value of only 65.92% and 42.11% respectively. Another study conducted by Qi-he *et al*. (2007) [13] using RSM with 6 variables (T, t, the volume of media, inoculation volume, seed age, shaking speed) on the enhanced production of elastase *B.licheniformis* ZJUEL31410 also obtained R squared values below 50%, and so was study by Fucinos *et al*. (2011) [14] using RSM with 2 variables (pH and T) on the model of stability obtained KLEST-3S, which showed R squared values 0.797.

To explore whether the optimized model from RSM could be applied to a large scale synthesis, the reaction volume should be increased by a factor of 10. This had been done by Liu *et al*. (2007) [15], who demonstrated that there was no significant difference between large and small scale syntheses.

Conclusions

The experiments indicated that medium containing 3.64% baker's yeast, 1.21% glucose, 0.13% skim milk and 76.79 rpm agitation speed was the best for this purpose, the enzyme activity was 1.273 ± 0.227 mU/mL and protein levels 0.248 ± 0.012 mg/mL.

The best media composition with C/N ratio 0.043, skim milk as an inducer and 77 rpm agitation might be applied to scaled-up fermentation to produce trypsin – like

protease and purification as well as characterization of trypsin-like protease from *L plantarum* FNCC0270 in future studies.

Acknowledgments

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