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OPTIMIZATION OF PRODUCTION OF PROTEASE BY *LACTOBACILLUS PLANTARUM SK (5)* FROM BEKASAM WITH RESPONSE SURFACE METHODOLOGY

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ABSTRACT

Lactobacillus plantarum SK (5) is one of lactic acid bacteria isolated from Indonesia fermented fish know as bekasam. The isolate was chosen to optimize its protease production by using response surface methodology. The aim of this study was to select the composition of medium having the highest specific protease activity produced from *L. plantarum* SK (5). The variables involved in this study were glucose, peptone, yeast extract, volume of inoculant, and pH. These variables would be optimized by central composite design (CCD) matrix of response surface methodology. The optimal cultural condition for protease production obtained with response surface methodology were glucose 6%, peptone 6%, yeast extract 7.5%, volume of inoculant 3 mL and pH 6.0. The specific protease activity under unoptimized conditions was 3.615 U/mg protein. Under the final optimized conditions, the predicted response from protease production was 6.393 U/mg protein and the observed validated experiment value was 6.503U/mg protein. The optimization of the production of protease with response surface methodology resulted in about two folds increase in the production of protease by *L. plantarum* SK (5).

Key words: *Lactobacillus plantarum*, proteases, optimization, bekasam.

INTRODUCTION

Proteases are the enzymes that hydrolyze the peptide linkages of proteins to oligopeptides and amino acid. Protease enzyme have applications in physiological and commercial fields (Rathakrishnan *et al*., 2013). Microbial proteases commercially used almost 60% compared with the enzyme from other types of the industrial enzyme's sales in the world. The world market is estimated to reach 7.6% per year for the enzyme (David *et al*., 2009). Applications protease in some industrial sectors such as in detergent, food, pharmaceuticals, chemicals, leather, paper and pulp (Gupta *et al*., 2002). Proteases sources widely spread as well as plants, animals, fungi and bacteria (Oyeleke *et al*., 2009). Microbial proteases have an advantage due to their productivity and thermostability, cheaper production cost and use of renewable resources (Burhan *et al*., 2003).

Recent research has focused on optimization the proteases production. Optimization efforts should be made to maximize the production of protease enzymes (El Enshasy *et al*., 2008). Optimization typically apply one-factor-at - a method where this method requires considerable time. There are some parameters that are optimized but in this method other parameters must remain constant while studying parameters at a time. In addition, effect of interaction of various parameters are also not considered. Therefore, the response surface methodology is being recommended for optimization purposes (Bas *et al*., 2007). Response surface methodology is a useful tool to study the effect of varying the response to them simul -taneously and can also be used to study the relationship between one more factor (independent variable) and the response (dependent variable) (Adinarayana dan Ellaiah, 2002). Thus optimization will be much easier to carry out.

MATERIALS AND METHODS

Microorganism strain and growth conditions:

Ten isolates of lactic acid bacteria (LAB) were previously isolated from bekasam (Desniar, 2013). Each culture has grown on deMan Rogosa Sharpe agar (MRSA) at 37°C for 48 h incubation.

Screening of protease producing strain: Primary screening of the isolates based on protease production was done by skim milk agar plate method (Dajanta *et al*., 2009). In addition, the selected isolate also carried out based on the highest of the activity of crude protease enzyme. The strain with significant skim milk hydrolytic zone on skim milk agar

plate and showed enzyme activity was selected for further optimization.

The growth curve and protease production of *L. plantarum* SK (5): Inoculation of two loopful of selected isolate into 50 mL deMan Rogosa Sharpe (MRS) supplemented with 1% skim milk and incubated at 37°C for 12 h. In amount of 1% culture (8.8 x 10^8 CFU/mL) was inoculated into 100 mL MRS with skim milk which used enzyme production medium. The culture was incubated at 37°C at 130 rpm (Pranomo *et al*., 2003) and collected every 3 hours until 24 h and each optical density of the culture measured at 600 nm. The supernatant consisted of crude protease was centrifuged at 10000 rpm for 5 min in Eppendorf MiniSpin with rotor F-45-12-11.

Protease assay: Protease activity was assayed by modified casein method of Walter (1984). The assay mixture consisted of 250 µL casein solution, 250µL 0.5M buffer tris-HCl pH 7.0, and 50µL crude protease then incubated at 37°C for 10min. The reaction was stopped with trichloroacetic acid 5 mM and then reincubated on 37°C for 10 min. Precipitates separated by centrifugation at 10000 rpm for 10 min in Eppendorf MiniSpin with rotor F-45-12-11. Supernatant was treated with 0.5M Na_2CO_3 and Folin's reagent to determine the tyrosine released by protease. The absorbance of the supernatant was measured at 57nm wavelength with spectrophotometer. One protease unit is the amount of enzyme required to release 1 µmol of tyrosine per minute under the conditions of assay. The activity of enzyme protease (U) was calculated according to the formula: U = ΔC/(V x t) (Units/mL), where ΔC = mg peptide produced by crude protease and V is volume (mL).

Total proteins measurement: Proteins were measured according to Bradford's method (Bradford, 1976) using bovine serum albumin as standard. The specific activity of enzyme protease (U) was calculated according to the formula: U = activity of enzyme protease (U) per total proteins (Units/mg protein).

Optimization of protease production: Optimization of protease production was done using response surface methodology. Response surface methodology was modeling techniques for multiple regression analysis to solve multivariable equations simultaneously using quantitative data obtained from a factorial design (Rao *et al*., 2000). The media components affecting the enzyme production were optimized by central composite design (CCD).

Table 1. Ranges of the independent variables used in response surface methodology

Variables	Level code variables				
	-Two	-One	0	+One	+Two
Glucosa (%)	4.0	5.0	6.0	7.0	8.0
Peptone (%)	4.0	5.0	6.0	7.0	8.0
Yeast extract (%)	5.5	6.5	7.5	8.5	9.5
Volume of inoculant (mL)	1.0	2.0	3.0	4.0	5.0
pH	4.0	5.0	6.0	7.0	8.0

The CCD is a statistical experimental design where each numeric factor was varied over 5 levels –alpha points (-2, +2), 1 factor (-1, +1) and one center point resulting in a total of 32 experiments. The design is shown in Table 1. The behavior of the system is explained by the following quadratic equation :

$$Y = \beta_o + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j \quad \text{(Eq 1)}$$

Where Y is the predicted response (specific enzyme activity), β_o is the intercept term, β_i, β_{ii}, β_{ij} are linear coefficient, X_i, X_i^2, $X_i X_j$ are coded independent variables. The regression equation was optimized for maximum value to obtain the optimum conditions using *Design-Expert*[R] 8.0' (Stat-Ease, Inc., Minneapolis USA).

The statistical model was validated for protease production under the conditions predicted by the model in shake flask conditions.

RESULTS AND DISCUSSION

Screening of protease producing strain: The selected isolate was a *L. plantarum* SK (5) exhibiting largest zone of hydrolysis of skim milk (Fig. 1).

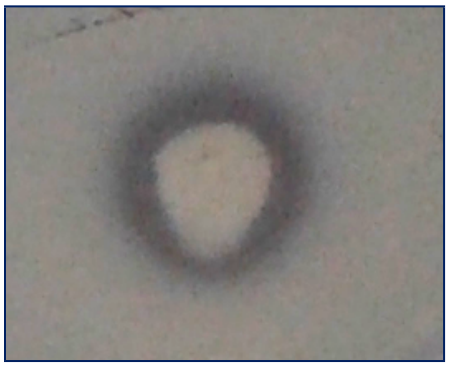

Fig.-1: Zone of hydrolysis of skim milk from *Lactobacillus plantarum on skim milk* agar media.

The clear zone around bacterial colonies showed the ability of proteolytic *L. plantarum* SK (5) to hydrolyze the skim milk. The protease activity of *L. plantarum* SK (5) under unoptimized conditions of crude enzyme for *L. plantarum* SK (5) was 0.443 U/mL protein and specific enzyme activity was 3.6151 U/mg protein (Table 2).

L. plantarum SK (5) is a Gram positive LAB. Some species of LAB are used for the production of yogurt and various types cheses, therefore, the study of the proteolytic system getting a lot of attention in recent years. The proteolytic system consists of cell envelope-associated proteinase (Prt P), specific peptide and amino acid transport systems and several cytoplasmic peptidases (Piuri *et al*., 2005).

The ability of LAB to produce extracellular protease is very important. This is because most of the LAB were isolated from fermented milk product that has some amino acids so as to be able to grow in a medium rich in protein expression dependent on the proteolytic system to degrade casein which is the main protein in milk (Kok, 1993; Visser, 1993). BAL has the ability to degrade casein by the proteolytic system for producing peptides and amino acids needed for growth (Savikoji *et al*., 2006).

Table 2. Enzyme activity and specific enzyme activity of lactid acid bacteria isolates

Isolates	Enzyme Activity (U/mL)	Specific Enzyme Activity (U/mg protein)
SK (5)	0.443	3.615
SK (15)	0.042	0.282
BP (5)	0.193	1.444
BP (8)	0.031	0.195
BP (20)	0.128	0.951
BI (2)	0.024	0.140
NS (9)	0.269	1.627
NS (6)	0.267	2.508

The growth curve and protease production of *L. plantarum* SK (5): The growth of *L. plantarum* SK (5) begins with the log phase is the phase of the synthesis of enzymes used by cells for metabolism metabolites. Pommerville (2011) states the log phase occurs when all of the cells in culture undergo binary fission. Every generation is passed, the number of cells increased two-fold and increased graphics in the form of a straight line or a logarithmic graph. Protease enzyme activity increased was found at the 12h incubation (Fig. 2). Proteolytic activity of the lactic acid bacteria is done at 37°C for 12 h incubation (Elfahri, 2012). According to Putranto (2006), *L. plantarum* express extracellular proteases with the highest activity on the 18 h incubation of 0.752 U/mL during the growth of bacteria (log). According to research of Desniar (2013), the rate of growth of *L. plantarum* bacteria reach a maximum point at the 16 h incubation. This may be due to the difference in treatment is done at the time of incubation. According to Wenge and Methews (1999), the growth and use of metabolism in the process of fermentation and metabolic pathways of lactic acid bacteria is influenced by various parameters such as temperature, pH, agitation speed and the level of dissolved oxygen.

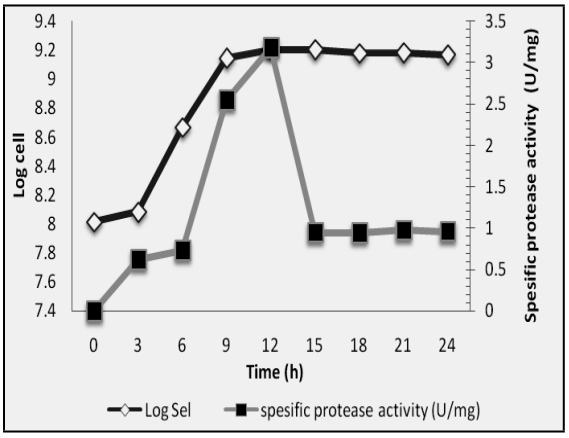

Fig. -2: Growth and protease production of L. *plantarum* SK (5) isolate on production medium supplemented skim milk.

Protease production decline occurred after the fermentation at the 12 h incubation when the stationary phase. Cohen (2011) stated that if the condition of essential nutrients lost during growth, the medium became too acidic or too alkaline then the growth rate will decrease and approach zero. So that the accumulation of toxic substances will be able to inhibit cell division.

Optimization of protease production

Determination of optimum model: The ANOVA analysis of the optimization study had F-value was 48.37. It showed that model was significant. Values of Prob > F less than 0.0500 indicate model terms are significant. The Lack of Fit F-value of 2.49 implied the Lack of Fit was not significant relative to the pure error (Table 3). There was a 15.94% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant of lack of fit was good.

The regression equation coefficient was calculated and the data was fitted to a second order polynomial equation. Thus the response (Y), protease production by the selected *Lactobacillus plantarum* SK (5) can be expressed in terms of the following regression equation.

$$Y = -130.1888 + 11.7A + 9.433B + 3.896C + 5.976D + 15.573E - 0.976A^2 - 0.7683B^2 - 0.251C^2 - 0.98D^2 - 1.25E^2 \quad \text{(Eq 2)}$$

A is concentration of glucose, B is concentration of peptone, C is concentration of yeast extract, D is volume of inoculant and E is pH.

Table-3: Analysis of variance (ANOVA) for response surface quadratic model

Source	Sum of Squares	df	Mean Square	F Value	P-Value Prob>F
Model	105.85	1.0	10.59	48.37	<0.0001
Residual	004.60	21	0.22		
Lack of Fit	004.08	16	0.26	2.49	0.1594
Pure Eror	000.51	5.0	0.10		
Cor Total	110.45	31			

R-Squared = 0.9584; Adeq Precision = 22.367; Pred R-Squared = 0.8556; C.V.= 16.71%

The Pred R-Squared of 0.8556 is in reasonable agreement with the Adj R-Squared of 0.9386. Adeq Precision measures the signal to noise ratio. A ratio greater than four is desirable. The ratio of 22.367 indicates an adequate signal. This model can be used to navigate the design space.

The optimal cultural condition for protease production obtained with response surface methodology were glucose 6%, peptone 6%, yeast extract 7.5%, volume of inoculant 3 mL, and pH 6.0. Under the final optimed conditions, the predicted response from protease production was 6.393 U/mg (Table 4). A source of nitrogen which a bit will not make of bacteria take nitrogen as a source of nutrients that will secrete proteases to degrade the source of other nutrients such as peptone as alternative nutrients (Puri et al., 2002).

All microorganisms need carbons sources in order to live as it is the food for them. The carbon source are one of nutritional factor that influenced the protease production (Adinarayana and Ellaiah, 2003). In addition, glucose is the substrate limiting the aerobic and anaerobic conditions (Schuler, *et al*., 1992). Giving concentration of glucose, peptone and yeast extract higher or lower as well as changes in pH and

volume of inoculum will affect the increase in protease activity. Padmavathi (2013) reported that carbon and nitrogen showed an effect to the protease activity. Glucose and yeast extract provide protease activity of 80 U/mL higher than most other sources of carbon and nitrogen. Yeast extract is a key nutrient that controls the biosynthesis enzyme and is required in small amounts so as to spur the production of the enzyme protease. Peptone is a source of nitrogen which provide a high influence on the growth of *L. acidophilus* (Elfahri, 2012). While the pH of the number concentrations of (H^+) and H^+ will give effect to the growth of microorganisms. The increase in pH will cause a decrease in protease activity.

Table-4: Central composite design for optimization and its response values

Run	A	B	C	D	E	Specific activity (U/mg)	Specific Activity Prediction (U/mg)
1	5	5	6.5	2	7	2.694	2.319
2	7	5	6.5	2	5	1.382	1.154
3	5	7	6.5	2	5	1.341	1.615
4	7	7	6.5	2	7	2.793	2.714
5	5	5	8.5	2	5	1.363	1.430
6	7	5	8.5	2	7	2.695	2.529
7	5	7	8.5	2	7	2.583	2.990
8	7	7	8.5	2	5	1.404	1.826
9	5	5	6.5	4	5	1.305	1.373
10	7	5	6.5	4	7	2.552	2.472
11	5	7	6.5	4	7	2.307	2.932
12	7	7	6.5	4	5	1.275	1.768
13	5	5	8.5	4	7	2.926	2.748
14	7	5	8.5	4	5	1.452	1.583
15	5	7	8.5	4	5	1.742	2.044
16	7	7	8.5	4	7	4.061	3.143
17	4	6	7.5	3	6	3.030	2.520
18	8	6	7.5	3	8	2.164	1.415
19	6	4	7.5	3	6	2.429	2.891
20	6	8	7.5	3	6	4.408	3.747
21	6	6	5.5	3	6	5.410	5.143
22	6	6	9.5	3	6	5.582	5.629
23	6	6	7.5	1	6	2.366	2.285
24	6	6	7.5	5	6	2.794	2.655
25	6	6	7.5	3	4	0.940	0.258
26	6	6	7.5	3	8	2.060	2.522
27	6	6	7.5	3	6	6.530	6.393
28	6	6	7.5	3	6	6.028	6.393
29	6	6	7.5	3	6	6.228	6.393
30	6	6	7.5	3	6	6.564	6.393
31	6	6	7.5	3	6	6.806	6.393
32	6	6	7.5	3	6	6.019	6.393

A: concentration of glucose. B: concentration of peptone. C: concentration of yeast extract. D: volume of inoculant and E refers to pH.

Response surface and optimum point: The optimum point on the response form a three-dimensional surface plots are located on the highest point (redness). It was clear that the provision of the concentration of glucose, peptone and yeast extract higher or lower as well as changes in pH and volume of inoculum will affect the increase in protease activity which can be seen from the curvature of the curve. Interactions between nitrogen sources with carbon sources influence on the activity of the protease enzyme. Interaction between peptone and glucose appears that the activity was moving toward optimum when the concentrations of peptone and glucose were reach 6% for each. Increasing and decreasing concentrations of peptone and glucose, the optimum activity also decreases (Fig. 3).

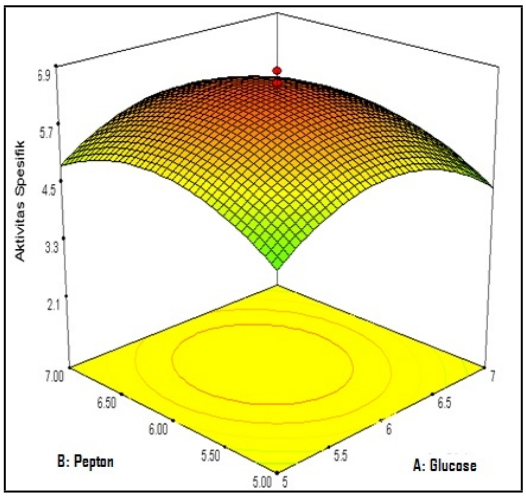

Fig.-3: Response surface plots of 3D that describe the influence of the independent variables (peptone and glucose) with response(specific enzyme activity (Umg/).

Interaction between pH and glucose also gives an influence on the activity of the protease enzyme. At the concentration of 6% glucose was achieved the optimum pH optimum. When the higher glucose concentration, pH value which results in optimum activity moving towards the pH between 6-6.5 (Fig. 4).

Optimum activity occurs at 3 mL inoculum and concentrations of peptone 6% (Fig. 5). While the interaction between pH and the concentration of peptone, optimum activity occurs at pH 6.0 and peptone concentration 6% (Fig. 6).

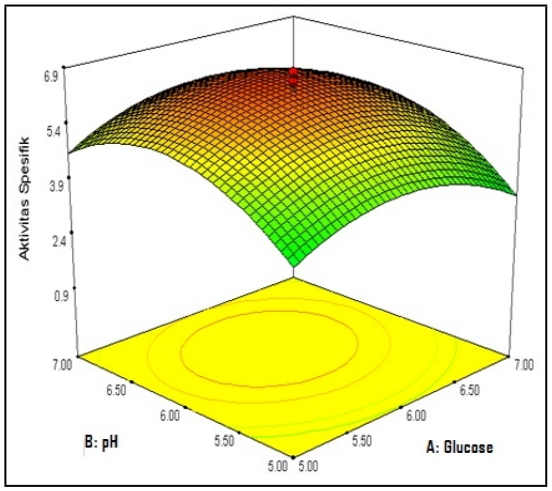

Fig.-4: Response surface plots of 3D that describe the influence of the independent variables (pH and glucose) with response (specific enzyme activity (U/mg).

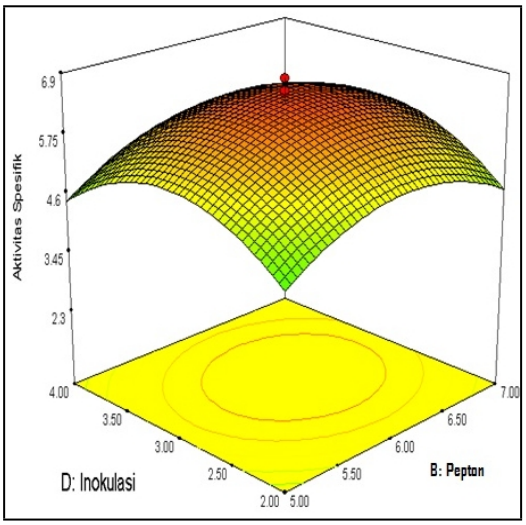

Fig.-5: Response surface plots of 3D that describe the influence of the independent variables (inoculation and pepton) with response (specific enzyme activity (U/mg).

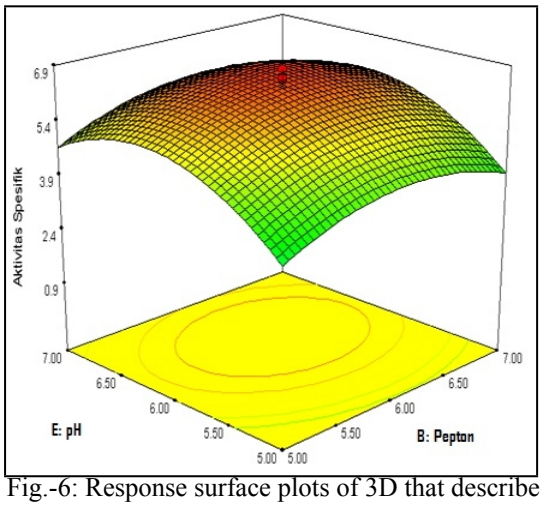

Fig.-6: Response surface plots of 3D that describe the influence of the independent variables (pH and pepton) with response (specific enzyme activity (U/mg).

Conclusions

Optimization of protease production by *Lactobacillus plantarum* SK (5) from bekasam was successful. The optimization of the production of protease with response surface methodology resulted in about two fold increase in the production of enzyme by *L. plantarum.* The optimal cultural condition for protease production obtained with response surface methodology were glucose 6%, peptone 6%, yeast extract 7.5%, volume of inoculant 3 mL and pH 6.0.

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