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Lipase Activity Enhancement of KC4J Mutant from Oil Palm Waste Using Response Surface Method and Partial Characterization

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Lipase has an important role in industry. The KC4J mutated isolates from oil palm waste had 100% similarity to *Aspergillus fumigatus* strain RA204, a fungus known produce lipase. The study aimed to increase lipase activity of KC4J mutant through media optimization using Response Surface Methodology (RSM) and partial characterization. The three variables of media composition (olive oil, soy flour, and pH were optimized using Central Composite Design (CCD). The lipase characterization measured the influence of pH, temperature and metal ions. The pH tested on range 6 to 12, while the temperature variation tested on 30 to 70 °C. The metal ions tested were Mg^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Fe^{2+} and K^{+} with concentrations of 1 mM and 10 mM. The production medium containing 1.25% of olive oil, 3.5% of soy flour and 7.5 pH resulting 11.25 U/mL of Lipase activity, which was higher than the previous media composition (10.00 U/mL). The results of CCD and quadratic analysis showed that the source of carbon, nitrogen and pH had an effect on lipase activity which showed R^2 0.93. The optimum lipase activity produced at pH 6 and on 60 °C, and the lipase stable at pH 6-8 and on 30-70 °C. All metal ions tested were able to increase lipase activity with Ca^{2+} ion gave the highest result.

Key words: Central Composite Design, KC4J mutant, lipase, oil palm waste

Lipase memiliki peran penting dalam industri. Mutan KC4J hasil mutasi isolat dari limbah kelapa sawit memiliki similaritas 100% dengan spesies *Aspergillus fumigatus* strain RA204 merupakan kapang yang dapat menghasilkan lipase. Produksi lipase dipengaruhi oleh beberapa faktor, seperti jenis mikroorganisme, sumber nutrisi dan faktor lingkungan. Penelitian bertujuan meningkatkan aktivitas lipase mutan KC4J melalui optimasi media dengan menggunakan Metodologi Permukaan Respon (RSM) dan karakterisasi parsial. Tiga variabel komposisi media yang digunakan adalah minyak zaitun, tepung kedelai dan pH. Ketiga variabel tersebut dioptimalkan dengan *Central Composite Design* (CCD), karakterisasi lipase yang dilakukan adalah pengaruh pH, suhu dan ion logam. Kisaran pH yang diuji yaitu pH 6-12, sedangkan variasi suhu 30-70°C. Ion logam yang diuji adalah Mg^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Fe^{2+} dan K^{+} dengan konsentrasi 1 mM dan 10 mM. Hasil penelitian menunjukkan bahwa media produksi yang mengandung 1,25% minyak zaitun, 3,5% tepung kedelai dan pH 7,5 mampu menghasilkan aktivitas lipase 11,25 U/mL, yang lebih tinggi dari komposisi media sebelumnya (10,00 U/mL). Hasil *Central Composite Design* dan analisis kuadrat menunjukkan bahwa sumber karbon, nitrogen dan pH berpengaruh terhadap aktivitas lipase yang menunjukkan R^2 0,93. Aktivitas lipase optimum pada pH 6 dan suhu 60°C, stabil pada pH 6-8 dan suhu 30-70 °C. Semua ion logam yang diuji dapat meningkatkan aktivitas relatif lipase. Ion Ca^{2+} dapat meningkatkan aktivitas relatif tertinggi dibandingkan ion lainnya.

Kata kunci: *Central Composite Design,* limbah kelapa sawit, lipase, mutan KC4J

Lipase (Triacylglycerol acylhydrolase, E.C. 3.1.1.3) is an enzyme that has great potential for commercial applications due to its broad selectivity and substrate specificity. Lipases can catalyze various reactions such as the hydrolysis of fats and oils to glycerol and fatty acids. Lipases can also catalyze esterification and transesterification reactions, so they can be used as catalyst for biodiesel production. (Jaeger and Eggert 2002; Ghaly *et al.* 2010, Sharma *et al.* 2011, Prasad *et al.* 2012, Andualema and Gessesse 2012). Conventional biodiesel production using acid or alkaline catalysts still requires several processes for final product purification and wastewater treatment, so alternative catalytic agents are needed to overcome these problems (Ghaly *et al.* 2010). Lipase is an alternative biocatalyst agent that can be used for biodiesel production. In addition, the lipase is more environmentally friendly (Kotogan *et al.* 2014). Some fungi can produce lipase, namely *Aspergillus niger*, *Aspergillus fumigatus*, *Candida rugosa*, *Colletotrichum gloeosporioides*, *Mortierellaechinospaera*, *Penicillium restricum*, *Rhizopus miehei* and *Rhizopus stolonifer* (Prabakaran *et al.* 2009; Kotogan *et al.* 2014; El-Batal *et*

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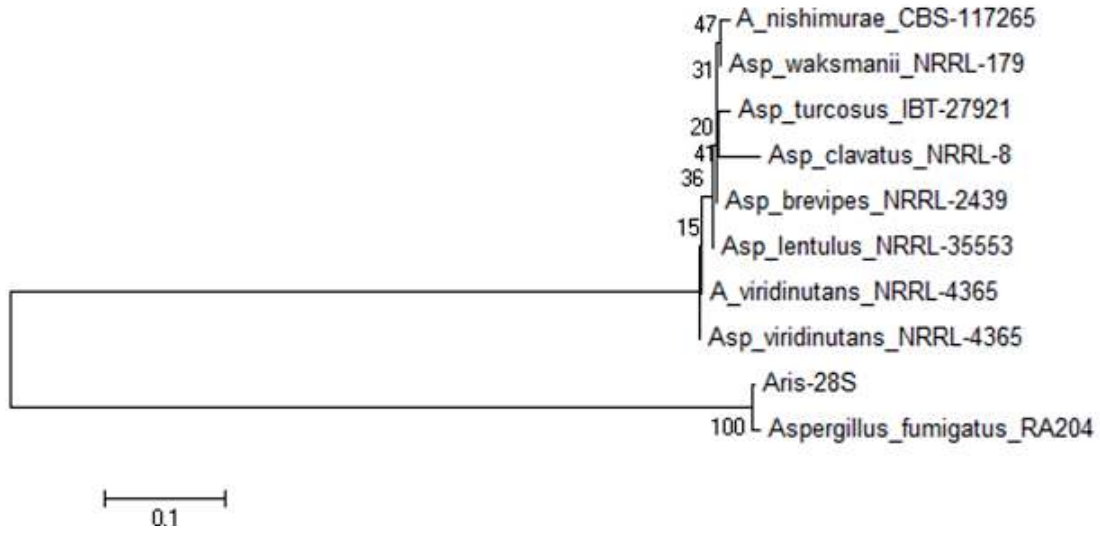

Fig 1 Position of KC4J mutant isolate (Aris28S) shown on the phylogenetic tree based on the 28S rRNA gene sequence using Maximum Likelihood (ML).The position of the substitution per nucleotide is shown in 0.1 bar.

al. 2014; El-Batal et al.). Types of microorganisms, nutrient sources and environmental factors influence lipase production (Lima *et al.* 2003; Pinheiro *et al.* 2008; Ulker *et al.* 2011; Kotogan *et al.* 2014). According to Khausik *et al.* (2010), lipase production increased by media optimization. The Response Surface Method (RSM) is one of the methods that can be used. This method used to determine the optimum conditions using Mathematical and Statistical analysis. This method used to obtain the best solution from a combination of several variables that affect the production process. Jia *et al.* (2015) proved that RSM can be used for optimization of lipase production. In addition, increasing lipase activity can also be done by optimizing pH and temperature and metal ions addition. Metal ions function as cofactors for lipases which can make lipases more stable in binding to the substrate (Palmer 1991; Dandavate *et al.* 2009; Iqbal and Rehman 2015). Mutations have been carried out with the use of ultraviolet (UV) light on C kernel mold isolates from oil palm waste, Malinping, Lebak, Banten, West Java, Indonesia. In Figure 1, the result of phenotypic and phylogenetic approaches (28s rRNA) KC4J isolate mutated by UV light has 100% similiarity with *Aspergillus fumigatus* strain Ra204.

Changes in the mean character that occur in the branches are indicated by the values contained in the Maximum Likelihood phylogenetic tree, so that the KC4J mutant isolate (Aris-28S) has the closest ancestor to *Aspergillus fumigatus* strain RA204. The maximum likelihood phylogenetic tree compiled was based on DNA changes (Reece *et al.* 2014; Indriawan *et al.* 2018). Reported by Indriawan *et al.* 2018 that mutated Kernel C isolates with UV light from oil palm waste whichwere fermented at room temperature (28-30 ºC) for 7 days in a 200 rpm rotary shaker able to produce lipase. The media used was fish meal as anitrogen source and olive oil as carbon source as well as an inducer (Murni *et al.* 2015). The KC4J mutant gave the highest lipase activity and transesterification activity with 10 U / mL and 0.114 U / mg, respectively. Therefore, it was expected that the KC4J mutant lipase can be used as catalyst in biodiesel production. This study aim to increase the activity of mutant KC4J lipase which is similar to *Aspergillus fumigatus* strain RA204 by optimizing the media using RSMand also performing partial characterization. Partial characterization of KC4J mutant lipase included the influence of pH, temperature and metal ions. The pH tested was on 6 to 12 range, while the temperature variation was tested at 30 up to70 °C. The metal ions tested were Mg^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Fe^{2+}, and K^{+} with concentrations of 1 mM and 10 mM. Reported by Djafar *et al.* 2010, that the optimization of the production medium needs to be done to determine the composition of the media that can produce optimum lipase activity, and lipase characterization is needed to determine the characteristics of lipase when applied to the industrial sector.

MATERIALS AND METHODS

Microorganisms. This study used KC4J mutant, a collection of the Bioindustrial Technology Laboratory,

Table 1 Independent variables range and level in lipase production optimization using RSM design

Independent variable	Variable ranges and levels				
	- α	-1	0	+1	+ α
X_1	-1.6817	-1	0	+1	+1.6817
X_2	-1.6817	-1	0	+1	+1.6817
X_3	-1.6817	-1	0	+1	+1.6817

LAPTIAB-BPPT, PUSPIPTEK, Banten, which is similar to *Aspergillus fumigatus* strain RA204. The mold culture was grown on slant Potato Dextrose Agar (PDA) medium that stored at 4 °C rejuvenated every 30 days.

Preparation of Inoculum and Liquid Media. The KC4J mutant was grown in a PDA slant test tube and incubated for 7 days at 28 °C. After incubation, 10 mL of sterile distilled water was put into the slant culture and scraped off using inoculation loop. The culture furthermore homogenized for 30 seconds and the spore suspension used to inoculate was adjusted to 10^7 spores/mL (El-Batal *et al.* 2015). Lipase production was carried out in 50 mL of liquid production medium on250 mL Erlenmeyer flasks based on the results of preliminary research (unpublished) which contain 3% of soy flour and 1% of olive oil on pH 7 which sterilized for 15 minutes at 121 °C. The spore suspension was inoculated into the flask with 10% (w / v) ratiofrom the total production medium. The fermentation process were conducted for 7 days on a rotary shaker at 200 rpm agitation in room temperature (28-30 °C) (Maia *et al.* 2001; Ellaiah *et al.* 2004; Pinheiro *et al.* 2008; El-Batal *et al.* 2015). Experimental design and analysis Experiments were carried out with RSMusing CCD experimental design. The design used three variables with two lipase activity as the measured response. Each variable consists five level of codes. The variables used were carbon source (X_1), nitrogen source (X_2) and pH (X_3), as shown in Table 1. Lipase production was carried out according to the variation in the combination determined by RSM. All data obtained were processed using RSM analysis. Based on CCD experimental design using Design Expert v. 7.0.0. Software (Statease, USA), all the data obtained were plotted on a second order polynomial equation. Furthermore, each coefficient of significance was seen based on the P and F values in the ANOVA. Each parameter has a significant effect if the P value gets smaller and the F value gets bigger (Demirel and Kayan 2012).

Lipase Activity Assay. Lipase activity assay can be done using Li *et al.* (2014) method with modifications. The substrate was made with composition: 1.5% of polyvinyl alcohol (PVA), 25% of olive oil, and distilled water, which were mixed until homogeneous. A total of 5 mL of the substrate was added with 4 mL of 0.05 M phosphate buffer solution, pH 6 and 1 mL of crude lipase extract.The mixture were incubated at 50 °C for 20 minutes in shaker (150 rpm). After the incubation completed, 5 mL of methanol was added to stop the reaction. 2 drops of phenolphthalein (PP) indicator were added to the solution and then titrated with 0.05 M NaOH. The titration was stopped when the color of the solution turned to and remained pink, and the titration volume was recorded. The blank solution was made in the same way as the sample, whilst methanol addition before incubation were to stop the lipase reaction.

Lipase Characterization. Lipase characterization was carried out by determining the optimum pH and temperature of the lipase, test the pH and temperature stability of the lipase, and verifythe effect of metal ions on lipase.

Optimum pH and Temperature. Determination of the optimum pH of lipase was carried out by testing lipase activity at pH 6, 7, 8, 9, 10, 11 and 12 using titration method (Li *et al.* 2014). Several buffer solutions used were: 0.05 M phosphate buffer solutions pH 6 and 7, 0.05 M tris-HCl buffer solutions pH 8 and 9, and 0.05 M glycine-NaOH buffer solutions pH 10, 11, and 12. The Lipase Activity assay was incubated at 50 °C with 200 rpm agitation for 20 minutes. Using Li *et al.* (2014) method with modification, determination of lipase optimum carried out at the optimum pH with different temperature variations (30, 40, 50, 60 and 70 °C).

pH and Temperature Stability. Lipase pH stability was carried out by incubating lipase in pH 6, 7, 8, 9, 10, 11 and 12 with one part of enzyme incubate in four parts of buffer solutions at optimum temperature. Sampling was carried out at 0, 30, 60 and 90 minutes. Also using Li *et al.* (2014) method with modification, the effect of temperature on lipase stability was determined by incubating the lipase at 30, 40, 50, 60 and 70 °C at the optimum pH. Sampling was carried out at 0, 30, 60 and 90 minutes.

Effect of Metal Ions. The effect of metal ions determined by testing the activity of lipases containing ions such as Mn^{2+}, Zn^{2+}, Ca^{2+}, K^{+}, Mg^{2+} and Fe^{2+}. The ion concentration used are 1 mM and 10 mM (Ghori *et al.* 2011; Mokodongan 2013).

RESULTS

Optimization of Media using RSM. The media used for the production of lipase contained olive oil, soy flour and was adjusted to pH 7. The composition and conditions of the media were optimized with a CCD design and the design response measured was lipase activity. In this paper, only design respondto lipase activity are reported, as can be seen in Table 2. The variables used were olive oil, soy flour and pH. RSM was used to examine the effect of each variable on the response. Multiple regression analysis was used to analyze the data obtained and the second order polynomial equation is described from the regression analysis below:

Lipase activity response

$Y = +10,34 + 0,051X_1 + 0,24X_2 + 0,30X_3 - 0,040X_1X_2 + 0,12X_1X_3 + 0,21X_2X_3 - 0,11X_1^2 - 0,028X_2^2 + 3,523E\text{-}003X_3^2$

The Y equation shows the responds to lipase activity; X_1, X_2 and X_3 show the independent variables of carbon source, nitrogen source and pH.

Data from CCD designthen analyzed by ANOVA with Design Expert v.7.1.5 program. Design Expert Program 7.1.5. used for model selection analysis based on the Sequential Model Sum of Square, model mismatch testing (Lack of Fit Test) and Model Summary Statistics. The results of model selection on the responds to lipase activity are shown on Table 3, whilst the ANOVA calculation on lipase activity shown in Table 4. The three-dimensional image of the interaction between olive oil media composition, soy flour and pH on lipase activity was shown in Figure 2.The actual and predictive values of research for lipase activity was shown in Figure 3.

Optimum pH and Temperature. The lipase activity test was carried out at several variations of pH and temperature, in order to obtain the optimum pH and temperature of the lipase. The pH variation used was pH 6-12, while the temperature variation used was 30-70 °C. Figure 4 shows that the highest lipase activity is at pH 6 at 11.50 U/mL and decreases at pH 7-9. Lipase activity stops at pH 10-12.

Figure 5 shows that lipase activity increases at 30-60 °C and decreases in lipase activity at 70 °C. The highest lipase activity was at 60 ° C at 13.44 U/mL.

PH and Temperature Stability. The effect of pH on the relative stability of lipase activity is presented in Figure 6 and the effect of temperature in Figure 7. The effect of pH on the stability of the relative activity of lipases was tested at pH 6-12.Relative lipase activity at 0 minutes, lipase had no activity.The 30th minute of lipase relative activity increased until the 60th minute and decreased by the 90th minute at all tested pHs.The highest relative lipase activity was at pH 6 min. 60, which was 129.08%.The relative activity of lipase tended to be stable at pH 6-8, but above pH 8, the relative activity of lipase was 0%.Lipase stability at pH 6 showed a relative lipase activity pattern that increased until the 60th minute incubation time and decreased at the 90th minute.KC4J mutant lipase similar to *Aspergillus fumigatus* strain RA204 has optimal lipase activity at acidic pH, namely pH 6 and stable at pH 6-8.

Effect of Metal Ions. The effect of metal ions on the relative activity of lipases is presented in Figure 8. All metal ions can increase the relative activity of lipases at concentrations of 1 mM and 10 mM and act as activators. The highest relative lipase activity was found in the addition of Ca^{2+} 1 mM ion concentration of 146.55%.

DISCUSSION

Optimization of media using RSM. The choice of model in Table 3 was based on the description of the sum of squares of the sequence model (Sequential Model Sum of Square) which is suggested to use the quadratic model versus two interaction factors (2FI), because it has a p value of 0.0305 or p 0.305% ($p < 5\%$) . This shows that the probability of error from the model was less than 5%, so that the model has a significant impact on explaining the effect of experimental factors or variables on the response. In the selection of the model mismatch test (Lack of Fit Test), it is suggested that a quadratic model with an F value of 0.52 and a p value of 0.75 indicates that the cascade model is not significant because the p value is 0.75. Model Summary Statistics suggested a quadratic model for the optimization of media composition.

The analysis of variance (ANOVA) model was used to determine the effect of each variable and the interaction between experimental variables on lipase activity. The model with an F value of 15.60 shows that mistakes do not play an important role and errors are likely to occur by 0.01% (Table 4). F value or

Table 2 Central Composite Design and the responds to lipase activity

Run	Olive oil (%)	Soybean powder (%)	pH	Lipase activity (U/mL)
1	1,50	3,00	7,00	9,94
2	0,75	3,50	6,50	10,13
3	1,25	3,50	6,50	9,81
4	0,75	3,50	7,50	9,94
5	1,00	3,00	7,00	10,44
6	1,00	3,00	7,00	10,19
7	1,00	3,00	7,00	10,50
8	0,75	3,50	7,50	10,81
9	0,75	2,50	6,50	9,81
10	1,00	3,00	6,00	9,75
11	1,00	4,00	7,00	10,63
12	1,00	3,00	7,00	10,19
13	1,25	2,50	6,50	9,94
14	1,00	3,00	7,00	10,5
15	0,50	3,00	7,00	9,81
16	1,00	3,00	8,00	10,88
17	1,25	3,50	7,50	11,25
18	1,25	2,50	7,50	10,25
19	1,00	3,00	7,00	10,13
20	1,00	2,00	7,00	9,75

Table 3 Analysis of model selection with the Sequential Model Sum of Squares, Lack of Fits Test and Model Summary Statistics on lipase activity responds

Sequential Model Sum of Squares						
Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Mean vs Total	2094,08	1	2094,08	12,72	0,0002	
Linear vs Mean	2,41	3	0,80	3,87	0,0352	
2FI vs Linear	0,48	3	0,16	4,49	0,0305	Suggested
Quadratic vs 2FI	0,31	3	0,10	0,53	0,7221	Aliased
Cubic vs Quadra	0,059	4	0,015			
Residual	0,17	6	0,028			
Total	2097,50	20	104,87			
Lack of Fit Tests						
Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Linear	0,86	11	0,078	2,62	0,1484	
2FI	0,38	8	0,048	1,61	0,3109	
Quadratic	0,078	5	0,016	0,52	0,7523	Suggested
Cubic	0,019	1	0,019	0,64	0,4591	Aliased
Pure Error	0,15	2	0,030			
Model Summary Statistics						
Source	Std. Dev.	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS	
Linear	0,25	0,7046	0,6492	0,5013	1,70	
2FI	0,20	0,8440	0,7720	0,6669	1,14	
Quadratic	0,15	0,9335	0,8737	0,7678	0,79	Suggested
Cubic	0,17	0,9508	0,8441	-0,2136	4,14	Aliased

Table 4 Analysis of Variance (ANOVA) for response to lipase el Summary Statistics on lipase activity responds

Source	Sum of Square	df	Mean Square	F Value	p-value Prob > F	
Model	3,19	9	0,35	15,60	<0,0001	Significant
X_1-Olive oil	0,042	1	0,042	1,85	0,2035	
X_2-Soybean powder	0,91	1	0,91	40,17	<0,0001	
X_3-pH	1,45	1	1,45	63,96	<0,0001	
X_1 X_2	0,013	1	0,013	0,56	0,4700	
X_1 X_3	0,11	1	0,11	4,87	0,0519	
X_2 X_3	0,35	1	0,35	15,54	0,0028	
X_1^2	0,29	1	0,29	12,56	0,0053	
X_2^2	0,019	1	0,019	0,85	0,3779	
X_3^2	3,120E-004	1	3,120E-004	0,014	0,9090	
Residual	0,23	10	0,023			
Lack of Fit	0,078	5	0,016	0,52	0,7523	Not significant
Pure Error	0,15	5	0,030			
Cor Total	3,41	19				

Std. Dev	0,15	R-Squared	0,9335
Mean	10,23	Adj R-Squared	0,8737
C.V. %	1,47	Pred R-Squared	0,7678
PRESS	0,79	Adeq R-Squared	12,544

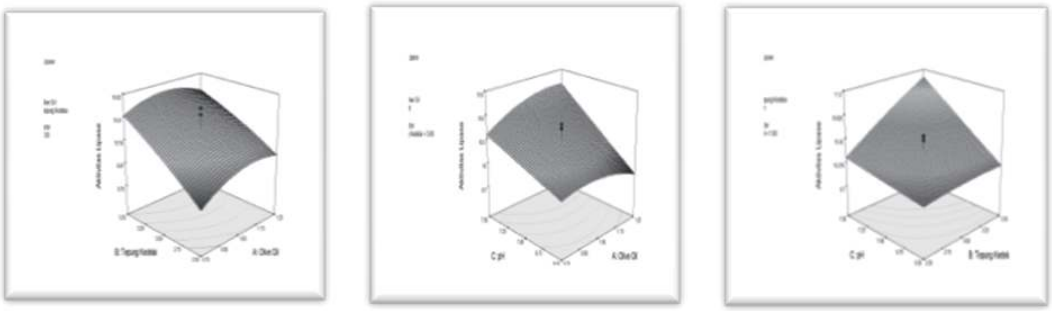

Fig 2 The interaction of factor variables of olive oil, soybean flour and pH on the response to lipase activity.

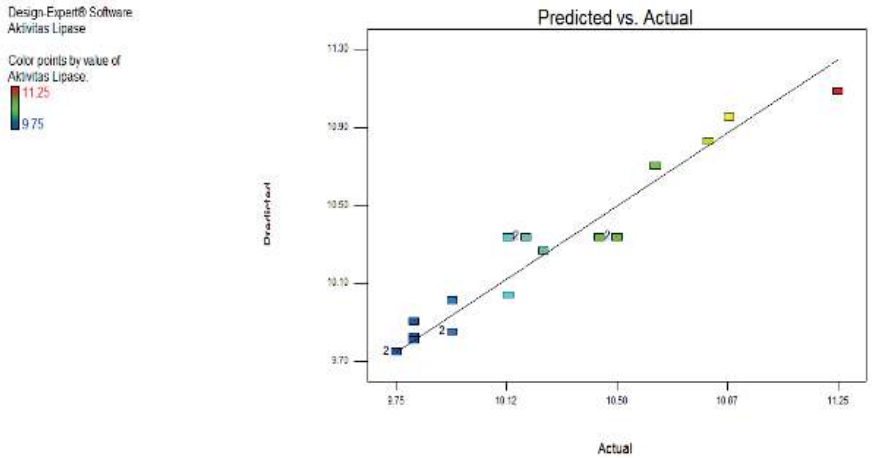

Fig 3 Distribution of research results and predictive value to lipase activity response.

probability less than 0.05 indicates that the model components are significant, B and C are significant components. Lack of fit F value of 0.52 indicates that the model is not significant to the response, there is a chance that the model is not suitable. Insignificant model mismatch can be interpreted well and the selected model is correct.

The interaction between media composition (Olive Oil, soy bean flour and pH) on lipase activity was shown in the form of a three-dimensional image. The highest desirability value of the research results was 1 with a media composition of 1.25% olive oil, 3.50% soybean flour, pH 7.00, 11.25 U/mL of lipase activity was obtained. The composition of media that has a desirability value of 1 has been verified in the laboratory. The verification results resulted: 11.50 U/mL of lipase activity, 3.02 mg/mL of protein content,0.95 grams of biomass, and 0.14 U/g of transesterification activity. RSM can be used for optimization of lipase production as reported by Kaushik *et al.* (2010) and Jia *et al.* (2015).One of RSM design are CCD, which maximizes both the precision and accuracy of the estimated extreme point of second-order response surface for the constructed model parameters fixed values (Coetzer *et al.* 2011).Research of variables or numerical factors of olive oil, soybean flour and pH gave the maximum effect to lipase activity response, because it had an R^2 value of 93.35%.The concentration of olive oil and soy flour are the main nutrients that regulate lipase biosynthesis. The highest concentration of these two nutrients can inhibit lipase synthesis, so that the appropriate concentration can increase the optimum lipase production. In addition, pH has an effect on the biosynthesis of the lipase. Too low and high pH can inhibit lipase synthesis and is supported by research Jayaprakash and Ebener (2012).

Optimum pH and Temperature. Based on these results, it is known that lipase activity is influenced by pH. pH 6.00 is the optimum pH because it has the highest lipase activity. According to Christakopoulus (1992), low or high pH can denature lipase thus decrease lipase activity. Lipase is a protein, changes in pH can cause protein molecules to ionize, thus changing the three-dimensional structure of lipases. These structural changes can cause disruption of the catalytic function of lipases. In addition, low pH hydrolysis will occur in unstable peptide bonds, while at high pH there will be irreversible denaturation and partial damage (Ghaima *et al.* 2014). The optimum pH obtained in this study is the same as the optimum pH obtained by Faloni *et al.* (2006) on *Aspergillus niger* lipase, which is pH 6.00. This is also supported by the results of research by Crueger and Crueger (1993), other *Aspergillus niger* strains are also optimum at pH 6.00.

Based on the data obtained, it is known that lipase activity is influenced by temperature.

According to Septiningrum and Moeis (2009), an increase in temperature will have a positive correlation with an increase in lipase activity before it reaches the optimum temperature, while at temperatures above the optimum, the lipase activity will decrease rapidly. The optimum temperature obtained in this study is quite high, namely 60 °C. The optimum temperature conditions increase the kinetic energy which accelerates the rotation and movement of the lipase molecules and the substrate, thus increasing the collision frequency which is the opportunity for both of them to react. If it is above the optimum temperature, the lipase activity will decrease due to changes in the tertiary structure of the protein in the lipase (Gomes *et al.* 2006). According to Daniel *et al.* (2010) stated that high temperatures can reduce lipase activity due to denaturation of the protein structure.

PH and Temperature Stability. The lipase characteristics are similar to the research of Mhetras et al.(2009), reported that *Aspergillus niger* lipase NCIM 1207 was stable at alkaline pH (pH 8-11), but had optimum activity at acidic pH. According to Colla *et al.* (2015), *Aspergillus flavus* has pH stability at pH 3.5-6.5 for 24 hours of incubation time with a residual activity of> 80%, while at pH 7-10 it has a residual activity of around 50%. pH can affect lipase stability by changing the electrostatic interaction of the lipase protein structure, which causes changes in amino acid ionization, secondary and tertiary structures of proteins (Sharma *et al.* 2002; Rajakumara *et al.* 2008).

The effect of temperature on the relative stability of lipase activity was tested at 30-70 °C. The relative activity of lipase at 0 minutes was 0%. The 30th minute of lipase relative activity increased until the 60th minute and decreased by the 90th minute at all tested temperatures. The highest relative lipase activity was at 60 °C in the 60th minute, which was 130.68%. Temperature 30-70 °C, the relative lipase activity tends to be stable. Lipase stability at 60 °C indicated that the relative lipase activity pattern increased until the 60th minute incubation time. The 0th minute there is no lipase activity. Lipases are stable at high temperatures (> 50 °C) probably due to the presence of polyamines in the protein structure. In addition, a high proportion of thermophilic amino acids, salt bridges and the number

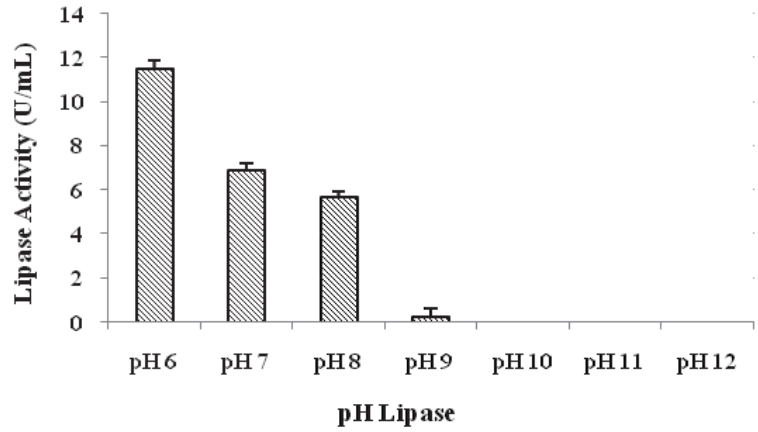

Fig 4 Lipase activity (U/mL) vs pH (6-12) KC4J mutant lipase similar to *Aspergillus fumigatus* strain RA204.

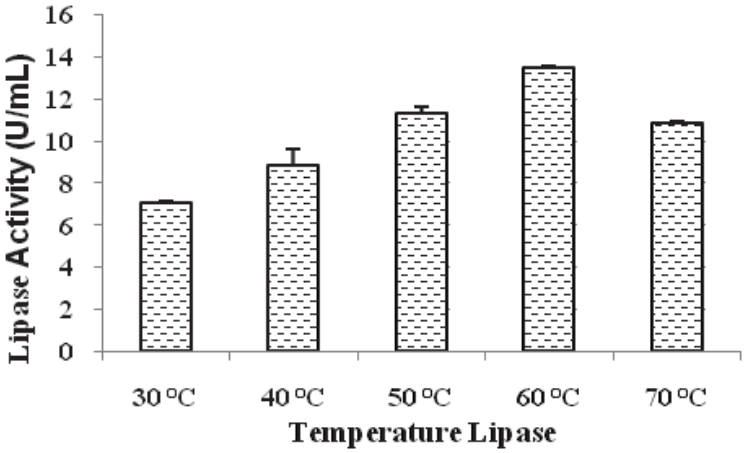

Fig 5 Lipase activity (U / mL) vs temperature (30-70^{0}C) KC4J mutant lipase similar to *Aspergillus fumigatus* strain RA204.

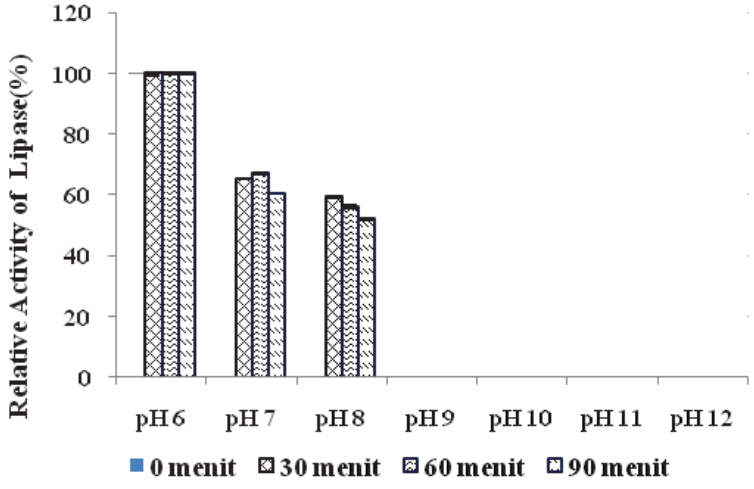

Fig 6 Effect of pH on the relative stability of lipase activity during 90 minutes of incubation.

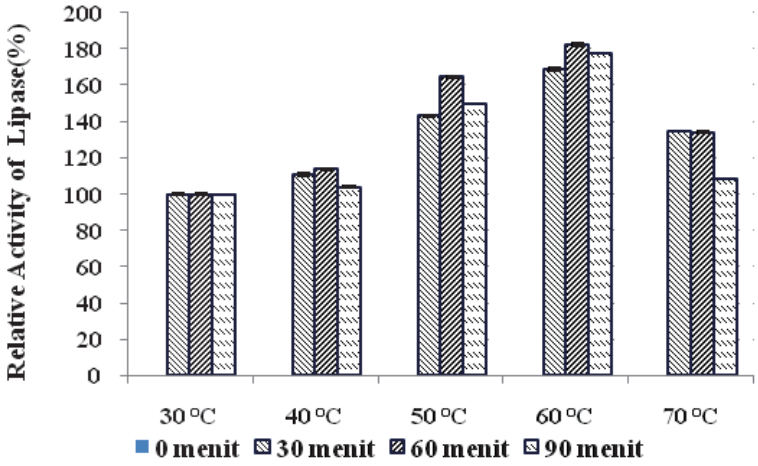

Fig 7 Effect of temperature on the relative stability of lipase activity during 90 minutes of incubation.

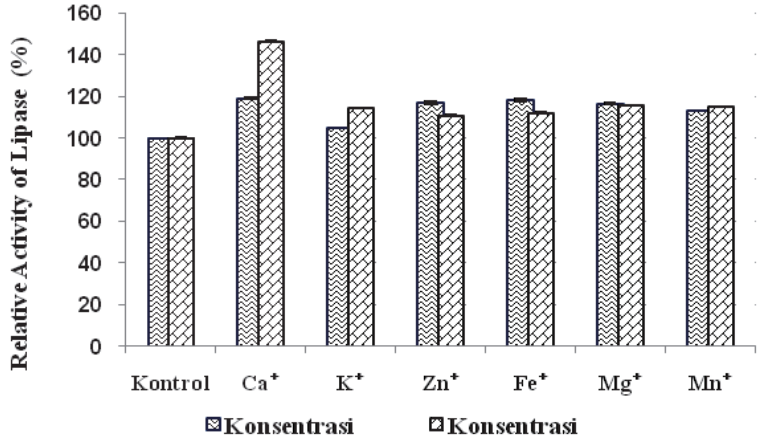

Fig 8 Effect of addition of 1 mM and 10 mM metal ions on the relative activity of lipase.

of hydrogen bonds can also affect the stability of lipases against temperature (Bora and Bora 2012).

Rhizopus oligosporus lipase can maintain residual activity of 80% at 25-30 °C and in *Rhizopus oligosporus* mutant, residual activity of lipase can be 100% at 20-50 °C. Increasing incubation temperature can cause lipase activity to be inhibited (Iftikhar *et al.* 2011) and *Aspergillus niger* NCIM 1207 is stable at 40 °C for 3 hours and at 50 ° C for 1 hour causing 52% loss of activity (Mhetras *et al.* 2009).

Effect of Metal Ions. Several metal ions are required for lipase to increase its activity. Metal ions at certain concentrations can act as activators or inhibitors for lipases.In addition, metal ions also function as cofactors for lipases and make lipases more stable when binding to the substrate (Palmer 1991).

In general, Ca^{2+} ions play an important role in increasing lipase activity and these ions can also influence structural changes rather than the catalytic role of lipases (Dandavate *et al.* 2009). In addition, these ions at a concentration of 1 mM can induce a conformational change in the lipase structure to become more stable, thus increasing lipase activity (Iqbal and Rehman 2015).

According to Glogauer *et al.* (2011), the ion concentration of 1 mM Ca^{2+}, Cu^{2+} and Mn^{2+} can increase the relative activity of lipase. The relative activity of *Rhizopus oryzae* lipase can also be increased with the addition of Ca^{2+} and Mg^{2+} ions (10 mM) (Dali *et al.* 2009). Lipases produced by other *Rhizopus oligospores* can also be increased by the addition of Ca^{2+}, Mg^{2+} and Mn^{2+} ions (5 mM) (Kareem *et al.* 2017). Shah and Bhatt (2012) and Wahyuni (2016), reported that lipase activity can be increased by the addition of ions such as Ca^{2+}, Mg^{2+}, Mn^{2+} and Fe^{2+}.

In conclusion, the R^2 value of 0.93 results from the Central Composite Design analysis and the quadratic model shows that carbon, nitrogen and pH sources have an effect on lipase activity. The composition of the medium for production of 1.25% olive oil, 3.5% soy

flour and a pH of 7.5 are the optimum conditions for lipase fermentation. The optimum lipase activity is at pH 6 and temperature 60 °C, relatively stable at pH 7-8 and temperature 30-60 °C. All of the ions tested could increase lipase activity and Ca^{2+} ions were the ones that could increase the highest lipase activity.

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