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Optimization of Lipases Production by*Bacillus licheniformis* F11.4 using Response Surface Methodology

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Lipase is a lipids hydrolyze enzyme which are widely used in various industries such as chemical, pharmaceutical, food industries, and detergents. *Bacillus licheniformis* F11.4 is one of the bacteria with potential source of lipase. This study aimed to obtain optimum production of lipase from *B. licheniformis* F11.4 by optimizing the composition of media and pH values with fish flour as a replacement for peptone and yeast extract based medium. Selection of the significant factors used a 2-level factorial design. The upper limit and lower limit of the selected factors was optimized using Central Composite Design (CCD) and the data analysis was performed using the Response Surface Methodology (RSM). Fermentation was carried out in erlenmeyer at initial pH 8 and a temperature of 37 °C, using a shaker incubator at 150 rpm. A fermentation system for lipases production is considered optimal when its desirability value closes to 1. By using numerical optimization, an optimal medium could be obtained, i.e. consisting of olive oil (OO): crude palm oil (CPO) 0.14 % (w/v) and fish flour 2% (w/v), at pH 8 and 150 rpm, which produced lipase with enzyme activity of 1.563 U mL^{-1} and protein level of 0.08 mg mL^{-1}. Furthermore, the results are verified in the Erlenmeyer, working volume of 50 mL, pH = 8, T = 37 °C, agitation 150 rpm, t=18 hours, the activity of lipase and protein levels are 1.568 ± 0.014 U mL^{-1} and 0.072 ± 0.006 mg mL^{-1} respectively.The results showed that the optimum condition lipase activity was 1.568 U mL^{-1} so that the the activity of only 75% compared to that before optimization.

Key words: *Bacillus licheniformis* F11.4, Central Composite Design, enzyme activity, protein content, Response Surface Methodology

Lipase merupakan enzim penghidrolisis lipida yang banyak digunakan di berbagai industri seperti kimia, farmasi, makanan, maupun aditif detergen. *Bacillus licheniformis* F11.4 adalah salah satu bakteri yang potensial sebagai sumber lipase. Penelitian ini bertujuan untuk mendapatkan produksi lipase yang optimal dari *B. licheniformis* F11.4 melalui optimasi komposisi media dan nilai pHdengan tepung ikan sebagai pengganti pepton dan yeast ekstrak dalam medium. Pemilihan faktor-faktor yang berpengaruh secara signifikan menggunakan desain faktorial 2 level. Batas atas dan batas bawah faktor-faktor yang terpilih selanjutnya dioptimasi menggunakan *Central Composite Design* (CCD) dan analisis data dilakukan dengan menggunakan *Respone Surface Methodology* (RSM). Fermentasi dilakukan di dalam erlenmeyer pH awal 8 dan suhu 37 °C, menggunakan shaker inkubator 150 rpm. Sistem fermentasi untuk produksi lipase dikatakan optimal jika nilai *desirability* mendekati 1. Dengan optimasi numerik nilai *desirability* mendekati satu, komposisi media fermentasi terbaik adalah minyak olive (OO) : minyak sawit mentah (CPO) = 0,14 % (b/v); tepung ikan = 2% (b/v) dan nilai pH = 8, agitasi 150 rpm, aktivitas enzyme dan kadar protein yang akan diperoleh masing-masing adalah 1,563 U mL^{-1}, dan 0,08 mg mL^{-1}. Selanjutnya hasil tersebut diverifikasi di dalam Erlenmeyer, volume kerja 50 mL, pH = 8, T = 37 °C, agitasi 150 rpm, t = 18 jam, memberikan nilai aktivitas lipase dan kadar protein masing-masing adalah 1,568 ± 0,014 U mL^{-1} dan 0,072 ± 0,006 mg mL^{-1}. Hasil penelitian menunjukkan bahwa pada kondisi optimum aktivitas lipase adalah 1,568 U mL^{-1} sehingga aktivitas hanya 75% dibandingkan dengan kondisi sebelum optimasi.

Kata kunci: aktivitas enzim, *Bacillus licheniformis* F11.4., Central Composite Design, aktivitas enzim, kadar protein, *Response Surface Methodology*

Lipases are triacylglycerol acyl-hydrolases (EC 3.1.1.3) that catalyze the hydrolysis of triacylglycerol to glycerol and fatty acids (Verma *et al*. 2012). The enzymes are capable of hydrolyzing the ester bonds of water-insoluble substrates at the interface between the substrate and water. It is well known that the reaction is reversible and the enzymes catalyze both ester synthesis and transesterification. Since lipases catalyze a number of different reactions, they have been widely used in such industries as food, chemical, and pharmaceutical, detergent industries as well as in leather processing industry, and also for producing better quality products as part of an eco-friendly process (Selvamohan *et al*. 2012; Hanifi *et al*. 2013; Francois*et al*. 2014). Lipases are ubiquitous enzymes

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synthesized by all biological system, including animals, plants, and microorganisms (bacteria, fungi, yeast, and actinomycetes). Microbial lipases have gained special attention due to their ability towards both extreme temperature and pH, organic solvents as well as chemo-, region-, and enantio-selectivity (Devi *et al*. 2012; Verma *et al*. 2012; Kumar *et al*. 2007). Among lipases producing bacteria, *Pseudomonas aeruginosa*, *Enterobacter aerogenes* and some species of *Bacillus* such as *B. subtilis*, *B. pumilis*, *B. licheniformis*, *B. thermoleovorans*, *B. stearothermophilus*, and *B. sphaericus* produce lipases suitable for biotechnological applications. Widely known as a multipurpose organism, *B. licheniformis* has gained popularity along with *B. subtilis*. *B. licheniformis*, which is commonly found in soil and on the feathers of ground dwelling birds (Maria *et al*. 2013), produces extracellular lipases. Lipases production by *B. licheniformis*is affected by many physicochemical (particularly pH and temperature) and nutritional factors (the nature and availability of carbon and nitrogen substrates as well as lipids).

In bacterial fermentation, peptone is usually used as the main source of nitrogen with yeast extract as the source of growth factors, including vitamins.To increase the production of lipases, olive oil is often used both as inducer and carbon substrate (Niyonzima *et al*. 2013). However, peptone, yeast extract, and olive oil are quite expensive and not very easy to obtain. In the present study, peptone and yeast extract were entirely substituted with fish flour as a source of nitrogen, then olive oil and crude palm oil (CPO) can be used as a carbon source or inducer in media production lipase (Limpon *et al*, 2012). To determine the optimal substrate composition as well as fermentation condition (pH, temperature), Response Surface Methodology (RSM) was used along with Central Composite Design (CCD) technique.

RSM is the most popular optimization method used in recent years. There are so many works based on the application of RSM in chemical and biochemical process (Deniz *et al*. 2007). RSM experimental design is an efficient approach to determine which explanatory variables (e.g. medium componentand/or fermentation condition) have impacts to the response variables of interests (e.g. growth rate, protein level or productivity) (Box *et al*. 2005), and there are several reports on application of RSM for the production of primary and secondary metabolites through microbial fermentation (Devi *et al*. 2012; Dash *et al*. 2002).

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 (Sematech 2010). This study aimed to obtain optimum production of lipase from *B. licheniformis* F11.4 through optimization of media composition and pH values with fish flour as a replacement for peptone and yeast extract based on Sharma media that has been modified (Sharma *et al*. 2012).

MATERIALS AND METHODS

Materials. *B. licheniformis* F11.4 is a collection of the Laboratory of Bioindustrial Technology- LAPTIAB, BPPT, Serpong. Fish flour from CV Pratama Hikmah Karya, Cimahi, Bandung, Indonesia; olive oil (OO) a trademark of Le Riche; crude palm oil (CPO) a product of PT Sinar Mas, Indonesia; tryptone, agar, peptone, yeast extract, NaCl from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Other ingredients and equipment are commonly used for bioprocess research.

Regeneration of the Microorganisms. A colony of *B. licheniformis* F11.4 was transferred from the stock culture onto Luria Bertani Agar and incubated at 37 °C or 50 °C, pH 7, for 16 h.

Propagation of the Microorganisms. One colony of regenerated *B. licheniformis* F11.4 was innoculated into Luria Bertani (LB) liquid media and incubated at 37 °C in a shaking incubator (Kuhner) with constant agitation at 150 rpm, for 8-16 h, up to optical density (OD) of 0.6-0.8. The suspension of bacterial cells were then used as bacterial starter in the production of lipases. The LB liquid media were prepared by dissolving into RO water adjusting its pH to 7 by adding HCl 1M or NaOH 1M, and sterilizing the mixture in autoclave for 20 min at 121 °C.

Production of Lipase. The bacterial starters (100 mL) were innoculated into liquid medium for lipase production (900 mL), incubated at 37 °C with initial pH of 8 and agitation at 150 rpm, for 18 h. The number of bacteria were minimum 10^7 cells mL^{-1}, determined prior to the incubation (hour zero). The fermentation medium used was described by Sharma with some modification (Sharma *et al*. 2012) consisted of 3% pepton ,1% yeast extract, 0.3% $NaNO_3$,0.01% KH_2PO_4, 1% olive oil (OO), and 1% CPO. In the modification, peptone and yeast extract were substituted with fish flour of equal protein content. The protein content was determined by using modified Lowry's method. In the present study, two kind of fish flours, one made of bony fish (P) and the other of more-fleshy fish (N), were compared. Regarding the inducer, the proportion of OO

and CPO was modified to 0.25% (w/v) and 0.75% (w/v), respectively, in accordance with the results of the preliminary study on OO and CPO requirementf o r lipase production by *B. licheniformis* F11.4.

Harvesting of Lipase: The enzyme harvesting were carried out by separating the supernatant from biomass through centrifugation at 10 000 rpm for 15 min at 4 °C. The supernatant, termed as crude (extracellular) enzyme fraction, was assayed for its lipase activity.

Assay for Lipase Activity. The crude enzyme's lipase activity was determined using spectrophotometric method. As the substrate, 3 mg of paranitrophenyl-palmitate (p-NPP) was dissolved in 1 mL isopropanol and mixed with 10 mg of arabic gum and 40 mg of triton X-100 in 9 mL of 0.05M Tris-HCl buffer solution of pH 8. Into 0.9 mL of substrate, 0.1 mL crude enzyme was added and then incubated at 37 °C in a thermoshaker with constant agitation at 300 rpm, for 30 min. The absorbance of the incubated mixtures was measured at λ410 nm against blank. Lipase activity was calculated using the following formula (Silva *et al*. 2005):

$$\text{Lipase activity (U mL}^{-1}) = \frac{C \times 1000}{MR\text{pNPP} \times t \times v}$$

Where C = concentration of p-NPP (mg mL-1) , t = time (minute), V = Volume of crude enzyme (m), and molecular mass of p-NPP (MR p-NPP) = 377.52.

Protein content of the crude enzyme was analyzed by spectrophotometric method using Bradford reagent (Bradford 1976).

Optimization Using RSM. In the present study, optimization using RSM was performed in two stages. In the first stage, two-level factorial design, the proportion of OO and CPO (0.1-0.5% w/v) as inducers, fish flour (1.8-5.4%) as the main source of nitrogen, and pH (8-10) were treated as independent variables with lipase activity as the response variable. The fermenting media also contained 3% $NaNO_3$ and 0.1% KH_2PO_4; and *B. licheniformis* F11.4. innoculated were incubated at 37 °C with constant agitation at 150 rpm. The data were analyzed with Design Expert (version 7.1.5, Stat-Ease Inc., USA) software. Media composition that gave the highest lipase activities were used to determine the upper limit and lower limit of independent variables for the second stage optimization experiment that used central composite design (CCD).

In the second stage, factorial design 2^3 which was expanded with 6 starting points and 6 center points were used, allowing 20 combinations of OO, CPO, and fish flour. For each combination, fermentation was carried out in a 125 mL erlenmeyer flask using 25 mL fermenting media of pH 8, containing 3% $NaNO_3$ and 0.1% KH_2PO_4, at 37 °C and 150 rpm for 18 h. Numerical optimization of the results was performed using the Design Expert software.The ideal desirability value in numerical optimization is 1.0. The results of the numerical optimization that close to the ideal desirability value are verified in the laboratory with a minimum of 6 replicates.

RESULTS

Optimization Using Response Surface Methodology. In the first stage, optimization of lipase production was conducted by using a two-level factorial design experiment toward the three affecting factors, i.e. OO:CPO was coded A, fish flour was coded B, and pH was coded C, with lipase activity as the variable response. The Design Expert software was used to generate the experimental designs, of which produced 12 experimental design models (Table 1). The statistical significance of the models in lipase activities of crude enzymes produced were evaluated using analysis of variance (ANOVA) and the results are presented in Table 2. From the first stage of research the two-level factorial, used to specify an upper limit and a lower limit on the central composite design (CCD) as shown in Table 3.

Based on the data in Table 3 of research second stage using composite design center (CCD) with 3 factors A, B, and C and two response variables are the activity and protein levels and resulting 20 combination of treatments as shown in Table 4. Further data enzyme activity and protein levels of 20 combinations of research results incorporated into the program Design Expert 7.1.5. and the results of the analysis can be seen in Fig 1 and Fig 2.

The results of ANOVA for lipase activity response can be seen in Table 5, and the interaction between the media composition and pH value on the response of lipase activity can be seen in Fig 3a, Fig 3b, and Fig 3c.

The test results of analysis of variance (ANOVA) response protein levels can be seen in Table 6, while the interaction between the media composition and pH value on the response of lipase activity can be seen in Fig 4a, Fig 4b, and Fig 4c.

The results of numerical calculation models media optimization and the pH value based on the central

Table 1. Generated experimental design models for lipase production by *B. licheniformis* F11.4

Run	OO:CPO [A]	Fish flour [B]	pH [C]	Activity, U mL^{-1}
1	0.50	5.40	10.00	0.969
2	0.50	5.40	8.00	0.511
3	0.10	1.80	8.00	1.198
4	0.30	3.60	9.00	0.695
5	0.50	1.80	10.00	0.463
6	0.50	1.80	8.00	1.150
7	0.30	3.60	9.00	0.691
8	0.10	1.80	10.00	0.618
9	0 .30	3.60	9.00	0.751
10	0.10	5.40	8.00	0.875
11	0.10	5.40	10.00	0.933
12	0.30	3.60	9.00	0.801

Factors affecting lipase production by *B. licheniformis* F11.4:
A = ratio of olive oil and CPO = OO/CPO, upper limit 0.5% (w/v), lower limit 0.1% (w/v)
B = fish flour P, upper limit 5.4 % (w/v), lower limit 1.8% (w/v)
C = pH, upper limit 10, lower limit 8

composite design (CCD) and Response Surface Method (RSM) using Design Expert 7.1.5 program delivering provide desirability 0.764 with composition 0.1% (w/v) OO:CPO; v2.04% (w/v) fish flour and pH 8. Desirability value approaching 1 (one) obtained by trial and error using Design Expert 7.1.5 program. From the trial and error were obtained twelve combination with desirability value = 0.921.

DISCUSSION

Factors that influence the production of lipase *B. licheniformis* F11.4. were determined by two level factorial experimental design. Response lipase *B. licheniformis* F11.4. was observed on hour to 8, 14, and 18. The result of the interaction between the concentration OO:CPO, the concentration of fish flour and pH values were analyzed using Analysis of Variance (ANOVA), selected to give the highest activity on the observation hours to 18. The analysis results in Table 2 show that the model of interaction between the concentration OO:CPO, the concentration of fish flour and the pH value to yield significant results.

From the analysis of variance, the value of F of the model is larger than F table (28.96> 4.87) and p-value = 0.0094 <α = 0.05 shows a model of a significant or unacceptable to see the estimated effect of each variable and interaction factor with the response. The coefficient of determination (R^2) 0.9854 shows a high correlation between the influence of factors on the response variable.

Point arc curve (curvature) also showed a significant result so that the model can be used to define the design of the central composite design of experiments process (Central Composite Design/CCD) to determine the optimal media composition in the production of lipase *B. licheniformis* F11.4. Of the two-level factorial experiments obtained the highest lipase activity of 1.198 U mL^{-1} in the combined treatment of 0.1% OO:CPO; 1.8% fish flour and 8 pH value is then used to determine an upper limit and a lower limit on CCD (Table 3).

From the central composite design (CCD) by Design Expert 7.1.5 program provides 20 combined treatment (Table 4). Results of analysis of variance Design Expert 7.1.5 in Table 5 and Table 6 is the response to enzyme activity and protein levels indicate that a significant model analysis with P values <5%, which means that the model can be used for process

Table 2. ANOVA of the factors affecting lipase production by *B. licheniformis* F11.4

Response 1 Activity

ANOVA for selected factorial model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	0.55	7	0.079	28.96	0.0094	significant
A-OO:CPO	*0.035*	*1*	*0.035*	*12.98*	*0.0367*	
B-fish flour	*2.485E-003*	*1*	*2.485E-003*	*0.92*	*0.4093*	
C-pH	*0.071*	*1*	*0.071*	*25.96*	*0.0146*	
AB	*1.953E-003*	*1*	*1.953E-003*	*0.72*	*0.4587*	
AC	*0.011*	*1*	*0.011*	*3.95*	*0.1410*	
BC	*0.40*	*1*	*0.40*	*146.33*	*0.0012*	
ABC	*0.032*	*1*	*0.032*	*11.83*	*0.0412*	
Curvature	0.029	1	0.029	10.85	0.0459	significant
Pure Error	8.147E-003	3	2.716E-003			
Cor Total	0.59	11				

Std. Dev.	0.052	R-Squared	0.9854
Mean	0.80	Adj R-Squared	0.9514
C.V. %	6.48	Pred R-Squared	N/A
PRESS	N/A	Adeq Precision	16.286

Case(s) with leverage of 1.0000: Pred R-Squared and PRESS statistic not defined

Table 3 The upper limit and lower limit factors for central composite design (CCD)

Factor	Name	Low Actual	High Actual
A	OO:CPO (% w/v)	0.05	0.18
B	Fish flour (% w/v)	0.8	3.1
C	pH	7	9

optimization of lipase production. Addition of test results Lack of Fit to the model can be seen that there are no inaccuracies models, it can be proved of value for Lack of Fit obtained P value 0.052 (Not significant) for the response to the activity and the P value 0.2136 (Not significant) for response to the protein content, meaning that the regression model is accepted. Second equation (quadratic) was obtained from Design Expert 7.1.5 (Equation 1), which explains the data response on enzyme activity.

$$Y = -20.024 + 5.773A + 0.338\,B + 5.219\,C + 0.148\,AB + 0.189\,AC + 0.075\,BC - 25.056\,A2 - 0.255\,B2 - 0.337\,C2 \quad \text{(Eq.1)}$$

Second equation (quadratic) was obtained from Design Expert 7.1.5 (Equation 2), which explains the data response on protein content.

$$Y = +2.197 - 8.314\,A + 0.209\,B - 0.411\,C + 3.512\,AB + 0.827\,AC - 0.032\,BC + 5.702\,A2 + 8.122\,B2 - 0.021\,C2 \quad \text{(Eq.2)}$$

The accuracy of the model can also be seen from a comparison of the actual value of research with model predictions. Prediction model (Predicted) expressed as a straight line and the actual results of the study (Actual) expressed as a box. From Fig 1 and Fig 2 can be seen that the actual value (of the box) scattered approach the predicted values (straight line), it indicates the

Table 4 The data activity (U mL^{-1}) and protein content (mg mL^{-1}) of were combined treatments of OO:CPO, Fish flour and the pH value, the result of the central composite design (CCD) by the program Design Expert 7.1.5.

Std	Run	Factor 1 A:OO :CPO %	Factor 2 B:Fish flour %	Factor 3 C:pH	Response 1 Activity U/mL	Response 2 Protein conten mg/mL
19	1	0.11	1.95	8.00	1.4003	0.0595
14	2	0.11	1.95	9.68	0.7001	0.0282
11	3	0.11	0.02	8.00	0.5008	0.0786
5	4	0.05	0.80	9.00	0.7044	0.2075
6	5	0.18	0.80	9.00	0.7075	0.2202
18	6	0.11	1.95	8.00	1.602	0.0521
7	7	0.05	3.10	9.00	0.6002	0.0131
13	8	0.11	1.95	6.32	0.5016	0.2181
15	9	0.11	1.95	8.00	1.4006	0.0588
20	10	0.11	1.95	8.00	1.6005	0.0855
4	11	0.18	3.10	7.00	0.6007	0.0812
17	12	0.11	1.95	8.00	1.7017	0.1671
8	13	0.18	3.10	9.00	0.7006	0.1024
16	14	0.11	1.95	8.00	1.5019	0.1087
12	15	0.11	3.88	8.00	0.7005	0.1077
1	16	0.05	0.80	7.00	1.0008	0.2525
9	17	0.01	1.95	8.00	0.8088	0.1637
3	18	0.05	3.10	7.00	0.6028	0.2825
2	19	0.18	0.80	7.00	1.0077	0.1257
10	20	0.22	1.95	8.00	1.7034	0.0982

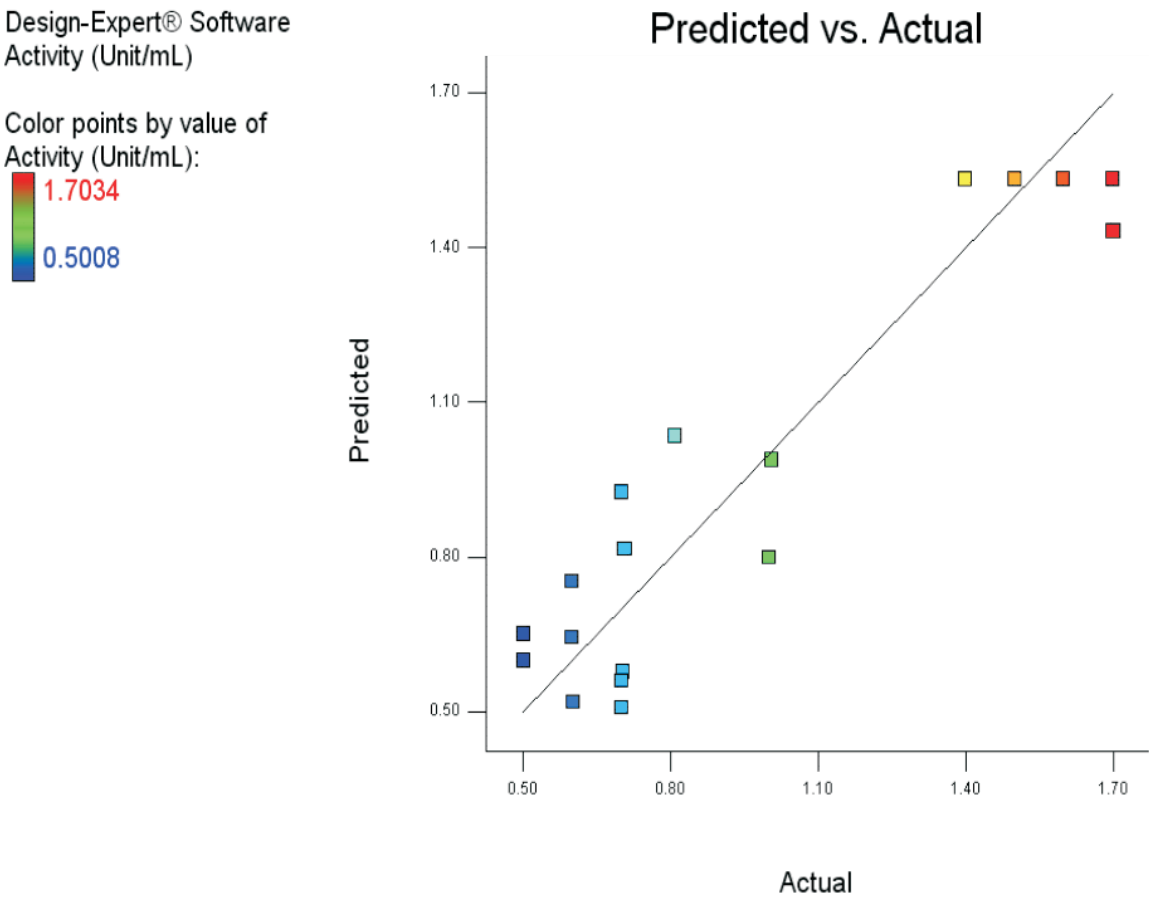

Fig 1 Distribution of actual and predicted values in the lipase production activity respon.

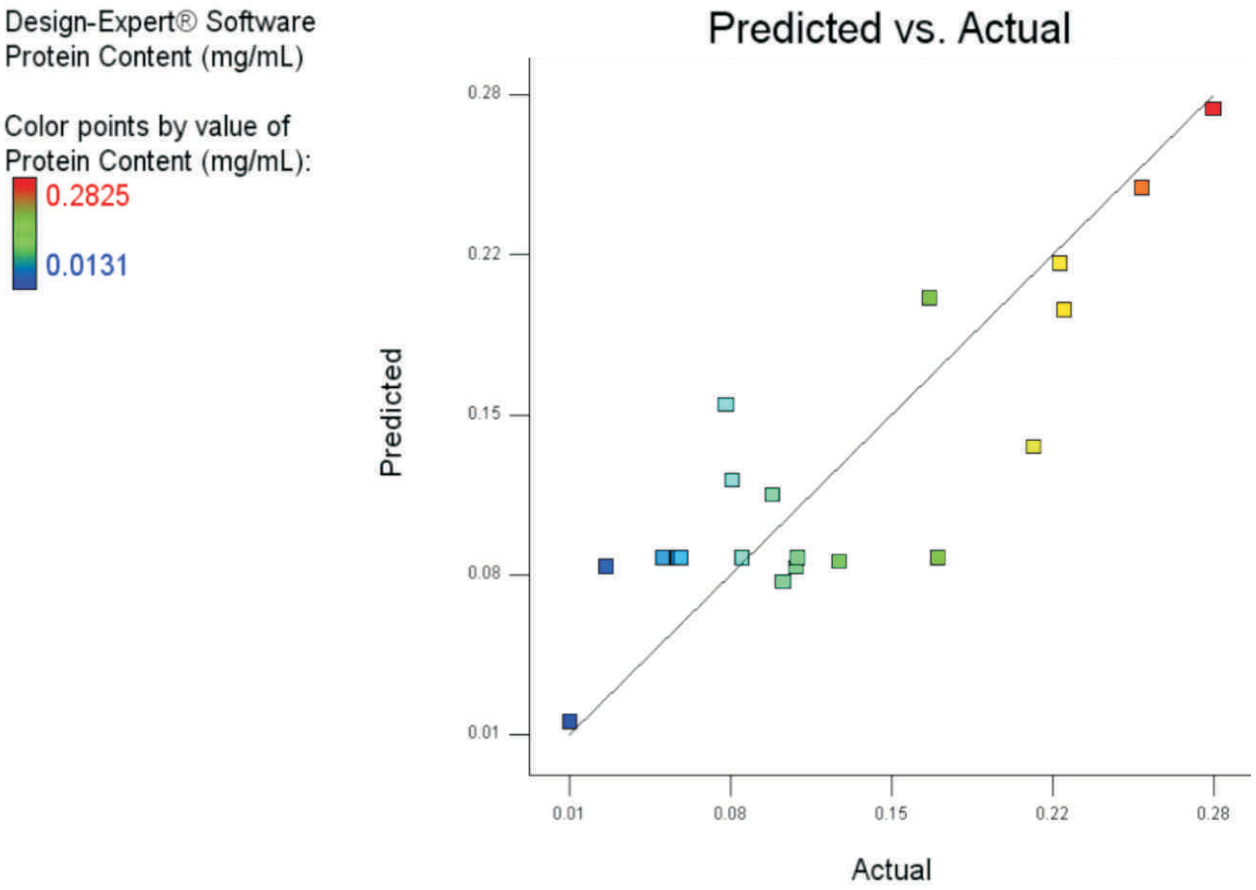

Fig 2 Distribution of actual and predicted values in the lipase production protein content respon.

deviation is low both in response to the activity as well as on the response of the protein content is evidenced by the standard deviation of ANOVA each is 0:21 and 0:06. A low standard deviation shows that the model has good accuracy or the model correspond (fit model). According Gaspersz (1995) the model is said to be proper if the assumption residual meet the assumption of normal distribution. If the residual plot spread randomly around zero and likely to approach a straight line (linear line) so as to be normally distributed.

ANOVA test results in Table 5 obtained determination coefficient (R^2) 0.8824 for the response to enzyme activity and in Table 6 for a response to the protein content obtained determination coefficient (R^2) 0.7312. It shows that 88% correlation activity response variable and 73.12% variable response to the production of lipase protein levels are influenced by independent variables (OO:CPO; fish flour: pH value). The optimum area of independent variables (factors) that produces the maximum response can be seen from the three-dimensional contour plot curves in Fig 3a that the interactions between fish flour, the pH of the enzyme activity showed that the parabolic curve is obtained. In Fig 3b; 3c and Fig 4a; 4b; 4c curve obtained shaped saddle (saddle point) so that the optimum point of the curve a little bit difficult to determine. The calculation numerical models of media optimization and the pH value is based on the CCD and the analysis of RSM provides desirability value 0.764. Trial and error using Design Expert 7.1.5 program there are twelve combinations that provide value desirability 0.921. As for the lower limit and upper limit, 0.01- 0,14 % (w/v) OO:CPO; 0.8 to 2 % (w/v) fish flour and pH 7.8 to8; value will be the activity of 1.563 U mL^{-1} and the protein content of 0.08 mg mL^{-1} with media compositions 0.14% (w/v) OO:CPO; 2 % (w/v) fish flour and pH 8.

Validation study was conducted in 125 mL Erlenmeyer, initial pH 8 fermentation time 18 h with the addition 0.3% $NaNO_3$ and 0.1% KH_2PO_4 with six replications, providing value lipase activity 1.568 ± 0.014 U mL^{-1} and 0.072 ± lipase protein content of 0.006 mg mL^{-1} or the value of a specific activity is 21.210 U mg^{-1}. From India reported by Sharma *et al.* 2012, produce lipase from *B. licheniformis* MTCC-10498, inducers tween 80 0.5% w/v, pH 7.5, a temperature of 55 °C, 150 rpm, fermentation time of 72 h the enzyme activity of 2.1 U mL^{-1}. From India was also reported by Devi *et al.* 2012, that for the

Table 5 ANOVA lipase activity response of experimental design CCD

Response 1 Activity (Unit/mL)

ANOVA for Response Surface Quadratic Model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	3.30	9	0.37	8.34	0.0013	significant
A-OO :CPO	*0.19*	*1*	*0.19*	*4.33*	*0.0640*	
B-Fish Flour	*0.025*	*1*	*0.025*	*0.56*	*0.4711*	
C-pH	*2.005E-003*	*1*	*2.005E-003*	*0.046*	*0.8352*	
AB	*9.746E-004*	*1*	*9.746E-004*	*0.022*	*0.8846*	
AC	*1.218E-003*	*1*	*1.218E-003*	*0.028*	*0.8711*	
BC	*0.060*	*1*	*0.060*	*1.37*	*0.2690*	
A^2	*0.16*	*1*	*0.16*	*3.68*	*0.0842*	
B^2	*1.64*	*1*	*1.64*	*37.38*	*0.0001*	
C^2	*1.64*	*1*	*1.64*	*37.36*	*0.0001*	
Residual	0.44	10	0.044			
Lack of Fit	*0.37*	*5*	*0.073*	*4.95*	*0.0520*	*not significant*
Pure Error	*0.074*	*5*	*0.015*			
Cor Total	3.74	19				

Std. Dev.	0.21	R-Squared	0.8824
Mean	1.00	Adj R-Squared	0.7765
C.V. %	20.91	Pred R-Squared	0.2277
PRESS	2.89	Adeq Precision	6.924

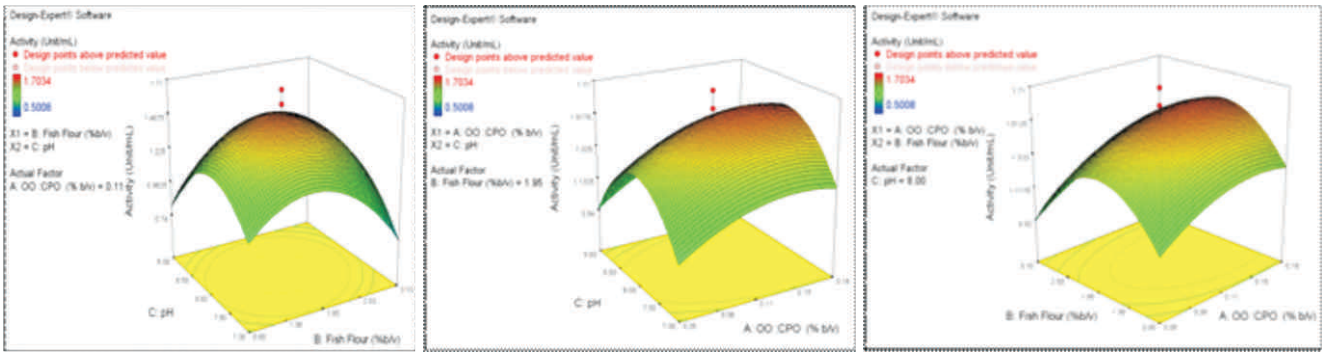

Fig 3 Interaction OO: CPO, fishflour, pH on lipase activity response analysis results Surface Method (RSM) using the program Design Expert 7.1.5

production of lipase *B. subtilis* with inducers tween 80 and using the method RSM. Media optimization results with yeast extract 9.3636 g mL^{-1}, CaCl 0.8986 g mL^{-1} and incubation periods 1.813 d, gives the activity enzyme 16.627 U min^{-1} mL^{-1}. Anahita *et al* (2011) from Malaysia also reported that the research of production the lipase *Acinobacter* sp. in submerged fermentations using RSM at the optimum condition at the fermentation time 24 h, T = 29 °C, pH = 6, the value of a specific enzyme activity of 32.2 U mg^{-1}.

The results of the optimization study *B. licheniformis* F11.4 lipase production gives the enzyme activity 1568 ± 0014 U mL^{-1}, lipase protein content of 0.072 ± 0.006 mg mL^{-1} and a specific enzyme activity of 21.77 U mg^{-1}. The optimum medium composition of fishflour 2% (w/v), olive oil 0.07% (w/v), CPO 0.5% (w/v), $NaNO_3$ 0.3% (w/v), and KH_2PO_4 0.1% (w/v) in the working volume of 50 mL

Table 6 Anova response lipase protein content of experimental design CCD

Response 2 Protein Content (mg/mL)

ANOVA for Response Surface Quadratic Model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	0.082	9	9.113E-003	3.02	0.0499	significant
A-OO :CPO	*8.279E-003*	*1*	*8.279E-003*	*2.75*	*0.1285*	
B-Fish Flour	*5.649E-003*	*1*	*5.649E-003*	*1.87*	*0.2010*	
C-pH	*0.020*	*1*	*0.020*	*6.52*	*0.0287*	
AB	*5.513E-007*	*1*	*5.513E-007*	*1.828E-004*	*0.9895*	
AC	*0.023*	*1*	*0.023*	*7.67*	*0.0198*	
BC	*0.011*	*1*	*0.011*	*3.67*	*0.0843*	
A^2	*8.365E-003*	*1*	*8.365E-003*	*2.77*	*0.1268*	
B^2	*1.659E-003*	*1*	*1.659E-003*	*0.55*	*0.4754*	
C^2	*6.559E-003*	*1*	*6.559E-003*	*2.18*	*0.1710*	
Residual	0.030	10	3.015E-003			
Lack of Fit	*0.021*	*5*	*4.102E-003*	*2.13*	*0.2136*	*not significant*
Pure Error	*9.643E-003*	*5*	*1.929E-003*			
Cor Total	0.11	19				

Std. Dev.	0.055	R-Squared	0.7312
Mean	0.13	Adj R-Squared	0.4892
C.V. %	43.73	Pred R-Squared	-0.5502
PRESS	0.17	Adeq Precision	6.640

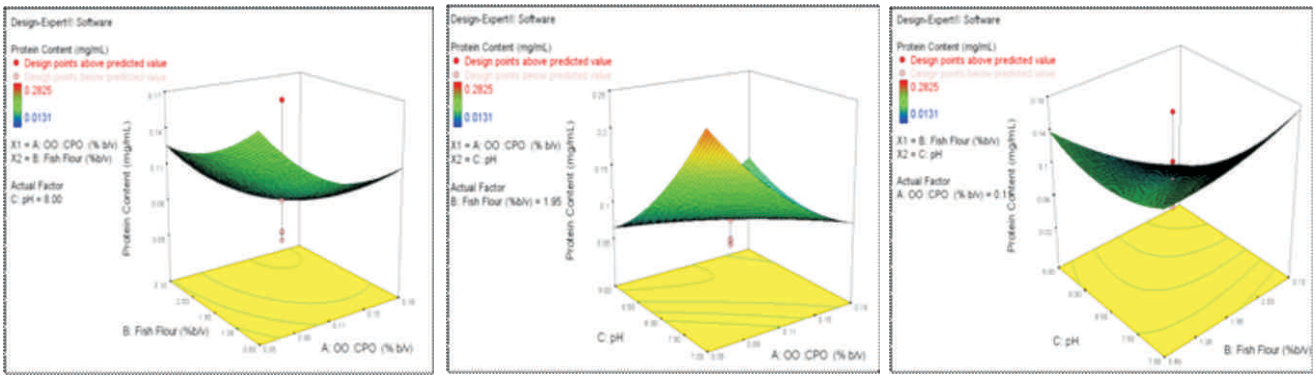

Fig 3 Interaction OO:CPO, fish flour, pH on lipase activity response analysis results surface method (RSM) using the program Design Expert 7.1.5

erlenmeyer with the operating conditions of pH 8, temperature 37 °C, agitation 150 rpm and fermentation time of 18 h. The increase in the activity of only 75% compared to before the optimization ie 1,176 U mL^{-1}.

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REFERENCES

Anahita K, Afshin E, Boon KB, and Oi ML.2011. Production of a solvent, detergent, and thermotolerant lipase by a newly isolated *Acintobacter* sp. in

Submerged and solid-state fermentations. J Biomed Biotechnol. Hindawi Publishing Corporation, Article ID 702179, 12 pages. doi:10.1155/2011/702179.

Bradford M. 1976.A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem.72(1-2):248-254. doi:10.1016/0003-2697(76)90527-3.

Box GEP, Hunter JS, Hunter WG . 2005.Statistic for experiments : design, innovation and discovery. New Jersey : John Willey & Sons.

Dash VV, Panda T . 2002. Optimization of microbiological parameters for enhanced griseofulvin production using response surface methodology. Bioprocess Engineering 22(1):45-49. doi:10.1007/PL00009099.

Deniz B, Ismail H. Boyacı. 2007. Modeling and optimization I: Usability of response surface methodology. Journal of Food Engineering 78(3):836-845. doi:10.1016/j.jfoodeng.2005.11.024.

Devi A, Devi KC, Rajendiran R. 2012. Optimization of lipase production using *Bacillus subtilis* by respone surface methodology. World Academy of Science, Engineering and Technology. Int J Biological, Veterinary, Agricultural and Food Engineering. 6 (9):840-845.

Francois N. Niyonzima and Sunil S.More. 2013. Screening and identification of a novel alkaline lipase producing bacterium. Int J Pharm Bio Sci. 4(2):1037-1045.

Gaspersz V. 1995. Teknik analisis dalam penelitian percobaan. Bandung (ID): Tarsito Pr.

Hanifi B, Mustafa D, Mehmet K.2013.Environmentally Friendly Detergent Production. Research in Civil and Environmental Engineering (RCEE).1(03):169-176.

Limpon B, Minakshi B. 2012. Optimization Of extracellular thermophilic highly alkaline lipase from thermophilic *Bacillus* Sp isolated from hotspring of arunachal pradesh, India. Braz J Microbiol. 43(1):30-42. doi:10.1590/S1517-83822012000100004 .

Maria G, Asma A, Afsheen A, Rashida RZ, Nadir NS, and Shah AUlQ.2013. Isolation and characterization of different strains of *Bacillus licheniformis*for the production of commercially significant enzymes. Pak J Pharm Sci. 26(4):691-697 691.

Niyonzima FN, More SS.2014. Concomitant production of detergent compatible enzymes by *Bacillus flexus* XJU-1. Braz J Microbiol. 45(3):903-910. doi:10.1590/S1517-83822014000300020.

Prasanth KMP, Valsa AK. 2007. Optimization of culture media and cultural conditions for the production of extracellular lipase by *Bacillus coagulans.* Indian J Biotechnol. 6(1):114-117.

Selvamohan T, Ramadas V, Sathya TA. 2012. International journal of modern engineering research (IJMER). 2(6):4231-4234.

Sematech. 2010. Engineering Statistic Handbook. http://itl,nist.gov/div898/handbook/pri/section3/pri3361.htm.

Sharma Chander K, Kanwar Shamsher S.2012.Purification of a Novel Thermophilic Lipase from *B. licheniformis* MTCC-10498. ISCA. J Biol Sci. 1(3):43-48.

Silva WDB, Mitidieri S, Schrank A, Vainstein MH. 2005. Production and Extraction ofan extracelluler lipase from the entomopathogenic fungus *Metharizumaniso-pliae*. Process Biochemistry 40(1):321-326. doi:10.1016/j.procbio.2004.01.005.

Verma N, Thakur S, Bhatt AK. 2012. Microbial lipases: industrial applications and properties (a review). International Research Journal of Biological Sciences. International Science Congress Association.ISSN 2278-3202. 1(8):88-92.