

Immobilization of Lipase Enzyme by Low Cost Material: A Statistical Approach

Md. Z. Alam, Devi R. Asih, Md. N. Salleh

Abstract—Immobilization of lipase enzyme produced from palm oil mill effluent (POME) by the activated carbon (AC) among the low cost support materials was optimized. The results indicated that immobilization of 94% was achieved by AC as the most suitable support material. A sequential optimization strategy based on a statistical experimental design, including one-factor-at-a-time (OFAT) method was used to determine the equilibrium time. Three components influencing lipase immobilization were optimized by the response surface methodology (RSM) based on the face-centered central composite design (FCCCD). On the statistical analysis of the results, the optimum enzyme concentration loading, agitation rate and carbon active dosage were found to be 30 U/ml, 300 rpm and 8 g/L respectively, with a maximum immobilization activity of 3732.9 U/g-AC after 2 hrs of immobilization. Analysis of variance (ANOVA) showed a high regression coefficient (R^2) of 0.999, which indicated a satisfactory fit of the model with the experimental data. The parameters were statistically significant at $p < 0.05$.

Keywords—Activated carbon, adsorption, immobilization, POME based lipase.

I. INTRODUCTION

THE palm oil mill effluent (POME) is a sludge waste generated by the palm oil mills during the processing of crude palm oil which has the physical appearance of a viscous brown liquid. It is predominantly organic and non-toxic with a highly unpleasant odor containing fine suspended solids at pH ranging between 4 and 5 [1]. POME consists of high concentrations of carbohydrates, proteins, nitrogenous compounds, lipids and minerals [2]. These compositions are a suitable source which had shown a successful fermentation of lipase production at pilot scale [3]. Although previous have study shown optimum production of lipase or other hydrolytic enzymes, further focuses should be on characterization and application of enzymes for make it more suitable to industrial applications.

Currently, low stability and activity or selectivity encountered with a number of enzymes has been the major restriction impeding the rapid expansion of industrial lipase technology on a large scale. Therefore, customization of lipases by chemical and physical modifications has more recently been attempted to improve their catalytic properties in hydrolysis and synthesis [4]. Moreover the application of microbial lipases at industrial level still restricted due to the

low stability of their native state compared to classical chemical catalysts state. Such improvement can be carried out by chemical, physical or genetical modifications of the native enzyme. Therefore, enzyme immobilization is one of the possible alternatives because of its enable reusability, flexible in operational and ease of product recovery from the enzyme.

The produced lipase was then immobilized with several support materials in order to improve its properties for suitability to industrial applications. Various numbers of support material types have been used in immobilization studies. The parameter selection of the best material support will influence the immobilization performance. The properties of material support for an enzyme carrier should be permeability, insolubility, large surface area, stability at chemical, mechanical and thermal states, high rigidity, suitable shape and particle size, and resistance to microbial attachment. Recent works on immobilization had successfully used cellulose [5], activated carbon [6], coconut fiber and rice husk as material supports. Based on previous works, among the support materials (cellulose, activated carbon, empty fruit bunch-EFB) activated carbon had shown high potential to immobilization of lipase [3]. To enhance the immobilization capacity, an optimization study was conducted to evaluate various parameters such as enzyme concentration, agitation and the dosage of the material support and finally validated the predicted models for industrial applications.

II. MATERIALS AND METHODS

A. Collection of Lipase Fermented from POME

A commercial microbial strain (*Candida cylindracea* ATCC 14830) was used in preparation of lipase at 300L of stirred tank bioreactor using palm oil mill effluent as a basal medium. Detail procedures of fermentation and collection of lipase at pilot scale production were carried out by the methods of Asih et al. [3] and Salihu et al. [7]. Lipase activity was carried out according to the method of Gopinath et al. [8] (2005). The cell free extract from the fermentation broth was assayed quantitatively using *p*-nitrophenyl palmitate (*p*NPP).

B. Lipase Immobilization

The cell free filtrate was obtained after centrifuging the fermentation broth at 5000×g for 10min followed by the microfiltration using hollow fiber membrane cartridge in QuickStand™ bench top system. Next the partly purified lipase was immobilized based on physical adsorption technique. The process conditions were: 0.2g of each material support, activated carbon in 25.0ml of partly purified lipase (22 U/ml) at room temperature ($\pm 30^\circ\text{C}$) with the agitation of

M. Z. Alam, D. R. Asih, M. N. Salleh with Bioenvironmental Engineering Research Centre (BERC), Department of Biotechnology Engineering, Faculty of Engineering, International Islamic University Malaysia (IIUM), Gombak, Kuala Lumpur, 50728, Malaysia (tel.: +603 61964571; fax: +603-61964442; e-mail:zahangir@iium.edu.my).

300 rpm for 2 hours of contact time. The immobilized lipase was then centrifuged at 10000 rpm for 10min and the supernatant solution was separated. The amount of adsorbed lipase was determined from the remaining activity in the supernatant solution.

C. Statistical Optimization Experiments for Immobilization Process

A strategy based on statistical experimental design which is the response surface methodology (RSM) through the face-centered central composite design (FCCCD) was performed to optimize the process parameters on the lipase immobilization. The Design Expert software (Version 6.0.8, Stat-Ease Inc., Minneapolis, USA) used with three significant factors namely enzyme concentration, agitation and the dosage of the material support to immobilize the lipase. A 20 set experimental was run with six replicated center points. The independent variables were studied at three different levels, low (-1), medium (0) and high (+1). The experimental design used for the study is shown in Table I. The remaining factor as time was maintained at the fixed time for two hours. Immobilization was applied in 100ml Erlenmeyer flasks containing 25ml semi purified lipase and carbon active dosages according to the design. The incubation was carried out at room temperature and various agitations in rpm. The residual lipase production obtained was taken as the response (Y).

The develop regression model was evaluated by analyzing the values of regression coefficients, analysis of variance (ANOVA), Fisher's F-test (overall model significance), R^2 . Furthermore, the fitted polynomial equation expressed in the form of contour and surface plots describing the relationship between the responses and the experimental levels of each of the variables utilized in this study.

D. Validation of the Experimental Model

The statistical model was validated with respect to all the three variables within the design space. Experiments predicted by the point prediction feature of the Design Expert software were conducted in triplicates. Six combinations of media constituents (Table II) were used to determine the lipase immobilization and the results were compared with the predicted values.

III. RESULTS AND DISCUSSION

A. Optimization of the Immobilization Process through Statistical Design

One of the most useful experimental statistical techniques in optimizing process is response surface methodology (RSM). It has been proved to be the most effective methods in improving the immobilization process and eliminating the limitations of a single factor optimization process [9], [10]. Face centered central composite design (FCCCD) under response surface methodology (RSM) was used with three significant factors which are enzyme concentration, agitation and activated carbon dosage. There are 20 experimental runs equipped with six replicate center points. The predicted carbon

adsorption activity obtained from the regression equation for the 20 combinations and the independent variables of three different levels, low (-1), medium (0) and high (+1) are shown in Table I.

The results demonstrated that highest amount of immobilization process (adsorption capacity) 3644.7 U/g was observed in the run 16 while the lowest result was observed in run 17 (821.75 U/g). This shows that the design matrix of FCCCD further improved the immobilization process, such that the difference between the lowest and the highest response (3644.7–821.75 U/g). The method of FCCCD improved the immobilization process where adsorption was 1956.7 U/g before optimization and adsorption of 3644.7 was achieved by optimization. The result enhancement proved that statistical based experimental design is a valuable tool in optimizing the factors. This method offers a number of important advantages such as the factor effects, obtaining the optimum values and also developing a system model with considerably less experimental requirements [11].

The relationship between dependent and independent variables is explained by the developed equation as follows:

$$Y(\text{carbon adsorption capacity, U/g}) = +1963.04 + 990.97A + 20.10B - 387.81C - 14.99A^2 - 12.84B^2 + 84.40C^2 + 19.08AB - 185.76AC - 8.79BC \quad (1)$$

where, the carbon adsorption capacity as yield (Y); A, B and C are independent variables (enzyme concentration, agitation and activated carbon dosage). Hence, the statistical analysis of the model was generated to evaluate the analysis of variance (ANOVA). This analysis also covers the Fisher's F-test and its associated probability values, and coefficient of determination R^2 . The corresponding analysis of variance for this media is presented in Table II.

The Model F-value of 4351.06 implies the model is significant ($P < 0.01$). There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicates model terms are significant. In this case A, B, C, C^2 , AB, AC are significant model terms. Values greater than 0.05 indicate the model terms are not significant. The corrective measures to estimate the regression equation are the multiple correlation coefficient R and the determination coefficient R^2 . The closer the R value to 1 the better the correlation between the experimental and predicted values. The result of this research showing the value R and R^2 were 0.998 and 0.9995. These values indicate a strong correlation between the experimental and predicted values. The value of R^2 indicates that 99.9% of the variability in the response variables could be explained by the model and only 0.1% of the deviation could not be explained by the model which shown the model are highly significant.

The 3D response surface plot is the graphical representation of the regression equation used to determine the optimum values of the variables within the range considered [12]. The 3D for interaction between factors (agitation, enzyme concentration and activated carbon dosage) is shown in Fig. 1.

The graphs were drawn to understand the interaction among the variables in order to find optimum conditions of immobilization. The 3D response surface also illustrated the main and interactive effects of the independent variables on

the dependent ones [13]. The maximum activated carbon adsorption as the response of immobilization about 3644.7 U/g was obtained near the center points of the response surface.

TABLE I
THE FCCCD RESULT OF THE EXPERIMENTAL MODEL WITH EXPERIMENTAL AND PREDICTED BY THE REGRESSION MODEL

Run	Enzyme concentration (U/mL)	Agitation (rpm)	Dosage (g/L)	Response adsorption capacity (U/g)	
				Experimental	Predicted
1	20 (0)	200 (0)	10 (0)	1956.7	1963.04
2	20 (0)	300 (+1)	10 (0)	1955.8	1970.31
3	20 (0)	200 (0)	10 (0)	1961.5	1963.04
4	10 (-1)	100 (-1)	12 (+1)	822.1	834.37
5	20 (0)	200 (0)	10 (0)	1974.96	1963.04
6	10 (-1)	100 (-1)	8 (-1)	1212.75	1220.89
7	20 (0)	200 (0)	10 (0)	1969.19	1963.04
8	30 (+1)	100 (-1)	8 (-1)	3533.6	3536.17
9	30 (+1)	200 (0)	10 (0)	2910.6	2939.02
10	20 (0)	200 (0)	10 (0)	1961.6	1963.04
11	10 (-1)	300 (+1)	8 (-1)	1231	1240.53
12	30 (+1)	300 (+1)	12 (+1)	2475.9	2467.41
13	20 (0)	200 (0)	12 (+1)	1650.6	1659.63
14	20 (0)	100 (-1)	10 (0)	1943.2	1930.10
15	30 (+1)	100 (-1)	12 (+1)	2416.5	2406.62
16	30 (+1)	300 (+1)	8 (-1)	3644.7	3632.13
17	10 (-1)	300 (+1)	12 (+1)	821.75	818.83
18	20 (0)	200 (0)	8 (-1)	2442.88	2435.26
19	20 (0)	200 (0)	10 (0)	1957.1	1963.04
20	10 (-1)	200 (0)	10 (0)	984.1	957.09

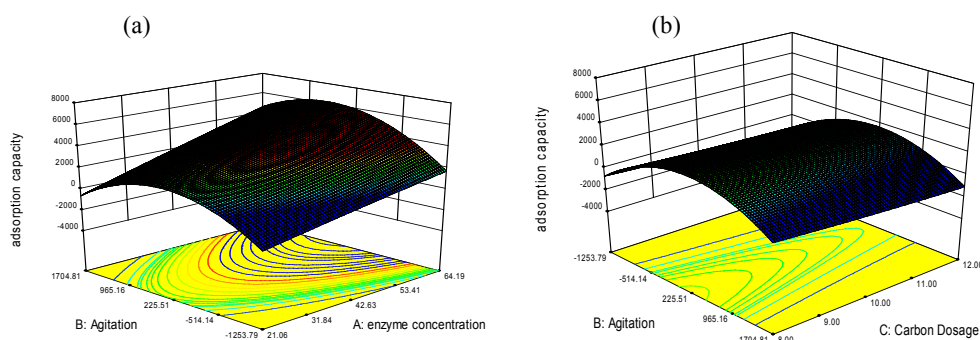


Fig. 1 3D response surface curves of the combined effects of parameters in immobilization; (a) agitation and enzyme level concentration at fixed level of activated carbon dosage; (b) Agitation and carbon dosage at fixed level enzyme concentration

TABLE II
THE ANALYSIS OF VARIANCE (ANOVA) FOR RESPONSE SURFACE QUADRATIC MODEL

Source	F-value	p-value > F
Model	4351.06	< 0.0001
Enzyme concentration, A	33058.76	< 0.0001
Agitation, B	13.61	0.0042
Activated carbon dosage, C	5063.08	< 0.0001
A ²	2.08	0.1799
B ²	1.53	0.2450
C ²	65.95	< 0.0001
AB	9.81	0.0107
AC	929.28	< 0.0001
BC	2.08	0.1796
Lack of fit	10.49	0.0110

*p < 0.01 indicate the model terms are highly significant.

B. Validation of the Immobilization Model

To check the adequacy of the model equation, some sets of confirmation experiments were carried out within the design space based on the optimum conditions established using the FCCCD for the lipase immobilization. To confirm the validity of the statistical experimental strategies and gain a better understanding of the lipase immobilization, experiments were performed within the design space. Good agreement between the predicted and experimental results verified with low standard deviation. The validity of the model and the existence of the optimal conditions through five sets of experiments were shown in Table III.

TABLE III
THE VALIDATION RESULT OF THE EXPERIMENTAL MODEL

Lipase (U/ml)	Agitation (rpm)	Carbon Dosage (g/L)	Carbon adsorption (U/g)		Error (%)
			Expt.	Pred.	
30	300	8	3732.9	3631.8	2.7
30	250	12	2495.2	2461.6	1.34
25	275	10	2496.4	2410.5	3.4
20	300	8	2496.31	2451.3	1.8
30	250	10	2995.7	2800.2	6.5

IV. CONCLUSION

The results show that activated carbon (AC) as a support material is able to immobilize lipase POME based media up to 94%. The effects of RSM technique enhanced the immobilization process as compared to the single factor optimization process. The results revealed that the optimum process parameters of highest carbon adsorption obtained were 30U/ml lipase concentration, 8g/L activated carbon dosage and 300 rpm of agitation. The predicted lipase immobilization was validated to be 3732.9 U/g-AC with the optimum process parameters which was 94% of immobilization. The findings show that activated carbon as a material support would be an alternative to more useful in industrial applications as cost would be comparatively cheaper than the conventional ones.

ACKNOWLEDGMENT

This work is being supported by the ERGS (ERGS 11-002-0002), MOHE and IIUM Endowment Fund (EDW B11-130-0608), the facilities of Biotechnology Engineering Department, IIUM, and Sime Darby Research Centre Sdn. Bhd. for providing the POME sample to conduct this project.

REFERENCES

- [1] S. Kathiravale, and A. Ripin, "Palm oil mill effluent treatment towards zero discharge", A paper presented at National Science and Technology Conference, held in July, 15-16; Kuala Lumpur, Malaysia, pp. 1 – 8, 1997.
- [2] T. Y. Wu, A. W. Mohammad, J. M. Jahim and N. Anuar, "Investigations on protease production by a wild-type *Aspergillus terreus* strain using diluted retentate of pre-filtered palm oil mill effluent (POME) as substrate", *Enzyme and Microbial Technology* vol. 39, pp.1223–1229, 2006.
- [3] D. N. Asih, M. Z. Alam and M. N. Salleh, "Pilot scale production and immobilization of lipase using palm oil mill effluent as basal medium", *Journal of Oleo Science*, 2014, Accepted
- [4] P. Villeneuve, J. M. Muderhwa, J. Graille and M. J. Haas, "Customizing lipases for biocatalysis: a survey of chemical, physical and molecular biological approaches", *Journal of Molecular Catalysis B: Enzymatic* vol. 9, pp.113–148, 2000
- [5] M. Karra-Châabouni, I. Bouaziz, S. Boufi, A. M. Botelho do Rego, "Physical immobilization of *Rhizopus oryzae* lipase onto cellulose substrate: Activity and stability studies", *Colloids and Surfaces B: Biointerfaces* vol. 66, pp. 168–177, 2008.
- [6] K. Ramani, E. Chockalingam and G. Sekaran, "Production of a novel extracellular acidic lipase from *Pseudomonas gessardii* using slaughterhouse waste as a substrate", *J Ind Microbiol Biotechnol.* vol. 37, pp.531–535, 2010.
- [7] A. Salihi, M. Z. Alam, M. A. AbdulKarim and H. M. Salleh, "Effect of process parameters on lipase production by *Candida cylindracea* in stirred tank bioreactor using renewable palm oil mill effluent based medium", *Journal of Molecular Catalysis B: Enzymatic*, vol. 72, pp. 187-192, 2011.
- [8] S. C. B. Gopinath, P. Anbu and A. Hilda, "Extracellular enzymatic activity profiles in fungi isolated from oil-rich environments", *Mycoscience*, vol. 46, pp.119–126, 2005.
- [9] Y. He and T. Tan, "Use of response surface methodology to optimize culture medium for production of lipase with *Candida* sp. 99-125". *Journal of Molecular Catalysis B: Enzymatic* vol. 43, pp.9–14, 2006.
- [10] N. Gupta, V. Sahai and R. Gupta, "Alkaline lipase from a novel strain *Burkholderia multivorans*: Statistical medium optimization and production in a bioreactor". *Process Biochemistry* vol. 42(4), pp.518 – 526, 2007
- [11] T. Y. Wu, A. W. Mohammad, J. M. Jahim, and N. Anuar, "Optimized reuse and bioconversion from retentate of pre-filtered palm oil mill effluent (POME) into microbial protease by *Aspergillus terreus* using response surface methodology", *J Chem Technol Biotechnol* 84: 1390–1396, 2009.
- [12] M. S. Tanyildizi, Ö. Dursun and E. Murat, "Optimization of α -amylase production by *Bacillus* sp. Using response surface methodology", *Process Biochemistry*, vol. 40, pp. 2291-2297, 2005.
- [13] Z. Long, J. Xu and J. Pan, "Significant Improvement of *Serratia marcescens* Lipase Fermentation, by Optimizing Medium, Induction, and Oxygen Supply", *Appl Biochem Biotechnol* vol. 142, pp.148–157, 2007.