FOUR-FACTOR RESPONSE SURFACE OPTIMIZATION OF THE ENZYMATIC SYNTHESIS OF WAX ESTER FROM PALM KERNEL OIL

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ABSTRACT

The synthesis of wax ester using refined, bleached and deodorized (RBD) palm kernel oil (PKO) and oley alcohol catalyzed by Lipozyme IM was carried out. Response surface methodology (RSM) based on a five-level, four-factor central composite rotatable design (CCRD) was used to evaluate the interactive effects of synthesis, of reaction time (5-20 h), temperature (20-50 ^{o}C), amount of enzyme (0.1-0.2 g) and substrate molar ratio (palm kernel oil to oleyl alcohol, 1:1-1:5) on the percentage yield of wax esters. The optimum condition conditions derived via RSM were reaction time 8.46 h, temperature 44.4 ^{o}C, amount of enzyme 0.182 g, substrate molar ratio 1 to 3.7. The actual experimental yield was 92.9 % under optimum condition, which good accordance to the maximum predicted value of 92.4 %.

Keywords: *response surface methodology, central composite rotatable design, palm kernel oil, lipozyme, alcoholysis, wax ester*

INTRODUCTION

Wax esters are long chain esters that are derived from fatty acids and alcohols with chain lengths of 12 carbons or more [1]. Wax usually refers to a substance that is a solid at ambient temperature and that, on being subjected to slightly higher temperatures, becomes a low viscosity liquid. Wax esters can be classified into solid wax esters (solid at room temperature) and liquid wax esters (liquid at room temperature). The properties depend on the carbon chain length and degree of unsaturation of the esters. Increasing the carbon chain length and saturation will increase the melting point of the esters.

Wax esters are important ingredients in the cosmetic formulations such as cleansers, conditioners and moisturizers in pharmaceuticals [2], as an anti foaming agent in the production of penicillin, a timed release in the production pharmaceutical tablet [3] and as lubricants, plasticizers and polishes [1,4]. This is due to their unique property of having excellent wetting behavior without the oily feeling.

Wax esters can be synthesized chemically [5, 6] and enzymatic methods [7]. However, the use of homogeneous chemical catalyst may lead to several problems such as corrosion of equipments, hazards of handling of the corrosive acids, high-energy consumption and degradation of esters [8, 9]. Meanwhile, the enzymatic synthesis has attracted attention because of the mild reaction conditions under which an environmentally friendly process. Furthermore, the use of immobilized enzymes can withstand high temperature and organic solvents that are normally used in industrial process.

Palm kernel oil is one type of oils/fats that can be extracted from oil palm fruit. Liquid at room temperature, these oils/fats can be fractionated into semi solid and liquid fractions to yield kernel stearin and kernel olein, respectively. They can also be processed through physical and chemical refining, to yield either refined, bleached and deodorized (RBD) or neutralized, bleached and deodorized (NBD) oils. Combination of these processes lead to various types of palm kernel oil products (POP) [10].

Enzymatic synthesis of wax esters from palm kernel oil and oleyl alcohol was studied using a commercial immobilized lipase. The conventional method of optimization involves varying one parameter at a time and keeping the others constants. This often does not bring about the effect of interaction of various parameters as compared to factorial design [11]. On the other hand, carrying out experiment with every possible factorial combination of the test variables is impractical because of the large number of experiments required.

Response surface methodology (RSM) is a useful model for studying the effect of several factors influencing the responses by varying them simultaneously and carrying out a limited number of experiments. RSM comprising a five-level, four-factor CCRD was used in our work to evaluate the interactive effects and to obtain the optimum conditions for the enzymatic synthesis of wax ester from palm kernel oil**.** The substrates and parameters studies were selected in previous study.

EXPERIMENTAL SECTION

Material

Immobilized lipase from *Mucor miehei* (Lipozyme) was produced by Novo Nordisk (Denmark). Palm oil (MW = 3 x average of saponification

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equivalent of palm kernel oil) was obtained from Southern Edible Oil Sdn. Bhd. (Malaysia). Fatty acid compositions of Malaysia palm kernel oil are Saturated: Caproic C6:0 (0.3%), Caprylic C8:0 (4.4%), Capric C10:0 (3.7%), Lauric C12:0 (48.3%), Myristic C14:0 (15.6%), Palmitic C16:0 (7.8%), Stearic C18:0 (2.0%), Monounsaturated: Oleic C18:1 (15.1%), Polyunsaturated: Linoleic C18:2 (2.7%) [12]. Oleyl alcohol was obtained from Fluka Chemika (Switzerland). Ester standards, oleyl laurate, oleyl myristate, oleyl palmitate, oleyl stearate, oleyl oleate, oleyl linoleate and methyl linoleate were obtained from Sigma Aldrich (USA). Hexane was obtained from J.T. Baker (USA). All other chemicals were of analytical grade.

Procedure

Experimental Design

A five-level-four-factor CCRD was employed in this study, requiring 30 experiments [13]. The fractional factorial design consisted of 16 factorial points, 8 axial points and 6 center points. The variable and their levels selected for the wax esters synthesis were: time (5 – 20 h); temperature (30 – 60 ^{o}C); amount of enzyme (1 - 2% w/v); substrate molar ratio (palm oil to oleyl alcohol, 1:1 - 1:5).

Synthesis and Analysis

Different molar ratios of palm kernel oil and oleyl alcohol were added to 10 mL *n*-hexane, followed by different amounts of enzyme. The mixture of palm kernel oil, oleyl alcohol and Lipozyme IM were incubated in a horizontal water bath shaker (150 rpm) at different reaction temperature and reaction times. Before sample analysis, linoleic methyl ester was added to each sample as internal standard. Injecting a micro liter aliquot in a splitess mode into a Shimadzu GC-9a, Japan. Gas chromatograph equipped with a flame ionization detector and a BPX-5 fused silica capillary column. Injector and detector temperature were set at 280 and 320 ^{o}C, respectively. The temperature program was 200 ^{o}C, hold for 2 min and increased up to 300 ^{o}C at rate of 10 ^{o}C/min and hold for 13 min. Helium was used as carrier gas. The concentrations of esters were calculated by equation: $C_{\mathbf{x}} = (A_{\mathbf{x}}/A_{IS}) \times (C_{IS} x D_{Rf\ IS}/D_{Rf\mathbf{x}})$, where C is the amount of component **x** or internal standard, A is area for component **x** or internal standard and D_{Rf} is detector response factor for component **x** or internal standard ($D_{Rf\ \mathbf{x}} = A_{\mathbf{x}}/C_{\mathbf{x}}$ and $D_{Rf\ IS} = A_{IS}/C_{IS}$). The percentage yield of ester was calculated by

$$\text{Yield (\%)} = \left(\frac{\text{mmol ester}}{\text{mmol palm kernel oil used}} \right) \times 100\%$$

Data Analysis

The data from the experiments performed are analyzed using Design Expert 6.06 version and then interpreted. Three main analytical steps: analysis of variance (ANOVA), a regression analysis and the plotting of response surface were performed to establish an optimum condition for the alcoholysis.

Triplicate experiments were set up for each run with all 30 runs performed in random order. The reactions were catalyzed by various amounts of Lipozyme. Concentration of PKO was kept constant at 1 mmol while concentration of oleyl alcohol was varied according to Table 5. The percentage yield of ester was determined using GC as described earlier**.**

Statistical and Graphical Analyses

The results of the full factorial experiments and CCRD were analyzed using Design Expert Version 6.0.4 (Stat-Ease Inc. Statistics Made Easy, Minneapolis, MN, USA) software. The linear, quadratic and cubic effects of each four factor under study, as well as their linear interactions, on alcoholysis were calculated. Their significance was evaluated by analysis of variance (ANOVA).

A surface, describes by a first- or a second-order polynomial equation was fitted to each set of experimental data points. First- and second-order coefficients were generated by regression analysis. The results of the full factorial experiments were used to establish first-order models. Second–order polynomials were fit to the experimental data of CCRD.

The goodness of fit of the models was evaluated by the determination of R^2 coefficients complemented by the graphic plot of predicted values by the model vs observed experimental values. High values of R^2 suggest a good fit of the model to the experimental data points.

Optimization of reaction and model validation

For every model selected, a set of optimal reaction condition was generated using the numerical optimization function in the Design Expert software based on an objective function called desirability (Design Expert Version 6.0.4 User Guide). The overall desirability (D) is the geometric (multiplicative) mean of all individual desirabilities (d_i) that range from 0 (least) to 1 (most). If any of the responses fell outside their desirability range, the overall function becomes zero. Experiments were then carried out under the recommended conditions and the resulting yields compared to those predicted by the software.

RESULT AND DISCUSSION

Experimental design

Experiments were conducted using a central composite design (2^4 + star) that helps in investigating linear, quadratic or cubic and cross product effects of four factors, each varied at five levels and also includes six center points for replication [14]. The factors studied

Table 1. Central composite rotatable quadratic polynomial model, experimental data, actual and predicted values for 5-level-4-factor response surface analysis of palm kernel oil.

No	Temp. (^oC)	Time (h)	Molar ratio (mmol)	Amount of enz. (g)	Act. Yield (%)	Pred. Yield (%)
1	30 (-1)	5 (-1)	2.0 (-1)	0.1 (-1)	39.6	33.4
2	50 (1)	5 (-1)	2.0 (-1)	0.1 (-1)	59.2	53.5
3	30 (-1)	15 (1)	2.0 (-1)	0.1 (-1)	77.7	66.4
4	50 (1)	15 (1)	2.0 (-1)	0.1 (-1)	66.6	71.2
5	30 (-1)	5 (-1)	4.0 (1)	0.1 (-1)	58.5	50.8
6	50 (1)	5 (-1)	4.0 (1)	0.1 (-1)	77.9	78.2
7	30 (-1)	15 (1)	4.0 (1)	0.1 (-1)	73.7	75.1
8	50 (1)	15 (1)	4.0 (1)	0.1 (-1)	81.9	87.4
9	30 (-1)	5 (-1)	2.0 (-1)	0.2 (1)	74.4	61.2
10	50 (1)	5 (-1)	2.0 (-1)	0.2 (1)	76.8	68.6
11	30 (-1)	15 (1)	2.0 (-1)	0.2 (1)	85.2	78.1
12	50 (1)	15 (1)	2.0 (-1)	0.2 (1)	70.3	70.2
13	30 (-1)	5 (-1)	4.0 (1)	0.2 (1)	84.8	73.2
14	50 (1)	5 (-1)	4.0 (1)	0.2 (1)	84.5	88.0
15	30 (-1)	15 (1)	4.0 (1)	0.2 (1)	83.6	81.5
16	50 (1)	15 (1)	4.0 (1)	0.2 (1)	81.8	81.1
17	20 (-2)	20 (0)	3.0 (0)	0.15 (0)	21.4	42.9
18	60 (2)	10 (0)	3.0 (0)	0.15 (0)	69.5	62.5
19	40 (0)	0.0 (-2)	3.0 (0)	0.15 (0)	37.7	54.7
20	40 (0)	20.0 (2)	3.0 (0)	0.15 (0)	83.2	80.8
21	40 (0)	10.0 (0)	1.0 (-2)	0.15 (0)	39.5	55.7
22	40 (0)	10.0 (0)	5.0 (2)	0.15 (0)	85.6	83.9
23	40 (0)	10.0 (0)	3.0 (0)	0.05 (-2)	76.2	78.4
24	40 (0)	10.0 (0)	3.0 (0)	0.25 (2)	87.5	99.9
25	40 (0)	10.0 (0)	3.0 (0)	0.15 (0)	86.6	87.1
26	40 (0)	10.0 (0)	3.0 (0)	0.15 (0)	86.8	87.1
27	40 (0)	10.0 (0)	3.0 (0)	0.15 (0)	85.0	87.1
28	40 (0)	10.0 (0)	3.0 (0)	0.15 (0)	91.8	87.1
29	40 (0)	10.0 (0)	3.0 (0)	0.15 (0)	86.3	87.1
30	50 (0)	10.0 (0)	3.0 (0)	0.15 (0)	85.9	87.1

were enzyme concentration, substrate concentration, temperature and reaction time.

The assay conditions for reaction parameter were taken at zero level (center point) and one level (+1 and –1). The design was extended up to $\pm \alpha$ (axial point) of 2. The center values for variables were based on previous studies and were carried out at least six times for the estimation of error.

The experiments were produced in random order and triplicate measurements of alcoholysis percentage yields and were run on each experiment. Experimental data for Lipozyme-catalyzed synthesis of ester from palm kernel oil with oleyl alcohol is given in Table 1. The predicted values were obtained from model fitting technique using the software Design Expert version 6.06 which is to be sufficiently correlated to the observed values.

Fitting model and ANOVA

Fitting of the data to various models (linear, quadratic and cubic) and their subsequent ANOVA showed that reactions of palm kernel oil with oleyl alcohol was most suitably described by quadratic polynomial model (Table 2).

The statistical equation, the quadratic polynomial of palm kernel oil alcoholysis, given by design expert, is as shown below:

$$\text{Yield (\%)} = 87.1 + 4.91\,A + 6.51\,B + 7.05\,C + 5.37\,D - 8.59\,A^2 - 4.83\,B^2 - 4.31\,C^2 + 0.52\,D^2 - 0.0760\,AB - 3.8\,AC - 3.17\,AD - 2.15\,BC - 4.03\,BD - 1.33\,CD \quad (1)$$

The tabular value of $F_{0.01(14,15)}$ for palm kernel oil alcoholysis was 3.56. implying the model are significant at 1% confidence level. This value was lower than the computed F-value of 3.74. The analysis of variance (ANOVA) indicated that the quadratic polynomial model was significant and adequate to represent the actual relationship between percentage yield and the significant variables, with small P-value (0.007).

A coefficient of determination (R^2) value of 0.777 showed equation to be not too highly reliable. However, this value is still above the 0.75 values generally considered to be sufficient for adequately representing

Table 2. ANOVA for joint test of palm kernel oil

Source	Sum of squares	Degree of freedom	Mean square	F- Value	Prob > F
Model	7056	14	504	3.74	0.00798[a]
Temperature (A)	578	1	578	4.29	0.0559[b]
Time (B)	1020	1	1020	7.56	0.0149[a]
Molar ratio (C)	1190	1	1190	8.86	0.00942[a]
Amount of Enzyme (D)	692	1	692	5.13	0.03871[a]
Residual	2020	15	135	-	-
Lack of fit	1990	10	199	34	0.00057[a]
Pure error	29.3	5	5.86	-	-
Cor Total	9070	29	-	-	-

[a] *Significant at "Prob>F" less than 0.05* [b] *Insignificant at "Prob>F" more than 0.05*

the real relationship among the factors [15]. Lower values of R^2 (0.565 - 0.720) has been reported by Kiran *et al*. [16] for lipase-catalyzed synthesis of steoryl and palmitoyl lactic ester as well as by Osario *et al*. [17] for Novozyme-catalyzed transesterification of palm oil stearin with soybean oil (R^2 =0.566).

Regression analysis

Coefficients of the full model of Eq. 1 was evaluated by regression analysis and tested for their significance. The insignificant coefficients were eliminated on the basis of P-value after examining the coefficients, and the model was finally refined. Subsequent regression analysis of each set of data then generated corresponding sets of coefficients for developing model equations (Table 3).

Negative values of coefficient estimates denote negative influences of the parameters on the reaction. It was observed that all the linear coefficients of palm kernel alcoholysis were positive effects. It is interesting to note that linear effects of temperature on these sections were not significance. It was clearly observed that from the whole linear coefficients, the molar ratio of palm kernel oil alcoholysis has the biggest effect with an estimated effect of 7.05. Increasing the molar ratio of palm kernel oil alcoholysis markedly increased the percentage yield. The next largest linear effects are reaction time and amount of enzyme. The variables; time, molar ratio and amount of enzyme are the most significant in the process. Temperature had a less significant effect on these reactions (Table 3). These results indicated that time, molar ratio and amount of enzyme were the more important variables.

Parameter effects

Interactive effect of reaction time and temperature

As shown in Table 3, the effect of the interactions between reaction time and temperature of palm kernel oil alcoholysis are negative effects. Meanwhile, Fig. 1 shows the response surface plots as function of time, temperature and interaction on ester synthesis from

Table 3. Values and significance of regression coefficients of coded factors for palm kernel oil alcoholysis.

Factor	Coefficient Estimate	Prob > F
Intercept	87.1	-
A	4.91	0.0559
B	6.51	0.0149*
C	7.05	0.0094*
D	5.37	0.0387*
A^2	-8.59	0.00149*
B^2	-4.83	0.0454*
C^2	-4.31	0.0707*
D^2	0.524	0.816
AB	-3.80	0.210
AC	1.85	0.533
AD	-3.17	0.293
BC	-2.15	0.471
BD	-4.03	0.185
CD	-1.33	0.654

*Significant at P-value less than 0.05

palm kernel oil alcoholysis at molar ratio 1:3 and amount of enzyme, 0.15 g.

The percentage yield increase with increased the incubation time but decrease if the temperature was above 50 ^oC (negative effect). Habulin *et al*. [18] proposed that Lipozyme at 50 ^oC existed in equilibrium between inactive and active forms. The alcoholysis reaction of palm kernel oil was carried out at temperatures ranging from 30 to 50 ^oC for 5 to 15 h (Fig 1). Increasing the reaction time has a positive effect. Percentage yields were at maximum on moderate temperature (40 ^oC) and incubation period at 10 h. At higher temperatures, an increase in reaction time may cause a slight decrease in the percentage yields of alcoholysis reaction (negative effect but not significantly). Yields cannot be enhanced at these elevated temperatures without affecting enzyme activity. Thermal inactivation at high temperature and long times could explain these results. The interrelationship between the conformational stability and enzyme activity explains that enzymes cannot exhibit stability at temperatures for above these [19].

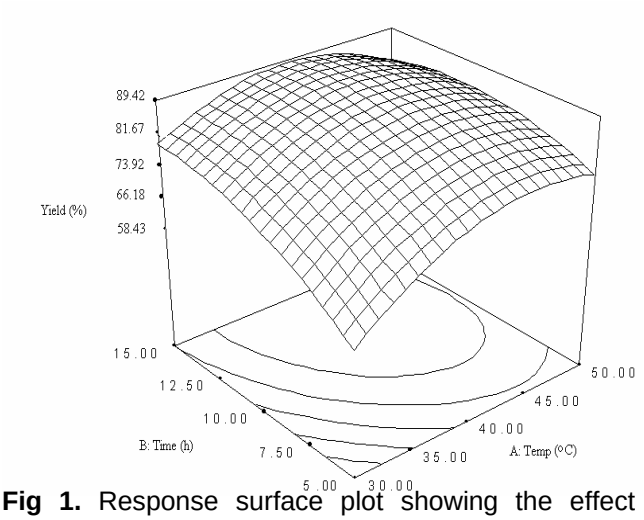

Fig 1. Response surface plot showing the effect of incubation time, temperature and their mutual effect on the synthesis of esters from palm kernel oil alcoholysis. Other variables are constant: enzyme, 0.15 g and molar ratio palm kernel oil: oleyl alcohol (1:3)

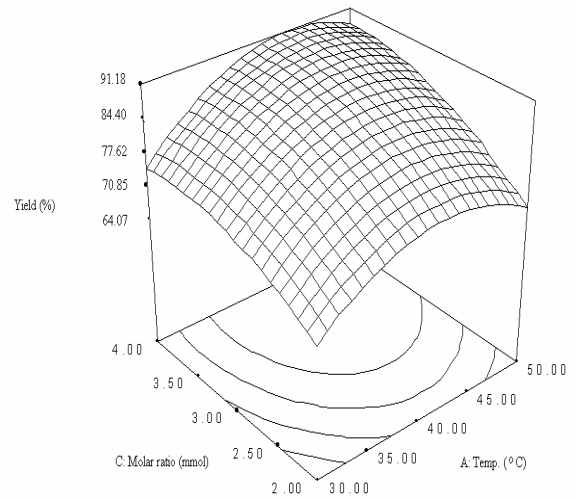

Fig 2. Response surface plot showing the effect of molar ratio, temperature and their mutual effect on the synthesis of esters from palm kernel oil alcoholysis. Other variables are constant: enzyme, 0.15 g and incubation time, 10 h.

Interactive effect of substrate molar ratio and temperature

The effect of varying molar ratio and reaction temperature on alcoholysis at a constant reaction time (10 h), and amount of enzyme at 0.15 g is shown in Fig. 2. The temperature versus substrate molar ratio plots for palm kernel oil alcoholysis showed the positive impact of temperature at high levels of the acyl donor. Low percentage yield was predicted at low substrate, low temperature and high substrate, low temperature. With increase in substrate concentration (1:1-1:3 mmol, oil:oleyl alcohol) for alcoholysis reaction, ester conversions increased at moderate temperature of 40 ^{o}C. Thereafter, the conversion decreased further up to optimum temperature.

Typical plot like this is dome shaped. Many lipases catalyzed esterification system exhibit this type of plots [20]. Similar model was shown by Gunawan et al [21]. In this plot, while in one axis there is a linear increase in alcoholysis, other axis showed the increment to be only up to extent, which decreases thereafter. This indicates that a critical temperature is involved up to which alcoholysis is favored and it is not so after that critical temperature. In general, the increment of substrate concentration lowered the alcoholysis capacity of lipases. This effect has been reported in the biosynthesis of isoamyl acetate isoamyl isovalerat [22] and ethyl esters of short-chain fatty acids [23]. Rodriguez-nogales *et al*. [24] mentioned that the lowest conversions at higher substrate concentrations are attributed to the accumulation of water during the progress of the reaction, which favors the backward reaction (hydrolysis). Alcohols are reported to be terminal inhibitor of lipases and their effects could increase at high temperatures due to the increase of substrate solubility in the reaction time [22].

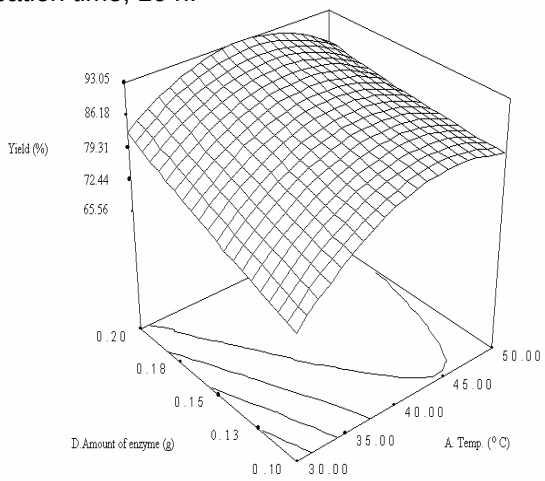

Fig 3. Response surface plot showing the effect of amount of enzyme, temperature and their mutual effect on the synthesis of esters of palm kernel oil alcoholysis. Other variables are constant: molar ratio palm oil: oleyl alcohol, (1:3) and incubation time, 10 h.

Interactive effect of varying amount of enzyme and temperature

Response surface plot depicting the interactions between temperature and biocatalyst amount (Fig. 3) with other parameters fixed at their center points, oil substrate = 1 mmol, reaction time = 10 h and hexane as a solvent. Similarly, the typical plot is dome shaped.

An increase in yield with increase in temperature and enzyme amount was observed. Moderate temperatures (40 ^{o}C) and amounts of enzyme (0.15 g) appear to be the least favorable conditions for the reactions. At low temperature, the negative effect of high medium viscosity retarding the reaction was offset by high enzyme stability while at high temperature; enzyme instability was compensated for by the benefits

of low viscosity. At intermediate temperatures then, insufficient lowering of medium viscosity coupled with deceasing enzyme stability may have led to observed minimum [25].

Thermal inactivation of enzyme was observed as the surface decrease when reaction temperature increases, reaching a minimum at the 45 ^{o}C. Chiang *et al.* [26] had published an increase in temperature up to 55 ^{o}C resulted in less alcoholysis there after at any given amount of enzyme, which was because of inactivation of enzyme at temperature over 55 ^{o}C. Similar result was reported by Gunawan *et al*.[27]

Palm kernel oil alcoholysis has a negative effect (Table 3) for their interaction but the value is non significant. Hari Khrisna *et al.* [22] had published a negative effect for temperature versus enzyme/substrate ratio profile for the Lipozyme-catalyzed synthesis of isoamyl isobutyrate. The authors reported that high temperatures reduced the operational stability of the enzyme, since no esterification activity was observed with the enzyme recovered after the reaction at 60 ^{o}C for 24h. Inactivation of amylase above 105 ^{o}C was also observed with all enzyme concentration in Govindasamy *et al.* [28] enzymatic hydrolysis of sago starch. Garcia *et al*. [29] reported the Novozyme-catalyzed synthesis of ester, where it was shown that temperature and catalyst interaction was positive and at high levels of catalyst concentration showed negative interaction between factors.

Interactive effect of reaction time and substrate molar ratio

Substrate molar ratio of oil to alcohol and reaction time were investigated in the range of 1: 1 - 1: 5 and 0 – 20 h for palm kernel oil alcoholysis. Fig 4 shows the response surface plot (in the significant ranges) as a function of time versus substrate molar ratio at temperature 40 ^{o}C.

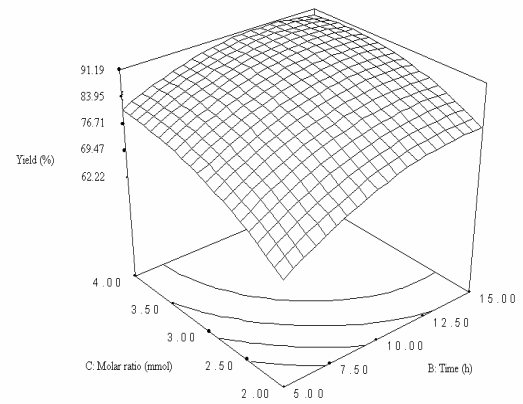

Fig 4. Response surface plot showing the effect of molar ratio, time and their mutual effect on the synthesis of esters from palm kernel oil alcoholysis. Other variables are constant: enzyme, 0.15 g and temperature, 40 ^{o}C

A reaction with moderate substrate molar ratio 1: 3 and highest reaction time favored maximal yield and decreases up to substrate molar ratio 1: 3.5. This may be due to, around critical molar ratio, competing alcohol binding reduces the formation of the acyl – enzyme complex and thereby the result was decreased in alcoholysis reaction [20]. As can be observed from the interactions, the effect of reaction time and substrate molar ratio are positive. However, the interaction between them is negative effect and significant (Table 3). The coefficient estimate of reaction time is higher compared with substrate molar ratio.

Interactive effect of reaction time and amount of enzyme

The response surface plots, as a function of time versus amount of enzyme at temperature 40 ^{o}C is shown in Fig 5. The esters production increased with increase in amount of enzyme and incubation time. The Figure also shows that the reaction rate is increased proportionally with enzyme loading. The influence of the reaction time and amount of enzyme is statistically significant in the range study. Alcoholysis of esters from palm kernel oil (Fig 5), at fixed 1: 3 molar ratio (oil: alcohol) and temperature 40 ^{o}C, the negative effect for interactions between reaction time and amount of enzyme occurs at long reaction time (7.5 h). Kiran *et al*. [16] detected in porcine pancreas lipase, the major activity loss was within 48 h of incubation time. Their studies found that for longer periods of incubation up to 10 h have clearly brought out the differential behavior of probable unfolding of the enzyme.

Interactive effect of varying amount of enzyme and substrate molar ratio

Fig 6 represents the effect of varying amount of enzyme and substrate molar ratio on palm kernel oil alcoholysis at 40 ^{o}C for 10 h. At low amount of enzyme

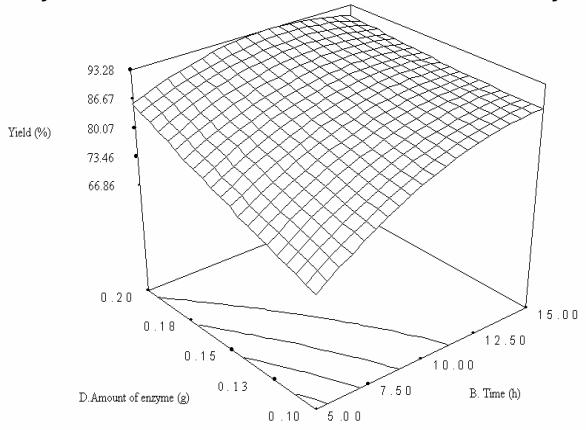

Fig 5. Response surface plot showing the effect of amount of enzyme, time and their mutual effect on the synthesis of esters from palm kernel oil alcoholysis. Other variables are constant: molar ratio palm oil: oleyl alcohol, (1:3) and temperature, 40 ^{o}C.

Table 4. Solutions of optimum conditions of palm kernel oil

Exp.	*Temp. (^{0}C)*	*Time (h)*	*Molar ratio (mmol)*	*Amount of enz. (g)*	*Predicted Yield (%)*	*Actual Yield (%)*
1	*42.5*	*9.25*	*3.59*	*0.191*	*93.9*	*92.5*
2	*44.2*	*10.8*	*3.78*	*0.157*	*91.8*	*92.3*
3	*38.5*	*8.81*	*3.24*	*0.2*	*92.7*	*89.7*
4	*37*	*9.96*	*2.64*	*0.2*	*89.2*	*87.6*
5	*44.4*	*8.46*	*3.7*	*0.182*	*92.4*	*92.9*

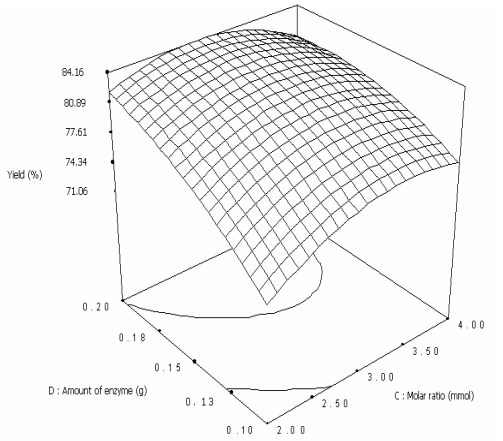

Fig 6. Response surface plot showing the effect of amount of enzyme, molar ratio and their mutual effect on the synthesis of esters from palm oil alcoholysis. Other variables are constant: incubation time, 5h and temperature, 50 ^{o}C.

and low molar ratio, the yield was lower. Increasing amount of enzyme will lead to increase of percentage yields. Reaction with high amount of enzyme and substrate molar ratio 1: 3-3.5 referred maximal percentage yields. Presence of larger amount of substrates generally increases the probability of substrate enzyme collision [30]. This relationship holds when there are no limiting factors such as a low as substrate concentration, presence of activators or inhibitors or mass transfer effect.

The percentage yields slight decrease at substrate molar ratio 1: 4. It is well known that hydrophilic solvents have a capability of stripping off even the essential water from the enzyme surface, leading to an insufficiently hydrated enzyme molecule and in turn to a decrease in enzyme activity [31]. However, two workers, (Shieh *et al*. [32] and Krishna *et al*., [22] reported that even at high substrate levels and low enzyme concentration, high conversion could be achieved which is relevant from the economic point. Considering the cost of enzyme is usually higher than that of substrate.

The percentage yield was increased with increasing molar ratio and amount of enzyme. This result indicated that Lipozyme was not denaturated by excess alcohol. Hari Krishna et al. [22] suggested that excess alcohol (as nucleophile) generally pushes the equilibrium of the reaction to the product side. The high solubility of product side in alcohol also minimizes the product side accumulation over the enzyme surface leading to high percentage yields.

Optimization of Reaction by RSM

The optimum conditions for the Lipozyme-catalyzed synthesis of esters from alcoholysis of palm kernel oil were predicted using the optimization function of the Design Expert Software. These are presented in Table 4 along with their predicted and actual values. On palm kernel oil alcoholysis, the values for the four parameters were taken from the contour plot which yielded values close to 90% esters, the maximum possible value for the production of esters.

The higher value was predicted to be near a combination of 0.191 g amount of enzyme and 3.59 substrate molar ratios (experiment 1). With the same reason as above, experiment 2 was choice of the optimum condition. However, for all the experiments, the predicted and experimental values matched closely. This indicates that the generated model adequately predicted the esters yield. Thus the optimum conditions for maximum esters production were successfully developed by shell design and RSM.

CONCLUSION

In this study, a five-level and four-factor CCRD has been applied to optimize the synthesis process of was ester. Comparison of predicted and experimental values revealed good correspondence between them, implying that empirical models derived from RSM can be used to adequately describe the relationship between the factors and response in Lipozyme-catalyzed synthesis of wax ester from palm kernel oil and oleyl alcohol. These models can then be used to predict wax ester yield under any given conditions within the experimental range.

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