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OPTIMIZATION OF LIPASE-CATALYZED ESTERIFICATION FOR THE PRODUCTION OF MEDIUM-CHAIN ACYLGCEROLS FROM PALM OIL FATTY ACID DISTILLATE: USING RESPONSE SURFACE METHODOLOGY

Zanariah Mohd Dom[1], Ling Tau Chuan[2] and Rozita Yusoff[2]

[1,]Department of Process and Food Engineering, Faculty of Engineering, UPM Serdang, Malaysia,
[2]Department of Chemical Engineering, Faculty of Engineering, UM Kuala Lumpur. Malaysia
Email: nana@eng.upm.edu.my

ABSTRACT

Optimization of lipase-catalyzed esterification for the production of medium-chain acylglycerols (MCA) from palm oil fatty acid distillate (PFAD) and glycerol was carried out in order to determine the factors that have significant effects on the reaction system and MCA yield. Oil palm mesocarp lipase was used as catalyze in the esterification reaction. Response surface methodology (RSM) was applied to optimize the reaction conditions. The reaction conditions, namely, the reaction time (30-240 min), enzyme load (0.5-1.5kg), silica gel load (0.2-1.0 kg), and solvent amount (200-600 vol/wt). Reaction time, enzyme loading and solvent amount strongly effect MCA synthesis ($p<0.05$). However, water absorbent (silica gel) loading did not have significant ($p>0.05$) influence on MCA yield. Best-fitting models were successfully established for MCA yield (R^2 =0.9133). The optimum MCA yield were get 75% by prediction and 75.4% by experimental run by using 6kg enzyme loading, a reaction time of 135min and a solvent amount of 350 vol/wt at 65^{o}C reaction temperature.. Verification experiments under optimized reaction conditions were conducted, and the results agreed well with the range of predictions.

Keywords*: Esterification, lipase, Medium-chain acylglycerols (MCA), optimization, Response surface methodology (RSM).*

INTRODUCTION

Acylglycerols (or glycerides) are formed of mono-, di- and triacylglycerol classes. In this context, one may cite the production and application of MCA where are formed of monoacylglycerols (MAG) and diacylglycerols (DAG) in several industrial segments. These glycerides are the most used emulsifiers in the food, cosmetic and pharmaceutical industries [1,2,3]. Often, mixtures of MAG and DAG are used in these applications, since they are cheap and give proper performance. Various methods have been reported for the production of MAG and DAG using lipase such as the hydrolysis of triolein, the glycerolysis of triacylglycerol, the esterification of fatty acids and glycerol in organic solvents and in a solvent-free system., The glycerol has been widely used in the industry for chemical production of food monoacylglycerols (MAG) and diacylglycerols (DAG) at high temperature. So that the enzymatic approach provides an alternative process due to its mild performance conditions, the regioselectivity of the lipases and the low environmental impact.

Lipase or acylglycerol acylhydrolase (EC.3.1.1.3) have been defined as enzymes that hydrolyze esters of long-chain fatty acids from acylglycerol. Today, lipases stand amongst the most important biocatalyst carrying out novel reactions in both aqueous and non-aqueous media. In addition to their biological significance, lipase hold tremendous potential for exploitation in biotechnology.

Palm oil fatty acid distillate (PFAD) is a by-product from the physical refining of crude palm oil [4,5]. In Malaysia alone, it was estimated that about 402,500 metric tons of PFAD was generated for the year 2002, and with the rapid increase in global palm oil production, the amount of PFAD produced will rise substantially [6]. Generally , PFAD consists of valuable compounds such as free fatty acids (FFA), vitamin E and phytosterols. Chong *et. al.* conducted an enzymatic esterification reaction by using glycerol and PFAD to produce medium-chain glycerides. It was reported that this lipase-catalyzed reaction produced mainly

diacylglcerols (DAG) followed by monoacyglycerols (MAG) and triacylglycerols (TAG) [7].This further verifies the PFAD as an important commodity which should be evaluated to expand its uses.

In this work, alternative and industrially feasible method to produce MCA for similar applications has been used and optimized in order to obtain the highest yield of MCA at a lower cost. PFAD was used as a source of medium-chain fatty acid to esterify with glycerol [7,8]. The used of a by-product from the palm oil industry enhances the economic viability of this work. As the esterification reaction was catalyzed by lipase, so alternatively, the use of a naturally-bound lipase from oil palm mesocarp can be cost effective because the biomass can be directly, thus eliminating isolation, purification and immobilization procedures. Besides, this naturally-bound lipase offer advantages such as increased stability to organic solvent, high temperature optimum and extreme pH reaction [9].

Response surface methodology (RSM) is an effective statistical technique commonly used for optimization studies. It uses quantitative data in experimental design to determine and simultaneously solve multivariate equations in order to optimize processes or products. The equations describe the effect of test variables on responses, establish the interrelationships among test variables and represent the combined effect of all the test variables in the response. The main advantage of using RSM in an optimization process is the reduced number of experimental runs needed to provide adequate data for statistically reliable results. This technique is much faster and less expensive in gathering research information than the conventional one-variable-at-a-time or full factorial experimentation.

The objectives of this work were to understand the effects and relationships among four factors, which are reaction time, enzyme load, silica gel load and amount of solvent, and to determine the optimum conditions for esterification of PFAD with glycerol.

MATERIALS AND METHODS

Materials

PFAD from Golden Jomalina Food Industries Sdn.Bhd., Selangor, Malaysia. Oil palm mesocarp fibre was a generous donation from the MPOB Experimental Palm Oil Mill in Labu, Negeri Sembilan. The fruit lets were from *Tenera* species where they have bigger fruit lets and thick mesocarps. Glutaraldehyde, isopropyl alcohol, natrium hydrogen phosphate, natrium hydroxide, oleic acid, methanol, ethanol, acetone and phenolptalien were purchased from Sigma-Aldrich Inc., USA. Technical grade of n-hexane was obtained from Kofa Chemical Co. Hexane, a type of organic solvent can dissolve long-chain fatty acids and has been used previously as the reaction medium for short-chain esterifications [10]. Palm cooking oil for hydrolysis experiment was 'Pisau' brand bought from the supermarket.

Identification of reaction products and estimation of the degree of esterification:

Quantitative analysis : Alkaline Titration

The concentration of free fatty acid in the sample of reaction product was quantified by titration with 0.5 M NaOH. All the sample analysis was perfomed in triplicate. The degree of esterification were calculated based on the following equation [8]:

$$\text{Amount of esterified free fatty acid, } \mu\text{mol} = (V_c - V_s)M \times 1000 \quad (1)$$

$$\text{Degree of esterification, } ED = \frac{\textit{Amount of esterified ffa, µmol}}{\textit{Amount of ffa incontrol reaction ,µmol}} \times 100\% \quad (2)$$

$$\text{Activity of lipase (U/g)} = \frac{\textit{Amount of esterified ffa, µmol}}{\textit{8h x 60min x 2g lipase}} \times 100\% \quad (3)$$

Where V_c = volume of NaOH used for the control, ml
V_s = volume of NaOH used for the sample,ml
M = molarity of NaOH solution
ED = degree of esterification.%

Qualitative analysis : Rapid thin-layer chromatographic analysis

Thin layer chromatography (TLC) technique has been widely used for the monitoring of lipase-catalyzed esterification reactions [7,11,12]. Esterification products (MAG, DAG and TAG) were identified by the aid of this simple method. This method facilitates a rapid separation and identification of fatty acids, which are retained at the origin of the chromatogram as their sodium salts from the various acylglycerols, which migrate together close to the solvent front. The TLC enables a rapid yet clear-cut separation of unesterified fatty acids and each of the different classes of acylglycerols that are likely to occur in a lipase-catalyzed esterification reaction. Since one single 4cm x 14cm plate may be subdivided into 3 lanes and the time of development is very fast (5-6 min), this technique is very convenient for the rapid assay of a large number of samples.

Optimization by RSM

The reaction mixture (10 litters), comprising 2.0kg PFADs, 0.335kg glycerol (as fatty/glycerol acid ratio 2:1) and hexane as a solvent, was first loaded into the feeding tank of the PBR. The mixture was heated at the optimum temperature of 65^{o}C. It was then pumped into the packed bed vessel and sprayed over the NIL bed. The reaction mixture was collected at the bottom of the vessel and then recycled between the water removal column and the main reactor, and finally to the product tank. Recycling continued until a satisfactory acylglycerol yield was achieved. The esterified product was then stored in the product tank. Silica gels were used as the water removal agent to prevent the reverse hydrolysis of esterified product caused by the reaction water.

Several parameters such as a reaction time, amounts of enzyme loading, silica gel and solvent were tested. A three-level four-factor fractional experimental design with 30 experiments was conducted for lipase-catalyzed esterification to evaluate the effect of multiple independent factors and their interactions. The factors and parameter ranges selected were based on the results from studies by Chong et al. (2006) [7] and preliminary screening tests (summarized in Table 1).

Table 1:Selected factors and ranges for lipase-catalyzed esterification process optimization using RSM

Factors	Ranges
Reaction time (min)	30-240
Enzyme amount (kg)	0.5-1.5
Solvent amount (vol/wt)	200-600
Silica gel amount (kg)	0.5-1.5

The experiments were conducted in random order and triplicate measurements of esterification percentage were run in each experiment. For generating response surfaces, the experimental data obtained were fitted to a second-order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum i^2 = 1 \sum_{j=i+1}^{3} \beta_{ij} X_{ij} + e \qquad (1)$$

Where
Y= percentage of MCA yield
X_i, X_j = independent factors,
B_o β_j, β_{jj} = regression coefficients,
e= error term

The design of this experiment was carried out by using the Design Expert® Version 7.1.6 (Stat-Ease, Inc., Minneapolis). Meanwhile, the Central Composite Design (CCD) was employed in this experiment with the quadratic models used to study the effects of four independent variables factors was chosen for the optimization were, namely; Reaction time, Enzyme loading and absorbent loading, and solvent amount on the response function of degree of esterification (MCA yield). The experiments performed were analyzed using design-expert in three analytical steps, which are analysis of variance (ANOVA), regression analysis and the plotting of the response surface to obtain the optimum condition for the esterification reaction. The design of the experiments is presented in Table 2.

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RESULTS AND DISCUSSIONS

Esterification activity of lipases

The esterification activity of lipases between various of temperature from 45°C to 75°C was compare in Fig. 1. From the figure, significant differences between the four different temperature were observed based on each respective esterification activity.

The activities of free lipase and immobilized lipase were investigated at various reaction temperatures. The change of the reaction temperature will affect enzymatic rate and functional group of substrate involved in the reaction. Therefore, reactions must be carried out to determine the optimum temperature in order to obtain the best yield. Temperature plays an important role in liquid viscosity and enzyme activity [13]. Generally, higher reaction temperature causes a decrease in viscosity of the reaction mixture and therefore, increases the rate of interaction between the substrates and enzyme molecule. The PFAD is in crystal form at temperatures below 45°C, which caused improper mixing of reaction mixture.

The optimum reaction temperature for most immobilized lipases ranging from 45°C to 65°C [14,15]. The highest degree of esterification was achieved at 65°C in this study. Further increase of temperature to 75°C has resulted in a drastic decrease in esterification. The decrease of esterification degree (acylglycerols yield) at this high temperature may be due to the denaturation of lipase at elevated heat energy.

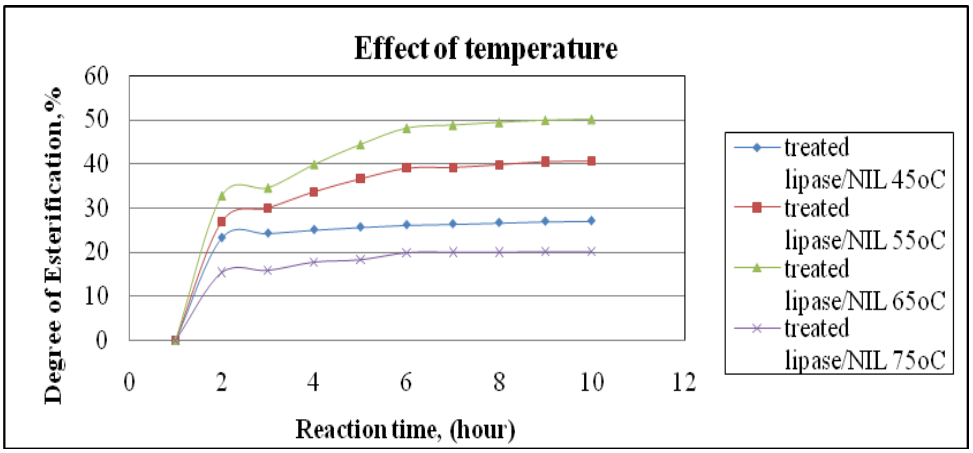

Fig. 1 :The effect of temperature on the degree of esterification with NIL.

Design of Experiment

Response surface methodology (RSM) was applied to the experimental model using four reaction parameters: reaction time, enzyme loading, water adsorbent loading and solvent amount. Table 1 shows the degree of esterification (acylglycerol yield) at each of the 30 runs.

Table 2: Experimental runs based on four-factor, three-level surfaceresponse method and responses obtained in the PBR

Run	Factor 1 Reaction time (min)	Factor 2 Enzyme loading (kg)	Factor 3 Adsorbent loading (kg)	Factor 4 Solvent amount (vol/wt)	Response/ Degree of Esterification (%)
1	30	2.00	0.5	600.00	35.0
2	30	2.00	0.5	200.00	27.3
3	30	2.00	1.5	600.00	37.0
4	30	2.00	1.5	200.00	36.5
5	30	10.00	0.5	200.00	23.0
6	30	10.00	0.5	600.00	42.0
7	30	10.00	1.5	200.00	23.2
8	30	10.00	1.5	600.00	45.0
9	75	6.00	1.0	400.00	48.0
10	135	2.00	1.0	400.00	23.1
11	135	6.00	0.0	400.00	38.2

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12	135	6.00	1.0	400.00	76.2
13	135	6.00	1.0	400.00	76.0
14	135	6.00	1.0	400.00	75.2
15	135	6.00	1.0	400.00	75.8
16	135	6.00	1.0	400.00	74.6
17	135	6.00	1.0	400.00	75.9
18	135	6.00	1.0	0.00	6.0
19	135	6.00	1.0	800.00	43.1
20	135	6.0	2.0	400.0	34.6
21	135	14.0	1.0	400.0	68.6
22	240	2.0	0.5	200.0	28.3
23	240	2.0	0.5	600.0	53.0
24	240	2.0	1.5	600.0	20.6
25	240	2.00	1.5	200.0	35.1
26	240	10.0	0.5	200.0	45.9
27	240	10.0	0.5	600.0	58.5
28	240	10.0	1.5	200.0	30.4
29	240	10.0	1.5	600.0	78.5
30	345	6.0	1.0	400.0	65.8

Model Fitting

Modeling of factors and responses was performed by response surface methodology (RSM) to predict the highest possible degree of esterification or acylglycerol yield. The underlying results for the models are listed in Table 2. A central composite rotatable design is generally the best design for response surface optimization (Montgomery, 1997). The best–fitting model was determined by regression and backward elimination. According to the models, the degree of esterification was affected by first-order variables (main effects) as well as second-order variables (interactions). All model coefficients (β) and probability values (P) were below 0.05 after the models were refined (Table 2). ANOVA demonstrated that the model was satisfactory with a coefficient of determination (R^2) for response of 0.9133. The actual and predicted responses were sufficiently correlated in Fig.2.

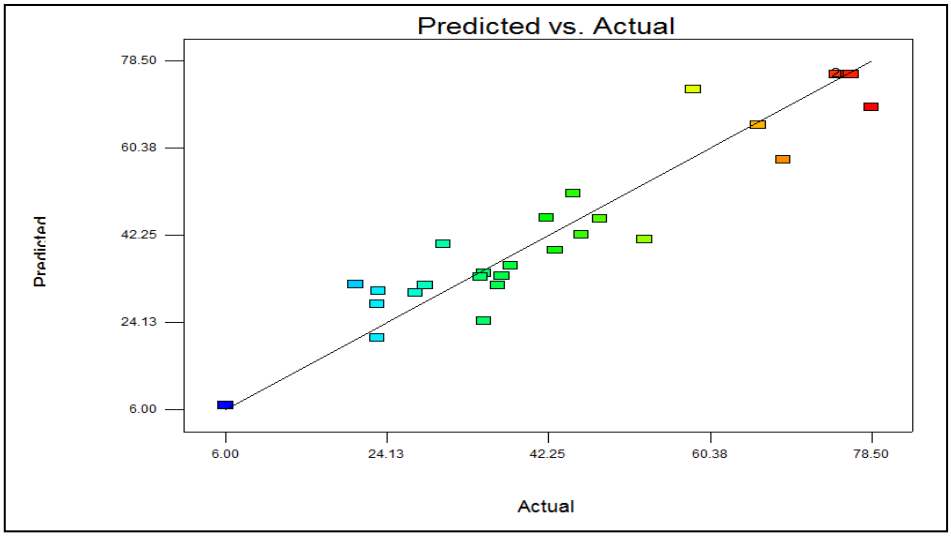

Fig. 2 :Relationship between predicted and actual responses of acylglycerolyield by the developed models. The solid line represents a linear regression line.

Main Effects of Parameters

The major influence of parameters can be evaluated from plots of main effects on the degree of esterification, as described by the analysis of variance (ANOVA) in Table 3. The “Model F-value” of 11.28 indicated that the model was significant. There was 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 indicated that the model terms were significant. Therefore, in this case, A, B, D, AB, BD, A2, B2, C2, D2 were significant model terms. On the other hand,

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values that were greater than 0.1 indicated that the model terms were not significant. The goodness of fit of model was checked by the determination coefficient, R^2. In this case, R^2 was 0.9133 for acylglycerol yield.

Reaction time, enzyme loading and solvent amount were the three factors tested that significantly influenced the degree of esterification (acylglycerol yield) in PBR. These first order coefficients had positive effects on acylglycerol yield. Increased enzyme load resulted in increased acylglycerol yield, until an optimal acylglycerol yield of 78.5% was obtained with an enzyme load of 10kg at 240 minutes reaction time and 600 vol/wt solvent. The acylglycerol yield improved with longer reaction time until an optimum was obtained after 135 min with an acylglycerol yield of 76% (Table 2). However, equilibrium conditions producing constant amount of acylglycerols were expected at a certain time and enzyme dosage. Increased enzyme load resulted in increased acylglycerol yield, until an optimal yield of 76% was obtained with an enzyme load of 6kg. The water absorbent loading was not crucial in a PBR since its effect on acylglycerol yield was insignificant (Table 3)

Table 3: Analysis of variance (ANOVA) for the model representing thedegree of esterification in PBR

Source	Sum of Squares	df	F-value	P-value
Model	11701.30	14	11.28	<0.0001
A-reaction time	571.35	1	7.71	0.0141
B-enzyme loading	1127.51	1	15.22	0.0014
C-adsorbent loading	8.05	1	0.11	0.7462
D-solvent	1569.78	1	21.19	0.0003
AB (time*enzyme)	391.05	1	5.28	0.0364
AC (time* adsorbent)	78.77	1	1.06	0.3188
AD(time*solvent)	29.43	1	0.40	0.5380
BC (enzyme*adsorbent)	30.53	1	0.41	0.5306
BD (enzyme*solvent)	433.68	1	5.85	0.0287
CD (adsorbent*solvent)	3.71	1	0.050	0.8260
A^2 (time*time)	703.83	1	9.50	0.0076
B^2 (enzyme*enzyme)	1680.81	1	22.69	0.0003
C^2 (adsorbent*adsorbent)	2862.42	1	38.64	<0.0001
D^2 (solvent*solvent)	4754.30	1	64.19	<0.0001
Residual	1111.07	15		
Std. Deviation	8.61			
R^2	0.9133			

Optimization

Based on the models generated, it can be construed that acylglycerol yield was influenced not only by first-order variables but also by second-order variables and parameter interactions. The complex relationship between reaction parameters and responses can be well-evaluated by contour plots giving good predictions of optimized conditions. Several optimal combinations are available to obtain the highest acylglycerol yield. Contour plots between different parameters were generated for aclglycerol (MAG and DAG) formation. A pattern with high effect of enzyme amount and reaction time and little effect of water absorbent was seen in Fig 3. The highest possible acylglycerol yield that could be established in this system was predicted to be 75%, requiring an enzyme load of 6kg, solvent amount of 460 vol/wt and reaction time of 135 min. Verification experiments under optimized reaction conditions were conducted, and the results agreed well with the range of predictions (Table 4).

Predicted optimal conditions must be interpreted in the context of industrial operations. The use of solvents increases expenses, therefore an extra step for solvent removal within the process is needed. Extra attention to safety issues is required as well. Accordingly, the lowest possible solvent amount is advantageous from an industrial point of view. A compromise in the solvent amount can easily be made without a dramatic reduction in the predicted acylglycerol yield (Fig.3). Therefore, a solvent amount that is lower than the predicted optimum is recommended. Table 4 shows the experimental result and the predicted optimum.

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Table 4: Experimental verification of model prediction

Run	Reaction time (min)	Enzyme loading (kg)	Solvent amount (vol/wt)	Predicted acylglycerol yield (%)	Experimental acylglycerol yield (%)
1.	135	6	460	76	77.3
2.	130	6	400	74	74.6
3	135	6	350	75	75.4

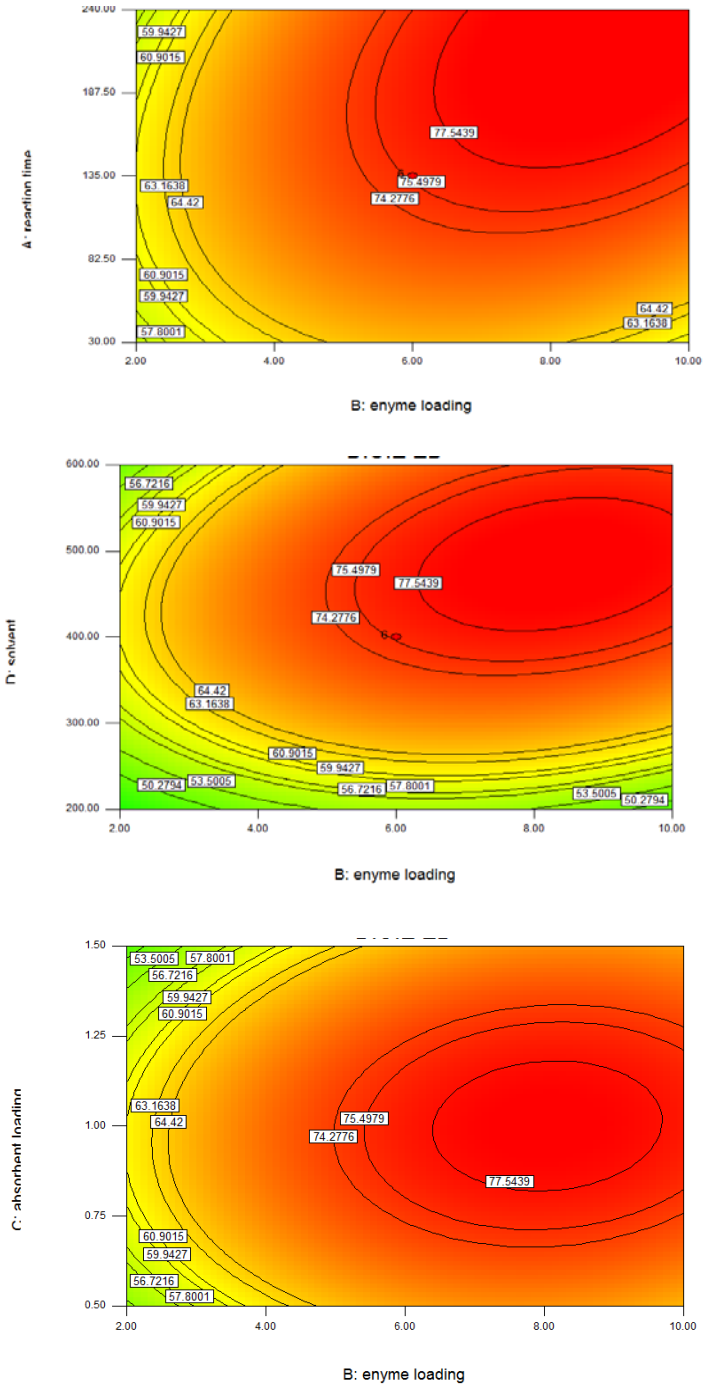

Fig. 3: Predicted responses from contour plots of aclyglycerols at optimized conditions: enzyme loading 6kg, solvent amount 460 vol/wt and reaction time 135min.

Analysis by Thin Layer Chromatography

Esterification products (MAGs, DAGs and TAGs) from the PBR were identified using Thin Layer Chromatography (TLC), a simple method which facilitates a rapid separation and identification of fatty acids. Fatty acids are immobilized as sodium salts and retained at the origin of the chromatogram whereas the various acylglycerols migrate together close to the solvent front (Figure 4). The chromatograms show the successful fractionation of esterified products by TLC.

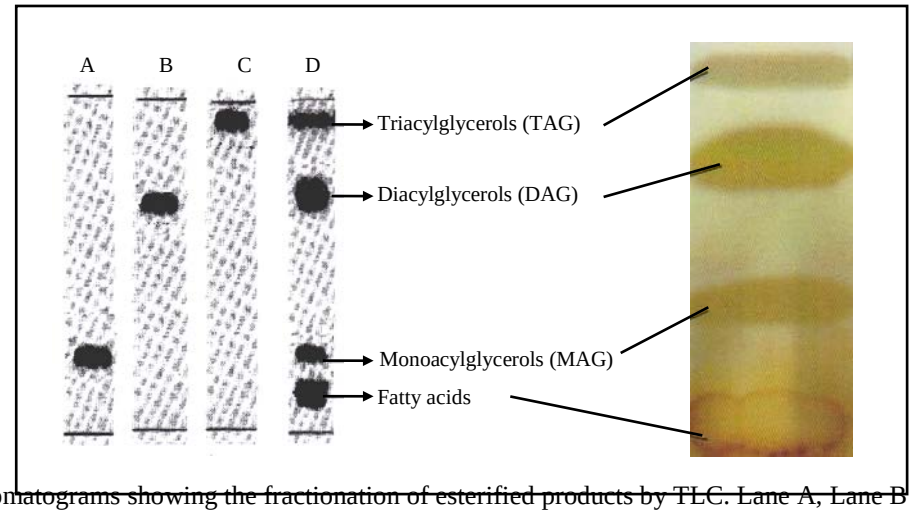

Fig. 4: Chromatograms showing the fractionation of esterified products by TLC. Lane A, Lane B and Lane C show the standard spots of monoacylglycerols (MAG), diacylglycerols (DAG) and triacylglycerols (TAG) respectively. Lane D is the optimum esterification product fractions from reaction at 65^{o}C.

CONCLUSIONS

An alternative method for production of medium-chain acylglycerols by using by- product from local food-processing industries (PFAD and OPML) were have successfully developed. The successful application for production of acyglycerol may have a very important both on both the local economy and environmental sectors in Malaysia.

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