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PRODUCTION OF MEDIUM-CHAIN ACYLGLYCEROLS BY LIPASE ESTERIFICATION IN PACKED BED REACTOR: PROCESS OPTIMIZATION BY RESPONSE SURFACE METHODOLOGY

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Abstract

Medium-chain acylglycerols (or glycerides) are formed of mono-, di- and triacylglycerol classes. In this study, an alternative method to produce MCA from esterifying palm oil fatty acid distillate (PFAD) with the presence of oil palm mesocarp lipase (OPML) which is a plant-sourced lipase and PFAD is also cheap by-product is developed in a packed bed reactor. The production of medium-chain acylglycerols (MCA) by lipase-catalysed esterification of palm oil fatty acid distillate with glycerol are optimize in order to determine the factors that have significant effects on the reaction condition and high yield of MCA. Response surface methodology (RSM) was applied to optimize the reaction conditions. The reaction conditions, namely, the reaction time (30-240 min), enzyme load (0.5-1.5 kg), 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 75% from the predicted value and 75.4% from the experimental data for 6 kg enzyme loading, a reaction time of 135min and a solvent amount of 350 vol/wt at 65ºC reaction temperature. Verification of experimental results under optimized reaction conditions were conducted, and the results agreed well with the predicted range. Esterification products (mono-, di- and triacylglycerol) from the PBR were identified using Thin Layer Chromatography method. The chromatograms showed the successful fractionation of esterified products in this alternative method of process esterification.

Keywords: Esterification, Oil palm mesocarp lipase, Medium-chain acylglycerols (MCA), Packed bed reactor, Response surface methodology (RSM).

Nomenclatures

B_o	Regression coefficient
ED	Degree of esterification, %
e	Error terms
M	Molarity of NaOH solution
V_c	Volume of NaOH used for the control, ml
V_s	Volume of NaOH used for the sample, ml
X_i, X_j	Independent factors
Y	Percentage of MCA yield, %

Greek Symbols

β_o, βj, βjj	Regression coefficients

Abbreviations

DAG	Diacylglycerols
MAG	Monoacylglycerols
MCA	Medium-chain acylglycerols
OPML	Oil palm mesocarp lipase
PFAD	Palm oil fatty acid distillate
RSM	Response surface methodology
TLC	Thin layer chromatography

1. Introduction

Medium-chain acylglycerols (or glycerides) are formed of mono-, di- and triacylglycerol classes. In this study, the production and application of medium-chain acylglycerols (MCA), which are forms of monoacylglycerols (MAG) and diacylglycerols (DAG) used in several industrial segments. The glycerides, are the common emulsifiers in the food, cosmetic and pharmaceutical industries [1-3]. MAGs are minor components which can be found in both animal fats and plant oils [4]. Due to their excellent emulsifying properties, MAGs are widely applied in various food processing fields as an emulsifier.

Their excellent emulsifying properties come from their unique chemical structure. Other than the excellent emulsifying properties, they also have less odour and taste. Less odour and taste are important properties to ensure that the flavour of food products are not affected with addition of MAGs into products. In additions, MAGs are also readily biodegradable and generally recognized as safe (GRAS) [5]. 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 [3]. Hence, in this study, oil palm mesocarp lipase which is a plant lipase is used as the enzymatic approach to provide an alternative process due to its mild performance conditions, the regioselectivity of the lipases and the low environmental impact.

In this works, plant based fatty acid is used. Palm oil fatty acid distillate (PFAD) is by-product from the physical refining of crude palm oil [6, 7]. Currently, PFAD

is selling to pharmaceutical and cosmetic field as drug carriers or as fuel in power plants and industrial boilers. Several studies on utilisation of PFAD are conducted such as using PFAD as feedstock to produce biodiesel, MAGs and DAGs.. Generally, PFAD consists of valuable compounds such as free fatty acids (FFA), vitamin E and phytosterols which has large purpose in many fields when extracted and it as an important commodity which should be evaluated to expand its uses.

In this work, alternative and industrially feasible method to produce medium-chain acylglycerols (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 [8, 9]. As the esterification reaction was catalysed 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 used, thus eliminating isolation, purification and immobilization procedures [10]. Besides, this naturally-bound lipase offer advantages such as increased stability of organic solvent, high optimum temperature and extreme pH reaction [10].

The objectives of this work were to understand the effects and relationships among four factors, which are reaction time, enzyme loading, silica gel loading and amount of solvent, by using response surface methodology (RSM) as an effective statistical technique for optimization studies for esterification of PFAD with glycerol.

2. Materials and Methods

2.1. 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. 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.

2.2. Immobilization of oil palm mesocarp lipase

In the preparation of mesocarp lipase, the pre-treatment of mesocarp fibre need to be done. Oil palm mesocarp fibre was first defatted by dispersing in n-hexane. The hexane was subsequently removed by filtration and the defatted mesocarp fiber was dried in an oven at 40ºC for 5 days. The dried defatted was immersed in 0.05 M phosphate buffer (pH 8) at 25ºC for 1 h. The immobilized oil palm mesocarp lipase was then washed with fresh buffer to remove the unabsorbed soluble enzyme. It was then oven dried at 40 ºC for 5 days and stored at –20°C for subsequent use.

2.3. Stability of solvents

The stability of untreated and immobilized lipase was investigated by incubating them in hexane (without shaking) for 16 hours at room temperature. A sample was taken every hour to calculate the degree of esterification, and then divided by the maximum degree of esterification at first hour. Based on this calculation, the relative degree of esterification at every hour can be obtained**.**

2.4. Esterification process

The esterification of PFAD and glycerol catalysed by oil palm mesocarp lipase (OPML) was performed in a PBR designed and fabricated in house (Fig. 1) [8]. The temperature of the PBR was controlled and maintained by the heating system and water jacket installed. In addition, a water-removal column filled with water-removal agent was used to remove the reaction water. The reactor was designed for down-flow in semi-continuous mode of operation. The reaction mixture (10 l) was optimized by using RSM, consisting of of PFAD, glycerol, and hexane as a solvent, was first loaded into the feeding tank of the PBR. The mixture was preheated to reach the reaction temperature (45, 55, and 65ºC). It was then pumped at a flow rate of 20 l/min to the PBR and sprayed over the surface of the oil palm mesocarp lipase bed. The estimated residence time for the reactant in the PBR was about 1 min. The reaction mixture collected at the bottom of the vessel and was then recirculated back to the top of the PBR via the water-removal column at the same flow rate. The estimated residence time of the partially esterified product in the water-removal column was about 0.1min. The recirculation of the partially esterified mixture was continued until a satisfactory degree of esterification was achieved. The esterified product was then stored in the product tank. Silica gels act as water-removal agents to prevent the reverse hydrolysis of esterified product caused by the reaction water.

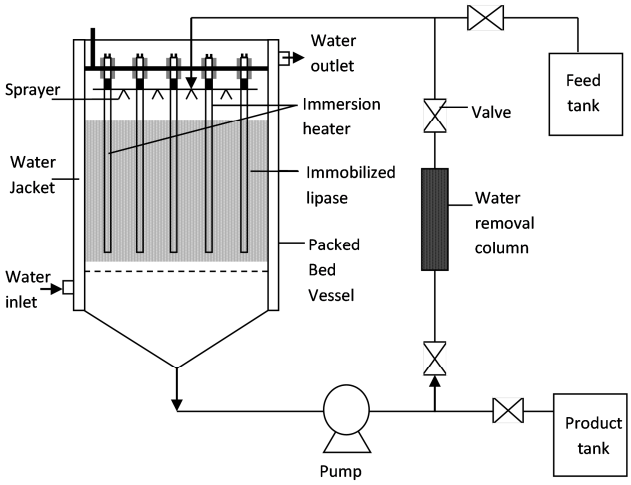


Fig. 1. Schematic Diagram of the Setup of PBR Use for the Esterification Process.

2.5. Identification of reaction products and estimation of the degree of esterification.

2.5.1. 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 performed in triplicate. The degree of esterification were calculated based on the following equations [8]:

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

$$ED = \frac{\text{Amount of esterified } ffa, \mu\text{mol}}{\text{Amount of } ffa \text{ in control reaction}, \mu\text{mol}} \times 100\% \quad (2)$$

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

where V_c is volume of NaOH used for the control (ml), V_s is volume of NaOH used for the sample (ml), M is molarity of NaOH solution, and ED is degree of esterification (%).

2.5.2. Qualitative analysis: Rapid thin-layer chromatographic analysis

Thin layer chromatography (TLC) technique has been widely used for the monitoring of lipase-catalysed esterification reactions [8, 11]. Esterification products (MAG, DAG and TAG) were identified by the aid of this simple method. This method was described by Sonwalker et al. [12]. TLC plates are activated by heating them at 105^{o}C for 30 minutes. The samples were spotted on the TLC plate. Then, it is developed in a solvent micture containing chloroform:acetone:methanol (90:8:2 v/v/v). By staining the spots with iodine vapour, the glycerides is determined [8]. 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.

2.6. Optimization by RSM

The reaction mixture (10 litres), comprising 2.0 kg PFADs, 0.335 kg 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 ere 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-catalysed 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. [8] and preliminary screening tests (summarised in Table 1).

Table 1. Selected Factors and Ranges for Lipase-Catalysed 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 measurements of esterification percentage were run in triplicate for each experiment. For generating response surfaces, the experimental data obtained were fitted to a second-order polynomial equation

$$y = \beta_\circ + \sum_{i=1}^{k} \beta_i x_i + \sum \sum_{i<j} \beta_{ij} x_i x_j + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \in \quad (4)$$

where y is represent the response, X_i and X_j are independent factors, $\beta_\circ, \beta_i, \beta_{ij}, \beta_{ii}$ are represent regression coefficient of intercept, linear, interaction and quadratic terms respectively, $\in$ represent the random experimental error [13].

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 analysed 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.

Table 2. Experimental runs based on Four-Factor, Three-Level Surface Response 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
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

3. Results and Discussion

3.1. Esterification activity of lipases

The esterification activity of lipases for various temperature ranged from 45°C to 75°C was compare in Fig. 2. From the figure, significant differences between the four different temperatures were observed based for 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 [14]. 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 [4, 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 [16].

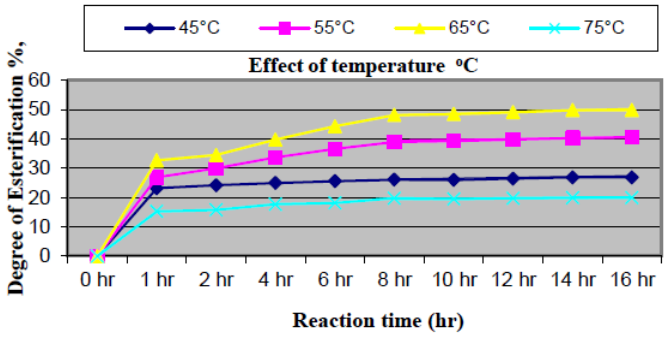


Fig. 2. The Effect of Temperature on the Degree of Esterification with NIL.

3.2. Stability of solvent

In order to effectively carry out bioconversions of lipophilic compounds, it is essential to introduce organic solvents into the reaction systems. The use of organic solvents helps improve the poor solubility in water of substrates or other reaction components of a hydrophobic nature. Organic solvents produce various physicochemical effects on enzyme molecules and the effects differ depending on the types of organic solvents and enzymes used [8]. The stability of untreated and immobilized OPML was investigated by incubation in hexane (without shaking) for 16 hours at room temperature. Hexane, atype of organic solvent can dissolve long-chain fatty acids and has been used previously as the reaction medium for short-chain esterification [8, 17]. Figure 3 shows that after 16 hours of incubation

in organic solvent, the immobilized OPML still maintained relative esterification activity at 80%, while that of untreated OPML reduced to 50%. The decrease in relative esterification activity was due to the stripping of essential monolayer of water surrounding enzyme molecules by hexane [7].

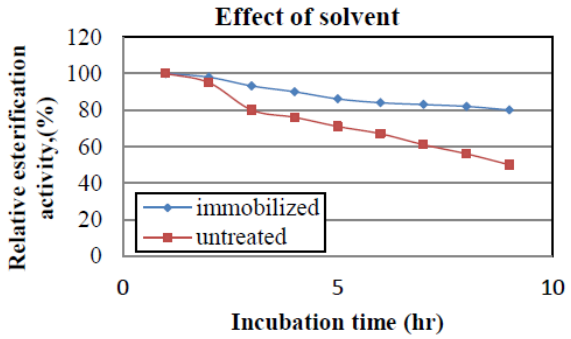


Fig. 3. Relative Esterification Activity of Untreated and Immobilized OPML after 16 Hours of Incubation in Hexane.

3.3. 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.

3.4. Model fitting

Modelling 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 [18]. The best–fitting model was determined by regression and backward elimination [18]. 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 (R2) for response of 0.9133. The actual and predicted responses were sufficiently correlated in Fig. 4.

3.5. 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, 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 of acylglycerol yield, until an optimal acylglycerol yield of 78.5% was obtained with an enzyme load of 10 kg at 240 minutes reaction time and 600 vol/wt solvent. The acylglycerol yield improved with longer reaction time until an optimum value 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 of acylglycerol yield, until an optimal yield of 76% was obtained with an enzyme load of 6 kg. The water absorbent loading was not crucial in a PBR since its effect on acylglycerol yield was insignificant (Table 3).

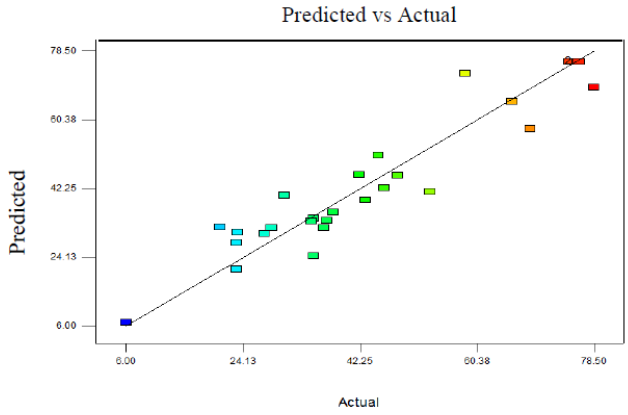


Fig. 4. Relationship between Predicted and Actual Responses of Acylglycerolyield by the Developed Models. The Solid Line represents a Linear Regression Line.

3.6. Optimization

Based on the models generated, it can be construed that acylglycerol yield was influenced not only by the first-order variables but also by the 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 [19]. 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 loading of 6 kg, 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).

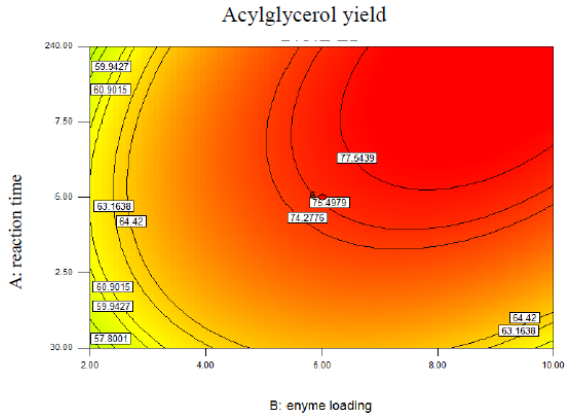
Predicted optimal conditions must be interpreted in the context of industrial operations. The use of solvents increases expenses, therefore an extra step for solvent removal within the process is needed [19]. Extra attention to safety issues is also required [19]. Accordingly, the lowest possible solvent 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. 5). Therefore, a solvent amount that is lower than the predicted optimum is recommended. Table 4 shows the experimental result and the predicted optimum.

Table 3. Analysis of Variance (ANOVA) for the Model Representing the Degree of Esterification in PBR.

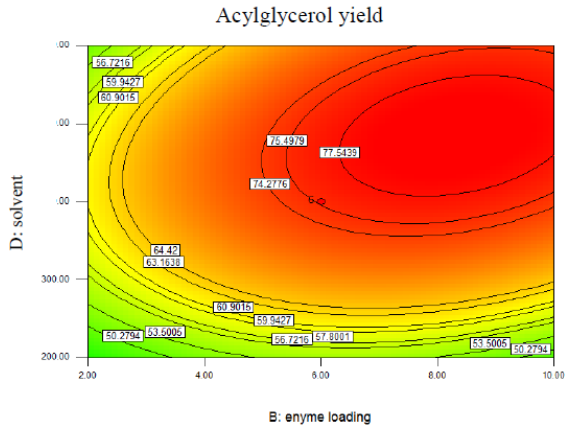
Source	Sum of Squares	df	*F*-value	*p*-value
Model	11701.30	14	11.28	<0.0001
A-reaction time	571.35	1	7.71	0.0141
B-enzyme loading	1127.51	1	15.22	0.0014
C-adsorbent loading	8.05	1	0.11	0.7462
D-solvent	1569.78	1	21.19	0.0003
AB (time*enzyme)	391.05	1	5.28	0.0364
AC (time* adsorbent)	78.77	1	1.06	0.3188
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			

Table 4. Experimental Verification of Model Prediction.

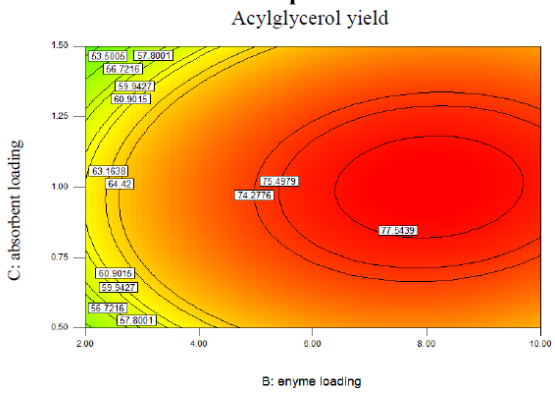
Run	Reaction time (min)	Enzyme loading (kg)	Solvent amount (vol/wt)	Predicted acylglycerol yield (%)	Experimental acylglycerol yield (%)
1.	135	6	460	76	77.3
2.	130	6	400	74	74.6
3	135	6	350	75	75.4



Counter plot A



Counter plot B



Counter plot C

Fig. 5. Predicted Responses from Contour Plots A, B and C of Aclyglycerols at Optimized Conditions: Enzyme Loading 6 kg, Solvent Amount 460 vol/wt and Reaction Time 135 min.

3.7. 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 (Fig. 6). The chromatograms show the successful fractionation of esterified products by TLC.

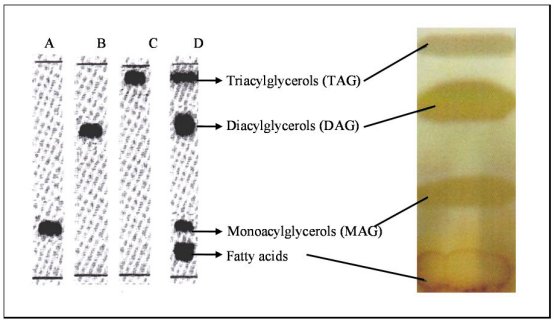


Fig. 6. Chromatograms Showing the Fractionation of Esterified Products by TLC. Lane A, Lane B and Lane C Show the Standard Spots of Monoacylglycerols (MAG), Diacylglycerols (DAG) and Triacylglycerols (TAG) Respectively. Lane D is the Optimum Esterification Product Fractions from Reaction at 65^{o}C.

4. Conclusions

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

- Packed bed reactor was chosen for the application of continuous lipase-catalysed esterification process to assess the performance of the process in a commercial reactor for large-scale implementation.
- Response Surface Methodology (RSM) was applied to optimize the parameters.
- Four factors, namely enzyme loading, water adsorbent loading, reaction time and solvent amount, were optimized to achieve high acylglycerol yield.
- Based on the optimization, the optimal parameters which produced a high yield of acylglycerols were 6 kg enzyme loading, 350 vol/wt solvent amount and 135 minutes reaction time.
- The highest acylglycerol yield was 75.4% based on actual experimental run, close to the predicted yield of 75%.
- Analysis by Thin Layer Chromatography illustrated that esterification products (MAG, DAG and TAG) were successfully identified.

In summary, it can be concluded that the Packed Bed Reactor is suitable for the implementation of large scale, continuous lipase-catalysed esterification process due to its simple operations, high reaction efficiency and high acylglycerol yield.

Acknowledgement

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