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OPTIMISATION OF ENZYMATIC DIRECTED INTERESTERIFICATION OF PALM OIL FOR HIGH YIELD OF TRIUNSATURATED TRIACYLGLYCEROL USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

Unsaturated fatty acids (USAFA), especially monounsaturated fatty acids (MUFA), has been shown to provide substantial health benefits. This study attempted to increase the triunsaturated (U_3) triacylglycerols (TAG) content in palm oil (PO) via lipase-catalysed directed interesterification (EDIE), to enable the production of a liquid fraction that is high in USAFA, especially MUFA. A response surface methodology with three factors and five levels in a central composite design was employed for optimisation of the EDIE conditions for the U_3 TAG production. The effects of reaction temperature (20°C-50°C), reaction time (12-28 hr) and enzyme load (2%-18%) on the U_3 TAG and by-products yields were investigated. Well-fitted quadratic and linear models were successfully established for both responses. All processing conditions affected significantly ($p<0.05$) U_3 TAG yield. By-products yield was affected by reaction time and temperature. Although increasing reaction temperature and enzyme load, and extending the reaction time resulted in a higher U_3 TAG yield, a higher amount of by-products were formed as well. The optimised processing conditions were a reaction temperature of 30°C and reaction time of 18 hr, when immobilised lipase enzyme load was set at 10% (w/w), and resulted in yields of about 27% of U_3 TAG and 23% of by-products.

Keywords: palm oil, triunsaturated triacylglycerol, unsaturated fatty acid, enzymatic directed interesterification, response surface methodology.

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INTRODUCTION

Monounsaturated fatty acids (MUFA), especially oleic acid (C18:1), are often regarded as healthy dietary fatty acids that can confer substantial health benefits, as compared to polyunsaturated (PUFA) and saturated (SAFA) fatty acids. Many studies indicate that MUFA as the predominant form of fatty acid promoted a healthy lipid profile and reduced plasma total cholesterol, low-density lipoprotein (LDL) cholesterol and triacylglycerol (TAG) levels

(Alvizouri-Muñoz *et al*., 1992; Gillingham *et al*., 2011; Schwingshackl and Hoffmann, 2012), thus offers significant protection against coronary heart disease/cardiovascular disease (Hu and Willett, 2002). A MUFA-enriched diet also displayed beneficial effects on glucose metabolism, by improving insulin action and lowering blood glucose levels (Gillingham *et al*., 2011; Qian *et al*., 2016; Sartorius *et al*., 2012; Schwingshackl *et al*., 2011), may play a protective role against mild cognitive impairment and, ultimately, dementia (Naqvi *et al*., 2011; Vercambre *et al*., 2010), and showed anti-inflammatory effects (Galland, 2010). As for guidelines for MUFA consumption, the Academy of Nutrition and Dietetics, as well as the Canadian Dietetic Association, both encourage the consumption of <25% MUFA, while the American Heart Association sets a limit of 20% (Schwingshackl and Hoffmann, 2012).

MUFA are commonly found in liquid vegetable oils. Among the richest sources of MUFA are olive oil, high oleic sunflower oil and high oleic soyabean oil. Palm oil (PO) which is extracted from oil palm fruit's mesocarp is also rich in MUFA. PO consists of almost 50% of unsaturated fatty acids (USAFA), mainly in the form of oleic acid. The oleic acid content of PO is around 37.3%-40.8% of the total PO fatty acids (Kushairi *et al*., 2018; Noor Lida *et al*., 2002; O'Brien, 2009), and is mainly in the form of POO and POP TAG molecules, where *O* is oleic acid and *P* is palmitic acid. Fractionation of PO results in two distinct fractions: the liquid fraction called palm olein (POo) and the solid fraction called palm stearin (POs). POO and other low-melting triunsaturated (U_3) TAG such as LOO/OLO, LLO/LOL and OOO (where *L* is linoleic acid) is concentrated in the POo fraction. However, POo also contains a high amount of disaturated-monounsaturated (S_2U) TAG, namely POP, PLP and POS, where *S* is stearic acid.

Looking at the fatty acid composition, it seems possible to restructure the TAG molecules of PO for a higher amount of U_3 TAG, especially OOO and OOL/OLO, which can later be concentrated in the POo fraction upon fractionation. About 6.4%-8.4% of U_3 TAG are present naturally in PO (Noor Lida *et al*., 2002; Siew, 2011). Modification of the TAG structure of PO to increase its U_3 TAG content, and, consequently, USAFA (especially the MUFA content in the POo fraction) is possible by interesterification (IE). IE, either chemical or enzymatic (EIE), is a process of rearranging the fatty acids within and between TAG molecules, leading to a variety of TAG species (Rozendaal, 1990). In general, IE is performed at a temperature higher than the melting point of the highest melting TAG component of the fat mixture, with the help of a chemical or enzymatic catalyst. IE can be random, specific or directed. Based on the law of probability, random IE, either chemical or enzymatic, leads to randomisation of fatty acids on the TAG molecules (Going, 1967; Klemann *et al*., 1994; Rozendaal, 1992). Specific IE is normally catalysed by an enzyme, *i.e.* lipase. Different categories of specific lipases have been identified, including positional specific (*sn*-1,3 and *sn*-2 specific) and fatty acid-specific lipases (Cowan, 2013). Using specific lipases, full randomisation of fatty acids within or between TAG molecules can be avoided, thus producing TAG with a specific structure. In the case of PO, IE, either random or specific, only leads to small changes in the TAG composition. Restructuring of the TAG molecules of PO, especially for a higher content of U_3 TAG (particularly OOO) is made possible via directed IE (DIE).

DIE is performed at a temperature just below the melting point of the highest melting TAG, normally below 50°C (De Lathauwer *et al*., 1981). DIE is initialised by melting the oils and fats before addition of a catalyst to expedite the reaction. Subsequently, the DIE temperature is reduced to a level that is low enough for the highest melting TAG, typically S_3 TAG, to crystallise out as they are formed. The high melting TAG are then withdrawn from the reaction phase (liquid phase) because when TAG crystallise, they are in the solid phase and can no longer play a part in the DIE reaction. To re-establish the reaction equilibrium, new high melting TAG are continuously being formed in the remaining liquid phase, which in turn also crystallise as a precipitate. The high melting TAG continue to form and crystallise until all TAG that can enter the solid phase are removed from the reaction. The segregation of SAFA into high melting TAG is in tandem with a tendency for USAFA to form very low melting (typically U_3) TAG. DIE eventually results in a liquid phase that has become much less saturated as it is rich in very low melting TAG, and a solid phase that is rich in high melting TAG (Boot *et al*., 1984; Hawley and Holman, 1956; Huyghebaert *et al*., 1994; Placek and Holman, 1957; Rousseau and Marangoni, 2008). Hence, with DIE, U_3 TAG in PO can be increased, which consequently improves USAFA content (especially MUFA) in POo fractions. The rearrangement of the TAG structure of PO via DIE may end up with a mixture that is rich in U_3 TAG especially OOO and OOL/OLO and S_3 TAG particularly tripalmitin (PPP), which can be separated out later by fractionation into the POo fraction which is high in oleic acid and the POs fraction which is rich in palmitic acid.

DIE can be carried out via a chemical (Lago and Hartman, 1986) or an enzymatic (MacKenzie and Stevenson, 2000) route. The main advantage of enzymatic over the chemical route is that the former is a 'green' and an environment-friendly process. Similar to other enzymatic IE processes, the efficiency of enzymatic DIE (EDIE) is influenced by several factors, such as the fat mixture composition,

type of enzyme, enzyme load, reaction time, reaction temperature and moisture content. This study was thus conducted to study and optimise the processing parameters for EDIE of PO to produce a maximum yield of U_3 TAG with a tolerable, if not a minimum, level of by-products, *i.e.* a mixture of free fatty acids (FFA), monoacylglycerols (MAG) and diacylglycerols (DAG), using response surface methodology (RSM). RSM is a collection of mathematical and statistical models used to develop, improve and optimise a product or process that requires only a minimum number of experimental runs as compared to the conventional single factor design (Bas and Boyacı, 2007). RSM enables an evaluation of the effects of multiple parameters, either alone or when interacting with each other, on response variables. The main advantage of RSM is that with a minimum number of experimental runs, it can provide sufficient information for statistically acceptable results (Mu *et al*., 1998). The predictive models derived from the statistical analysis of the experiment can then be utilised to construct the response surface curves for each of the selected responses. Analysis of the response surface curves allows for the identification of the optimum process parameters (Martinčič *et al*., 2008).

This study is of significance as information on DIE, especially on EDIE of PO is scarce. Information on the utilisation of RSM to determine the optimum EDIE conditions for a maximum U_3 TAG yield from EDIE of PO is also not available. Hence, the present study aimed to optimise the reaction conditions of EDIE of PO, namely reaction time, reaction temperature and enzyme load in order to obtain maximum yield of U_3 TAG using RSM.

MATERIALS AND METHODS

Materials

Refined, bleached and deodorised (RBD) PO was procured from Keck Seng (M) Berhad (Masai, Johor, Malaysia). Commercial immobilised Lipozyme TLIM *Thermomyces lanuginosus* lipase was purchased from Novozymes A/S (Bagsvaerd, Denmark). The TAG standards used for elucidation of the TAG species were purchased from Sigma Aldrich Inc. (Sigma Chemical Co., USA). All chemicals and solvents were of analytical and high performance liquid chromatography (HPLC) grades, respectively.

Methods

Response surface methodology experimental design. Design Expert 10.0.3.1 software (Stat-Ease Inc, Minnesota, USA) was used to optimise U_3 TAG yield and minimise the amount of by-products formed during EDIE of PO catalysed by Lipozyme TLIM lipase. Three factors and five levels central composite design (CCD), comprising 20 experimental runs and six replicates at the centre point, were used. The independent variables studied included reaction time (*A*), reaction temperature (*B*) and enzyme load (*C*). The experiment variables in coded and actual units are shown in *Table 1*. The response variables were U_3 TAG and by-products yields. The U_3 TAG and by-products yields were analysed using HPLC with an evaporative light-scattering detector (ELSD).

The 20 experimental runs were performed at random. Analysis of variance (ANOVA) and multiple regression analysis with backward elimination at a 95% significance level ($p<0.05$) were used to evaluate the data obtained from the experiments. The data fitted well with the second-order regression equation shown below, where Y represents the responses which are U_3 TAG and by-products yields, β_0 is the intercept, while β_i, β_j and β_{ij} are the linear, quadratic, and the interaction coefficients, respectively. X_i and X_j are the levels of independent variables.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j$$

Contour and three-dimensional response surface plots were created to demonstrate the relationships between the independent variables and responses. Subsequently, numerical optimisation was used to locate the 'sweet spot' for the responses of interest.

Lipozyme TLIM lipase conditioning. Lipozyme TLIM lipase was chosen as the catalyst in this study as it is commonly used for the enzymatic IE reaction, is low-priced, of food grade and possesses high IE activity (Lee *et al*., 2015; Zhang, 2007). The Lipozyme TLIM lipase was pre-conditioned to eliminate extra moisture before the EDIE reaction was conducted.

TABLE 1. EXPERIMENT VARIABLES IN CODED AND ACTUAL UNITS

Independent variable	Symbol	Coded variable				
		$-\alpha$	-1	0	+1	$+\alpha$
Reaction time (hr)	A	12.0	15.0	20.0	25.0	28.0
Reaction temperature (°C)	B	20.0	25.0	35.0	45.0	50.0
Enzyme load (w/w %)	C	2.0	5.0	10.0	15.0	18.0

Water, approximately 5% (w/w) in immobilised enzyme, has to be removed to prevent hydrolysis of the oil substrate as well as to prevent FFA and partial glycerides such as MAG and DAG from forming (Saw and Siew, 2014). Lipozyme TLIM lipase was conditioned by reacting the lipase (10% w/w) with PO at 70°C for 30 min before draining the PO from the reaction system. This step was repeated five times with fresh RBD PO. After the fifth reaction, PO contained only about 3.0% FFA compared with 18% FFA in PO from the initial reaction. The FFA was assessed by a titration method defined in the AOCS Official Method Ca 5a-40 (Firestone, 2009). The FFA was expressed as the percentage of palmitic acid. The treated enzyme was then used for the subsequent EDIE reaction.

Enzymatic directed interesterification of palm oil. Accurately weighed moisture-free PO was melted at 80°C for 45 min under vacuum in a temperature-controlled stirred vessel. The oil temperature was then reduced to the selected reaction temperature. The reaction was initialised following the addition of the pre-conditioned Lipozyme TLIM lipase once the desired reaction temperature was reached. When the desired reaction time was achieved, the reaction was stopped by rapidly heating the reaction mixture to a high temperature in a microwave oven. The desired reaction temperature, enzyme load and reaction time were in accordance with the RSM design. The mixture containing the melted EDIE PO and enzyme was then quickly vacuum-filtered to remove the enzyme. The EDIE PO samples were kept at -20°C for further analysis.

Triacylglycerol composition. TAG composition was measured using Waters HPLC Model Alliance e2695 (Waters, United Kingdom), which was equipped with Waters ELSD model ELSD 2424. A Merck KGaA column (Darmstadt, Germany) Purospher® Star RP-18e (250 mm × 4 mm) with 5 μm particle size was used to separate out the TAG. Column temperature was set at 35°C. The samples were first melted at 70°C and dissolved in acetone at a concentration of 10% (v/v) (100 µl of sample into 900 µl of acetone), and then passed through a 0.2 µm PTFE membrane filter to remove impurities. The 10 µl samples were then injected into the column. Separation was carried out with a mobile phase containing a mixture of acetone and acetonitrile (Merck, Darmstadt, Germany) at a gradient composition of 35%:65%, 65%:35%, 85%:15%, 35%:65% and 35%:65% of acetone: acetonitrile for 0, 10, 15, 20 and 25 min, respectively. The mobile phase flow rate was set at 1.5 ml min^{-1} with a total run time of 25 min. Individual TAG (OLL, OLO, OOO, PLL, PLO, POO, SOO, PLP, POP, POS, PMP, PPP and PPS) peaks were identified by comparing the peaks elution times with those of pure TAG standards, and quantified using the peak area normalisation method.

By-products (free fatty acid, monoacylglycerol and diacylglycerol) quantification. Quantification of the by-products (FFA, MAG and DAG) in the EDIE PO was done by subtracting the total TAG area (%) from the total chromatogram area (100%). The TAG was quantified according to the method described earlier.

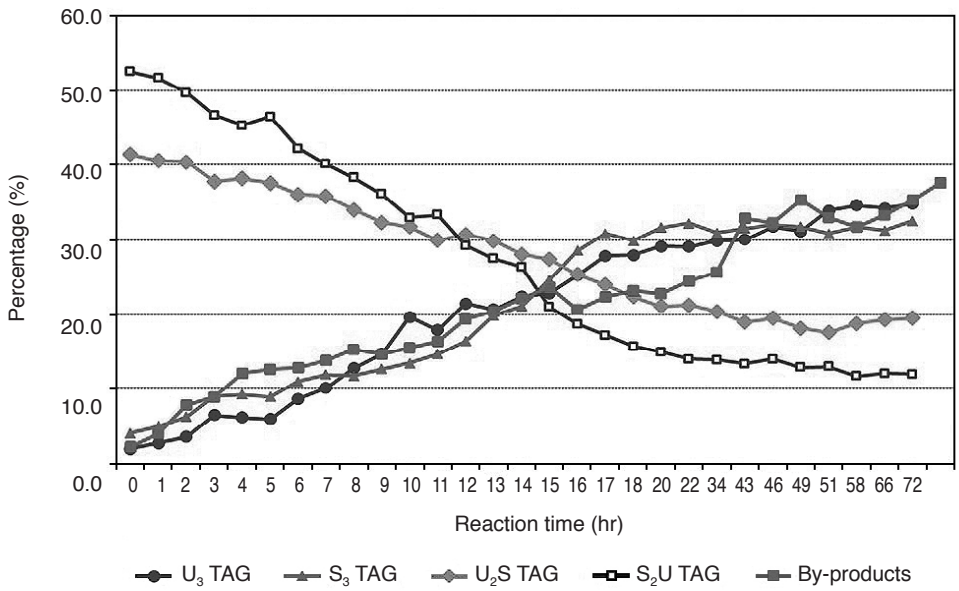
RESULTS AND DISCUSSION

Screening of the Range of Enzymatic Directed Interesterification Reaction Parameters

Before performing RSM, screening of the important parameters affecting the EDIE reaction, namely the reaction temperature, reaction time and enzyme load, was executed to determine the operational and range of interest for the formation of U_3 TAG. Reaction temperature in the range of 25°C-45°C was chosen for the RSM design as it was observed that EDIE did not take place efficiently if the reaction temperature was too low (<25°C) or too high (>45°C). It was found that when enzyme load and reaction temperature were fixed, U_3 TAG yield was the highest when reaction temperature was around 25°C-35°C. Reaction time of 15-25 hr was selected for the RSM design as it was observed that even though total U_3 TAG increased as reaction time was prolonged, extending the reaction time also increased the by-products. A high amount of by-products is not desirable. Shorter reaction time (<15 hr) on the other hand resulted in a too low yield of U_3 TAG. Enzyme load of 5%-15% was chosen as the Lipozyme TLIM lipase works well in this range. Enzyme load of 15% was set as the maximum limit because a higher enzyme load did not have much of an effect on U_3 TAG yield, and, at the same time, it also incurred a higher operational cost. An example of the amounts of U_3 TAG and by-products produced throughout the 72 hr of EDIE of PO in the presence of 10% Lipozyme TLIM lipase at 30°C is shown in *Figure 1*. It was observed that the increase in U_3 TAG yield occurred simultaneously with an increase in S_3 TAG and by-product yields and at the expense of U_2S and S_2U TAG that were initially present in high amounts in PO.

Model Fitting

CCD of five levels and three factors was adopted to determine U_3 TAG and by-products yields. CCD for the 20 experimental runs and data for two responses, namely U_3 TAG and by-products yields, are shown in *Table 2*.



Note: TAG – triacylglycerol.

Figure 1. Amounts of triunsaturated (U_3), diunsaturated-monosaturated (U_2S), monounsaturated-disaturated (S_2U), trisaturated (S_3) triacylglycerols and by-products (free fatty acids, monoacylglycerols and diacylglycerols) throughout the 72 hr of enzymatic directed interesterification of palm oil produced in the presence of 10% Lipozyme TLIM lipase at 30°C.

TABLE 2. CENTRAL COMPOSITE DESIGN FOR 20 EXPERIMENTAL RUNS AND DATA FOR TWO RESPONSES, NAMELY TRIUNSATURATED TRIACYLGLYCEROLS AND BY-PRODUCTS (free fatty acids, monoacylglycerols and diacylglycerols) YIELDS

Std	Run	Independent variable			Response	
		A	B	C	U_3 TAG (%)	By-products (%)
4	1	25.0	45.0	5.0	9.4	30.8
10	2	28.0	35.0	10.0	26.8	33.4
1	3	15.0	25.0	5.0	7.3	20.7
12	4	20.0	50.0	10.0	5.8	32.1
8	5	25.0	45.0	15.0	17.9	34.2
19	6	20.0	35.0	10.0	27.9	26.8
18	7	20.0	35.0	10.0	29.0	24.3
13	8	20.0	35.0	2.0	7.8	23.8
2	9	25.0	25.0	5.0	12.8	27.4
5	10	15.0	25.0	15.0	23.2	18.3
14	11	20.0	35.0	18.0	28.8	28.7
16	12	20.0	35.0	10.0	27.4	25.6
17	13	20.0	35.0	10.0	28.0	24.1
7	14	15.0	45.0	15.0	12.8	20.6
6	15	25.0	25.0	15.0	30.3	30.1
11	16	20.0	20.0	10.0	20.2	24.3
15	17	20.0	35.0	10.0	29.7	25.9
3	18	15.0	45.0	5.0	7.8	21.8
9	19	12.0	35.0	10.0	18.1	20.1
20	20	20.0	35.0	10.0	27.8	23.8

Note: A - reaction time (hr), B - reaction temperature (°C), C - enzyme load (w/w %), U_3 TAG - triunsaturated triacylglycerols.

The best-fitting models were analysed through multiple linear regression with backward elimination in which factors and interactions that were not significant were removed from the models. Out of the four models, namely linear, two-level factorial, quadratic and cubic, EDIE for U_3 TAG yield fitted well with the quadratic model (modified model). By-products yield fitted best with the linear model. The goodness of fit model was determined by the coefficient of determination, the R^2. While a good model has a high R^2, depending only on the value of regular R^2 can lead to poor prediction. The R^2 increases when more variables are added to the model, whether or not these variables are statistically significant. Thus, better predictions are made by considering both the adjusted-R^2 and

predicted-R^2 values (Lee *et al*., 2015). However, the adjusted-R^2 and the predicted-R^2 plateau when too many insignificant variables are added to the model. The rule of thumb to follow is that the difference between the adjusted-R^2 and predicted-R^2 values should be not more than 0.2. The values of R^2, adjusted-R^2 and predicted-R^2 for U_3 TAG and by-products yields were 0.9911, 0.9831 and 0.9475, and 0.8733, 0.8495 and 0.7865, respectively. The difference between the adjusted-R^2 and predicted-R^2 values for U_3 TAG and by-products yields was less than 0.2. The F-values of the models were 123.97 for U_3 TAG yield and 36.75 for by-products yield, implying that both models were significant. The model terms are significant when the value of 'Probability > F' is less than 0.05 while values higher than 0.05 show insignificant model terms. In the case of U_3 TAG yield, A, B, C, BC, A^2, B^2, C^2, and, in the case of by-products, *A*, *B* were significant model terms, where *A* represents reaction time, *B* reaction temperature and *C* enzyme load. The quadratic model for U_3 TAG yield and the linear model for by-products yield had a lack of fit F-value of 2.64 and 2.76, respectively, which imply that the lack of fit was not significant ($p>0.05$). The ANOVA for U_3 TAG and by-products yields are shown in *Tables 3* and *4*, respectively. The ANOVA data showed that both the quadratic model with backward elimination and the linear model were highly significant and sufficient to show the relationship between both responses and the variables in this study. An equation expressed in terms of coded factors can be utilised for predicting the response for any given level of a factor. The equation is also useful for identifying the relative impact of each factor by comparing the factor coefficients. The second order coded equations for U_3 TAG and by-products yields are as follows:

$$Y_{U_3\ TAG\ Yield} = +28.38 + 2.53A - 3.71B + 6.14C - 2.49BC - 2.52A^2 - 6.21B^2 - 4.14C^2$$

$$Y_{by\text{-}products\ Yield} = +25.84 + 4.75A + 1.78B + 0.79C$$

Degree of Three Parameters on Enzymatic Directed Interesterification Reaction of Palm Oil for Triunsaturated Triacylglycerol Production

The ANOVA of quadratic model representing U_3 TAG yield from EDIE of PO are shown in *Table 3*. All three factors, namely reaction time, reaction temperature and enzyme load, significantly affected U_3 TAG yield. The formation of U_3 TAG was positively influenced by reaction time and enzyme load, while negatively influenced by reaction temperature. Increase in time and enzyme load increased the U_3 TAG yield but increase in the reaction temperature above 35°C reduced the U_3 TAG yield. All factors had a significant effect on U_3 TAG yield, with a p-value of <0.0001 ($p<0.05$). This finding is in agreement with the findings of Cao *et al*. (2013), Öztürk *et al*. (2010) and Xu *et al*. (1998). There was a higher reaction conversion rate with a higher amount of lipase, which is in agreement with the findings of Cao *et al*. (2013) and Paula *et al*. (2016). In most enzymatic IE reactions, enzyme mainly speeds up the reaction and does not affect the conversion rate, as reported by Lee *et al*. (2015). However, in the case of batch EDIE of PO, the accumulation of S_3 TAG particularly PPP might have slowed down the enzyme activity especially when the enzyme load was too low, thus resulting in a low yield of U_3 TAG. From the 20 trial

TABLE 3. ANOVA OF QUADRATIC MODEL REPRESENTING TRIUNSATURATED TRIACYLGLYCEROL YIELD FROM ENZYMATIC DIRECTED INTERESTERIFICATION OF PALM OIL

Source	Sum of squares	DF	Mean squares	F value	p-value Prob > F
Model	1 528.05	9	169.78	123.97	< 0.0001
A	84.11	1	84.11	61.42	< 0.0001
B	181.07	1	181.07	132.21	< 0.0001
C	493.92	1	493.92	360.65	< 0.0001
A*B	4.35	1	4.35	3.18	0.1050
A*C	3.25	1	3.25	2.37	0.1544
B*C	49.50	1	49.50	36.14	0.0001
A*A	78.71	1	78.71	57.47	< 0.0001
B*B	479.20	1	479.20	349.90	< 0.0001
C*C	212.84	1	212.84	155.41	< 0.0001
Residual	13.70	10	1.37	NA	NA
Lack of fit	9.94	5	1.99	2.64	0.1549
Pure error	3.76	5	0.75	NA	NA
Cor total	1 541.75	19	-	-	-
R^2	0.9911	-	-	-	-
Adjusted-R^2	0.9831	-	-	-	-
Predicted-R^2	0.9475	-	-	-	-

Note: A - reaction time (hr), B - reaction temperature (°C), C - enzyme load (w/w %); DF - degree of freedom, NA - not available. ANOVA - analysis of variance.

runs (*Table 2*), the highest U_3 TAG yield obtained was 30.3% at 25°C in the presence of 15% enzyme load and for a 25-hr reaction time. Meanwhile, the lowest yield obtained was 5.8% when the reaction time was 20 hr, the reaction temperature was 50°C and the enzyme load was 10.0%. The relationship between reaction time and reaction temperature as well as the relationship between reaction time and enzyme load exhibited antagonistic effect on the U_3 TAG yield. Meanwhile, interaction between reaction temperature and enzyme load showed to have synergistic effect on the U_3 TAG. As for by-products yield, it was significantly influenced by reaction time (p-value <0.0001) and reaction temperature (p-value = 0.0022), but not by enzyme load (p-value = 0.1268). The ANOVA of linear model representing by-products yield from EDIE of PO is demonstrated in *Table 4*.

Single Factor Response

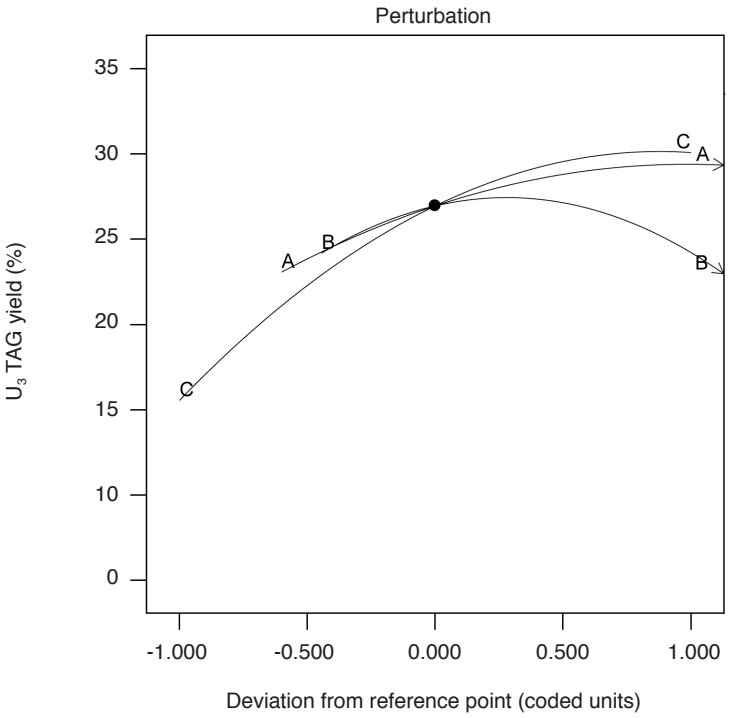
Figure 2 illustrates the perturbation graph of the effect of single factors on U_3 TAG yield. Reaction time significantly affects the product yield in an enzymatic IE process; thus, there was a rise in U_3 TAG yield when the reaction time of the EDIE of PO was increased. When the reaction temperature was fixed at 30°C and enzyme load at 10%, U_3 TAG yield reached its maximum point in about 20 hr. Any further increase in reaction time had little effect on U_3 TAG yield, as the reaction might have reached its equilibrium state. These findings are in agreement with the findings of Chobanov and Topalova (1979) who reported that U_3 TAG yield in lard increased with a more extended reaction time of EDIE. U_3 TAG yield in lard reached its maximum after approximately 18-20 hr at a reaction temperature of 28°C, with little increase in U_3 TAG yield when the reaction time was extended. Similarly, in the case of EDIE of PO, a further increase in the reaction time after U_3 TAG yield had reached its maximum resulted in a small but progressive increase in U_3 TAG yield. However, extended reaction time also led to the formation of a high amount of by-products, probably because of acyl migration, which is in agreement with the findings of Hamam and Budge (2010), Lee *et al*. (2015) and Zhang (2007). The extended reaction time may also lead to randomisation of FA in the glycerol backbone (Zhang, 2007).

Additionally, reaction temperature of EDIE PO was also a crucial factor that affected U_3 TAG yield. There was an increase in U_3 TAG yield when reaction temperature was increased until it reached a saturation point. This finding is in agreement with that of Mu *et al*. (1998) who found that increased reaction temperature of enzymatic IE improved the production of specific structured TAG containing essential fatty acids and medium-chain fatty acids. The best reaction temperature for EDIE of PO for producing high U_3 TAG yield was between 25°C and 35°C, with 30°C being the optimum temperature. Nevertheless, U_3 TAG yield started to decrease when reaction temperature was higher than 35°C. Temperatures below 25°C and above 35°C resulted in a reduction in U_3 TAG yield. According to Novozymes, the optimum reaction temperature for Lipozyme TLIM lipase is in the region of 20°C-50°C. Usually, for IE of oils and fats, the optimum reaction temperature when Lipozyme TLIM is used as the catalyst is in the range of 50°C-75°C, as reported by Elibal *et al*. (2011) and Zhang *et al*. (2001). A reaction temperature of EDIE PO that was too low reduced the lipase activity which subsequently led to a lower amount of U_3 TAG. An increase in reaction temperature above 35°C retarded the formation of U_3 TAG. These findings agree with that of Placek and Holman (1957) who stated that an EDIE reaction must be carried out at a temperature below the melting point of the fat. The melting point of PO is about 35.6°C (Tarmizi *et al*., 2008). Similar results

TABLE 4. ANOVA OF LINEAR MODEL REPRESENTING BY-PRODUCTS (free fatty acids, monoacylglycerols and diacylglycerols) YIELD FROM ENZYMATIC DIRECTED INTERESTERIFICATION OF PALM OIL

Source	Sum of squares	DF	Mean squares	F value	p-value Prob > F
Model	346.40	3	115.47	36.75	< 0.0001
A	296.59	1	296.59	94.41	< 0.0001
B	41.66	1	41.66	13.26	0.0022
C	8.15	1	8.15	2.59	0.1268
Residual	50.27	16	3.14	NA	NA
Lack of fit	43.16	11	3.92	2.76	0.1362
Pure error	7.11	5	1.42	NA	NA
Cor total	396.67	19	-	-	-
R^2	0.8733	-	-	-	-
Adjusted-R^2	0.8495	-	-	-	-
Predicted-R^2	0.7865	-	-	-	-

Note: A - reaction time (hr), B - reaction temperature (°C), C - enzyme load (w/w %), DF - degree of freedom, ANOVA - analysis of variance.



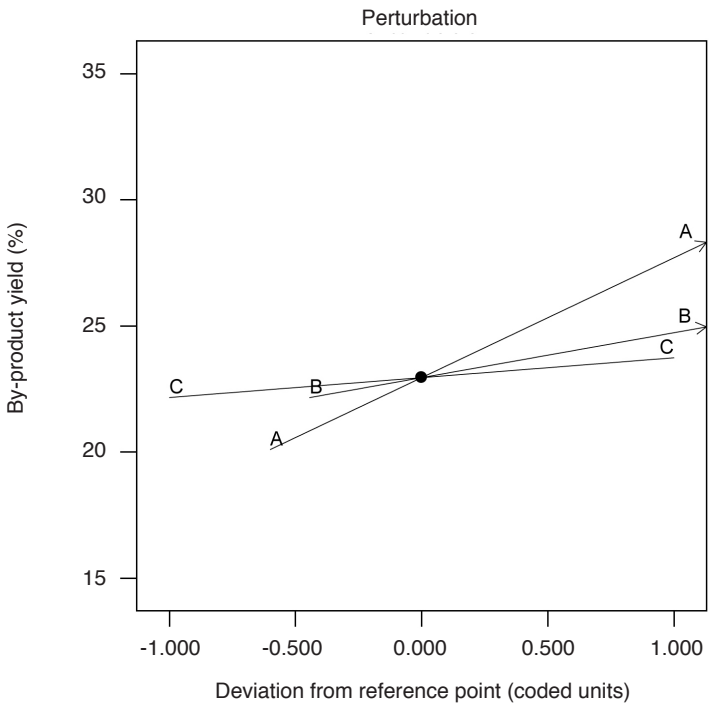
Note: A - reaction time, B - reaction temperature, C - enzyme load.

Figure 2. Perturbation graph showing the effect of single factors on triunsaturated triacylglycerols (U_3 TAG) yield.

have been reported by Chobanov and Topalova (1979) who found that to obtain a higher U_3 TAG yield from EDIE of lard, EDIE was best carried out at about 28°C. In the case of EDIE of PO, if the reaction was carried out at a temperature higher than 35°C, it will not result in a high yield of U_3 TAG because the high reaction temperature will impede the precipitation of the high melting TAG that were formed during EDIE. As a result, the IE will be directed towards randomisation instead.

A higher amount of Lipozyme TLIM lipase stimulated the conversion of S_2U and U_2S TAG to U_3 TAG until it reached a saturation point at about 10% enzyme load. There was little change in U_3 TAG yield when enzyme load was increased from 10% to 18% (w/w). This finding is in agreement with those of Cao *et al*. (2013) and Huang *et al*. (2010). Cao *et al*. (2013) found that the conversion of fatty acid methyl esters and the yield of U_3 TAG increased with the amount of lipase enzyme. Huang *et al*. (2010) showed that the optimum Lipozyme TLIM lipase load for obtaining the highest yield of lard-based biodiesel was 8% and that there was no significant increase in yield even when the additional enzyme was added. In contrast, Lee *et al*. (2015) reported that enzyme load was only responsible for speeding up the EIE reaction but had no effect on the yield of palm-based medium- and long-chain TAG. A low enzyme load, however, resulted in a lower conversion of S_2U and U_2S TAG into U_3 TAG. For example, a reaction time of 15 hr and an enzyme load of 6% resulted in the formation of about 15.0% U_3 TAG, whereas when enzyme load was increased to 12.0%, about 25.0% U_3 TAG can be achieved.

FFA, MAG, and DAG were the by-products formed in this study. The by-products must be kept to a minimum at the end of the reaction in order to obtain a pure TAG mixture consisting of a high amount of U_3 TAG. *Figure 3* shows the perturbation graph of the effect of the single factors on by-products yield. Only two factors, *i.e*., reaction time and reaction temperature, significantly ($p<0.05$) affected by-products yield (*Table 4*). Based on the regression coefficient, by-products yield was strongly affected by reaction time followed by reaction temperature. The by-products yield significantly increased with reaction time. For example, at 10% enzyme load and a reaction temperature of 30°C, by-products yield was 20.0%, 24.8%, and 29.6% at a reaction time of 15, 20 and 25 hr, respectively. The rise in the by-products yield in relation to increases in reaction time was due to acyl migration. According to Mu *et al*. (1998), acyl migration created a major issue in a batch-wise IE reaction and resulted in a high yield of by-products. A high reaction substrate to enzyme ratio prolonged the time required by the reaction to reach equilibrium, and, consequently, resulted in acyl migration. It was observed that an increase in reaction temperature resulted in a modest but significant increase in by-products yield, which is in line with findings of Lee *et al*. (2015) and Mu *et al*. (1998). Mu *et al*. (1998) reported that raising



Note: A - reaction time, B - reaction temperature, C - enzyme load.

Figure 3. Perturbation graph showing the effect of single factors on total free fatty acids (FFA), monoacylglycerols (MAG) and diacylglycerols (DAG) [by-products] yield.

reaction temperature was followed by only a small increase in acyl migration which in turn resulted in lower by-products yield. A modest and insignificant increase in the by-products yield was also noticed when enzyme load was increased, which in turn may be due to the silica coating of the Lipozyme TLIM lipase that is hydrophilic, thus promoting a hydrolysis reaction. Hence, TAG is likely to be hydrolysed into either FFA, MAG, or DAG when the lipase enzyme is present in higher amounts (Lee *et al*., 2015).

Relationship between the Reaction Parameters

Figure 4 shows the contour and response surface plot of the effect of interaction between enzyme load and reaction time on U_3 TAG yield. The response surface plot demonstrates that enzyme load played a significant role in the conversion of S_2U and U_2S TAG to U_3 TAG. A higher enzyme load resulted in a higher conversion rate, whereby U_3 TAG yield reached its maximum point within a shorter period. For instance, at an enzyme load of 14.0%, 28.0% of U_3 TAG yield can be achieved in about 16.5 hr, as compared with 20 hr at an enzyme load of 8.0%. The higher conversion rate at higher enzyme load may be due to a higher availability of activation sites for the substrate to react, leading to a higher conversion rate of TAG to partial glycerides that are subsequently used as a substrate for U_3 TAG formation. It was also observed that an enzyme load below 7% did not have a significant effect on U_3 TAG yield, even when the reaction time was extended. For example, at a 6% enzyme load, extending reaction time from 15 to 25 hr only resulted in a 5% increase in U_3 TAG yield.

Figure 5 shows the contour and response surface plot of the relationship between reaction temperature and reaction time and how they affected U_3 TAG yield. At a high reaction temperature up to 35ºC, a shorter time was required for the reaction system to reach a higher yield of U_3 TAG, indicating that the reaction equilibrium state was reached faster at a higher temperature. In turn, U_3 TAG yield reached its maximum point faster at higher reaction temperatures, showing a higher conversion rate from S_2U and U_2S TAG into U_3 TAG. For example, the time taken to obtain a U_3 TAG yield of 28.0% at reaction temperatures of 27°C and 30°C was approximately 23 and 19 hr, respectively. This finding on the direct effect of reaction temperature on the reaction rate supports the theory of the Arrhenius equation which describes the temperature dependence of a reaction rate. Lee *et al*. (2015) and Xu *et al*. (1998) also observed similar results in their studies on the effect of reaction temperature and reaction time on specific structured lipids yield produced by EIE.

Figure 6 shows the contour response surface plot of the effect of the relationship between reaction temperature and enzyme load on U_3 TAG yield. The contour plot shows that a high enzyme load up to about 17.0% and a high reaction temperature up to about 37.0°C resulted in a higher U_3 TAG yield of

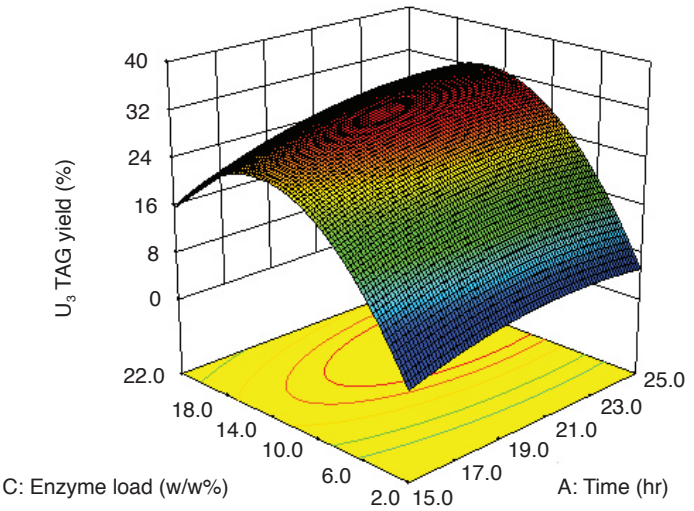


Figure 4. Contour and response surface plot showing the effect of interaction between enzyme load (w/w %) and reaction time (hr) at a reaction temperature of 30°C on triunsaturated triglycerides (U_3 TAG) yield.

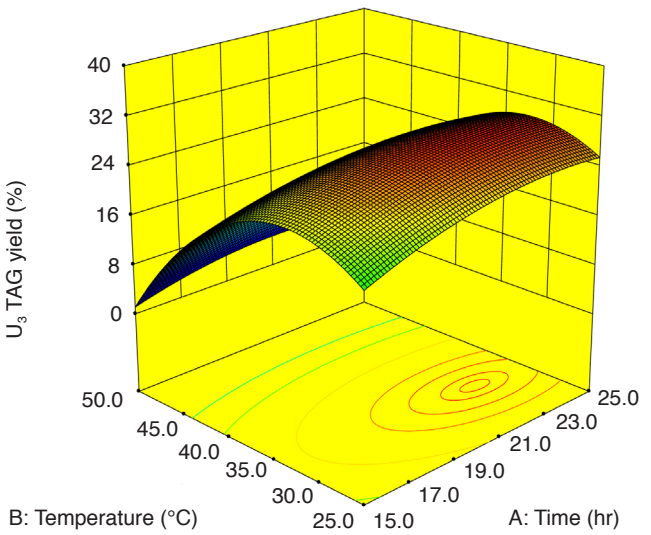


Figure 5. Contour and response surface plot showing the effect of interaction between reaction temperature (°C) and reaction time (hr) at enzyme load of 10% on triunsaturated triglycerides (U_3 TAG) yield.

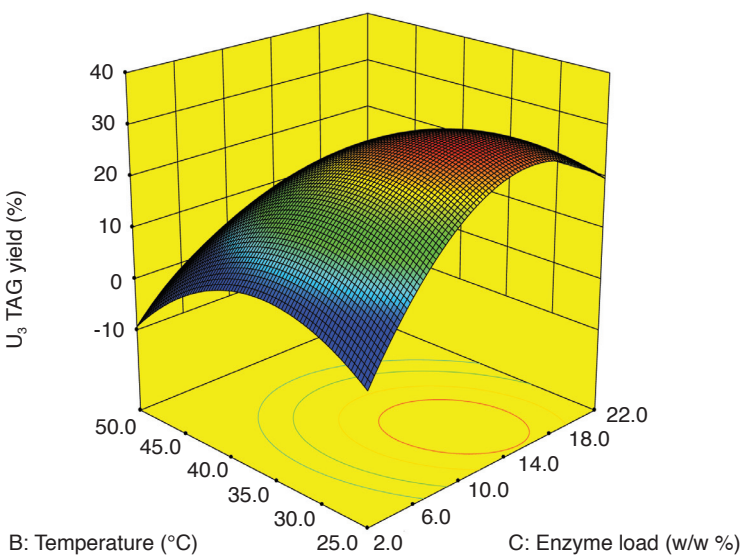


Figure 6. Contour and response surface plot showing the effect of interaction between enzyme load (w/w %) and reaction temperature (°C) at reaction time of 18 hr on triunsaturated triglycerides (U_3-TAG) yield.

about 30.3%. The conversion of S_2U and U_2S TAG to U_3 TAG was higher in the presence of a higher amount of Lipozyme TLIM lipase, at a reaction temperature range of 25.0°C to about 40.0°C. At reaction temperatures above 40.0°C, an increase in enzyme load did not have any significant effect on U_3 TAG yield, which was less than 22.0% at a reaction temperature of more than 40.0°C. It was also observed that at a high enzyme load, the maximum U_3 TAG yield was achievable even at a considerably lower reaction temperature. For example, at 13%-17% enzyme load, 27.0% U_3 TAG yield was achieved at a reaction temperature of only 25.0°C. These findings suggest that increasing the dosage of Lipozyme TLIM lipase and/or reaction temperature does not necessarily mean that a higher amount of U_3 TAG can be attainable, as U_3 TAG yield is also dependent on other processing parameters.

A clear interpretation of the effects of EDIE parameter interactions on by-products yield can be seen in the response contour plots in *Figures 7*, *8* and *9*. *Figure 7* shows that a higher amount of by-products was formed when EDIE of PO was carried out at a higher reaction temperature and for a longer reaction time. A minimum amount of by-products can be achieved when the EDIE reactions were performed at lower temperatures, shorter reaction time and in the presence of less enzyme. Similar interaction trends were observed between reaction time and enzyme load (*Figure 8*), and between reaction temperature and enzyme load (*Figure 9*).

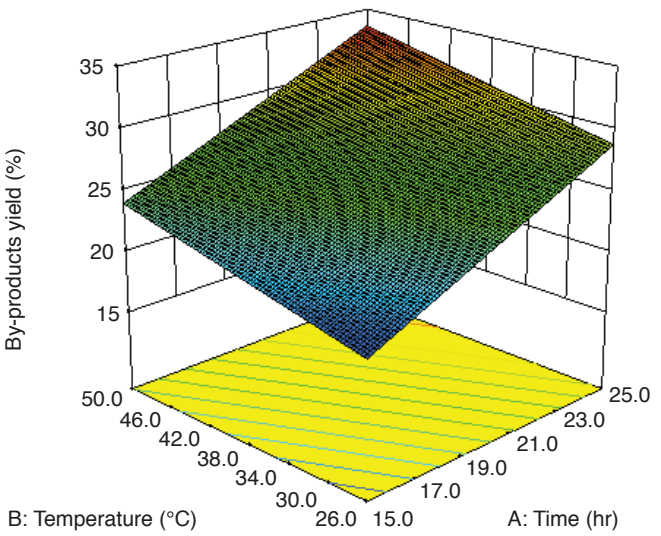


Figure 7. Contour and response surface plot of the effect of interaction between reaction temperature (°C) and reaction time (hr) at 10% enzyme load on by-products (free fatty acids, monoacylglycerols and diacylglycerols) yield.

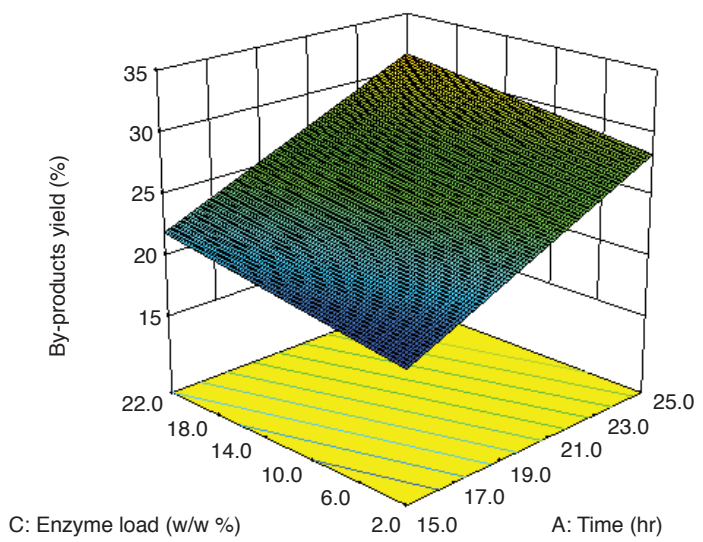


Figure 8. Contour and response surface plot of the effect of interaction between enzyme load (w/w %) and reaction time (hr) at reaction temperature of 30°C on by-products (free fatty acids, monoacylglycerols and diacylglycerols) yield.

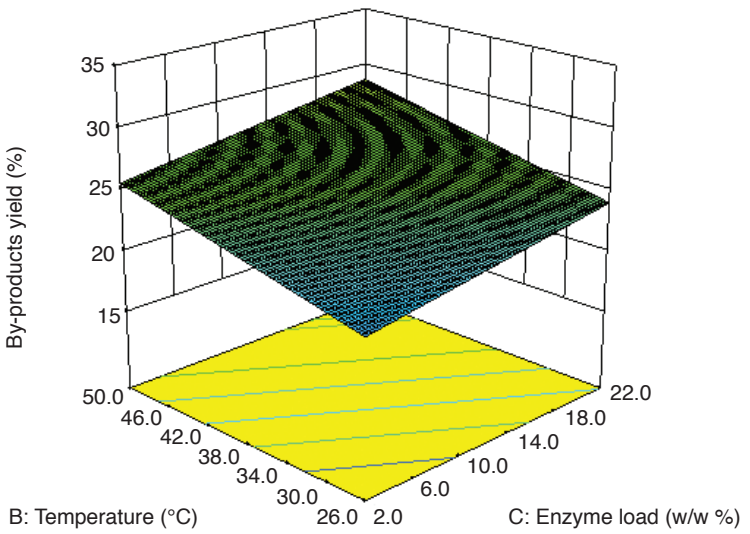


Figure 9. Contour and response surface plot of the effect of interaction between reaction temperature (°C) and enzyme load (w/w %) at reaction time of 18 hr on by-products (free fatty acids, monoacylglycerols and diacylglycerols) yield.

Optimisation of EDIE of PO Reaction Conditions for the Highest Triunsaturated Triacylglycerol Yield

The optimised EDIE reaction conditions were determined using the mathematical optimisation function in the Design Expert software to obtain the maximum yield of U_3 TAG and to have the least possible amount of by-products. EDIE of PO reaction conditions which employed the lowest amount of lipase enzyme for the production of the highest amount of U_3 TAG and the least possible amount of by-products within the shortest time was considered the most cost-efficient. Therefore, in determining the optimum reaction parameters, the enzyme load was fixed at 10% as the earlier ANOVA results had shown that an enzyme load of more than 10% had only a modest effect on U_3 TAG yield. Based on the contour plots, a set of approximate reaction conditions which met the criteria of optimising U_3 TAG yield and minimising the amount of by-products formed were predicted.

The optimisation contour plot of the effects of reaction time and reaction temperature at 10% enzyme load on the U_3 TAGs and by-product yields is shown in *Figure 10*. The most optimum reaction conditions when enzyme load was fixed at 10% were a reaction time of 18 hr and a reaction temperature of 30.1°C. These conditions were predicted to be able to produce EDIE PO containing 27% U_3 TAG with about 23% of by-products. These optimised conditions gave a desirability value

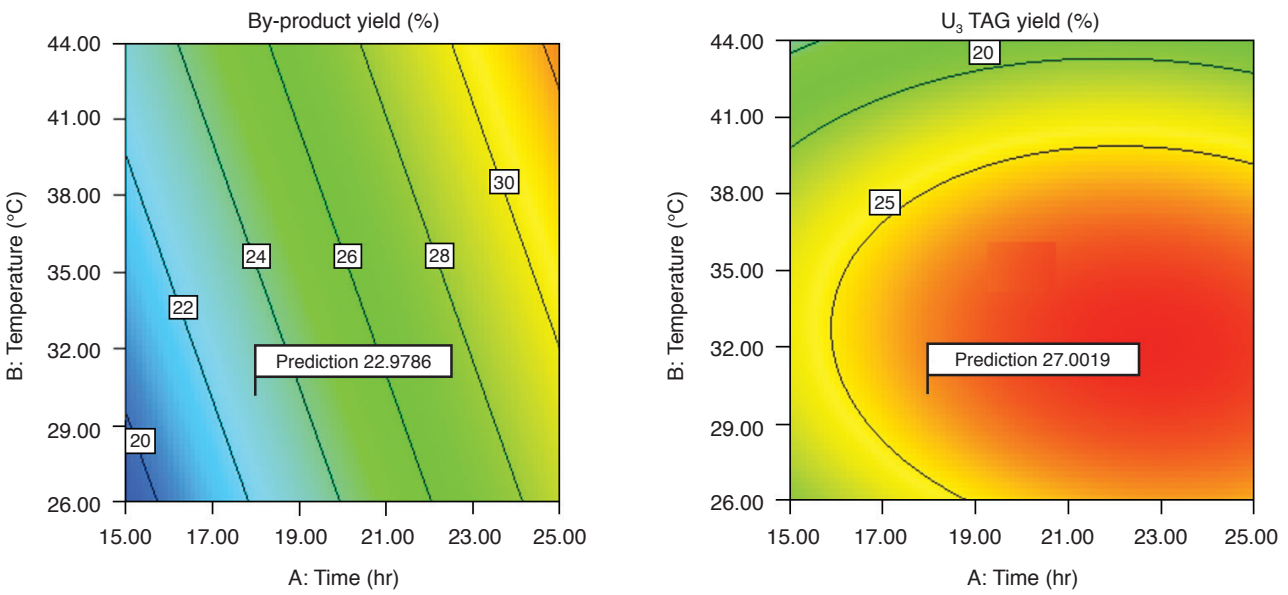


Figure 10. Contour plot of the effect of reaction time and reaction temperature at 10% enzyme load on the triunsaturated triacylglycerol (U_3 TAG) and by-product (free fatty acids, monoacylglycerols and diacylglycerols) yields.

TABLE 5. TRIUNSATURATED TRIACYLGLYCEROL (U_3 TAG) AND BY-PRODUCTS (free fatty acids, monoacylglycerols and diacylglycerols) YIELDS FROM THREE VALIDATION RUNS USING THE OPTIMISED REACTION CONDITION AS COMPARED TO THE PREDICTED VALUE OF RESPONSE SURFACE METHODOLOGY OPTIMISATION

Validation run	U_3 TAG yield (%)	By-products yield (%)
1	26.67	21.01
2	28.21	22.19
3	28.88	24.36
Predicted value	27.00	22.97

of 0.881. Desirability is an objective function that reflects the desirable ranges for each factor and is defined as the geometric means of all transformed factors (Dalvand *et al*., 2014). Validation runs were carried out to validate the predicted results. *Table 5* shows the yields of U_3 TAG and by-products from three validation runs using the optimised reaction conditions compared with the predicted values of RSM optimisation. The yields of U_3 TAG and by-products obtained from the validation runs were very close to the predicted yields.

CONCLUSION

U_3 TAG content in PO can be intensified via EDIE. RSM exploiting the CCD design with three factors and five levels ($-\alpha$, -1, 0, +1, $+\alpha$) was suitable for optimising the EDIE reaction conditions of PO for the production of U_3 TAG. Well-fitted quadratic and linear models were successfully built for U_3 TAG (R^2 of 0.9911) and by-products (R^2 of 0.8733) yields, respectively. U_3 TAG yield was affected significantly ($p<0.05$) by all the three processing factors: reaction time, reaction temperature and enzyme load. The U_3 TAG was strongly influenced by enzyme load, followed by reaction temperature and reaction time. By-products yield was affected significantly ($p<0.05$) by reaction time and reaction temperature. Extending reaction time and increasing reaction temperature resulted in higher U_3 TAG yield, but this was accompanied by higher by-products formation. The RSM optimised processing conditions for EDIE of PO to produce the utmost U_3 TAG and the least by-products were: a reaction temperature of 30.1°C, a reaction time of 18 hr and a Lipozyme TLIM lipase load of 10% (w/w). It was predicted that these processing conditions would result in a U_3 TAG yield of about 27% and by-products yield of about 23%. Verification runs gave similar U_3 TAG and by-products yields as predicted in the optimisation process.

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