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Synthesis of Medium and Long-Chain Structured Lipids from Oil Blend of Palm Olein and Palm Kernel Olein by *Geobacillus sp.* Strain ARM Lipase

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Abstract: Development of Structured Lipid (SL) a new generation of lipids with high nutritional and health value remains an interesting area in functional food processing. Design of new SL by enzymatic modification has revealed many interesting findings and the search for more potent biocatalyst is an urgent need to fulfill the consumers demand. In this research, we aimed to investigate and evaluate the ability of sn-1, 3 regioselective thermostable lipase from locally isolated *Geobacillus* sp. strain (designated as ARM lipase) in catalyzing the transesterification of Palm Olein (POo) and Palm Kernel Olein (PKO) a source rich in oleic, palmitic and lauric acid. Study conducted showed that the chemical composition of oil blend POo: PKO particularly the Fatty Acid (FA) chain length and distribution of FA on the glycerol backbone was altered, resulting in the production of Medium and Long Chain length of acylglycerol (MLCT) and Diacylglycerol (DAG). Two important parameters were investigated, reaction time and temperature. Both time and temperature of reaction have direct influence on the conversion of POo:PKO into MLCT and DAG which increases with an increase in time and temperature up to 10 h and 55°C, respectively. A gradual increment of MLCT and DAG was observed at 35-50°C and TAG reached its highest conversion with 12% MLCT and 16% DAG at 55°C after 10 h reaction. The result indicated the proficiency of ARM lipase to catalyze reaction at high temperature and remains stables for prolong reaction time. Analysis on the physical changes of the reacted oil blend showed that the oil blend has sharp melting profile and accelerated crystallization behavior. These attributes are critical for production of confectionery, margarine and shortening. ARM lipase demonstrates high competency as a new biocatalyst for lipid processing industry.

Key words: 1,3 regioselective, thermostable, lipase, medium and long chain structured lipids, reaction, ARM, margarine

INTRODUCTION

The potential biological function and nutritional perspective of Structured Lipids (SL) a new generation of healthy fats have primarily attracted health conscious consumers. The synthesis of low calorie lipid which is commonly categorized into several types Short-and Long Chain acyl residues (SLCTs), Medium-and Long Chain acyl residues (MLCTs) and Diacylglycerols (DAGs) has recently grow into an important area in oil processing industry. SL can be considered as an ideal lipid substitute only if it contains essential fatty acids, unsaturated fatty acids and gives no harmful effects to the consumers (Cao *et al*., 2013). The tailor-made SL constituting of Medium Chain Fatty Acid (MCFA) which can be developed by modification and restructuring the position and composition of fatty acids in its naturally occurring form is recognized to be safer and healthier as it can be easily hydrolyzed by lipases, absorbed into the intestines, hence, reducing the accumulation of body fats and problems associated with obesity (Wang *et al*., 2012). Additionally, incorporation of Long Chain Fatty Acid (LCFA) in SL provides health and nutritional benefits.

As consumers continuously demand for healthier food products, synthesizing SL from natural oils by lipase-mediated modification is economically feasible and more 'greener' compared to conventional chemical reaction using other high-cost substrates. Enzymatic transesterification, specifically lipase-catalyzed reaction prevails in many aspects over chemical reactions.

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Enzyme-mediated catalysis reaction is known for high specificity with fewer side products and can be conducted at mild and moderate reaction conditions (Palla and Carrine, 2014).

Furthermore, the nature of lipase exhibiting high specific activity, substrate specificity and stability in extreme conditions such as temperature and organic solvents makes it a potent biocatalyst in lipid processing. Substrate specificity of lipases is one of the key criteria in lipid modification. Lipase with regioselective property acts only on primary ester bonds (1, 3-specific lipases) and represents a good candidate for production of specific SLs (Palla and Carrin, 2014). For instance, sn-1, 3 specific lipase from *Rhizomucor miehei* was used in the production of SLs while the enzymatic synthesis of 1, 3-dioleoyl-2-Palmitoylglycerol (OPO), one of the components of human milk fat was carried out using 1, 3 specific *Aspergillus oryzae* lipase (Palla and Carrin, 2014; Cai *et al*., 2015). Enzymatic modification allows a selective incorporation of a desired acyl moiety onto a specific position of Triacylglycerides (TAG) (Lee *et al*., 2015).

While positional specificity is important to target certain fatty acids in triglycerides for restructuring the fatty acid position and composition, the limited number of 1,3 specific lipase available for synthesis of SLs has restricted the application of 1,3 specific lipase, hence, leading to a relatively expensive process. Development of new lipases for lipid modification is thus a never-ending quest in order to provide vast options and alleviate the production cost. Several reported lipases used in the synthesis of SL include *Pseudomonas cepacia* lipase for the production of 1,3-dioleoyl-2-Palmitoylglycerol (OPO) and immobilized lipase from *Rhizomucor miehei,* Novozymes for MLCT synthesis (Koh *et al*., 2011).

Another important factor in the bioconversion of lipids by lipase is the type of substrate used. Many SLs have been predominantly produced from vegetable oils such as canola, flaxseed and soybean oil (Wang *et al*., 2012; Khodadadi *et al*., 2013; Palla and Carrin, 2014). Oil palm, a major plantation crop in the country and many other tropical regions is a source of palm oil and palm kernel oil. Until recently, palm oil has been known as the highest edible oil worldwide. After fractionation, the liquid portion is known as palm olein and palm kernel olein, respectively. Palm oil which constituted of a balanced composition between saturated and unsaturated fatty acids namely palmitic (C16) and oleic acid (C18) contains high nutritional value and offers greater advantages compared to other vegetable oils. Palm kernel oil on the other hand, resembles coconut oil with high content of lauric acid (C12) and exhibits greater stability at high temperature and better shelf-life. A study conducted on modification of palm oil has succeeded to produce OPO an important infant formula (Chen *et al*., 2004).

MATERIALS AND METHODS

Bacterial sources: *E. coli* TOP10 harboring recombinant pTrcHis/ARM lipase in 80% glycerol stock was obtained from Enzyme and Microbial Technology Research Center, Universiti Putra Malaysia.

Preparation of crude enzyme: The bacterial culture was grown in 5 L Erlenmayer flask containing 1 L of LB broth medium supplemented with 50 μg/mL of ampicilin on a rotary shaker (200 rpm) at 37°C. The culture was induced with 1 mM IPTG (Isopropyl-β-D-thiogalactopyranoside) when optical density O. D_{600nm} reached approximately 0.5 and further incubated for 17 h at 37°C. Following incubation, cells (20 mL) were harvested by centrifugation (10, 000 xg , 10 min, 4°C) and the pellet was resuspended with 20 mL of 50 mM Tris-HCl pH 8.0 before sonication (Branson 250 sonifier: 20 intermittent cycles, output 2, duty cycle 28-30) and cleared by centrifugation at 10, 000 xg for 10 min at 4°C. The soluble fraction containing the crude lipase was analyzed for lipolytic activity prior to immobilization.

Lipase activity assay: Lipase activity assay was performed according to Kwon and Rhee (1986), calorimetric method with slight modification. Reaction mixture containing 10 μL of crude lipase, 0.05 g of immobilized lipase, 2.5 mL of olive oil emulsion (1:1, v/v), 20 μL of 0.02 M $CaCl_2$ and 990 μL of 50 mM Tris-HCl buffer were incubated in a water bath shaker at an agitation rate of 200 rpm for 30 min at 60°C. The enzymatic reaction was terminated by adding 1 mL of 6 M HCl and 5 mL isooctane followed by vigorous vortexing. The upper layer of isooctane (4 mL) containing the liberated fatty acid was transferred into a test tube containing 1 mL copper-pyridine reagent for analysis. The amount of free fatty acid released was measured by the absorbance reading at 715 nm. Oleic acid standard curve was used to determine the lipolytic activity. One unit of lipase activity is defined as the rate of 1 μmole fatty acid released per minute. The enzymatic assay was performed in triplicates.

Immobilization of crude ARM lipase: The crude lipase suspension was immobilized using simple adsorption technique onto different support matrices namely chitosan, amberlite of each support matrix was added to 10 mL crude enzyme solution (containing about

3000 U). To aid the adsorption process, the mixture was incubated in a water bath shaker at 20°C for 2 h. The immobilized lipase was vacuum-filtered through whatman No. 1 filter paper and washed with 20 mL of distilled water to remove any non-adsorbed lipase. The washing process was repeated, until the collected supernatant contained no protein trace as measured by Bradford protein assay.

The dried immobilized lipase was stored in a -80°C freezer prior to lyophilized using Christ Alpha 1-2 LD freeze dryer for 24 h. The enzymatic activity of the immobilized lipase (0.05 g) was measured and stored in a -20°C freezer before use (Rahman *et al*., 2008; Branco *et al*., 2010). The best support matrix for ARM lipase was chosen based on the highest activity performed by the immobilized lipase.

Enzymatic transesterification of palm olein and palm kernel oil: Enzymatic transesterification of the blend RBD POo and RBD PKO with mass (w/w) ratio 50:50 containing 50 g of total lipids was carried out in a solvent-free media, following the method described by Lee *et al*. (2015) with slight modification. The blend was stirred at 350 rpm and temperature was maintained with water bath. Five sets of oil blend were prepared for different temperatures (35,40, 45,50 and 55°C). The reaction started with the addition of immobilized ARM lipase of 250 U once the desired temperatures were achieved. Reacted samples were taken on every hour and further centrifuged and filtered with 0.45 µm PTFE membrane filter to separate the enzyme. Samples were stored at -20°C for further analyses.

Determination of fatty acids composition: Preparation of Fatty Acids Methyl Ester (FAME) was carried out according to O'fallon *et al*. (2007) method. Oil sample (20 µL), 0.7 mL 10 N KOH in water and 5.3 mL methanol were placed in 15 mL centrifuge tube. The tube was incubated at 55°C water bath for 1.5 h and vigorously shaken at every 20 min. Following incubation, the mixture was left cooled to room temperature under cold tap water and 0.58 mL of 24 N H_2SO_4 was added. Sample was then mixed by inversion until precipitated K_2SO_4 present. It was then incubated again for 1.5 h at 55°C water bath with 5 sec hand shaking every 20 min. Hexane (3 mL) was added and vortexed for 5 min. The hexane layer containing FAME was transferred into chromatography vial for Gas Chromatography (GC) analysis.

Analysis of the derivatized FAME was conducted using Agilent (Santa Clara California, USA), GC A2890 equipped with Flame Ionization Detector (FID) (Lee *et al*., 2015). The capillary column was BPX70 70% Cynopropyl Polysilphenylene-siloxane (SGE Analytical Science, Ridgewood Victoria, Australia), 30 m in length with internal diameter of 0.32 µm. The injector and detector temperature were maintained at 250 and 280°C, respectively while the carrier gas was nitrogen. The split ratio was 15:1. Oven temperature was programmed as follows the column was held at 150°C for 0.5 min increased to 180°C at a rate of 10°C/min, 220°C at a rate of 1.5°C/min and 260°C at a rate of 30°C/min and finally held at 260°C for 5 min. Duplicate measurements for each sample was performed. Results were expressed as mean±standard deviation.

Determination of TAG composition: A waters HPLC e2695 (Milford Massachusetts, USA) equipped with Evaporative Light Scattering Detector (ELSD) was used to determine the TAG class composition. The separation of TAG was done following the method outlined by Lee *et al*. (2015). The column used was a pre-coated silica reversed phase C18 HPLC column, Lichro CART 5 µm (4 mm×25 cm) from Merck (Darmstadt, Germany). The gradient mobile phase was a mixture of acetone (A) and acetonitrile (B) while the flow rate was 1 mL/min with a total run time of 55 min. The gradient phase was set as follows: 0-8 min (100% B), 8-15 min (25% A, 75% B), 15-35 min (25% A, 75% B), 35-40 min (100 % B), 40-65 min (100 % B). Column temperature was maintained at 35°C. The injection volume was 15µL of 5% (wt/vol) sample in acetone. TAG peaks were identified by calculating the ECN value. Reference standards were triododecanoate, tripalmitin and trioleate.

Thermal profile analysis using Differential Scanning Calorimetry (DSC): A Perkin Elmer DSC 8000 (Waltham Massachusetts, USA) was used for examination of melting and crystallization of profile of the oil blend before and after transesterification. Nitrogen was used to purge the thermal analysis system at a flow rate of 20 mL/min. An empty hermetically sealed aluminium pan was used as reference. Sample of around 5.0-6.0 mg was weighed into an aluminium pan and sealed with cover. Prior to analysis of crystallization behavior, the sample was heated at 80°C for 5 min to melt all crystals and nuclei. It was then cooled to -50°C at a rate of 5°C/min and held at -50°C for 10 min to fully crystallize the sample and obtain the crystallization thermogram. The sample was heated from -50-80°C at a rate of 5°C/min and held at 80°C for 10 min to record the melting thermogram (Lee *et al*., 2015). All measurements were performed with duplicate analysis.

RESULTS AND DISCUSSION

Immobilization of crude 1,3-regioselective ARM lipase: Crude ARM lipase was immobilized using simple

Table 1: Immobilization of ARM lipase onto different supports

Supports	Protein adsorbed (%)	Activity (μ mole min^{-1}/g^{-1} or U/g)
Celite	33.39	11.96
Amberlite	29.77	45.42
Chitosan	6.74	55.58

Total amount of protein offered for immobilization to 1 g of support 19 mg; lipase loading: 3016.67 U/g support

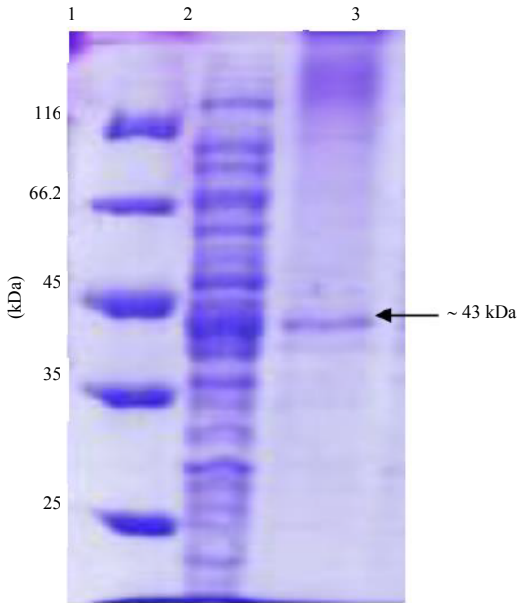

Fig. 1: SDS-PAGE analysis of thermostable 1,3 regioselective ARM lipase before and after immobilization on chitosan. Lane 1: Unstained Protein Molecular Weight Marker, Lane 2: crude enzyme, Lane 3: immobilized enzyme. Arrow indicates the target protein (ARM lipase) at approximately 43 kDa

physical adsorption method using several support matrices prior to enzymatic transesterification on both palm olein and palm kernel olein. Immobilization has been employed in many studies to improve enzyme stability (Nawani *et al*., 2006; Suescun *et al*., 2015) and is a strategy use to facilitate enzyme reuse, hence, reducing the processing cost (Adlercreutz, 2013). Screening for the suitable support for crude ARM lipase immobilization was made by comparing the protein amount adsorbed and activity performed by immobilized lipase on three different supports (celite, amberlite and chitosan). Table 1 depicts the percentage of protein adsorbed and lipolytic activity of the immobilized lipase.

Of the three supports, celite showed the highest percentage of adsorption (33.39%), followed by amberlite and chitosan with 29.77 and 6.74%, respectively. Conversely, result showed that the lipolytic activity of ARM lipase did not correspond to the percentage of adsorption. Chitosan-immobilized lipase displayed highest activity, 55.58 U/g as compared to amberlite and celite-immobilized lipase demonstrating a complete reversed trend to the adsorption percentage. Catalytic activity is one of the criteria evaluated in immobilized lipase preparation and achieving a high specific activity is extremely important to ensure the enzyme works at its maximum performance (Adlercreutz, 2013). Chitosan has been reported as a suitable support carrier for many lipases. Xie and Wang (2012) reported on the use of magnetic chitosan microsphere as a carrier for immobilization of commercial Candida rugosa lipase Lipase from *Talaromyces thermophilus* which was immobilized on chitosan pre-treated with glutaraldehyde, preserved its catalytic activity almost intact and offering maximum immobilization capacity (76 and 91%, respectively) (Romdhane *et al*., 2013).

Further investigation on the bound protein was carried out by analyzing the SDS-PAGE profile. Interestingly, chitosan-immobilized lipase showed a significant reduction of impurities leaving almost a purified ARM lipase as clearly seen in Lane 2 (Fig. 1) as compared to its crude free enzyme (Lane 1). A distinct band with a correct size of ARM lipase (43-kDa) was observed on the gel. This result suggested that immobilization has remarkably facilitated the purification of ARM lipase. Besides, this qualitative analysis explains the high specific activity exhibited by chitosan-immobilized lipase due to the decline of impurities in the sample. Chitosan has been known for its excellent properties as a lipase support and is relatively economical (Foresti and Ferreira, 2007).

Enzymatic synthesis of SLs from POo: PKO blend

Fatty acid composition: Fatty acid composition of POo: PKO (50:50 w/w) before and after transesterification is shown in Table 2. Data revealed no significant difference in fatty acid composition at any reaction temperature which indicated that enzymatic modification did not change the overall fatty acid composition of the oil. The fatty acid proportion of the synthesized SLs is similar to that of the original blend, thus, confirming the efficacy of the reaction and displaying no degradation to physical factors such as heat and air during the process. This result is in agreement with other reported work where enzymatic interesterification causes the exchange of fatty acid composition within and between the TAGs, hence, the overall distribution remains unchanged (Reshma *et al*., 2008).

PKO is known to have high content of short and medium chain saturated fatty acids such as Caprylic (C8), Capric (C10) and lauric acid (C12) and contain low amount of unsaturated fatty acids, oleic

Table 2: Fatty acid composition of POo:PKO (50:50 w/w) before and after reaction at different temperatures

	Amount (%)					
Types of fatty acid	Blend[2]	35°C	40°C	45°C	50°C	55°C
C8	1.357±0.00	1.374±0.06	1.449±0.01	1.308±0.06	1.496±0.11	1.495±0.01
C10	1.489±0.02	1.434±0.04	1.435±0.01	1.489±0.10	1.575±0.07	1.429±0.00
C12	23.51±0.01	23.64±0.01	23.43±0.04	23.29±0.03	23.34±0.04	23.44±0.03
C14	8.618±0.01	8.722±0.01	8.739±0.03	8.775±0.05	8.623±0.01	8.767±0.05
C16	24.49±0.01	24.51±0.00	24.46±0.05	24.59±0.01	24.25±0.00	24.44±0.03
C18	4.047±0.00	4.092±0.03	4.056±0.17	4.118±0.05	4.021±0.03	4.094±0.10
C18:1	29.88±0.03	29.64±0.02	29.76±0.10	29.75±0.04	29.28±0.03	29.68±0.01
C18:2	6.618±0.01	6.593±0.02	6.667±0.06	6.672±0.01	6.564±0.01	6.662±0.01
SFA	63.51	63.77	63.57	63.57	63.31	63.67
MUFA/PUFA	36.49	36.23	36.43	36.42	35.84	36.34

Types of fatty acid SFA = Saturated Fatty Acid, MUFA/PUFA= Mono/Polyunsaturated Fatty Acid. Each value in the table represents the mean±standard deviation; Types of oils. Blend = POo: PKO blend without enzyme added (control)

(C18:1) and linoleic (C18:2). To improve the quality and usefulness of PKO, the oil is often interesterified with other oil such as palm stearin for margarine production. In this research, blending of PKO with POo was seen to increase the Monounsaturated Fatty Acid (MUFA) and Polyunsaturated Aatty Acid (PUFA) content, since, the latter oil contains approximately 41.5% of C18:1 (oleic) and 11.6% of C18:2 (linoleic). High MUFA (in this case C18:1) diet is advantageous compared to PUFA (C18:2). The oil blend which composed of almost an equal percentage of lauric acid (23.51%) and oleic acid (29.88%) could be exploited for the production of more nutritious and wholesome oil.

Acylglycerol composition: Fats and oils have complex composition of acylglycerols (TAG, DAG, MAG and free fatty acids). Precise identification and quantification of the acylglycerols are vital to correlate the effect of modification on food lipid physical properties such as solid fat content and melting point (Neff *et al*., 2002; Lee *et al*., 2015). Lipase-mediated transesterification can be evaluated by looking at the changes in TAG concentration or formation of new TAGs. Apart from the changes in TAG concentration, the percentage of Free Fatty Acids (FFA%) is equally important to monitor the transesterification reaction, since, it occurs alongside hydrolysis (Ghazali *et al*., 1995; Ming *et al*., 1999).

Using HPLC, TAG species was determined according to, retention time and Equivalent Carbon Number (ECN). The TAG composition of oil blend PKO: POo was evaluated before and after the transesterification reaction up to 10 h. Based on HPLC chromatogram (Fig. 2a), the initial composition of the oil blend consisted of several major TAGs of PKO; CaLaLa, LaLaLa, LaLaM, LaLaM/LaLaP and POo; OOO, POO and PPO. The proportion of POo and PKO has linear correlation with TAG composition in the blend (Lida *et al*., 2002).

Changes in TAGs composition were more substantial at higher temperature and longer incubation time when the oil blend was subjected to transesterification reaction using ARM lipase. Figure 2b shows the TAG species observed after 10 h of the enzymatic reaction carried out at 55°C. The yield of LaLaLa, CaLaLa, LaLaM, LaLaM/LaLaP, LaLaO, POO and PPO dominated the TAG species detected. Several MLCT species can be seen after the enzymatic reaction including LaLaO, LaLaP, LaLaM suggesting that the transesterification has randomized and restructured the FFA composition of the reacted oil blend. The incorporation of palmitic acid (C16) and oleic acid (C18:1) into the major PKO fatty acid lauric acid (C12) generates a good blend with better functionality. Additionally, a new peak of DAG was also spotted after the enzymatic reaction. DAG is one of the important components in healthy food oil.

Effect of reaction time: Further interpretation of HPLC results was carried out to observe the composition of TAGs at two profound variables temperature (35-55°C) and reaction time (0-10 h). As shown in Fig. 3, MLCT production was influenced by the reaction time where prolonged enzymatic reaction (6-10 h) improved the product yield, especially at 55°C. At lower reaction temperature (40, 45, 50°C), almost a consistent yield of MLCT with marginal increment was observed throughout the total reaction time. The MLCT which include LaLaO, LaLaM/LaLaP, LaOL/LaPL, LaMO, LaMP and LaPP is a type of structured lipid consisting of both Medium Chain Fatty Acids (MCFA) and Long Chain Fatty Acids (LCFA) in a TAG. MCFA exhibits greater value than the LCFA due to its higher solubility and smaller molecular size which allows the absorption, transport and metabolism much easier than the latter form of fatty acids (Hamam, 2013). Nevertheless, the incorporation of LCFA will provide the essential fatty acid to the body (Lee *et al*., 2015).

MLCT reached its maximum yield within 4-7 h and further increase in reaction time has no effect to the yield except for reaction conducted at 55°C. This signifies that the transesterification has reached its steady state within

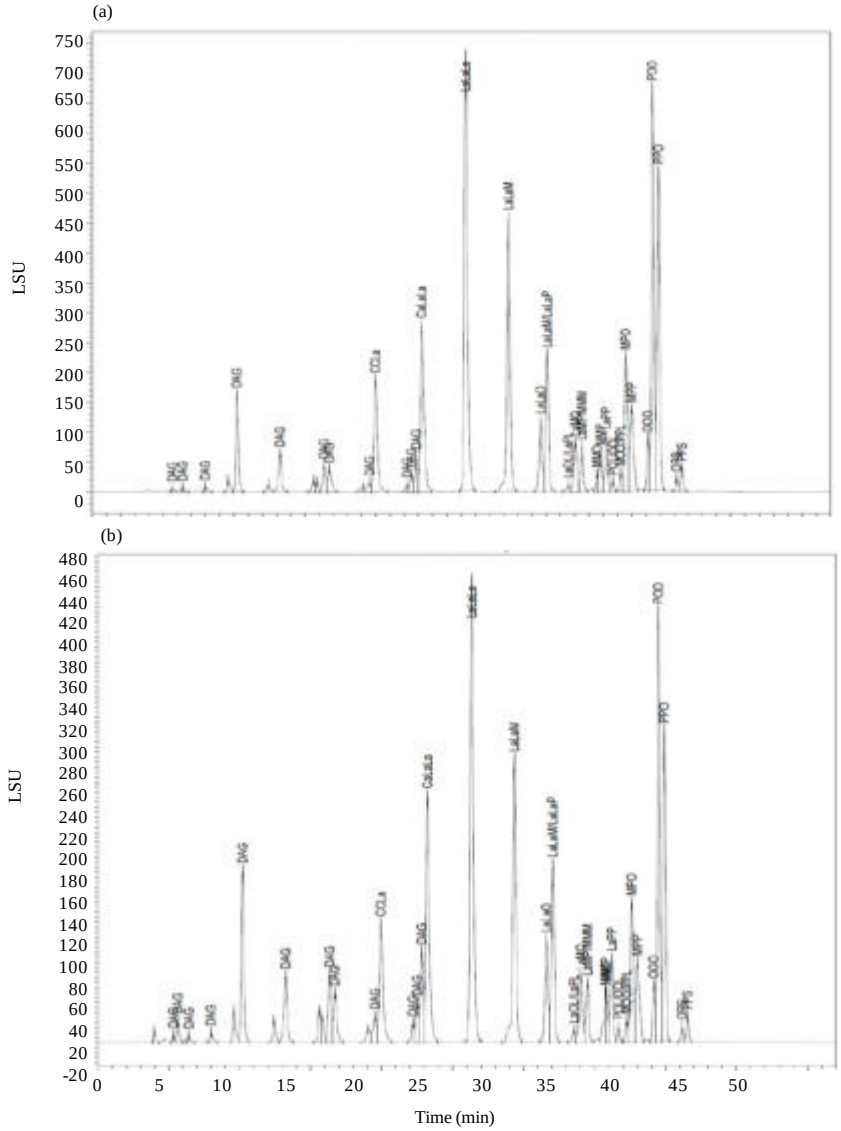

Fig. 2: HPLC chromatogram showing POo: PKO (50:50 w/w) before and after enzymatic transesterification reaction; a) Before enzymatic reaction and b) After enzymatic transesterification reaction at 55°C, 10 h. MAG/DAG = mono and diacylglycerol, C = Caprylic, Ca = Capric, La = Lauric, M = Myristic, P = Palmitic, O = Oleic, L = Linoleic

the period. Meanwhile, the concentration of DAG, a by product of hydrolysis was also found to dramatically increase with the increasing of reaction time starting from 2 up to 8 h of reaction. This could be due to the presence of water trace after prolonged reaction. Several methods of producing DAG have been reported including partial hydrolysis of TAG, esterification of FFA or its derivatives with glycerol or a combination of both methods (Guo and Sun, 2007). Watanabe *et al.* (2003) reported on the synthesis of 84% 1,3 DAG using immobilized 1,3-regiospecific lipase Lipozyme RM1M. The FFA/MAG yield on the other hand, showed an increasing trend throughout the reaction time but of lower amount compared to the former two products. A slight deviation from the general trends was observed at 35°C (during 9-10 h), 45°C (during 9-10 h) and 50°C (during 10 h), suggesting that FFAs liberated from previous hours could be re-esterified to form TAG in the extended time (data not shown). This is in agreement to a report suggesting that prolong reaction time will increase the formation of by products (free fatty acids and glycerol) and cause randomization of fatty acids in the glycerol backbone (Lee *et al.*, 2015). In food industry, oil with these by products need to be subjected with additional refining processes and hence, the cost of production will increase.

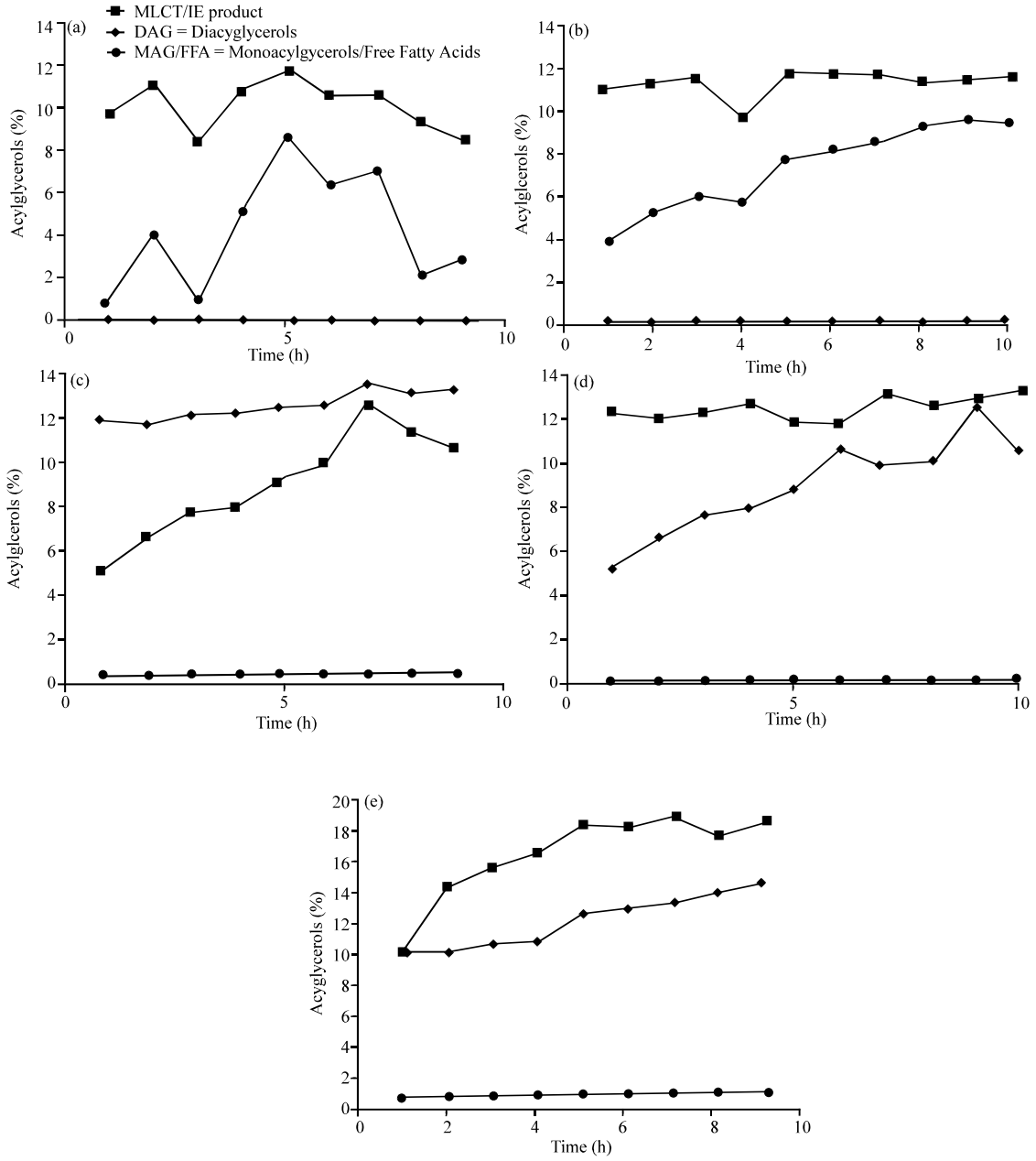

Fig. 3: Acylglycerol composition (%) of the oil blend at various reaction temperatures; a) 35°C; b) 40°C; c) 45°C; d) 50 °C°) 55 °C and e(-♦-) MLCT/IE product = Medium-and Long-Chain Triacylglycerols/interesterification product, (-▲-) DAG = Diacylglycerols, (-▲-) MAG/FFA= Monoacylglycerols/Free Fatty Acids

Effect of reaction temperature: For temperature variable, oil blend which has been enzymatically transesterified at 55°C showed a significant increase in MLCT yield, especially, at 10 h with 14.5% (Fig. 3e). In parallel with that of MLCT, the DAG yield was also increased. The yield of MLCT and DAG indicates that 1, 3- specific ARM lipase has successfully promoted fatty acids redistribution of the reacted blend, producing 1,2- or 2,3- diacylglycerols (1,2-or 2,3-DAGs). MLCT and DAG are products of enzymatic transesterification and partial hydrolysis, respectively. The use of 1,3- specific lipase in the reaction could be advantageous in incorporating Free Fatty Acids (FFA) present in the reaction medium to the sn-1 and sn-3 positions. Wang *et al.* (2012)

suggested that the metabolic and physical properties of SLs are influenced by the regiospecific positions of FA in the TAG molecules. Many 1,3 specific lipases have been reported in the production of Sls such as 1,3 *Rhizomucor miehei* lipase which was used in the acidolysis of sunflower oil and palmitic-stearic acid mixture (Palla and Carrin, 2014). Lipase NRRLY-7723 from *Pseudomonas lynferdii* exhibited 1,3-regiosepecificity toward the TAG of borage oil (Kim *et al*., 2010).

Figure 3a, at lower reaction temperature, 35°C, the MLCT concentration is relatively low demonstrating a poor catalytic activity of ARM lipase in catalyzing the transesterification of the oil blend. The synthesis of MLCT, however, gradually improved as the reaction temperature increased from 40-55°C with 5°C interval (Fig. 3 b-d). The results clearly depicted that reaction carried out at 55°C exhibited the highest conversion of TAG into MLCT and DAG. Apart from MLCT, DAG oil has been regarded as novel functional lipid (Xu *et al*., 2016). The dramatic increase in the reaction rate is suggested by the enhancement of lipolytic activity at high temperature. ARM lipase has been earlier reported to exhibit optimum temperature at 60°C (Ebrahimpour *et al*., 2011). Interestingly, the enzyme displayed excellent stability even when subjected to 10 h reaction at 55°C. Similar finding on 1,3-DG production was also reported at temperature between 45 and 55°C using commercial lipase, Novozym 435. Temperature was described to play significant role in acyl migration (Hamam, 2013). Apart from the intrinsic stability of ARM lipase at elevated temperature, immobilization may have synergistically improved its functional stability and performance.

Melting and crystallization behavior: The thermal profile of fats and oils is dependent on the distribution of TAG species. Lacking of specific melting point as PO consists of high and low melting point Triacylglycerols (TAGs), a temperature range where the oil melts is important to depict the re-arrangement of TAGs in the oil (Zhang *et al*., 2013). Using Differential Scanning Calorimetry (DSC) as an analytical tool to study the physical properties of POo:PKO blend, melting and crystallization behaviors were analyzed at different temperature transition during cooling and heating.

Several parameters including the onset temperature, enthalpy (heat flow status) and end set temperature were measured during the cooling stage (crystallization) of oil blend. The crystallization profile is described by the beginning of fat crystal formation due to reorganization of molecules in the presence of high saturated FA and the development of aggregation and molecules compaction (Saadi *et al*., 2012). Figure 4 shows the crystallization and melting curve of POo:PKO blend at 50: 50 (w/w) measured before and after the enzymatic reaction. The crystallization behavior of the oil blend was somewhat different after 10 h of enzymatic reaction as compared to the blend prior to reaction. Initially, DSC thermogram revealed two crystallization peaks with one sharp peak (Fig. 4a) at temperature range of 0.99°C±0.33 and 7.33°C±0.21. Although, similar pattern of crystallization profile was observed at different reaction temperatures, a marginal shift of the crystallization peak was detected as the temperature increased. At the highest reaction temperature, 55°C the exothermic peak was visible at 4.1°C±0.31 and 9.45°C±0.36. This could be an indication of redistribution of FA in the oil blend, hence, resulting in a slight difference in the physical behavior as compared to the initial blend. Such observation is often seen in oil samples wherethey display multiple exotherms when cooled in DSC (Tan and Man, 2002). This is true for the case of Poo: PKO blend when not subjected to enzymatic transesterification where a single exothermic peak is associated with another peak with small temperature difference. Crystallization temperature and composition of the blend has been reported to significantly influence the mechanism of crystallization (Rashid *et al*., 2012).

As for the melting profile, the curve was found more broad and irregular than the crystallization curves. Frederick *et al*. (2008) suggested that the amount of crystallized fat and the presence of polymorphic transitions are depicted in the melting profiles. Prior to enzymatic transesterification reaction, the original blend melted in one broad fraction associated with a shoulder where the endothermic peak was detected at 19.38°C with T_{on} 1.38°C and T_{off} 25.10°C. The broadening effect may be due to broad TAG distribution in the oil blend where it consists of high and low melting point TAGs (Ebrahimpour *et al*., 2011). As can be seen in Fig. 4b when the temperature increased, the shoulder began to disappear leaving a smooth broad peak with a slight shift towards lower temperature except for reaction conducted at 40°C. At 55°C, the endothermic peak was detected in a temperature range around 0.29 and 22.88°C.

The shift in melting temperature of the oil blend was probably influenced by the altered percentage of saturated and unsaturated fatty acid as previously described in Table 2 and Fig. 2. High melting peak is often attributed by the presence of SSS (Saturated-Saturated-Saturated) while low melting peak corresponds to UUU (Unsaturated-Unsaturated Unsaturated) content of TAG species. Meanwhile the melting peak for mixture of saturated and unsaturated fatty acid lies between the two extremes. The broad melting peak observed in the original blend indicates the

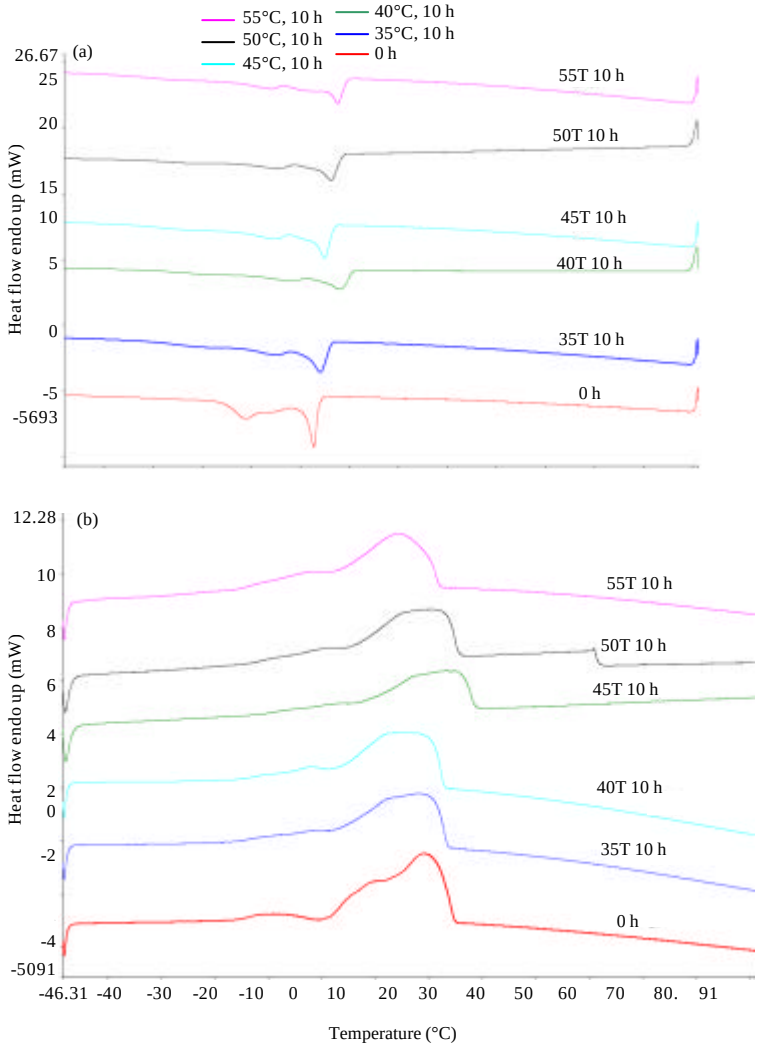

Fig. 4: DSC thermograms of POO: PKO blend (50:50 w/w) before and after enzymatic reaction at various temperatures, 35-55°C with 5°C interval; a) Cooling curves and b) Melting curves. Lines are indicated as follows: (-) 55 °C, 10 h; (-) 50 °C, 10 h; (-) 45 °C, 10 h; (-) 40 °C, 10 h; (-) 35°C, 10 h; (-) 0 h = before enzymatic transesterification

presence of SSS (LaLaLa, LaLaM, LaLaP), SSU (LaLaO), SUU (POO) and SSU (PPO) which occur in various quantity. As the enzymatic reaction took place, the yield of LaLaO, LaLaP and LaLaM became more significant, hence, varying the melting behavior.

CONCLUSION

In this research, we aim to synthesize new structured lipids deriving from oil blend of palm oil derivatives palm olein and palm kernel olein by lipase-catalyzed transesterification using locally isolated, immobilized thermostable 1,3-regioselective lipase from *Geobacillus* sp. strain ARM (designated as ARM lipase). To our best knowledge, literature reporting on blending of the palm oil and palm kernel oil derivatives is relatively limited. Oil blending is one of the effective ways to produce SLs for specific utilizations. Additionally, discovery of new local microbial lipase capable of synthesizing SLs at high temperature may contribute to development of competent biocatalyst for industrial exploitation.

ABBREVIATIONS

SL = Structured lipid
POo = Palm Olein
PKO = Palm Kernel Olein
MCFA = Medium Chain Fatty Acid
LCFA = Long Chain Fatty Acid
MLCT = Medium and Long Chain Triacylglyceride
DAG = Diacylglyceride
FA = Fatty Acid
TAG = Triacylglycerol

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