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Synthesis of Cocoa Butter Alternative via Enzymatic Transesterification using *Rhizopus oryzae* Lipase Immobilised on Sago

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Rhizopus oryzae lipase (ROL) is a valuable biocatalyst for modifying oils and fats, especially in creating cocoa butter alternatives. However, its application in high-temperature industrial processes is limited by thermal stability. Also, water-restricted reactions like lipase-catalysed transesterification require a dried enzyme form. This study explored the use of immobilised ROL on sago, a porous and biocompatible support, for the enzymatic synthesis of structured lipids resembling cocoa butter. ROL was immobilised on sago using an optimised spray-drying method and applied in the transesterification of palm mid-fraction, olive oil, and stearic acid blends. The product developed using a 1:1:1 ratio emerged as the best cocoa butter alternative based on chemical and temperature analyses. High-performance liquid chromatography analysis of the enzymatic product showed reduced palmitoyl-oleoyl-palmitoyl glycerol and increased palmitoyl-oleoyl-stearoyl glycerol and stearoyl-oleoyl-stearoyl glycerol. Gas chromatography confirmed that stearic acid incorporation increased linearly with its molar ratio the reaction. The slip melting point of the synthesised alternative was 36.2°C, with solid fat content at 7.73 % at 30°C and 2.02 % at 40°C. Differential scanning calorimetry indicated two melting peaks below 37°C. This study highlights the successful use of immobilised ROL on sago to produce potential cocoa butter alternatives, advancing plant-based substitutes for the confectionery industry.

Keywords: *Rhizopus oryzae* lipase; immobilised enzyme; biocatalyst; transesterification; cocoa butter alternative

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Enzymes are well-known biocatalysts that have been in popular demand with the increased interest in natural and sustainable products. One of many enzymes that are commonly used in industries is lipase. Lipases in general are very versatile enzymes that catalyse both the hydrolysis and the synthesis of ester bonds. Therefore, it has been heavily used in industries that focus on food processing, oleo-chemicals, detergents, dairy, pharmaceuticals, cosmetics, biodiesel, and waste management [1]. The reactions and substrate preferences of lipases are very much influenced by the source from which it is isolated [2]. Among the commercially available lipases is the *Rhizopus oryzae* lipase (ROL), a 1,3-specific lipase that prefers medium and long-chain fatty acids. ROL is often chosen for food-related use and research as it is classified as a food-grade enzyme [3].

Like any other enzymes, lipases are very sensitive to the environment and prone to degradation. Thus, to prolong the catalytic lifespan of the enzyme, especially during extreme conditions in industrial applications, transportation and storage, a lot of research has been done to stabilise the enzyme [4, 5]. One way to achieve this is through enzyme immobilisation, a powerful technique that is strategized

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to improve protein properties by bonding or trapping an enzyme on a selected surface or support [6]. On top of enhancing catalytic activity and structural stability, enzyme immobilisation proved to have other advantages like easy to handle and store, better product recovery, fast termination of reactions, controlled products] formation, and reusable [6-9]. There are various methods to immobilise enzymes, either by physical methods like adsorption, encapsulation, and entrapment, or by chemical methods like covalent binding and cross-linking [9, 10]. Depending on the immobilisation method and the support used, enzymes can be immobilised either reversibly or irreversibly [7].

The properties of the immobilisation support have a great influence on the efficiency of the immobilised enzyme. The ideal properties of the support are physically and chemically durable, inert, high loading capacity, cheap, easy to obtain, and can increase enzyme specificity and activity [5, 7, 11]. One organic material that matches those characteristics is sago. On top of that, sago is biodegradable and eco-friendly, making it a great choice as an immobilisation support [12, 13]. Sago is traditionally consumed as food, and only recently the usage has been diversified through scientific research [13-18]. The usage of sago as the immobilisation support for ROL lipase is perfect for applications in food-related industries like oil and fat modification and confectionery.

Fats and oil, or lipids, can be modified structurally by introducing new fatty acids or reorienting the positions of fatty acids on the glycerol backbone to produce a new or different lipid, often known as structured lipids [19]. Structured lipids can be synthesised by transesterification reaction using either chemical or enzyme, like lipase [20]. The goal of structured lipids is to enhance the characteristics of natural lipids, either of plant or animal origin, to better suit human needs, or to produce lipids that are facing scarcity like cocoa butter. Cocoa butter has a unique physicochemical property that is responsible for its distinctive melting and flavour profile. The desirable organoleptic characteristics make cocoa butter the superior fat to be used in confectionery and cosmetics [21]. The search for cocoa butter alternatives (CBA) that can function as a replacement for cocoa butter, whether used exclusively or blended with cocoa butter in chocolate and other confectionery products, can address the issues of cocoa butter shortage that is due to limited supply, variability in quality, high demand, and price fluctuations [22].

This study aimed to expand the applications of immobilised lipase on sago by producing a cocoa butter alternative. This initiative is hoped to provide more opportunities for lipase applications as well as sago applications.

EXPERIMENTAL

Immobilisation of ROL

ROL was immobilised according to a previously reported protocol [12]. A 1 % w/v solution of sago flour in distilled water was heated at 80°C for 30 minutes using a water bath (Memmert). A total of 650 unit of ROL (Lipase DF "Amano" 15 by Amano enzyme) were then added to 100 mL of the sago solution. The mixture was spray-dried using a BÜCHI Mini Spray Dryer B-290. The inlet temperature of the spray-dryer was 100°C, the aspirator was 35 m^3/h, and the pump was 2 mL/min. The immobilised enzyme was kept at 10°C until used.

Table 1. Ratios of the oil blend for transesterification reactions.

Sample No.	Ratio of PMF:olive oil:stearic acid
1	1:0:1
2	1:0:2
3	1:0:3
4	1:0.5:1
5	1:0.5:2
6	1:0.5:3
7	1:1:1
8	1:1:2
9	1:1:3

Synthesis of a Cocoa Butter Alternative using Immobilised ROL

Cocoa butter alternative, or structured lipid that mimics cocoa butter, was synthesised by enzymatic transesterification reaction using immobilised ROL. The substrates used in this reaction were palm mid-fraction (PMF) with an iodine value of 45, olive oil (Bertolli), and stearic acid (Sigma). The substrates were mixed with a different weight ratio (**Table 1**) and were melted at 60°C. Then, hexane (1 mL per 1 g of fat), molecular sieve (450 mg), and enzyme (10 % w/w of the total weight of substrates) were added. The reaction mixture was incubated at 60°C, 200 rpm, for 12 hours in a water bath shaker (Memmert). The enzyme was filtered using Whatman paper No. 1 to stop the reaction. The free fatty acids that remained in the reaction were removed by deacidification using alkaline extraction [23]. The purified product was subjected to further analyses. The cocoa butter reference material IRMM-801 (European Commission JRC, Italy) was used as the reference standard in the analyses.

Triacylglycerols Composition Determination

High-performance liquid chromatography (HPLC) was carried out to detect the triacylglycerol (TAG) profiles of the samples. The results were analysed to determine the products of the transesterification reactions. HPLC was done using Waters 2695 system in combination with a refractive index detector (Waters 2414), and Lichrospher Star RP-18 column, 5 µm (i.d. 4.6 x 250 mm) (Merck). A volume of 20 µL of the sample (10 % (v/v) in chloroform) was injected. The oven temperature was set at 30°C. The mobile phase used was acetone and acetonitrile (63.5:36.5) and the flow rate was 1.0 mL/min. TAGs were identified based on the reference data in the software. The cocoa butter reference material IRMM-801 (European Commission JRC, Italy) was used as the reference standard.

Fatty Acid Composition Determination

The samples were methylated into fatty acid methyl ester (FAME) before determination using gas chromatography-mass spectrometry (GC-MS) using a direct method [24]. The fatty acid composition of the FAME was determined by gas chromatography (Agilent 7890A Series) using a HP-88 GC column (Agilent). The initial oven temperature was 70°C, held for 2 minutes, subsequently increased to 230°C at a rate of 20°C/min, and then held for 15 minutes. Helium was used as the carrier gas at a flow rate of 20 cm/s. Both the injector and the detector were set at 230°C. The split ratio was 30:1. Fatty acids were identified using the database built in the GC/MSD ChemStation Software.

Slip Melting Point Determination

The slip melting point determination (SMP) was performed according to the American Oil Chemists' Society (AOCS) method Cc 3-25. An open-ended capillary tube was dipped into the melted homogenised sample to take up 10 mm of the sample in the capillary tube. The capillary tube was chilled against ice before being placed in a test tube and incubated at 10°C for 16 h. After incubation, the capillary tube was tied to a thermometer and dipped into a beaker of 10°C distilled water on an MS-H280-Pro magnetic hotplate and stirrer (Biologix, China). The temperature of the water was increased at the rate of 1°C/min until the sample in the capillary tube began to rise. The temperature at which the sample rises was observed. The experiment was done in triplicates.

Solid Fat Content Determination

The solid fat content (SFC) measurement was carried out using a Bruker Minispec (Model Mq 20) pulse Nuclear Magnetic Resonance (pNMR) spectrometer. The non-stabilised method according to MPOB Test Method p4.8 (2004) was used. The sample was put in the NMR tube and was melted at 70°C for 30 min, followed by chilling at 0°C for 90 min, and then tempering at each measuring temperature for 30 min prior to measurement. SFC measurements were taken at 5°C intervals over the range of 0-40°C. Melting, chilling, and holding of the samples were carried out in pre-equilibrated water baths with an accuracy of 0.1°C.

Thermal Analysis

Thermal analysis was carried out on a differential scanning calorimeter (Mettler Toledo DSC 823). Approximately 410 mg of molten sample was placed in a standard DSC aluminium pan and then hermetically sealed. An empty identical pan was used as a reference. The samples were heated to 80°C at a rate of 10°C/min and held for 15 minutes to destroy the thermal history of the samples. Then, cooled to −40°C at a rate of 10°C/min and held for 15 min. Then, the samples were heated back to 80°C at a rate of 5°C/min and held for 15 minutes. The cocoa butter reference material IRMM-801 (European Commission JRC, Italy) was used as the reference standard.

Statistical Analysis

The data represents mean ± standard error where the n value equals to three biological replicates. The data were statistically analysed by one-way ANOVA and Tukey's HSD post-hoc test using R version 3.1.0. The significant difference was based on a 95 % level of confidence ($p<0.05$).

RESULTS AND DISCUSSION

Synthesis of a Cocoa Butter Alternative using Immobilised ROL

Lipase mainly catalyses the hydrolysis of long-chain triacylglycerols, however, in water-limiting conditions, lipase reaction is reversed to esterification and transesterification. Immobilizing lipase not only stabilises the enzyme, but it also makes the lipase suitable for water-sensitive reactions due to its dry form [12]. In this research, enzymatic transesterification was catalysed by the immobilised ROL on sago and using Palm mid-fraction (PMF), olive oil and stearic acid as the substrate. Transesterification is a reaction that exchanges the acyl groups between the substrates, either with alcohol, acid, amine, or another acyl [25]. Here, two types of reactions happened during the transesterification, that is, interesterification (equation 1) and acidolysis (equation 2), where (a) is PMF, (b) is olive oil, and (c) is stearic acid. The products of these reactions were unique fats that are structurally different from the fats used as the substrates.

$$\underset{(a)}{R1COOR2} + \underset{(b)}{R3COOR4} \rightarrow R3COOR2 + R1COOR4 \quad (1)$$

$$\underset{(a)}{R1COOR2} + \underset{(c)}{R3COOH} \rightarrow R3COOR2 + R1COOR4 \quad (2)$$

PMF TAG composition is similar to cocoa butter, but the thermal characteristics are slightly different and therefore, it was chosen as the primary substrate. To adjust the thermal characteristics of PMF so that it is closer to the cocoa butter while maintaining the similarity of the chemical characteristics, olive oil and stearic acid were added to the reaction. The substrate ratios were manipulated aiming to obtain the reaction product that mimics cocoa butter's chemical and thermal characteristics. The chemical characteristics were analysed using HPLC and GC-MS, and the physical characteristics determined were the slip melting point, solid fat content, and thermal analysis.

Triacylglycerols Composition Determination

Table 2 shows the TAG composition of the transesterification products obtained by HPLC. There were 12 types of TAG detected by HPLC, but the main focus is on the percentage of di-saturated TAGs, that is, palmitoyl-oleoyl-palmitoyl glycerol (POP), palmitoyl-oleoyl-stearoyl glycerol (POS), and stearoyl-oleoyl-stearoyl glycerol (SOS), which constituted the majority component of cocoa butter. These TAGs are responsible for the distinguished melting profiles, crystallisation, and polymorphisms of cocoa butter that are desirable in confectionery applications, especially in chocolates [26].

PMF has almost the same chemical composition as cocoa butter and also a sigmoidal solid fat content (SFC) curve that is distinctive for cocoa butter, hence making it a good candidate as a cocoa butter alternative [26, 27]. PMF used in this research is made of 49 % POP, 9 % POS, and 0.6 % SOS (**Table 2**). However, a cocoa butter equivalent should have 21 % POP, 40 % POS, 27 % SOS, and a trace of other TAGs [27]. PMF lacks SOS, which is needed for β crystal formation in cocoa butter, while POP and POS are responsible for β′ crystals [26]. The β crystal is crucial for achieving the desirable texture, gloss and organoleptic characteristics of high-quality chocolate, while β′ crystals contribute to stability and a softer texture in certain confectionery products. The balance between these crystals ensures optimal product characteristics such as firmness, mouthfeel, and resistance to defects like fat bloom. To increase the amount of POS and SOS to resemble cocoa butter, therefore stearic acids were incorporated in the reaction.

The addition of stearic acid to the reaction mixture has encouraged the formation of POS and SOS from POP, which is the major component of PMF. However, the analysis of one-way ANOVA and Tukey's HSD test showed that the total di-saturated TAGs (SSU: POP, POS, SOS) for all transesterified products were still significantly lower than cocoa butter. The total amount of SSU of the transesterified products was reduced in comparison to the PMF due to a major reduction of POP, which has contributed to the increase of POS and SOS. The ratio of 1:0:2 had the closest reading of POP to cocoa butter, while 1:1:3 had the best reading of SOS, and none has come close to the POS reading of cocoa butter. However, with the increase of stearic acid, the total tri-saturated TAGs (SSS) were significantly higher than cocoa butter. The presence of tri-saturated TAGs would affect the slip melting point, solid fat content profile, and the thermal analysis by differential scanning calorimeter (DSC). Also, it will give an undesirable waxy mouthfeel effect when used in food products. This occurrence was also encountered by another researcher where the tri-saturated TAGs was 6.18 % after transesterification, and it was overcome by doing fractionation to remove the excess tri-saturated TAGs as well as the tri-unsaturated (UUU) and mono-saturated (SUU) [28]. After the fractionation, the majority that was left were the desired POP, POS, and SOS.

This study used another approach to reduce the tri-saturated TAGs, that is by adding olive oil to the reaction mixture. The addition of olive oil was anticipated to reduce the tri-saturated TAGs while driving the formation of POS and SOS as olive oil is low in tri-saturated TAGs and rich in sn-2 oleic acid (40 % oleoyl-oleoyl-oleoyl glycerol (OOO) and 24 % palmitic-oleoyl-oleoyl glycerol (POO)). **Table 2** shows that the supplementation of olive oil effectively reduced the total amount of tri-saturated TAGs (SSS),

as the OOO contributed by the olive oil reacted with the stearic acid in the reaction mixture, converting it into SOS.

Previous studies have used stearic acid to modify PMF to make cocoa butter alternatives, but the outcomes varied. For example, a previous research has produced a cocoa butter equivalent with 23.4 % POP, 38.5 % POS, and 20.2 % SOS with the use of 1.6 ratio of stearic acid/PMF [27]. However, the cocoa butter equivalent produced contained diacylglycerols, which required further purification process. Another research has altered the TAG composition of soft PMF with stearic acid (with a ratio of 75:25) using *Rhizomucor miehei* lipase and achieved the highest conversion to 12 % POP, 31 % POS, and 17 % SOS [29]. Another successful modification of the TAG composition in PMF, closely resembling that of cocoa butter, was achieved using a palmitic and stearic fatty acid mixture with the substrate ratio of 1:2, resulting in 30.7 % POP, 40.1 % POS, and 14.5 % SOS [30].

Table 2. Triacylglycerols compositions of the transesterification products (%).

TAG	CB	PMF	Olive oil	1:0:1	1:0:2	1:0:3	1:0.5:1	1:0.5:2	1:0.5:3	1:1:1	1:1:2	1:1:3
POP	18.55 ± 2.29^{bc}	48.90 ± 0.21^{a}	3.36 ± 0.25^{g}	21.7 ± 0.05^{b}	18.00 ± 2.00^{bd}	13.40 ± 2.61^{cdf}	15.80 ± 0.03^{bde}	13.80 ± 0.13^{bf}	10.00 ± 0.91^{efg}	10.20 ± 1.32^{dfg}	7.55 ± 1.37^{fg}	6.14 ± 2.96^{fg}
POS	44.74 ± 2.92^{a}	9.02 ± 0.09^{e}	1.26 ± 0.31^{f}	27.90 ± 0.09^{bc}	29.50 ± 0.53^{b}	28.90 ± 0.63^{b}	20.90 ± 0.03^{d}	21.90 ± 0.31^{d}	23.30 ± 0.29^{cd}	19.30 ± 0.40^{d}	19.70 ± 0.89^{d}	19.50 ± 1.11^{d}
SOS	30.31 ± 1.19^{a}	0.58 ± 0.29^{e}	0.24 ± 0.08^{e}	14.30 ± 0.55^{cd}	21.00 ± 1.42^{acd}	24.50 ± 2.44^{ac}	10.70 ± 0.16^{de}	16.20 ± 0.40^{bcd}	21.30 ± 0.44^{acd}	13.30 ± 1.56^{ce}	21.70 ± 4.61^{acd}	29.10 ± 7.22^{ab}
UUU	0.71 ± 0.62^{e}	2.50 ± 0.07^{de}	53.00 ± 0.42^{a}	0.96 ± 0.14^{e}	0.722 ± 0.38^{e}	3.21 ± 2.67^{ce}	9.29 ± 0.40^{bcd}	7.98 ± 0.63^{bcd}	6.39 ± 0.57^{de}	10.70 ± 0.30^{b}	9.60 ± 2.38^{bc}	7.36 ± 2.67^{be}
SSS	0.87 ± 0.41^{e}	6.49 ± 0.52^{bd}	0.36 ± 0.17^{e}	13.10 ± 0.62^{a}	14.30 ± 0.91^{a}	14.20 ± 0.74^{a}	7.16 ± 0.19^{bd}	8.00 ± 0.44^{bc}	8.39 ± 0.20^{b}	4.92 ± 0.16^{d}	5.05 ± 0.98^{cd}	5.43 ± 0.94^{bd}
SUU	3.39 ± 1.17^{h}	22.95 ± 0.50^{f}	36.90 ± 0.08^{ab}	18.10 ± 0.08^{g}	16.70 ± 1.76^{g}	14.00 ± 1.27^{g}	33.30 ± 0.07^{bc}	29.80 ± 0.23^{cd}	24.20 ± 1.21^{ef}	39.30 ± 0.12^{a}	32.80 ± 0.68^{bc}	27.70 ± 1.30^{de}
SSU	95.03 ± 1.84^{a}	68.06 ± 0.21^{b}	5.63 ± 0.33^{e}	67.80 ± 0.56^{b}	71.50 ± 0.67^{b}	68.60 ± 3.34^{b}	50.20 ± 0.14^{cd}	54.30 ± 0.04^{cd}	56.60 ± 1.69^{c}	44.70 ± 0.25^{d}	50.60 ± 3.24^{cd}	56.60 ± 4.70^{c}

Note: Ratio: PMF:olive oil:stearic acid;

CB: cocoa butter, PMF: palm mid-fraction; P: palmitic acid; S: stearic acid; O: oleic acid; UUU: tri-unsaturated TAGs; SSS: tri-saturated TAGs; SUU; mono-saturated TAGs; SUS: di-saturated TAGs.

Values are means ± SE, n = 3. Means in a row without a common superscript letter differ (P<0.05) as analysed by one-way ANOVA and the Tukey's HSD test.

Table 3. Fatty acids composition of the transesterification products.

	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Saturated fatty acid	Unsaturated fatty acid
Olive oil	12.06 ± 0.42^{ij}	3.15 ± 0.49^{i}	74.84 ± 0.42^{a}	8.96 ± 0.49^{a}	15.21 ± 0.91^{h}	83.8 ± 0.91^{a}
PMF	49.46 ± 0.08^{a}	7.86 ± 0.42^{h}	36.62 ± 0.08^{c}	6.06 ± 0.42^{bc}	57.32 ± 0.50^{f}	42.68 ± 0.50^{c}
Commercial CB	25.15 ± 0.57^{c}	38.54 ± 0.40^{d}	32.60 ± 0.57^{e}	2.41 ± 0.40^{f}	63.69 ± 0.97^{cd}	35.01 ± 0.97^{ef}
CB standard	24.37 ± 0.50^{cd}	38.02 ± 0.44^{de}	33.68 ± 0.50^{de}	2.77 ± 0.44^{ef}	62.39 ± 0.94^{ce}	36.45 ± 0.94^{df}
1:0:1	29.93 ± 0.52^{b}	36.14 ± 0.57^{ef}	29.40 ± 0.52^{f}	4.53 ± 0.57^{bde}	66.07 ± 1.09^{bc}	33.93 ± 1.09^{fg}
1:0:2	22.61 ± 0.34^{de}	47.09 ± 0.17^{b}	26.23 ± 0.34^{g}	4.07 ± 0.17^{cdf}	69.7 ± 0.51^{ab}	30.3 ± 0.51^{gh}
1:0:3	19.95 ± 0.51^{fg}	51.38 ± 0.07^{a}	24.80 ± 0.51^{g}	3.87 ± 0.07^{df}	71.33 ± 0.58^{a}	28.67 ± 0.58^{h}
1:0.5:1	22.00 ± 0.51^{ef}	35.29 ± 0.22^{f}	37.22 ± 0.51^{c}	5.49 ± 0.22^{bd}	57.29 ± 0.73^{f}	42.71 ± 0.73^{c}
1:0.5:2	17.56 ± 0.50^{h}	43.89 ± 0.49^{c}	33.52 ± 0.50^{de}	5.03 ± 0.49^{bd}	61.45 ± 0.99^{de}	38.55 ± 0.99^{de}
1:0.5:3	13.74 ± 0.27^{i}	51.49 ± 0.55^{a}	30.21 ± 0.27^{f}	4.56 ± 0.55^{bde}	65.23 ± 0.83^{cd}	34.77 ± 0.83^{ef}
1:1:1	18.98 ± 0.28^{gh}	33.17 ± 0.42^{g}	41.66 ± 0.28^{b}	6.19 ± 0.42^{b}	52.15 ± 0.70^{g}	47.85 ± 0.70^{b}
1:1:2	13.40 ± 0.41^{ij}	46.20 ± 0.20^{b}	35.15 ± 0.41^{cd}	5.25 ± 0.20^{bd}	59.6 ± 0.61^{ef}	40.4 ± 0.61^{cd}
1:1:3	11.39 ± 0.08^{j}	51.20 ± 0.35^{a}	32.63 ± 0.08^{e}	4.77 ± 0.35^{bde}	62.59 ± 0.43^{ce}	37.4 ± 0.43^{df}

Note: Ratio = PMF:olive oil:stearic acid.

CB: cocoa butter, PMF: palm mid-fraction.

Values are means ± SE, n = 3. Means in a column without a common superscript letter differ (P<0.05) as analysed by one-way ANOVA and the Tukey's HSD test.

Fatty Acids Composition Determination

Results for the Fatty acids composition are summarised in **Table 3**. The samples were made of four main fatty acids, that is, palmitic acid, stearic acid, oleic acid, and linoleic acid. It was shown that the increase of olive oil in the reaction leads to the increase of unsaturated fatty acids (oleic acid and linoleic acid) as they were contributed by the major TAGs of olive oil, OOO and POO. When stearic acid was added to the reaction, other fatty acids (palmitic acid, oleic acid, and linoleic acid) were displaced. The analysis of one-way ANOVA and Tukey's HSD test showed that with the increasing amount of free stearic acid in the substrate ratios, the amount of stearic acid in the samples has significantly increased while palmitic acid has significantly decreased. This result complies with the reduction of POP, as shown previously in **Table 2**. The reduction of palmitic acid could be refrained by adding free palmitic acid as the substrate to the reaction mixture [30]. Another study has also used a combination of palmitic acid and stearic acid to convert olive oil into a cocoa butter-like fat by acidolysis reaction using Lipozyme IM [31]. The excess palmitic acid added to the reaction competes with the stearic acid during the enzymatic reaction and slows down the conversion of POP into POS and SOS. Overall, the ratio of saturated and unsaturated fatty acids in samples 1:0:1, 1:0.5:2, 1:0.5:3, and 1:1:3 was not significantly different to cocoa butter and could be suitable as the cocoa butter alternatives.

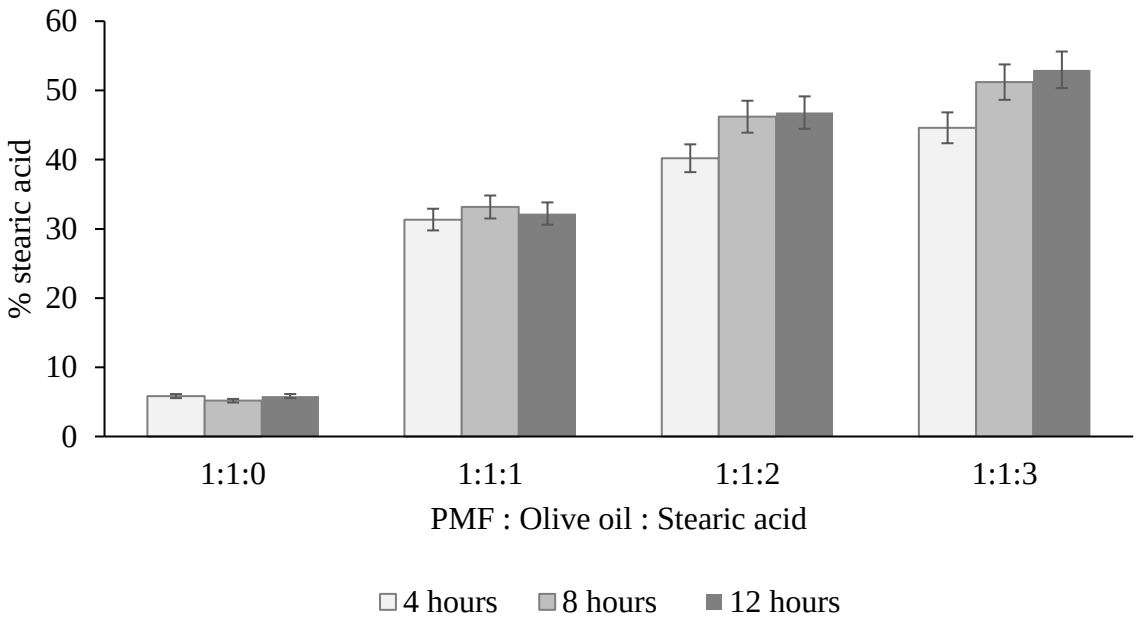

Figure 1. Percentage of stearic acid in the samples after the transesterification reactions with different reaction durations. The ratios of the substrates used in the reaction were 1:1:0, 1:1:1, 1:1:2, and 1:1:3 of PMF:olive oil:stearic acid. Data represents mean ± SE (n=3).

Figure 1 depicted that the amount of stearic acid incorporated increased linearly with the molar ratio of the stearic acid added to the enzymatic reaction and according to the analysis of one-way ANOVA and Tukey's HSD test, the increments were significant. Additionally, it seems that the reaction has not achieved substrate saturation and more stearic acid could be added to the reaction. However, that was not the intention of this research since the successive increase of stearic acid would result in the increaseof tri-saturated TAGs and that would be unfavourable for a cocoa butter alternative. From **Figure 1**, the rate of stearic acid uptake can also be observed. The incorporations of stearic acid showed no significant improvement when the reaction was incubated longer than 8 hours. Similar results were observed by another researcher during transesterification of PMF and stearic acid using *Rhizomucor miehei* lipase, where the highest substrate conversion rate was during the first 6 hours, following that was an equilibrium state from 12 to 14 hours before a decrease of TAG content was observed by the end of the reaction time (20 hours) [32]. As the incubation prolonged, the lipids were hydrolysed and hence the TAG content was reduced.

Slip Melting Point Determination

The slip melting point (SMP) of a fat sample is the temperature when the fat in a capillary becomes soft and fluid enough to slip up the capillary when heated [33]. During that heating process, the crystalline matter of the fat reduced until the crystal network lost its cohesion and became fluid. The SMP generally indicates that there was about 4 % of solid fats residual [34]. The SMP result presented in **Figure 2** shows that with the increase of stearic acid amount added to the enzymatic reaction, the SMP also increased. A higher temperature of SMP was due to a higher amount of saturated fatty acids in the TAGs. On the contrary, as the amount of olive oil increased, the SMP was reduced. This is because the addition of olive oil increased the amount of unsaturated fatty acids, which has a lower melting temperature. Based on the results gathered, the ratio 1:1:1 is the most suitable to be used as a cocoa butter alternative as the SMP was below 37°C, and statistically not significant from the cocoa butter's reading when analysed by the one-way ANOVA and Tukey's HSD test. However, a product with a slightly higher SMP is still acceptable for different usage, for example, cocoa butter improver (Palmy 400) and cocoa butter replacer (Melano STH), which have SMP of 37 − 41°C and 38.5 − 40.5°C, respectively [35]. These products were meant to improve the heat resistance of chocolate for moulding and enrobing.

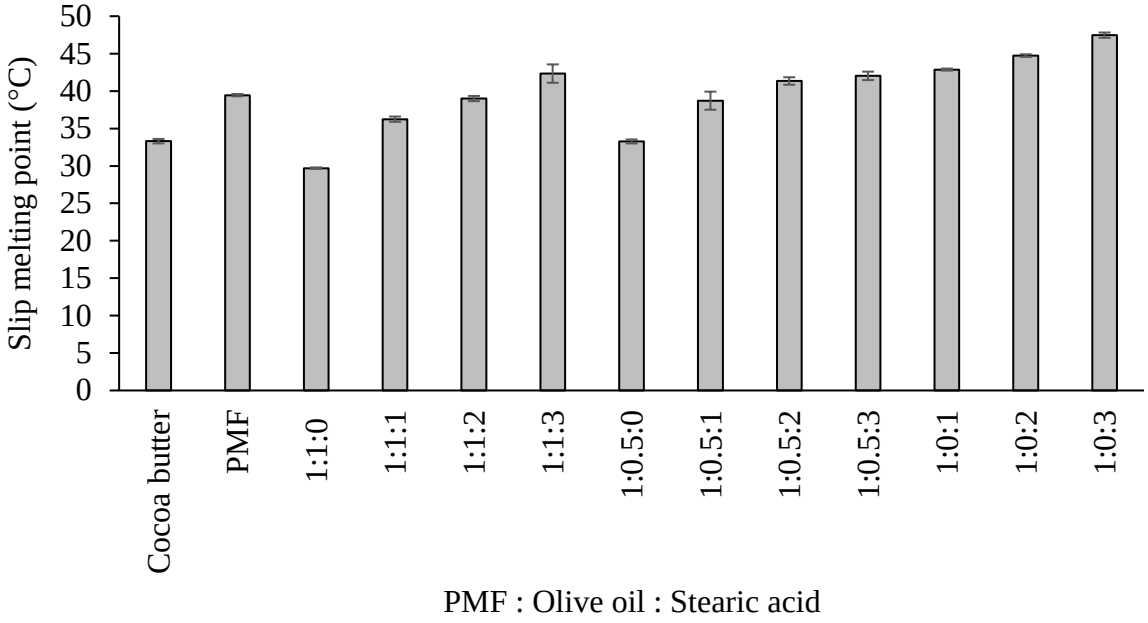

Figure 2. Slip melting points of the transesterification products. The ratios represent the substrate used, PMF: olive oil:stearic acid. Data represents mean ± SE (n=3).

Solid Fat Content Determination

Solid fat content (SFC) profile generally gives information about the hardness of a lipid sample at different temperatures and reveals its melting behaviour [36, 37]. A good cocoa butter alternative should mimic the SFC profile of the cocoa butter. Normally, the SFC curve of cocoa butter is divided into four parts; the temperatures below 25°C define its hardness, temperatures between 25°C to 30°C indicate its heat resistance, temperatures between 27°C to 33°C represent the main melting phase that brings a cooling sensation in the mouth and releases flavour, and finally SFC at temperatures above 35°C causes a waxy mouthfeel [38]. The SFC profile of cocoa butter in **Figure 3** is consistent with reported results by other researchers, there was a rapid drop in SCF at 20°C to 35°C, and the SFC approached 0 % at 37°C [39, 40]. That profile is responsible for the prominent features of cocoa butter that it is hard and brittle at room temperature and melts at body temperature. Presumably, this is due to the high content of di-saturated TAGs, that is, POP, POS, and SOS [26].

Compared to cocoa butter, the SFC curve of PMF has lower SFC at a temperature below 25°C, and slightly higher SFC at a temperature above that. At 35°C and 40°C, the SFC was 4.54 % and 3.72 %, respectively. A fat that has an SFC of less than 3.5 % at 33°C and above has no waxy mouthfeel [37]. Therefore, the addition of olive oil to PMF was expected to solve that matter. However, the presence of olive oil seems to lower the overall SFC, as can be seen in the results of PMF:olive oil:stearic acid ratio of 1:0:1 and 1:1:1 in **Figure 3**, where the PMF and stearic acid concentration remained constant. The presence of olive oil in the transesterification reaction has lowered the di-saturated TAGs (POP, POS, and SOS) and increased the mono-saturated TAGs (**Table 2**). The effect on SFC can be seen at temperatures between 15°C to 25°C as the percentage of solid fat decreased, which is the main melting temperature of those TAGs [26, 39]. This made the transesterification products slightly softer than PMF.

On the other hand, the increase of stearic acid concentration seems to increase the overall SFC, as can be seen in the results of PMF:olive oil:stearic acid ratio of 1:1:0, 1:1:1, 1:1:2, and 1:1:3 in **Figure 3**. The stearic acid supplemented into the transesterification products has affected the SFC at critical temperatures, above 35°C. This is because according to **Table 2**, the addition of stearic acid did not only increase the POS and SOS TAGs as desired but also increased the tri-saturated TAGs (SSS), which have a high melting temperature [26]. A high solid fat content at temperatures above 35°C is not favourable in the chocolate industry as it will produce a waxy mouthfeel, also known as the "fatty residue", that can easily be detected during a sensory evaluation [41].

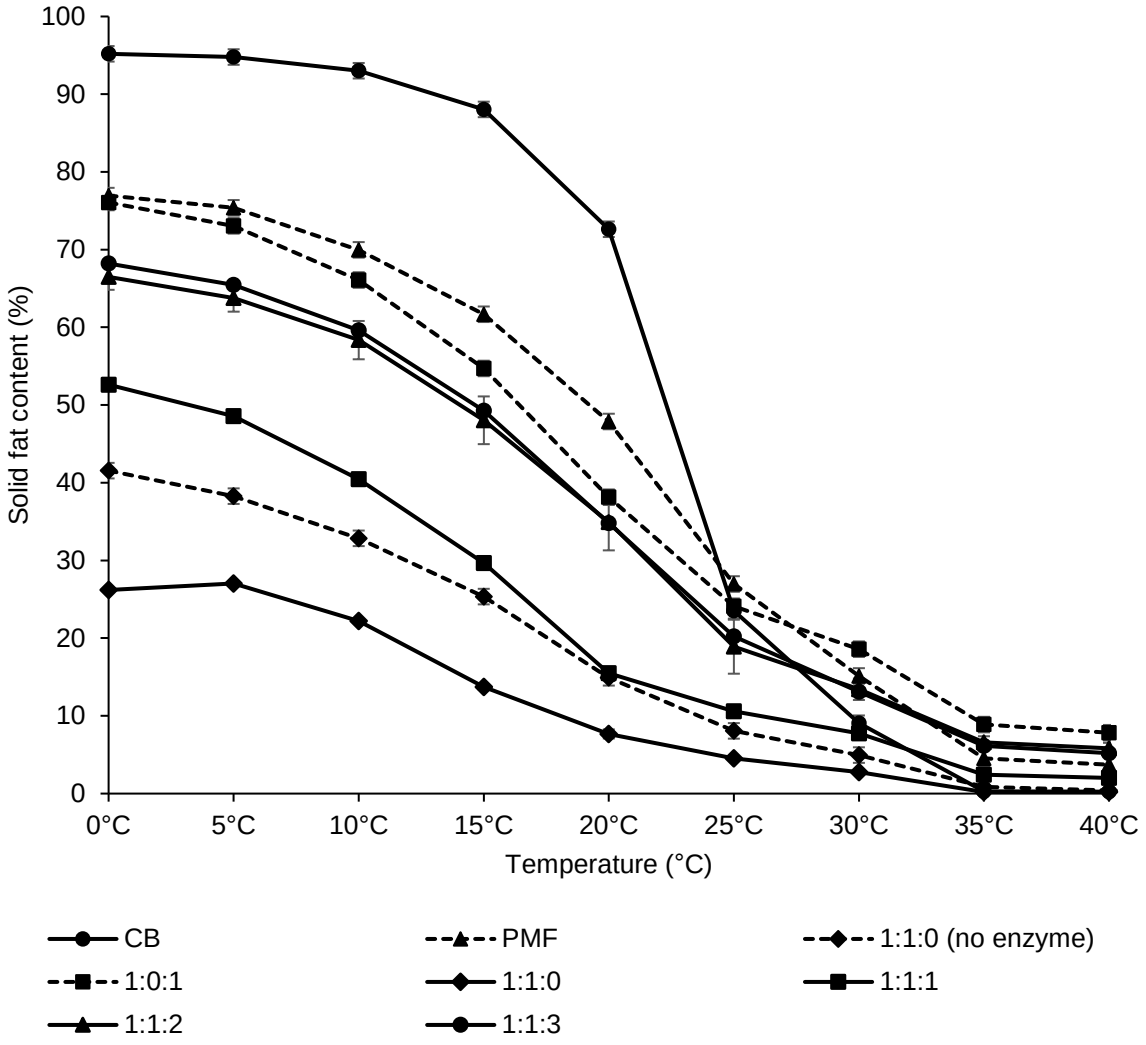

Figure 3. Solid fat content profiles of the transesterification products with substrate ratio of 1:0:1, 1:1:0, 1:1:1, 1:1:2, and 1:1:3 of PMF:olive oil:stearic acid, cocoa butter (CB), and palm mid-fraction (PMF). Data represents mean ± SE (n=3).

Based on the results in **Figure 3**, the most suitable sample for cocoa butter alternative is 1:1:1 as the SFC was close to cocoa butter at 30°C, 35°C, and 40°C with 7.73 %, 2.44 %, and 2.02 %, respectively. Moreover, the SFC at 25°C must be more than 10 % to avoid oil exudation, where the SFC for 1:1:1 was 15.50 % [34]. The right SFC of confectionary fat to make chocolate is 63 % at 20°C, 40 % at 25°C, and 0 % at 37°C [42]. Although the SFC of 1:1:1 is low at a lower temperature, it still has the potential to be a cocoa butter alternative and could be used in combination with cocoa butter for a different purpose other than chocolate, such as for confectionery fillings, which only require less than 50 % of SFC at 20°C [42].

Thermal Analysis

The crystallisation and melting profiles of the transesterification products obtained by differential scanning calorimeter (DSC) are summarised in **Table 4**. The thermal profiles provide information on the amounts of TAGs with different crystallisation or melting temperatures and also the presence of polymorphisms in the sample. Melting is an endothermic process, while crystallisation is exothermic. The crystallisation and melting thermograms of cocoa butter showed a single peak with high enthalpy. This is because cocoa butter predominantly consists of di-saturated TAGs (POP, POS, and SOS). PMF, on the other hand, showed a broad crystallisation and melting temperature range, which consists of two peaks, and so did all the transesterification products. This indicates that the samples had a wide range of TAG content with a broad range of crystallisation and melting temperatures [33]. Focusing on the endothermic peaks, peak 1 in PMF and transesterification products represent di-saturated, mono-saturated, and tri-unsaturated TAGs. Peak 2, which has higher onset temperatures and smaller peak areas, represents the tri-saturated TAGs, which have a higher melting temperature [27]. Similar DSC melting profiles have also been reported by other researchers [26, 27, 30, 33]. As highlighted in the

HPLC result, the percentage of tri-saturated TAGs of PMF and the transesterification products were more than in cocoa butter, and it was reflected here with the shift of peak 2 towards higher temperature with the increase of stearic acid additions. These results relate to the SFC results where there was also some percentage of SFC left at 40°C for PMF and the transesterification products. The high amount of mono-saturated and tri-unsaturated TAGs in PMF and the transesterification products seems to affect peak 1 by shifting them towards the lower temperature. These results were also reflected in the SFC as PMF and the transesterification products have lower SFC at temperatures below 25°C compared to cocoa butter. The analysis of one-way ANOVA and Tukey's HSD test showed that the melting peaks of all the transesterified products were significantly different to cocoa butter, meaning that none of the products have similar melting profiles as cocoa butter. However, the ratio 1:1:1 of PMF:olive oil:stearic acid showed to be the most promising cocoa butter alternative as the melting peaks were less than 37°C. The melting peak of the transesterification product using just PMF and stearic acid (1:0:1) exceeded 37°C, which is 42.43°C, and therefore, not very suitable to be used as a cocoa butter equivalent. Cocoa butter has a melting point around body temperature, approximately 37°C, a property that is responsible for the smooth, creamy texture characteristic of high-quality chocolate as it melts in the mouth. An alternative that fails to melt at this temperature may result in a waxy or gritty texture, leading to a less desirable sensory experience [38]. These products might not meet the chocolate industry's requirement as a cocoa butter equivalent since the transesterification products seem to show unsatisfactory melting behaviour [30]. Nonetheless, the product might still pass as a cocoa butter alternative under the category of non-lauric cocoa butter replacer or as a cocoa butter improver [43].

Table 4. Summary of DSC parameters.

Sample		Crystallisation			Melting		
		Onset temperature (°C)	Peak temperature (°C)	Enthalpy (J/g)	Onset temperature (°C)	Peak temperature (°C)	Enthalpy (J/g)
Cocoa butter		20.24 ± 0.08^{ef}	14.54 ± 0.33^{ef}	82.94 ± 0.31^{a}	12.60 ± 0.012^{f}	21.80 ± 0.19^{f}	83.94 ± 0.19^{a}
PMF	peak 1	25.83 ± 0.52^{d}	22.40 ± 0.58^{c}	15.43 ± 0.16^{i}	1.47 ± 0.40^{i}	12.50 ± 0.35^{k}	52.59 ± 0.33^{f}
	peak 2	10.62 ± 0.47^{j}	3.20 ± 0.07^{i}	49.87 ± 0.57^{d}	22.36 ± 0.09^{d}	28.08 ± 0.20^{e}	30.17 ± 0.36^{i}
Olive oil		-39.94 ± 0.10^{m}	-39.85 ± 0.27^{k}	33.33 ± 0.30^{e}	-13.66 ± 0.56^{k}	-4.50 ± 0.52^{m}	76.00 ± 0.47^{b}
1:1:0	peak 1	19.25 ± 0.32^{fg}	16.11 ± 0.09^{d}	8.11 ± 0.34^{k}	-4.72 ± 0.49^{j}	3.48 ± 0.05^{l}	43.61 ± 0.42^{h}
(no enzyme)	peak 2	3.94 ± 0.24^{l}	-2.90 ± 0.04^{j}	31.03 ± 0.18^{f}	12.55 ± 0.28^{f}	17.11 ± 0.16^{hi}	22.40 ± 0.22^{j}
1:0:1	peak 1	31.53 ± 0.04^{c}	28.00 ± 0.39^{b}	17.58 ± 0.27^{h}	-3.43 ± 0.42^{j}	14.28 ± 0.46^{j}	54.82 ± 0.52^{e}
	peak 2	16.90 ± 0.18^{h}	11.28 ± 0.234^{g}	49.81 ± 0.12^{d}	34.47 ± 0.30^{a}	42.43 ± 0.09^{a}	16.26 ± 0.18^{k}
1:1:0	peak 1	18.16 ± 0.17^{gh}	14.42 ± 0.23^{ef}	6.52 ± 0.17^{k}	-3.04 ± 0.07^{j}	3.58 ± 0.14^{l}	57.73 ± 0.10^{d}
	peak 2	5.53 ± 0.53^{k}	-3.24 ± 0.02^{j}	32.36 ± 0.50^{ef}	15.09 ± 0.41^{e}	19.72 ± 0.01^{g}	13.15 ± 0.23^{l}
1:1:1	peak 1	32.59 ± 0.03^{c}	28.17 ± 0.30^{b}	12.95 ± 0.47^{j}	-4.83 ± 0.28^{j}	11.56 ± 0.19^{k}	66.70 ± 0.35^{c}
	peak 2	15.30 ± 0.14^{i}	8.03 ± 0.38^{h}	64.94 ± 0.40^{c}	26.09 ± 0.01^{c}	33.41 ± 0.08^{d}	14.31 ± 0.31^{l}
1:1:2	peak 1	36.52 ± 0.30^{a}	28.73 ± 0.16^{b}	18.04 ± 0.17^{h}	6.95 ± 0.43^{h}	16.03 ± 0.44^{i}	44.76 ± 0.39^{h}
	peak 2	19.22 ± 0.38^{fg}	13.60 ± 0.45^{f}	77.20 ± 0.10^{b}	30.14 ± 0.48^{b}	38.74 ± 0.05^{b}	10.26 ± 0.19^{m}
1:1:3	peak 1	34.81 ± 0.42^{b}	31.06 ± 0.03^{a}	20.51 ± 0.56^{g}	9.83 ± 0.19^{g}	17.87 ± 0.33^{h}	49.23 ± 0.45^{g}
	peak 2	21.00 ± 0.13^{e}	15.54 ± 0.17^{de}	82.31 ± 0.46^{a}	26.73 ± 0.52^{c}	35.68 ± 0.25^{c}	14.18 ± 0.33^{l}

Note: Ratio = PMF/olive oil/stearic acid. Values are means ± SE, n = 3.
Means in a column without a common superscript letter differ (P<0.05) as analysed by one-way ANOVA and the Tukey's HSD test.

CONCLUSION

The results validated the potential of immobilised ROL for modifying oils and fats into sustainable cocoa butter alternatives. Additionally, the immobilised ROL demonstrated the capability to perform lipase reverse reactions, such as transesterification, under water-restricted conditions, even at a high temperature (60°C). To further enhance this research, the transesterification product could undergo a fractionation process to produce a higher-quality cocoa butter alternative, as adjusting the substrate ratio alone is insufficient. This research opens new possibilities for the use of sago and immobilised lipase in developing high-quality, plant-based lipid products.

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REFERENCES

1. Guerrand, D. (2017) Lipases industrial applications: Focus on food and agroindustries, *OCL - Oilseeds and Fats. Crops and Lipids*, **24(4)**, 1–7.

2. Haki, G. D. and Rakshit, S. K. (2003) Developments in industrially important thermostable enzymes: A review. *Bioresource Technology*, **89(1)**, 17–34.

3. Ghosh, B. and Ray, R. (2011) Current Commercial Perspective of *Rhyzopus oryzae*: A Review. *Journal of Applied Sciences*, **11(14)**, 2470–2486.

4. Minteer, S. D. (2011) Enzyme Stabilization and Immobilization. *Humana Press, New Jersey*.

5. Yabuki, S. (2017) How to lengthen the long-term stability of enzyme membranes: Trends and strategies. *Catalysts*, **7(36)**, 1–16.

6. Boudrant, J., Woodley, J. M. and Fernandez-Lafuente, R. (2020) Parameters necessary to define an immobilized enzyme preparation. *Process Biochemistry*, **90**, 66–80.

7. Brena, B., González-Pombo, P. and Batista-Viera, F. (2013) Immobilization of Enzymes: A literature survey, in *Immobilization of Enzymes and Cells*. *Eds J. M. Guisan, Humana Press, New Jersey*.

8. Ferreira, M. M., Santiago, F. L. B., Silva, N. A. G. d., Luiz, J. H. H., Fernandéz-Lafuente, R., Mendes, A. A. and Hirata, D. B. (2018) Different strategies to immobilize lipase from Geotrichum candidum: Kinetic and thermodynamic studies. *Process Biochemistry*, **67**, 55–63.

9. Filho, D. G., Silva, A. G. and Guidini, C. Z. (2019) Lipases: Sources, immobilization methods, and industrial applications. *Applied Microbiology and Biotechnology*, **103(18)**, 7399–7423.

10. Christensen, M. W., Andersen, L., Husum, T. L. and Kirk, O. (2003) Industrial lipase immobilization. *European Journal of Lipid Science and Technology*, **105(6)**, 318–321.

11. Datta, S., Christena, L. R. and Rajaram, Y. R. S. (2013) Enzyme immobilization: An overview on techniques and support materials. *3 Biotech*, **3**, 1–9.

12. Mohtar, N. S., Abdul Rahman, M. B., Mustafa, S., Mohamad Ali, M. S. and Raja Abd. Rahman, R. N. Z. (2019) Spray-dried im- mobilized lipase from *Geobacillus* sp. strain ARM in sago. *PeerJ*, **7**, e6880.

13. Zhu, F. (2019) Recent advances in modifications and applications of sago starch. *Food Hydrocolloids*, **96**, 412–423 (April, 2019).

14. Ahmad, A. N., Lim, S. A., Navaranjan, N., Hsu, Y. I. and Uyama, H. (2020) Green sago starch nanoparticles as reinforcing material for green composites. *Polymer*, **202,** 122646 (February, 2020).

15. Krishnamurthy, A. and Amritkumar, P. (2019) Synthesis and characterization of eco-friendly bioplastic from low-cost plant resources. *SN Applied Sciences*, **1(1432)**, 1–13.

16. Wannaphatchaiyong, S., Suksaeree, J., Waiprib, R., Kaewpuang, A., Saelee, W. and Pichayakorn, W. (2019) Gelatin/gelatinized sago starch bio-membranes as a drug delivery system using rubber latex as pasticizer. *Journal of Polymers and the Environment*, **27(11)**, 2380–2394.

17. Azfaralariff, A., Fazial, F. F., Sontanosamy, R. S., Nazar, M. F. and Lazim, A. M. (2020) Food-grade particle stabilized pickering emulsion using modified sago (*Metroxylon sagu*) starch nano-crystal. *Journal of Food Engineering*, **280**, 109974 (February, 2020).

18. Maryam, Kasim, A., Novelina and Emriadi (2020) Preparation and characterization of sago (*Metroxylon* sp.) Starch nanoparticles using hydrolysis-precipitation method. *Journal of Physics: Conference Series*, **1481(1)**, 1–9.

19. Houde, A., Kademi, A. and Leblanc, D. (2004) Lipases and their industrial applications. *Applied Biochemistry and Biotechnology*, **118**, 155–170.

20. Akoh, C. C. and Min, D. B. (2002) Food Lipids: Chemistry, Nutrition, and Biotechnology (2nd ed.). *Marcel Dekker, New York*.

21. Kim, B. H. and Akoh, C. C. (2015) Recent research trends on the enzymatic synthesis of structured lipids. *Journal of Food Science*, **80(8)**, C1713–C1724.

22. Zhang, Z., Song, J., Lee, W. J., Xie, X. and Wang, Y. (2020) Characterization of enzymatically interesterified palm oil-based fats and its potential application as cocoa butter substitute. *Food Chemistry*, **318**, 126518 (September 2019).

23. Lee, K. T. and Akoh, C. C. (1998) Characterization of enzymatically synthesized structured lipids containing eicosapentaenoic, docosahexaenoic, and caprylic acids. *JAOCS, Journal of the American Oil Chemists' Society*, **75(4)**, 495–499.

24. O'Fallon, J. V., Busboom, J. R., Nelson, M. L. and Gaskins, C. T. (2007) A direct method for fatty acid methyl ester synthesis: Application to wet meat tissues, oils, and feedstuffs. *Journal of Animal Science*, **85(6)**, 1511–1521.

25. Borrelli, G. M. and Trono, D. (2015) Recombinant lipases and phospholipases and their use as biocatalysts for industrial applications. *International Journal of Molecular Sciences*, **16(9)**, 20774–20840.

26. Biswas, N., Cheow, Y. L., Tan, C. P. and Siow, L. F. (2016) Blending of palm mid-fraction, refined bleached deodorized palm kernel oil or palm stearin for cocoa butter alternative. *JAOCS, Journal of the American Oil Chemists' Society*, **93(10)**, 1415–1427.

27. Undurraga, D., Markovits, A. and Erazo, S. (2001) Cocoa butter equivalent through enzymic interesterification of palm oil midfraction. *Process Biochemistry*, **36(10)**, 933–939.

28. Naessens, L. (2012) Production of cocoa butter equivalent through enzymatic acidolysis. *Master Thesis, Ghent University, Belgium*.

29. Gibon, V. (2011) Enzymatic interesterification of oils. *Lipid Technology*, **23(12)**, 274–277.

30. Mohamed, I. O. (2013) Lipase-catalyzed acidolysis of palm mid fraction oil with palmitic and stearic fatty acid mixture for production of cocoa butter equivalent. *Applied Biochemistry and Biotechnology*, **171(3)**, 655–666.

31. Çiftçi, O. N., Fadiloğlu, S. and Göğüş, F. (2009) Conversion of olive pomace oil to cocoa butter-like fat in a packed-bed enzyme reactor. *Bioresource Technology*, **100(1)**, 324–329.

32. Mojovic, L., Siler-Marinkovic, S., Kukic, G. and Vunjak-Novakovic, G. (1993) *Rhizopus arrhizus* lipase-catalyzed interesterification of the midfraction of palm oil to a cocoa butter equivalent fat. *Enzyme and Microbial Technology*, **15(5)**, 438–443.

33. Sonwai, S., Kaphueakngam, P. and Flood, A. (2014) Blending of mango kernel fat and palm oil mid-fraction to obtain cocoa butter equivalent. *Journal of Food Science and Technology*, **51(10)**, 2357–2369.

34. Lai, O. M., Ghazali, H., Cho, F. and Chong, C. (2000) Physical and textural properties of an experimental table margarine prepared from lipase-catalysed transesterifed palm stearin: Palm kernel olein mixture during storage. *Food Chemistry*, **71**, 173–179.

35. Fuji Oil Asia (2018) Hard Butters for Chocolate. *Fuji Oil Asia Pte. Ltd*. https://www.fujioil.asia/product/oil/oil_1.html.

36. Jahurul, M. H. A. A., Zaidul, I. S. M. M., Nik Norulaini, N. A., Sahena, F., Abedin, M. Z., Mohamed, A. and Mohd Omar, A. K. (2014) Hard cocoa butter replacers from mango seed fat and palm stearin. *Food Chemistry*, **154**, 323–329.

37. Sonwai, S., Ornla-Ied, P. and Aneknun, T. (2015) Lauric fat cocoa butter replacer from krabok (*Irvingia malayana*) seed fat and coconut oil. *Journal of Oleo Science*, **64(4)**, 357–365.

38. Torbica, A., Jovanovic, O. and Pajin, B. (2006) The advantages of solid fat content determination in cocoa butter and cocoa butter equivalents by the Karlshamns method. *European Food Research and Technology*, **222(3–4)**, 385–391.

39. Biswas, N., Cheow, Y. L., Tan, C. P. and Siow, L. F. (2018) Physicochemical properties of enzymatically produced palm-oil-based cocoa butter substitute (CBS) with cocoa butter mixture. *European Journal of Lipid Science and Technology*, **120(3)**, 1–9.

40. Kadivar, S., De Clercq, N., Mokbul, M. and Dewettinck, K. (2016) Influence of enzymatically produced sunflower oil based cocoa butter equivalents on the phase behavior of cocoa butter and quality of dark chocolate. *LWT – Food Science and Technology*, **66**, 48–55.

41. Quast, L. B., Luccas, V. and Kieckbusch, T. G. (2011) Physical properties of pre-crystallized mixtures of cocoa butter and cupuassu fat. *Grasas y Aceites*, **62(1)**, 62–67.

42. Biswas, N., Cheow, Y. L., Tan, C. P., Kanagaratnam, S. and Siow, L. F. (2017) Cocoa butter substitute (CBS) produced from palm mid-fraction/palm kernel oil/palm stearin for confectionery fillings. *JAOCS, Journal of the American Oil Chemists' Society*, **94(2)**, 235–245.

43. Talbot G. (2017) Properties of cocoa butter and vegetable fats, in *Beckett's Industrial Chocolate Manufacture and Use (5th edition). Eds. S. Beckett, M. Fowler and G. Ziegler, Wiley & Sons, New Jersey*.