Journal of Oil Palm Research
DOI: https://doi.org/10.21894/jopr.2022.0045

THE SYNTHESIS OF *sn*-2 PALMITATE AS HUMAN MILK FAT SUBSTITUTE FROM PALM OIL FRACTIONS BY ENZYMATIC INTERESTERIFICATION – A REVIEW

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ABSTRACT

Human milk fat substitute (HMFS) is structured lipids with characteristics similar to human milk fat used in infant formulas. HMFS is designed to contain 60%-70% palmitic acid at the sn-2 *position and unsaturated fatty acids at the* sn-*1,3 positions in the triacylglycerol structure. HMFS is synthesised by enzymatic interesterification of vegetable oils or animal fats. Generally, the type of HMFS synthesised is* sn-2 *palmitate, with the main triacylglycerol being 1,3-dioleoyl-2-palmitoylglycerol (OPO). Palm oil fractions are used as raw material to synthesise* sn-2 *palmitate because it contains high palmitic acid. This article reviews the synthesis of* sn-2 *palmitate based on palm oil fractions via enzymatic interesterification. It gives a detailed description of the potential of palm oil fractions as substrates, lipases as biocatalysts, methods and reactors synthesis, and fractionation to increase the triacylglycerol of OPO. The information presented in this review can be used to develop future strategies for the synthesis of palm-based HMFS.*

Keywords: enzymatic interesterification, human milk fat substitute, lipase, palm oil, *sn*-2 palmitate.

Received: 22 December 2021; **Accepted:** 13 June 2022; **Published online:** 5 August 2022.

INTRODUCTION

Human milk fat contains 32.0%-52.0% saturated fatty acid, 30.0%-50.0% monounsaturated fatty acid, and 2.5%-13.8% polyunsaturated fatty acid (Monaco *et al.*, 2016). The primary fatty acids contained in human milk fat are 30.0%-35.0% oleic acid, 20.0%-30.0% palmitic acid, 7.0%-14.0% linoleic acid, and 5.7%-8.0% stearic acid. Besides these fatty acids, the human milk fat also contains long-chain polyunsaturated fatty acids (LCPUFA) and they include docosahexaenoic acid, eicosapentaenoic acid, and arachidonic acid contained in with a concentration of less than 1.0% (Ferreira-Dias and Tecelão, 2014; Wei *et al.*, 2019). In human milk fat, about 60.0%-70.0% of palmitic acid is distributed at the *sn*-2 position and unsaturated fatty acids (oleic acid, linoleic acid, docosahexaenoic acid, eicosapentaenoic acid, and arachidonic acid) at the *sn*-1,3 positions (He *et al.*, 2017; Wei *et al.* 2019).

Human milk fat substitutes (HMFSs) are structured lipids with the distribution of fatty acids similar to human milk fat (Sahin *et al.*, 2005a; 2005b), commonly used as fat in infant formulas. HMFS has a similar function as human milk fat, increasing the permeation of calcium and fat, making the stool softer, and reducing obstipation (Zou *et al.*, 2017). HMFSs are classified into four types, which include *sn*-2 palmitate (β-palmitate), LCPUFA, medium-chain fatty acid (MCFA), and milk fat globule membrane supplements. *Sn*-2 palmitate is the most common type of HMFS synthesised and it contains 1,3-dioleoyl-2-palmitoylglycerol (OPO), one of the primary triacylglycerols in human milk fat (Wei *et al.*, 2019).

In general, *sn*-2 palmitate is synthesised using oils and fats containing palmitic acid at the *sn*-2 position including, tripalmitin (Ilyasoglu *et al.*, 2011;

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2013; Liu *et al.*, 2015; 2017; Tecelão *et al.*, 2010; Wang *et al.*, 2016; Yüksel and Yeşilçubuk, 2012; Zheng *et al.*, 2017), lard (Qin *et al.*, 2014; Wang *et al.*, 2010; Yang *et al.*, 2003; Zhang *et al.*, 2016), catfish oil (Zou *et al.*, 2016a; 2016b), oil from *Nannochloropsis oculata* (He *et al.*, 2017), butterfat oil (Rønne *et al.*, 2005) and palm oil fractions (Ghosh *et al.*, 2016; Karabulut *et al.*, 2007; Nagachinta and Akoh, 2013). Also, palmitic acid (Robles *et al.*, 2011; Turan *et al.*, 2013) and ethyl palmitate (Turan *et al.*, 2013) are used as palmitic acyl donors to enhance palmitic acid at the *sn*-2 position of oil containing high unsaturated fatty acids.

Palm oil fractions are the best substrates for HMFS synthesis among the vegetable oils synthesis (Mat Dian *et al.*, 2017). However, palm oil fractions contain high palmitic acid at the *sn*-1,3 positions (Lasekan *et al.*, 2017). The presence of palm oil fractions in infant formula would contribute to the physiological function of the body such as reduction of intestinal permeation of fat, palmitic acid, calcium and lower bone mass (Chen *et al.*, 2019; Koo *et al.*, 2006). Thus, palm oil fractions need to be modified so that more palmitic acid will be at the *sn*-2 position, hence resembling human milk fat. This article aims to scientifically update the usage of palm oil fractions for *sn*-2 palmitate synthesis as HMFS. This article will cover the potential of palm oil fractions as substrates, lipases as biocatalysts, reactors and methods synthesis, and fractionation to increase OPO content in HMFS. Prospects of palm-based *sn*-2 palmitate synthesis were also discussed.

PALM OIL FRACTIONS AS SUBSTRATES FOR *sn*-2 PALMITATE SYNTHESIS

Palm oil fractions can be used as a substrate for *sn*-2 palmitate synthesis because of their high content of palmitic acid and oleic acid (*Table 1*) and can act as acyl donors for both acids. Apart from palm oil fractions, palm kernel oil is also utilised as a source of MCFA (especially lauric acid) for the HMFS synthesis (Hasibuan and Ijah, 2016; Karabulut *et al.*, 2007; Zou *et al.*, 2011; 2012b).

The palm oil fractions contain tripalmitin, a palmitic acid-containing triacylglycerol at the *sn*-2 position. Palm stearin is a solid palm fraction that contains high palmitic acid and tripalmitin compared to palm oil and palm olein (*Table 2*). Hence palm stearin is often used as a substrate for *sn*-2 palmitate synthesis (Wei *et al.*, 2019). In the palm oil industry, palm stearin can be fractionated through dry fractionation to produce soft palm stearin and hard palm stearin (Hasibuan and Siahaan, 2013). Hard palm stearin has palmitic acid higher than palm stearin and soft palm stearin. Hard palm stearin contains the main triacylglycerol, namely tripalmitin of 51.0% (Ibrahim *et al.*, 2006). Although palm stearin is rich in tripalmitin, it also contains

TABLE 1. FATTY ACIDS OF PALM OIL FRACTIONS

Fatty acids (%)	Palm oil*	Palm olein*	Palm stearin*	Soft palm stearin**	Hard palm stearin**	Palm kernel oil*
C6:0	ND	ND	ND	ND	ND	ND-0,8
C8:0	ND	ND	ND	ND	ND	2.4-6.2
C10:0	ND	ND	ND	ND	ND	2.6-5.0
C12:0	ND-0.5	0.1-0.5	0.1-0.5	0.01-0.25	0.14-0.32	45.0-55.0
C14:0	0.5-2.0	0.5-1.5	1.0-2.0	0.97-1.21	1.06-1.36	14.0-18.0
C16:0	39.3-47.5	38.0-43.5	48.0-74.0	50.94-54.84	76.36-81.04	6.5-10.0
C16:1	ND-0.6	ND-0.6	ND-0.2	ND-0.76	ND-0.09	ND-0.2
C17:0	ND-0.2	ND-0.2	ND-0.2	ND	ND	ND
C17:1	ND	ND-0.1	ND-0.1	ND	ND	ND
C18:0	3.5-6.0	3.5-5.0	3.9-6.0	4.44-5.46	3.61-4.87	1.0-3.0
C18:1	36.0-44.0	39.8-46.0	15.5-36.0	30.82-34.06	11.62-13.66	12.0-19.0
C18:2	9.0-12.0	10.0-13.5	3.0-10.0	6.91-8.71	1.95-3.33	1.0-3.5
C18:3	ND-0.5	ND-0.6	ND-0.5	ND-0.21	ND-0.09	ND-0.2
C20:0	ND-1.0	ND-0.6	ND-1.0	ND-0.18	ND-0.12	ND-0.2
C20:1	ND-0.4	ND-0.4	ND-0.4	ND-0.06	ND-0.09	ND-0.2
C20:2	ND	ND	ND	ND	ND	ND
C22:0	ND-0.2	ND-0.2	ND-0.2	ND	ND	ND
Iodine value (IV)	50-56	≥56	≤48	39.90-43.14	14.77-19.33	14.1-21.0

Note: ND- not detected.
Source: *Codex Alimentarius (2001), **Hasibuan and Siahaan (2013).

triacylglycerol with oleic acid at the *sn*-2 position (*Table 2*). When triacylglycerol containing oleic acid at the *sn*-2 position is acidolysed with oleic acid using a specific lipase of *sn*-1,3 will produce triolein (Wang *et al.*, 2020), which is not a *sn*-2 palmitate product. *Table 3* shows that palm stearin has palmitic acid content at the *sn*-2 position ranging from 23.0%-70.1%. Thus, the palmitic acid content at the *sn*-2 position in palm stearin needs to be increased to produce a good substrate for HMFS synthesis (Hasibuan *et al*., 2021b).

Technologies for improving the positioning of palmitic acid at the *sn*-2 position of palm stearin are solvent fractionation (Ghosh *et al.*, 2016; Lee *et al.*, 2010; Wang *et al.*, 2019; Zou *et al.*, 2012a), enzymatic interesterification (Jiménez *et al.*, 2010a; 2010b) or chemical interesterification (Zou *et al.*, 2011; Zou *et al.*, 2012b). Palm stearin is fractionated using acetone as a solvent to produce a tripalmitin-rich triacylglycerol (92.0%) (Lee *et al.*, 2010) and palmitic acid (88.57%–92.3%) (Ghosh *et al.*, 2016; Wang *et al.*, 2019; Zou *et al.*, 2012a). In general, the palm stearin solvent fractionation process condition is carried out at an acetone ratio of 5-9 and a fractionation temperature of 20°C-40°C for 3-24 h.

Enzymatic interesterification between palm stearin (60.0% palmitic acid and 23.0% palmitic acid at the *sn*-2 position) with palmitic acid using Novozyme 435 produces a product with 68.0%-75.0% palmitic acid at the *sn*-2 position (Jiménez *et al.*, 2010a; 2010b). Meanwhile, chemical interesterification of palm stearin (41.7% palmitic acid at the *sn*-2 position) resulted in a product with 58.0% palmitic acid at the *sn*-2 position (Zou *et al.*,

TABLE 2. TRIACYLGLYCEROLS COMPOSITION OF PALM OIL FRACTIONS

Triacylglycerols (%)	Palm oil*	Palm olein*	Palm stearin*	Soft palm stearin**	Hard palm stearin**	Palm kernel oil*
CCLa						6.8
CLaLa						9.9
LaLaLa						21.2
LaLaM						17.0
LaLaO						5.3
LaMM						8.8
PLL				1.0	ND	
MMM	0.4	0.6	0.2	ND	ND	
LaLaP						1.2
LaMO						4.6
MPL	2.4	3.7	1.0	0.1	ND	
LaMP						4.6
LaOO						3.8
LaPO						4.3
LaPP+MMO						
OOL	0.7	0.8	0.1	0.8	ND	
MMP	1.8	2.6	0.8	ND	ND	0.7
MOO						2.0
POL	10.1	15.8	5.3	6.9	0.9	
PPL	9.8	11.2	7.8	8.1	3.8	0.6
MPP	0.6	ND	2.3	ND	ND	
OOO	4.1	5.6	1.8	4.1	4.5	1.4
POO	24.2	36.3	12.0	18.0	3.0	1.9
PPO	31.1	17.1	29.8	32.0	26.4	1.1
PPP	5.9	0.1	29.2	20.6	51.0	0.1
SOO	2.3	3.6	0.8	ND	ND	0.4
PSO	5.1	2.5	3.8	4.9	2.9	0.4
PPS	0.9	ND	5.2	3.5	7.4	
SSO	0.5	ND	ND	ND	ND	

Note: ND-not detected; L-lauric acid; M-myristic acid; O-oleic acid; P-palmitic acid; S-stearic acid.
Source: *Tan and Man (2002); ** Ibrahim *et al.* (2006).

2012b). In other studies, Zou *et al.* (2011) reported chemical interesterification of palm stearin (56.8% palmitic acid at the *sn*-2 position) and manufactured a product with 69.8% palmitic acid at the *sn*-2 position.

Palm oil fractions can also produce palmitic acid and oleic acid as palmitic and oleic acyl donors for the *sn*-2 palmitate synthesis. Both are manufactured by hydrolysis of palm oil fractions, then separated from other fatty acids (Esteban *et al.*, 2011; Jimenez *et al.*, 2010a). Besides palmitic acid, ethyl palmitate can also be used as a palmitic acyl donor (Pina-Rodriguez and Akoh, 2009; Turan *et al.*, 2013), produced through the esterification of palmitic acid with ethanol. Likewise, ethyl oleate is an oleic acyl donor (Lee *et al.*, 2010). Palmitic acyl donors are used as a substrate for improving palmitic acid at the *sn*-2 position of oil and fat. Meanwhile, acyl oleic donors are used to increase *sn*-1,3 oleic acid of oils and fats that contain high tripalmitin.

TABLE 3. FATTY ACIDS AT THE *sn*-2 POSITION OF PALM STEARIN

Fatty acids (%)	Total	*sn*-2
C12:0	ND - 0.9	ND - 0.3
C14:0	1.3 - 1.7	ND - 1.0
C16:0	68.8 - 70.1	23.0 - 70.1
C18:0	4.8 - 5.2	0.7 - 2.9
C18:1	18.7 - 29.0	30.9 - 65.2
C18:2	3.9 - 7.5	8.3 - 12.6
C18:3	ND - 0.3	ND - 0.1

Source: Jimenez *et al*. (2010a, 2010b); Zou *et al*. (2011; 2012b).

LIPASE FOR PALM-BASED *sn*-2 PALMITATE SYNTHESIS: TYPES AND SOURCES

Lipase (*triacylglycerol hydrolase*, EC 3.1.1.3) is a biocatalyst that naturally acts on carboxylate ester bonds to catalyse the hydrolysis of triacylglycerol (Araújo *et al.*, 2016). This substrate is insoluble in water, and the reaction usually occurs at the organic-water interface, where lipase works best (Adlercreutz, 2013). In non-aqueous media, lipase catalyses esterification, acidolysis, alcoholysis, and interesterification (Araújo *et al.*, 2016; Rodrigues and Fernandez-Lafuente, 2010; Speranza and Macedo, 2012). Lipase has serine-histidine-aspartate catalytic active sites, which is responsible for its catalytic activity (Ortiz *et al.*, 2019; Fernandez-Lafuente, 2010; Rodrigues and Fernandez-Lafuente, 2010). Lipase shows variable stability against the extreme pH conditions, the appearance of organic solvents, and ionic liquids (Kapoor and Gupta, 2012).

Lipase is an excellent biocatalyst for synthesising structured lipids, and triacylglycerol with fatty acids at a specific position (Iwasaki and Yamane, 2000). HMFS is a structured lipid produced using various types of enzymes, substrates, and acyl donors (Soumanou *et al.*, 2013). The lipases commonly used for HMFS synthesis are Novozyme 435, Lipozyme RM IM, and Lipozyme TL IM (*Table 4*).

Lipozyme TL IM is obtained from *Thermomyces lanuginose* and is immobilised using silica. Lipozyme TL IM can maintain activity at 55°C-60°C (Fernandez-Lafuente, 2010) and shows positional specificity at the *sn*-1,3 (Soumanou *et al.*, 2013). Lipozyme RM IM is derived from *Rhizomucor miehei* and is immobilised using Duolite ES 562. Lipozyme RM IM is highly specific in the choice of substrate, stereospecific, regioselective, active and stable (Rodrigues and Fernandes-Lafuente, 2010; Zou *et al.*, 2014). Lipozyme RM IM also shows positional specificity at the *sn*-1,3 (Soumanou *et al.*, 2013). Novozyme 435 is generated from *Candida antarctica* lipase B and is immobilised using acrylic resin. Novozyme 435 is one of the most stable commercial lipases commonly used for various reactions (Ortiz *et al.*, 2019). Novozyme 435 can be used at 60°C-70°C (Soumanou *et al.*, 2013). When the substrate is triacylglycerol, Novozyme 435 does not show positional specificity (Jiménez *et al.*, 2010a; 2010b; Soumanou *et al.*, 2013).

METHODS FOR PALM-BASED *sn*-2 PALMITATE SYNTHESIS

Interesterification is an accepted oil and fat modification technique by redistributing the fatty acid groups between and within the triacylglycerol. After the interesterification of the substrate, the product has a distinct chemical composition and improved physical characteristics (Pacheco *et al.*, 2015). Enzymatic interesterification can be carried out non-specifically and specifically (Silva *et al.*, 2012). Non-specific enzymatic interesterification is a random process similar to chemical interesterification. Meanwhile, specific enzymatic interesterification is an acyl exchange process to a particular position, mainly at the *sn*-1,3 position using regioselective lipase (Gibon *et al.*, 2009).

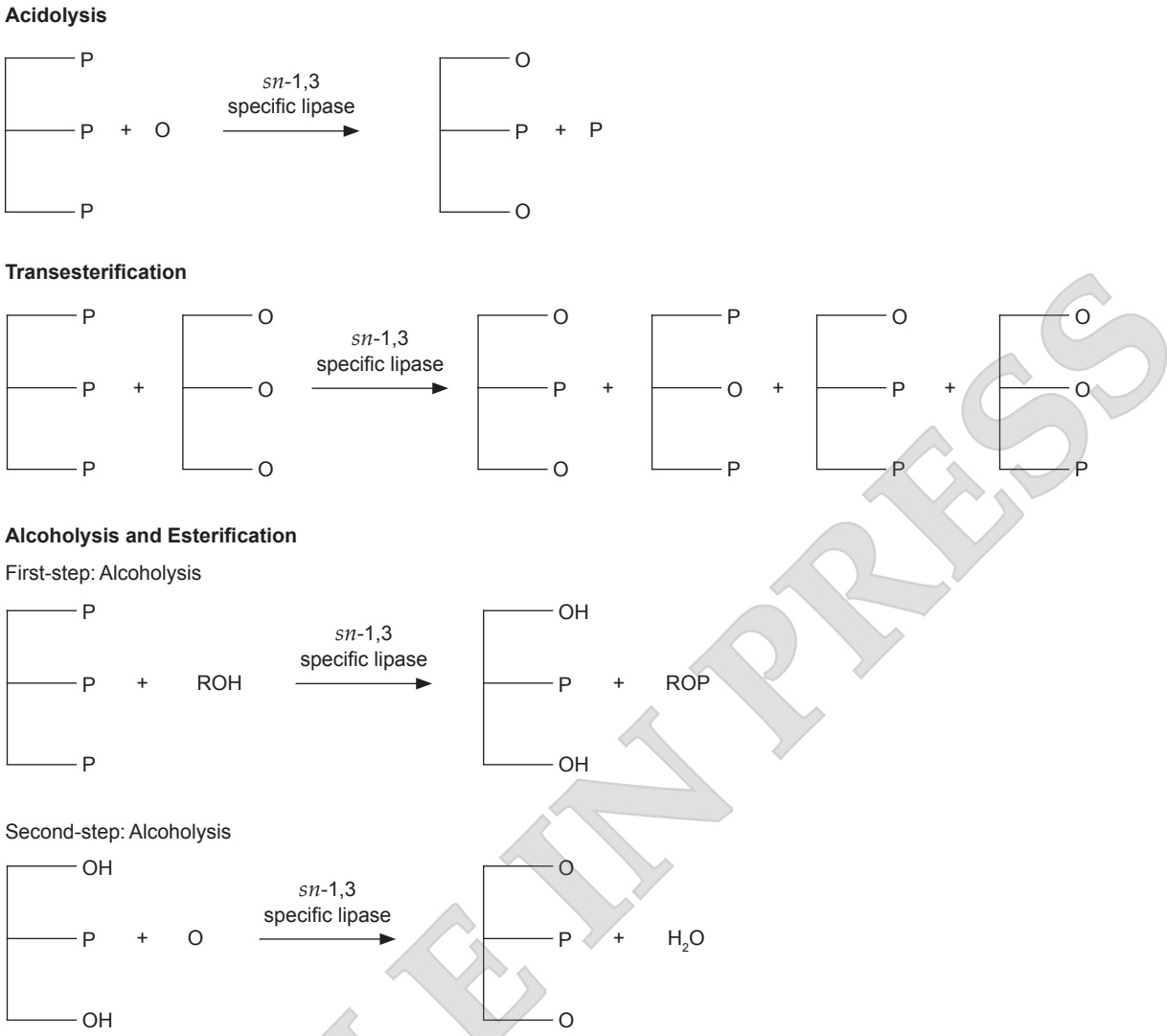
In general, *sn*-2 palmitate synthesis (especially OPO) is fabricated by a one-step reaction including transesterification or acidolysis and two-step reactions such as alcoholysis and esterification or two-step acidolysis (Hasibuan *et al*., 2021) (*Figure 1*). Apart from oleic acid (monounsaturated fatty acid, MUFA), fatty acids that are incorporated at the *sn*-1,3 position of *sn*-2 palmitate are fatty acids of MCFA (Karouw *et al.*, 2012), LCPUFA (Ghosh *et al.*, 2016; Nagachinta and Akoh, 2012; 2013), MUFA and LCPUFA (Wang *et al.*, 2019; Zou *et al.*, 2012) or MCFA, MUFA and LCPUFA (Hasibuan and Ijah, 2016; Karabulut *et al.*, 2007; Zou *et al.*, 2011; 2012b). Process conditions for palm-based *sn*-2 palmitate

TABLE 4. CONDITION REACTION FOR THE SYNTHESIS OF PALM-BASED HMFS

Type of reaction	Lipase	Substrate	Solvent system	Mode of operation	Lipase loading (%)	Substrate ratio (mol)	Temperature (°C)	Time (hr)	Characteristics of product	Reference
Transesterification	Lipozyme TL IM	Palm oil, palm kernel oil, olive oil, sunflower oil, and marine oil	Solvent-free system	Batch	10	4.0:3.5:1.0:1.5:0.2	60	6	23.00% palmitic acid and distributed at *sn*-2 41.5%	Karabulut *et al.* (2007)
Transesterification	Lipozyme TL IM	Palm stearin fractionates and ethyl oleate	Solvent-free system	Batch	10	1:5.5	50	3	31.43% OPO, 80.60% palmitic acid at the *sn*-2 position, and 64.90% *sn*-1,3 oleic acid	Lee *et al.* (2010)
Transesterification	Lipozyme TL IM	Palm stearin fractionates and PUFA-rich fish oil	Solvent-free system	Batch	10	2:1	60	12	75.98% palmitic acid at the *sn*-2 position, 0.27% arachidonic acid, 3.43% eicosapentaenoic acid, 4.25% docosahexaenoic acid, and melting point of 42°C	Ghosh *et al.* (2016)
Transesterification	Novozyme 435	Palm stearin, palm kernel oil, soybean oil, olive oil, and tuna fish oil	Solvent-free system	Batch	10	2.9:3.4:1.5:2.0:0.2	60	4	Fatty acids composition resembles human milk fat, melting point of 28°C	Hasibuan and Ijah (2016)
Acidolysis	Lipozyme RM IM	Palm stearin and a mixture of fatty acid from rapeseed oil, sunflower oil, palm kernel oil, stearic acid, and myristic acid	Solvent-free system	Batch	10.7	1:14.6	57	3.4	29.70% palmitic acid and 62.80% palmitic acid at the *sn*-2 position	Zou *et al.* (2011)
Acidolysis	Novozym 435	Palm olein, docosahexaenoic acid, and arachidonic acid	Hexane	Batch	10	1:18	60	24	Docosahexaenoic acid +arachidonic acid incorporated in triacylglycerol 25.25% (w/w) and docosahexaenoic acid +arachidonic acid incorporated at *sn*-2 17.20% (w/w)	Nagachinta and Akoh (2012)
Acidolysis	Novozym 435	Palm olein and a mixture of fatty acid (23.23% docosahexaenoic acid, 31.42% gamma-linoleic acid, and 15.12% palmitic acid)	Hexane	Batch	10	1:2	60	22.7	35.11% palmitic acid at the *sn*-2 position, 3.75% docosahexaenoic acid, and 5.03% gamma-linoleic acid	Nagachinta and Akoh (2013)

TABLE 4. CONDITION REACTION FOR THE SYNTHESIS OF PALM-BASED HMFS (continued)

Type of reaction	Lipase	Substrate	Solvent system	Mode of operation	Lipase loading (%)	Substrate ratio (mol)	Temperature (°C)	Time (hr)	Characteristics of product	Reference
Acidolysis	Lipozyme RM IM	Palm stearin and fatty acid from rapeseed oil	Solvent-free system	Batch	8	1:10	60	4	39.60% palmitic acid and 70.50% distributed at *sn*-2	Zou *et al.* (2012a)
Acidolysis	Lipozyme RM IM	Palm stearin and a mixture of stearic acid, myristic acid, fatty acid from rapeseed oil, sunflower oil, and palm kernel oil	Solvent-free system	Packed bed reactor		1:9.5	58	2.7	28.80% palmitic acid and 53.20% distributed at *sn*-2	Zou *et al.* (2012b)
Acidolysis	Lipozyme RM IM	Palm stearin fractionates and fungal oil from *Mortierella alpina* ALK (1:0.3) and oleic acid	Solvent-free system	Batch	8	1:6	60	6	Oleic acid incorporated 53.50% in triacylglycerol, fatty acid at *sn*-2: 68.70% palmitic acid, 9.80% arachidonic acid, and 7.9% oleic acid	Wang *et al.* (2019)
Acidolysis	Lipozyme RM IM immobilized on macroporous acrylic resin	Palm stearin fractionates, oleic acid and linoleic acid	Solvent-free system	Batch	8	1:8:4	60	4	OPO and OPL 69.26%, 87.75% palmitic acid at the *sn*-2 position	Wang *et al.* (2020)
Alcoholysis and esterification	Lipozyme RM IM	2-monoacylglycerol from alcoholysis of palm stearin, then esterified with lauric fatty acid methyl ester from coconut oil	Hexane	Batch	10	1:3	50	12	MCFA 43.86% with lauric acid 39.37%, 24.18% palmitic acid at the *sn*-2 position	Karouw *et al.* (2012)
Two-step acidolysis	Acidolysis 1: Novozyme 435 Acidolysis II: Lipase DF (Rhizopus oryzae)	Acidolysis 1: Palm stearin and palmitic acid Acidolysis II: Palmitic acid-rich triacylglycerol from palm stearin and oleic acid	Hexane	Batch stirred tank reactor	Acidolysis I: 9.6 g lipase × h/g triacylglycerol Acidolysis II: 0.4 g lipase × h/g triacylglycerol	Acidolysis I: 1:3 Acidolysis II: 1:6	37	Acidolysis I: 24 Acidolysis II:1	67.20% *sn*-1,3 oleic acid and 67.80% palmitic acid at the *sn*-2 position	Esteban *et al.* (2011)



Note: P-palmitic, O-oleic, ROH-alcohol, ROP-palmitic acid alkyl ester.

Figure 1. Mechanism of reaction for sn-*2 palmitate synthesis using* sn-*1,3 specific lipase (especially OPO).*

synthesis are presented in *Table 4*. *Sn*-2 palmitate synthesis can be performed in a solvent or solvent-free system. However, the solvent-free system is advisable for HMFS synthesis in terms of food safety and low production costs (Ferreira-Dias *et al.*, 2019).

Transesterification. Transesterification is a reaction between 1) triacylglycerol and triacylglycerol or 2) triacylglycerol and esterified fatty acids (Hassim *et al.*, 2018; Wei *et al.*, 2019). In the first type of reaction, Karabulut *et al.* (2007) conducted the interesterification of a mixture of palm oil, palm kernel oil, olive oil, sunflower oil, and marine oil using Lipozyme TL IM. The product contained 23.0% palmitic acid and 41.5% palmitic acid at the *sn*-2 position. Ghosh *et al.* (2016) reported transesterification between palm stearin fractionated with PUFA-rich fish oil with Lipozyme TL IM produced HMFS with 75.98% palmitic acid at the *sn*-2 position, 0.27% arachidonic acid, 3.43% eicosapentaenoic acid, and 4.25% docosahexaenoic acid. In the synthesis using the second reaction, Lee *et al.* (2010) reported transesterification of palm stearin fractions and ethyl oleate using Lipozyme TL IM. The product contained 31.43% OPO, 80.6% palmitic acid at the *sn*-2 position, and 64.9% oleic acid at the *sn*-1,3 position.

Acidolysis. Acidolysis is a reaction of triacylglycerol and fatty acid (Hassim *et al.*, 2018; Wei *et al.*, 2019). Acidolysis between triacylglycerol contains high palmitic acid at the *sn*-2 position from palm stearin with MUFA, MCFA, and LCPUFA using Lipozyme RM IM has been reported by Zou *et al.* (2012a; 2012b) and Wang *et al.* (2019). Meanwhile, Nagachinta and Akoh (2012) used Novozyme 435 for acidolysis of palm olein with docosahexaenoic acid, and arachidonic acid to produce triacylglycerol with 25.25% (w/w) docosahexaenoic acid+arachidonic

acid incorporation and 17.20% (w/w) docosahexaenoic acid+arachidonic acid at the *sn*-2 position. Nagachinta and Akoh (2013) also reported Novozyme 435 for acidolysis between palm olein with palmitic acid, docosahexaenoic acid, and gamma linoleic acid to produce triacylglycerol containing palmitic acid at the *sn*-2 position, docosahexaenoic acid, and gamma linoleic acid 35.11%, 3.75% and 5.03%, respectively.

Two-step process. The two-step process can be carried out by alcoholysis of triacylglycerol using a specific lipase *sn*-1,3 to produce *sn*-2 monoacylglycerol and then *sn*-2 monoacylglycerol esterified with fatty acid (Wei *et al.,* 2019) or esterified fatty acid (Karouw *et al.,* 2012). This method produces high yield and purity (Soumanou *et al.,* 2013). The two-step process can also be conducted using two-step acidolysis, as reported by Esteban *et al.* (2011). First, palm stearin is acidolysed with palmitic acid using Novozyme 435 to produce triacylglycerol containing high palmitic acid at the *sn*-2 position. Second, triacylglycerol is acidolysed with oleic acid using lipase DF from *Rhizopus oryzae*. The product had 67.80% palmitic acid at the *sn*-2 position and 57.20% distributed at the *sn*-2 position.

REACTORS FOR PALM-BASED *sn*-2 PALMITATE SYNTHESIS

The challenge in structured lipid synthesis by enzymatic interesterification is that production costs are relatively high, so it is necessary to use continuous processes and cost-effective catalysts (Bourlieu *et al.*, 2009; Jala and Kumar, 2018). The selection of reactors is essential to fabricate high product yields. A batch and continuous reactor are used in the enzymatic interesterification for *sn*-2 palmitate. The batch reactor is usually suitable for operation at a laboratory scale, whereas the continuous system is very appropriate for an industrial scale. The optimal reaction conditions for palm-based *sn*-2 palmitate synthesis in a batch reactor are enzyme load of 8.0%-10.0% (w/w of the total substrate), temperature 40°C-60°C, and reaction time 3-24 hr.

Continuous enzymatic interesterification is an economical technology for large-scale production because of its minimal costs, ease of operation, and being able to control the fatty acid distribution due to the selectivity and regiospecific of lipases (Silva *et al.*, 2012). The continuous system commonly used in enzymatic interesterification is a packed bed reactor (Soumanou *et al.,* 2013; Zou *et al.,* 2012b). The advantages of a packed bed reactor over batch reactors are due to relatively high enzyme stability, ease of operation on large scales, high reaction rates, and mass transfer, thereby reducing the occurrence of acyl migration (Sen *et al.,* 2016; Zou *et al.,* 2012b). A packed bed reactor is best applied continuously on an industrial scale to minimise the labour and costs of the processes (Nielsen *et al.,* 2006). Zou *et al.* (2012b) used a packed bed reactor in acidolysis between interesterified palm stearin with a mixture of stearic acid, myristic acid and fatty acids from rapeseed oil, sunflower oil, and palm kernel oil. The optimum conditions obtained using the response surface methodology approach were a substrate ratio of 9.5 mol/mol with a residence time of 2.7 hr at 58°C. The final product contained 28.8% palmitic acid and 53.2% palmitic acid at the *sn*-2 position.

ENHANCING OF *sn*-2 PALMITATE-RICH HMFS THROUGH FRACTIONATION

Karabulut *et al.* (2007) reported on the enzymatic interesterification of a mixture of palm oil, palm kernel oil, olive oil, sunflower oil, and marine oil was not optimum enough to produce a product with high palmitic acid at the *sn*-2 position. Fractionation can be applied in HMFS products obtained through enzymatic interesterification to increase triacylglycerol containing high palmitic acid at the *sn*-2 position (Hasibuan *et al*., 2021c).

Fractionation of the acidolysis product between butterfat and a mixture of fatty acids from rapeseed oil and soybean oil using acetone as solvent at a ratio of 2.5, temperature of 0°C for 3 hr was reported by Sørensen *et al.* (2010). The product contained 56.12% palmitic acid at the *sn*-2 position. This value was higher than the product obtained from butterfat's acidolysis fractionated first (47.26%). In another study, Lee *et al.* (2015) reported the OPO content enhanced from 25.2% to 53.3% after fractionation at 22°C for 12 hr of the interesterified palm oil and camellia oil.

FUTURE OUTLOOK: CHALLENGES AND OPPORTUNITIES IN *sn*-2 PALMITATE SYNTHESIS

Betapol is commercial *sn*-2 palmitate developed by Loders Croklaan in 1995 through acidolysis of the tripalmitin-rich palm stearin with oleic acid from high oleic sunflower oil using Lipozyme RM IM as a biocatalyst (Wei *et al.,* 2020). Palm oil fractions will continue to be developed as a substrate for *sn*-2 palmitate production because of the following advantages; high palmitic acid content, abundant availability, and low price (Hasibuan, 2021a). In addition, palm oil is one vegetable oil that does not contain cholesterol (Gesteiro *et al*., 2019). Palm stearin as a substrate in the *sn*-2 palmitate synthesis is very interesting. However, the melting point of palm stearin is relatively high, so in the enzymatic process, it is necessary to add organic solvents (such

as hexane) or be carried out at a sufficiently high reaction temperature (Jimenez *et al*., 2010a; 2010b).

The addition of solvents can lead to an increase in production costs and potential toxicity. In addition, the use of solvents is not recommended in terms of food safety (Ferreira-Dias and Tecelão, 2014). Thus, the production of HMFS in solvent-free systems is preferred in terms of food safety, cost, environmental friendly and ease of product purification (Ferreira-Dias and Tecelão, 2014; Tecelão *et al*., 2019). However, the reaction in a solvent-free system needs to be carried out at a high temperature (Hasibuan, 2021a), which affects the lipase stability. For this reason, the reaction in a solvent-free system can use immobilised enzymes, which have higher stability than the original free suspended enzymes. Enzyme immobilisation can prevent denaturation and leakage of enzymes so that the number of batches or the duration of synthesis can be increased (Adlercreutz, 2013).

Several studies reported that the use of commercial immobilised lipases such as lipases of Novozyme 435, Lipozyme TL IM, and Lipozyme RM IM in the synthesis of HMFS show good activity and stability (Hasibuan, 2021a). Jimenez *et al*. (2010a) reported that lipase *Alcaligenes* sp. immobilised on diatomaceous earth remained stable for at least 11 times using acidolysis between palm stearin and palmitic acid at a mole ratio of 1:3, reaction temperature of 65°C and reaction time of 24 hr. In addition, Esteban *et al*. (2011) reported that the lipase *Rhizopus oryzae* immobilised on Accurel MP1000 remained stable for at least ten times usage in acidolysis between palm stearin high in palmitic acid at the *sn*-2 position and oleic acid at a mole ratio of 1:6, 50°C for 19 hr. Exploring new biocatalysts with high catalytic activity and operational stability through isolation and genetic engineering is of interest for future research (Wei *et al*., 2020). Efficient and stable biocatalysts will reduce operating costs (Tecelão *et al*., 2019).

In contrast to lard, catfish oil, and butterfat oil, palm stearin has high *sn*-1,3 palmitic acid, requiring a high ratio of fatty acyl donors. The use of a high acyl donor ratio is unattractive because of the difficulty of the separation process (Zou *et al*., 2016b), so the cost for separation after processing is high (Zhang *et al*., 2016). Thus, it is necessary to develop specific lipases to synthesise palm-based HMFS using palm stearin with low acyl donor ratios. Faustino *et al*. (2016) reported that the acidolysis between tripalmitin and fatty acids from camelina oil at a low fatty acid mole ratio of 1:1.2 using lipase *Rhizopus oryzae* immobilised on Lewatit VPOC 1600 at a reaction temperature of 65°C could use up tripalmitin at 62.7% w/w.

Specialised treatments such as crystallisation fractionation are important to enrich palmitic acid at the *sn*-2 position. Acyl donors (MUFA, MCFA, and PUFA) will continue to be explored to produce palm-based HMFS resembling human milk fat (Hasibuan *et al*., 2021c). In addition, palm-based HMFS formulation for infant formula needs to be developed according to the baby's needs (age and condition) and regulations related to infant formula. Commercial formula is divided into three stages depending on the age of the baby, namely infant formula (0-6 months, stage 1), follow-up formula (6-12 months, stage 2), growth formula (12-36 months, stage 3). The rules fthe formulas of the various stages may differ (Wei *et al.* (2019).

The relevant regulations enacted by several authorities to regulate infant formula include the Codex Alimentarius Commission (CAC), the US Food and Drug Administration (FDA), the European Commission (EC), and the National Health Commission of the People's Republic of China (NHC). CAC, EC and NHC require that α-linolenic acid is required to be more than 50 mg/100 kcal, the amount of lauric acid and myristic acid should not exceed 20.0% of the total fatty acids, *trans*-fatty acids should be less than 3.0% of the total fatty acids, erucic acid should be less than 1.0% of total fatty acids and eicosapentaenoic acid levels should be no more than docosahexaenoic acid levels. Docosahexaenoic acid is recommended as the essential constituent (4.8-12 mg/100 kJ). The EC and NHC recommend that docosahexaenoic acid do not exceed 2.0% and 0.5% of total fatty acids, respectively, and arachidonic acid levels should not exceed 1.0%. The EC recommended that the ω-3 and ω-6 LCPUFAs be less than 1.0% and 2.0% of the total fatty acids, while the CAC allowed the addition of LCPUFAs, respectively. CAC requires no commercial hydrogenated fats and oils to be used as raw materials. The EC also states that sesame seed oil and cottonseed oil are not allowed in infant formula because of potential allergens (Wei *et al*., 2019).

CONCLUSION

Primarily, palm stearin is used as a substrate for *sn*-2 palmitate synthesis for HMFS. Triacylglycerol rich in palmitic acid at the *sn*-2 position of palm stearin needs to be increased to be an excellent substrate for the production of HMFS with palmitic acids at the *sn*-2 position >60%. The selection of enzyme types and loading, substrate types, synthesis methods, and reactor configuration is essential to improve the efficiency of HMFS synthesis. Generally, the optimal reaction conditions for palm-based *sn*-2 palmitate synthesis in a batch reactor are enzyme load of 8%-10% (w/w of the total substrate), temperature of 40°C-60°C, and reaction time of 3-24 hr. The OPO content in palm-based HMFS produced by enzymatic interesterification can be increased using fractionation. The challenge in HMFS synthesis is

the high production cost. In addition, the resulting product must resemble HMF, hence the complete set of acyl donors from fatty acids such as MUFA, MCFA and PUFA must be present in HMF. The reduction in the production cost of HMFS can be accomplished through the exploration of acyl donors and novel lipase enzymes with high catalytic activity and stability at a low cost.

ACKNOWLEDGEMENT

The author wishes to thank the Indonesian Oil Palm Research Institute.

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