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Enzymatic Synthesis of Human Milk Fat Substitute - A Review on Technological Approaches

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SUMMARY

Human milk fat substitute (HMFS) is a structured lipid designed to resemble human milk fat. It contains 60-70 % palmitic acid at the *sn*-2 position and unsaturated fatty acids at the *sn*-1,3 positions in triacylglycerol structures. HMFS is synthesized by the enzymatic interesterification of vegetable oils, animal fats, or blend of oils. The efficiency of HMFS synthesis can be enhanced through the selection of appropriate substrates, enzymes, and reaction methods. This review focuses on the synthesis of HMFS by lipase-catalyzed interesterification. This work provides a detailed overview of biocatalysts, substrates, synthesis methods, factors influencing the synthesis, and purification process of HMFS. Major challenges and future research in the synthesis of HMFS are also discussed. This review can be used as an information for developing future strategies in producing HMFS.

Key words: human milk fat substitute, interesterification, lipase, *sn*-2 palmitate

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INTRODUCTION

Fat is one component in human milk that gives half of the energy required for babies (*1-3*). Human milk contains 3-5 % fat containing 98 % of triacylglycerol (TAG) (*4-6*). The main fatty acids of TAG of human milk are 30-35 % oleic acid, 20-30 % palmitic acid, 7-14 % linoleic acid, and 5.7-8 % stearic acid (*5*). A 60-70 % of palmitic acid is located at the *sn*-2 position whereas unsaturated fatty acids (*i.e.,* oleic acid, linoleic acid, ω-3 polyunsaturated fatty acids (PUFAs)) are at the *sn*-1,3 positions in the TAG structure of human milk fat (HMF) (*6*). Palmitic acid at the *sn*-2 position has a significant importance especially in the absorption and metabolism of lipid and nutrition for infants (*7,8*).

The dominant TAGs in HMF are 1,3-dioleoyl-2-palmitoyl-glycerol (OPO; 16-29 %) and 1-oleoyl-2-palmitoyl-3-linoleoylglycerol (OPL; 13-20 %) (*9*). TAGs with palmitic acid at *sn*-2 position are very helpful in effectively promoting the absorption of calcium ions, leading to the formation of softer stools and hence reducing the possibility of constipation (*1,6,10,11*). TAGs containing palmitic acid at *sn*-2 position can also increase early bone mineralization and growth, affect the composition of intestinal microflora, decrease the level and severity of intestinal inflammation and influence neurobiology which includes modulation of early infant crying (*12*).

The composition and distribution of fatty acids in HMF are used as a basis to develop an alternative fat as an ingredient for infant formulas. As sources of nutrients, infant formulas are an alternative of human milk when the volume of human milk is limitedly produced by breast-feeding mother (*13,14*). Fats commonly used for infant formulas are vegetable oils or animal fats, especially bovine milk fat (*3*). However, the composition and distribution of fatty acids in vegetable oils and mammalian milk fats differ from that of HMF (*4*). In vegetable oils, palmitic acid is mainly (> 80 %) esterified to *sn*-1,3 positions (*9*). Meanwhile, animal fat such as cow milk fat has similar palmitic acid content with HMF, but the percentage of palmitic acid esterified at *sn*-2 position is only about 40 % (*4,14*). Hereby, the modifications of vegetable oils, animal fats, or blends of oils are performed to mimic the composition and distribution of fatty acids found in HMF (*15,16*). This modified fat is so-called human milk fat substitute (HMFS) (*14,17*).

HMFS has been successfully developed and commercialized as energy supplements and considered as sources of essential fatty acids, and as nutritional supplements in infant formulas (*4*). Based on the categories of the LIPID MAPS Structure Database, HMFSs are classified into four types based on their lipid structure: namely (i) *sn*-2 palmitate (palmitic acid at the *sn*-2 position) such as OPO, (ii) long-chain polyunsaturated fatty acids, such as docosahexaenoic acid (DHA, 22:6 ω-3), eicosapentaenoic acid (EPA, 22:5 ω-3) and arachidonic acid (ARA, 20:4 ω-6), as well as (iii) medium-

chain triacylglycerols (MCT) (which has medium-chain fatty acids (MCFAs) with 6-12 carbon length) and (iv) milk fat globule membrane supplements (*4*).

Synthesis of HMFS is conducted by the enzymatic interesterification of oils and fats. The enzymatic interesterification operates relatively at low temperature, considered as cost-efficient and environmentally friendly method (*18*). The interesterification utilizes lipase as a biocatalyst, which has specificity and selectivity to produce desired lipids with relatively low by-products (*19*). Hereby, the changes in the structure of TAGs can be specifically regulated at *sn*-1,3, *sn*-2, or unspecified position (*20-24*).

The development of structured lipids using enzymatic process technology has several challenges especially for tailoring higher catalytic efficiency and enzyme stability, which are important for overall productivity (*25*). HMFS contains palmitic acid at *sn*-2 position above of 70.0 % is produced by acidolysis in solvent system between tripalmitin and a mixture of hazelnut fatty acids and stearic acid using Lipozyme RM IM (*15*), or tripalmitin and fatty acids from hazelnut oil and gamma-linoleic acid (GLA) using Lipozyme RM IM and Lipozyme TL IM (*16*). He *et al.* (*6*) reported that acidolysis of TAG from *Nannochloropsis oculata* and fatty acids from *Isochrysis galbana* using Novozyme 435, Lipozyme TL IM, Lipozyme RM IM, and recombinant *Candida antarctica* Lipase B (recombinant CAL-B) in solvent-free system produced HMFS containing 59.38–68.13 % palmitic acid at *sn*-2 position.

The reported studies on HMFS production highlighted on the exploration of the use of new oils and fats, finding more cost-effective catalysts, synthesis methods, reactor configurations and purification process. Wei *et al.* (*4*) reviewed the achievements and trends of development of HMFS, with focus on nutritional bases, preparation methods, and applications of HMFS. As an additional to the previous comprehensive review (*4*), this work emphasizes more on the utilization of lipase as biocatalyst and factors for lipase-catalyzed of HMFS synthesis. This review starts with the biocatalysts used for producing HMFS, followed by substrates, methods and reactor configurations, factor influencing the synthesis and purification of HMFS, with a specific objective to increase the efficiency of HMFS synthesis. Additionally, the developments of HMFS production including challenges and opportunities for future research of HMFS are also presented in this work.

LIPASE FOR HMFS SYNTHESIS

Lipase (triacylglycerol acyl-hydrolase, EC 3.1.1.3) is commonly used for oil or fat hydrolysis. In non-aqueous media, lipase can also catalyze the esterification, acidolysis, alcoholysis, and interesterification (*19,26-28*). The lipase-catalyzed interesterification involves the reversible reaction of simultaneous hydrolysis and esterification reactions (*29*). A small amount of water is important for

non-aqueous enzymatic catalysis for maintaining enzyme's active conformational structure during non-covalent interactions (*30*). The excess of water has to be removed to shift the progress of the reaction from hydrolysis to esterification, thus enhancing the reaction yield. When the hydrolysis prevails over esterification, by-products such as glycerol, free fatty acid (FFA), monoacylglycerol (MAG) and diacylglycerol (DAG) are obtained which eventually hampers the separation process.

As part of non-aqueous reaction, the esterification of HMFS can be carried out by lipase as the biocatalyst. The sources of lipase are mostly from microorganisms. The commercial lipases available on the market and mostly studied in recent years for producing HMFS are derived from *Rhizomucor miehei*, *Thermomyces lanuginosa*, *Candida antarctica*, *Candida parapsilosis,* recombinant lipase B from *Candida antarctica*, *Candida lipolytica, Candida* sp. 99-125, *Rhizopus oryzae*, *Alcaligenes sp.*, and *Mucor miehei* (*9,23*).

Lipases having regiospecificity and regioselectivity are of interest as the reaction yield can be tuned up by having these properties. Additionally, the use of an immobilized lipase with a maintained biocatalytic activity at an industrial scale is required for multiple uses and ensuring the economic viability of the process (*31,32*) and thus, lowering production costs (*33,34*). The immobilized lipase sometimes has a higher stability as compared to the native from freely suspended enzyme (*35*).

Selectivity and/or specificity of lipases as biocatalysts for HMFS synthesis

Lipases present an obvious functional properties compared to chemical catalysts which is their specificity includes: (i) substrate specificity: ability of the lipase to hydrolyse preferentially a type of acylglycerol; (ii) fatty acid specificity or typoselectivity: ability to target a certain fatty acid or group of fatty acids; (iii) positional specificity or regioselectivity: ability to distinguish the two external positions of the TAG glycerol backbone; (iv) stereospecificity: ability to distinguish between *sn*-1 and *sn*-3 positions of TAG molecule (*26*). The incorporation of fatty acids into a TAG structure is influenced by many factors including the geometry of the binding sites of the lipases, free energy changes between the substrate and products, the variation of pH values, the effect of the chain length of fatty acids on solubility of the water and the physical state (*23*).

The most commonly commercialized lipases used for HMFS synthesis are Lipozyme RM IM (from *Rhizomucor miehei*), Lipozyme TL IM (from *Thermomyces lanuginosa*), and Novozyme 435 (from *Candida Antarctica*). The three enzymes are immobilized with support materials such as ion-exchange resin (Lipozyme RM IM), silica gel (Lipozyme TL IM), and microporous anionic resin (Novozyme 435) (*9*). These enzymes are specific for the interesterification reaction of oils and fats.

Lipozyme RM IM and Lipozyme TL IM are enzymes that show high regiospecificity at the *sn*-1,3 positions (*23*), while Novozyme 435 does not show positional specificity (*23,36,37*).

Generally, Lipozyme RM IM is used in acidolysis reaction (*15,16,38,39,40-52*). In addition, several studies also use Lipozyme RM IM in transesterification reactions (*42,53,54*). Meanwhile, Lipozyme TL IM is usually used in transesterification reaction (*55-58*). Some reported works also use Lipozyme TL IM in acidolysis reaction (*6,16,59,60,61*). Rodrigues & Fernandez-Lafuente (*19*) revealed that Lipozyme RM IM showed a higher activity in acidolysis, whereas Lipozyme TL IM in alcoholysis or transesterification. This can be due to the fact that Lipozyme RM IM is classified as an esterase and this enzyme is more specific for TAG containing low molecular weight fatty acids at pH 5.3 than at pH 8.0 (*19*), while Lipozyme TL IM is quite stable with the maximum activity at a pH around 9 (*62*).

Tecelão *et al.* (*59*) reported that the use of Lipozyme RM IM in acidolysis of tripalmitin and oleic acid or PUFAs produced HMFS containing palmitic acid at the *sn*-2 position of 79.9 %, higher than Lipozyme TL IM of 75.6 %. Lipozyme RM IM (68.13 %) is also superior compared to Lipozyme TL IM (59.38 %) in HMFS synthesis containing high palmitic acid at *sn*-2 position in acidolysis of microalgae oil from *Nannochloropsis oculate* and *Isochrysis galbana* (*6*). Lee *et al.* (*58*) reported that Lipozyme TL IM was used for transesterification between palm stearin and ethyl oleate at a molar ratio of 1:5.5, temperature of 50 °C for 3 h. The produced HMFS had 31.43 % OPO content and 80.6 % palmitic acid at *sn*-2 position. Karabulut *et al.* (*57*) produced HMFS containing 23.0 % palmitic acid and 41.5 % palmitic acid at *sn*-2 position of TAGs by transesterification between palm oil, palm kernel oil, olive oil, sunflower oil, and marine oil at a weight ratio of 4.0:3.5:1.0:1.5:0.2, temperature of 60 °C for 6 h using Lipozyme TL IM.

Mostly, Novozyme 435 is used for HMFS synthesis in the interesterification of oils and fats that improves palmitic acid content in the *sn*-2 position with palmitic donors such as palmitic acid, ethyl palmitate, or palm oil fractions. Generally, palm oil fractions contains high palmitic acid distributed at the *sn*-1,3 positions (*63*). The incorporation of fatty acid by acidolysis or transesterification using Novozyme 435 is affected by substrates. Novozyme 435 is a highly versatile catalyst, catalyzes a wide variety of different substrates due to its highly enantioselectivity (*59*). Robles *et al.* (*64*) used Novozyme 435 for acidolysis of tuna fish oil and palmitic acid, and produced TAG contained 57 mol% palmitic acid and 17 mol% DHA at *sn*-2 position. Turan *et al.* (*65*) also used Novozyme 435 in acidolysis and transesterification reactions between hazelnut oil with palmitic acid or ethyl palmitate in a solvent-free system. The optimum conditions were using ethyl palmitate at a molar ratio of 1:6, temperature of 65 °C and reaction for 17 h. Hereby, HMFS with 48.6 mol% palmitic

acid and 35.5 mol% palmitic acid at *sn*-2 position was obtained. Novozyme 435 is used in acidolysis of palm oil and a mixture of DHA and ARA to produce HMFS with DHA+ARA incorporated at *sn*-2 position of 17.20 % (*66*). Acidolysis of palm olein and a mixture of DHA, GLA and palmitic acid using Novozyme 435 produced HMFS contained 35.11 % palmitic acid at the *sn*-2 position (*67*). Novozyme 435 is also used in transesterification of a mixture of palm stearin, palm kernel oil, soybean oil, olive oil, and tuna fish oil to produce HMFS with fatty acid composition resembling HMF (*68*).

Reusability of lipase

Enzyme immobilization is carried out to prevent denaturation and enzyme leakage so that the number of batches or the period of synthesis can be increased. Enzyme is immobilized through adsorption, entrapment, covalent coupling, or cross-linking (*35*). The enzyme immobilization yields (*i.e.,* loading and recovered activity) are strongly dependent on the properties of the solid support such as the surface area, the number of accessible sides for binding, the porosity, and the pore size (*32*). In addition, the hydrophilicity of the enzyme support is a factor that affects the reaction performances and the hydrophilicity of the support could be a beneficial side-effect of the immobilization (*69*).

Reusability of an immobilized lipase is very important issue to evaluate the operational stability (*6,59*), considered as a major factor in determining the suitability of its utilization at industries (*70*). Table 1 (*6,31,32,36,38,39,59,60,70*) shows the reusability of lipases for HMFS synthesis. Reusability of lipases depends on the immobilization technique, inherent thermal property of enzyme, reaction temperature, and operational time. A gradual decrease of enzyme's activity may be observed after several reaction batches carried out. This is due to the denaturation (*71*) and/or loss of lipase immobilized during the reaction (*70*). In addition, the loss of enzyme's activity may be due to a progressive dehydration occurring along the reaction (*32*). The multiple uses of the immobilized lipases can be expected due to the construction of the support that can protect the enzymes from mechanical inactivation and simultaneously inhibit lipase leakage (*72*).

Zheng *et al.* (*70*) mentioned that *Candida lipolytica* immobilized in magnetic multi-walled carbon nanotubes (*CLL*@mMWCNTs) had a better activity and stability than Lipozyme RM IM and Lipozyme TL IM for interesterification between tripalmitin and oleic acid. Reusability of *CLL*@mMWCNTs was higher than that of Lipozyme RM IM, which was proven by OPO content of 1.5-fold higher than Lipozyme RM IM when reused for 20 cycles (1 cycle was for 2 h). Immobilization of *C. lipolytica* on mMWCNTs via hydrophobic and cation-exchange interaction prevented the extensive conformational changes due to typical thermal denaturation (*70*). Tecelão *et al.* (*32*)

reported *Rhizopus oryzae* lipase immobilized in Accurel® MP 1000 or in Lewatit® VP OC 1600 presented the best performance about 4-fold higher than Eupergit® C regarding oleic acid incorporation on tripalmitin. The immobilization of *Rhizopus oryzae* lipase in Accurel® MP 1000 and in Lewatit® VP OC 1600 by physical adsorption can be carried out. After the immobilization, glutaraldehyde is added to promote stable crosslink between the lipase and the matrix, as well as to promote intermolecular bonds between enzyme molecules. Meanwhile, immobilization of *R. oryzae* lipase on Eupergit® C can also be performed through direct enzyme binding on support via oxirane groups. However, enzymes immobilized on Eupergit® C through their different groups (amino, sulfhydryl, hydroxyl, phenolic) can block the substrate access to the enzyme's active site, or can even lead to enzyme denaturation (*32*). Conclusively, as reported by Idris and Bukhari (*73*), materials and techniques for immobilization affect the conformational structure of enzymes related to catalytic properties.

SOURCES AND TYPES OF SUBSTRATES FOR HMFS SYNTHESIS

Oils, fats, or single TAG molecules can be used as substrates for HMFS synthesis (Table 2) (*6,39,44,47-51,64,65,67,74-79*). Several reported sources of palmitic acid for HMFS synthesis are tripalmitin (*76*), lard (*47*), palm stearin (*51,74*), basa catfish oil and its fraction (*50*), oil from *Nannochloropsis oculata* (*6*), palm oil (*77*), and palm olein (*67*). Meanwhile, oils that can be used as sources of oleic acids are high oleic sunflower oil (*78*), hazelnut oil (*65*), tea seed oil (*47*), rapeseed oil (*48*), and olive oil (*75*). Several sources for linoleic acid can be sunflower oil and soybean oil (*44*). Flaxseed oil (*44*) and camelina oil (*76*) can be used as linolenic acid sources. Oils such as coconut oil (*44*) and palm kernel oil (*48*) can be used as MCFAs. Sources of EPA and DHA are fish oil (*64,79*), algal oil (*48*), microalgae oil from *Schizochytrium* sp. (*39*), and DHA single cell oil (*75*). Microbial oil is one of the sources of arachidonic acid (*48*). In addition, substrates as a single fatty acid donor can be used for the synthesis of HMFS are palmitic or ethyl palmitic acid (*65*) (as a palmitic acyl donor), oleic acid (*74*), or ethyl oleate (*58*) (as an oleic acyl donor), linoleic acid (*74*), GLA (*16*), EPA, DHA (*45*), ARA (*66*) (as PUFA donor), and myristic acid (*51*), caprylic acid, capric acid (*40*) (as an acyl MCFA donor).

The type of substrate is one of the important factors in the synthesis of HMFS. The composition of the raw material of the substrate that undergoes the interesterification process in the synthesis of HMFS has a significant influence on the final product. In the synthesis of HMFS containing palmitic acid at a high *sn*-2 position, it is better to use a substrate containing high palmitic acid at *sn*-2 position. Zhang *et al*. (*31*) produced HMFS containing palmitic acid at the *sn*-2 position of 79.51 % by acidolysis

between lard (palmitic acid at the *sn*-2 position of 81.92 %) and oleic acid (a mole ratio of 1:2) at 45 °C for 10 h using additives β-cyclodextrin, and *Candida* sp. 99-125 of 10 % (*m*/*m* of the total substrate). Yang *et al*. (*38*) also reported the production of HMFS with palmitic acid at the *sn*-2 position of 71.1 % obtained from acidolysis between lard (palmitic acid at the *sn*-2 position of 67.3 %) and fatty acids from soybean oil (a mole ratio of 1:2.4), at 61 °C for 1 h using Lipozyme RM IM of 13.7 % (*m*/*m* of the total substrate). In another study, Zou *et al*. (*49*) produced HMFS containing palmitic acid at the *sn*-2 position of 58.43 % by acidolysis between solid fraction of basa catfish oil (palmitic acid at the *sn*-2 position of 60.42 %) and fatty acids from high oleic acid sunflower oil (a mole ratio of 1:6) using Lipozyme RM IM of 12 % (*m*/*m* of the total substrate) at 50 °C for 2 h. Meanwhile, the use of basa catfish oil (palmitic acid at the *sn*-2 position of 49.3 %), which was acidolyzed with fatty acids from sesame oil (a mole ratio of 1:3), using Lipozyme RM IM of 8 % (*m*/*m* of the total substrate) at 40 °C for 2 h yielded HMFS containing palmitic acid at the *sn*-2 position of 48.3 % (*50*).

The use of palm stearin containing different levels of palmitic acid also affects the final quality of the product. Zou *et al*. (*51*) produced HMFS containing palmitic acid at the *sn*-2 position of 62.8 % from acidolysis between palm stearin (palmitic acid at the *sn*-2 position of 56.8 %) and a mixture of fatty acids from rapeseed oil, sunflower oil, palm kernel oil, stearic acid, and myristic acid (a mole ratio of 1:14.6), at 57 °C for 3.4 h using Lipozyme RM IM of 10.7 % (*m*/*m* of the total substrate). Meanwhile, Wang *et al*. (*74*) reported the use of fractionated palm stearin containing tripalmitin > 90 % (palmitic acid at the *sn*-2 position of 91.96 %), which was acidolyzed with a combination between oleic acid and linoleic acid at a mole ratio of 1:8:4, at 60 °C for 4 h using lipase NS 40086 of 8 % (*m*/*m* of the total substrate) produced HMFS with palmitic acid at the *sn*-2 position of 87.75 %. In another study, Faustino *et al*. (*76*) acidolyzed tripalmitin (purity > 85 %) with fatty acids from camelina oil (a mole ratio of 1:2) at 60 °C for 24 h using *R. oryzae* lipase immobilized on Lewatit VP OC 1600 of 5 % (*m*/*m* of the total substrate) yielding HMFS containing palmitic acid at the *sn*-2 position of 67.7 %.

Melting points of some substrates for HMFS synthesis are 66-68 °C for tripalmitin (*76*), 30.3-48 °C for lard (*44,80*), 58-61 °C for palm stearin (*51,55,78)*, 64.5 °C for fractionated palm stearin (*58*), and 63 °C for palmitic acid (*36*). The substrate's melting point influences the enzymatic interesterification to obtain the optimal target product. Lee *et al*. (*80*) reported that transesterification between lard (27.1 % palmitic acid) and olive oil (73.3 % oleic acid) or camellia oil (81.6 % oleic acid) at 40 °C for 12 h using Lipozyme IM-20 of 8.33 % (*m*/*m* of the total substrate) in isooctane solvent produced HMFS containing OPO of 12.9 % or 15.4 %, respectively. Meanwhile, transesterification of palm oil (44.3 % palmitic acid) with olive oil or camellia oil resulted in HMFS containing 21.8 % or 25.2 % OPO. Despite having a high palmitic acid at the *sn*-2 position, interesterification of lard produces

OPO content that is lower than palm oil. It is related to the low reaction temperature used (40 °C), lower than the melting point of lard which is 48 °C therefore the solubility of lard is low in isooctane at 40 °C (*80*).

METHODS AND REACTOR OF LIPASE-CATALYZED HMFS SYNTHESIS

Synthesis of HMFS can be carried out either through one-step reaction (*i.e.,* acidolysis or transesterification) or two-step reaction (*i.e.,* alcoholysis-esterification, acidolysis-acidolysis, or transesterification-acidolysis). The reaction schemes of lipase-catalyzed reaction for the synthesis of HMFS are shown in Fig. 1.

Acidolysis is a reaction between TAG and FFA (*4*). Enzymatic acidolysis is usually considered as a reversible two-step reaction: hydrolysis of the ester bonds with the released fatty acid followed by esterification of FFA to the backbone of glycerol (*5*). Table 3 (*6,15,16,31,38,39,43-52,59,61,66-68,74,75,80*) shows the reacting conditions for the HMFS production, where the resulting HMFS product generally contains palmitic acid at the *sn*-2 position of more than 60 %. The main by-product of the acidolysis reaction is FFAs, which can be removed through neutralization (*64,81*), liquid-liquid extraction (*82*), or molecular distillation (*83,84*). In acidolysis, there is usually acyl migration in which fatty acids at the *sn*-2 position migrates to either *sn*-1 or *sn*-3 position (*9*). This entails, various mixed TAGs as by-products are obtained in the reacting mixture. In general, acyl migration increases the separation complexity of the target HMFS from other by-products (*23*). Hence, the recovery of structured TAG (*i.e.,* target HMFS) is relatively lower than those obtained by the two-stage process (*9*).

Transesterification is a reaction between TAG and TAG or ester (*4*). HMFS-based transesterification reaction is carried out with TAGs which are rich in palmitic acid at the *sn*-2 position, using *sn*-1,3 regioselective lipase. In the transesterification reaction, the final product is a mixture of different TAGs with similar physical properties. To some extent, it is difficult to purify and obtain the target HMFS. Nevertheless, this issue is specific, depending on the type and proportion of oil (*5,9*). Table 4 (*42,54-58,65,68,85-91*) summarizes the reacting conditions of HMFS synthesis by transesterification.

The one-step enzymatic process has been used in many studies due to its simplicity, but the drawbacks of one-step enzymatic process are: (i) difficulties in converting intermediate DAGs into desired HMFS resembling HMF, and (ii) complexity in purification process due to the presence of by-products (*23*). To overcome these drawbacks, a multi-step enzymatic process such as alcoholysis-esterification has been proposed (*9,23*). The synthesis of HMFS *via* multi-step enzymatic process

results in a higher OPO purity (74-95 %), compared to one-step enzymatic process (about 43 %). However, this approach has also bottlenecks especially for the reaction complexity and high solvent consumption (*44*).

The two-step process for the HMFS synthesis can be alcoholysis route followed by the esterification reaction (*41,92,93*) (Table 5). The two-step process is proposed in the synthesis of HMFS to overcome the drawbacks of acidolysis and transesterification reaction. This method exploits the regioselectivity of lipase at *sn*-1,3 (*9,23*). Two-step synthesis consists of alcoholysis of TAG using *sn*-1,3 specific lipase to produce *sn*-2 MAG rich in palmitic acid. Forth, *sn*-2 MAG rich in palmitic acid esterified with FFAs (*4,94*) or esterified fatty acids (*92*). Generally, the final product from interesterification between *sn*-2 MAG rich in palmitic acid and oleic acid contains 92-94 % palmitic acid at *sn*-2 position and 83-89 % oleic acid at *sn*-1,3 positions, while the yield of OPO reaches 70-72 % (*9*). The alcoholysis and followed by esterification process avoids the acyl migration and obtains a purely structured TAG (HMFS) (*5,9,23*). However, this process is not commonly used in industrial production due to the complexity of the steps that leads to an increased overall cost (*9*).

Two-step synthesis of HMFS can be also carried out through two-step acidolysis (*60,64*) (Table 4). Esteban *et al.* (*60*) conducted acidolysis of palm stearin and palmitic acid at a molar ratio of 1:3, temperature 37 °C in solvent system using Novozyme 435 produced TAGs containing a high palmitic acid at the *sn*-2 position (74.5 %). After the first acidolysis, the resulted TAGs were used as intermediates for the second acidolysis with oleic acid at a molar ratio of 1:6 using *R. oryzae*, *Mucor miehei*, RM IM, TL IM, and *Alcaligenes sp.* lipases. The final product contained 67.8 % palmitic acid at *sn*-2 position and 67.2 % oleic acid at *sn*-1,3 positions (*60*). In addition, the two-step interesterification (transesterification-acidolysis) for the synthesis of the DHA-containing amaranth oil structured lipid was carried out by Pina-Rodriguez and Akoh (*95*). First, a customized amaranth oil was produced by transesterification of amaranth oil and ethyl palmitate using Novozyme 435. The second step, acidolysis of customized amaranth oil with DHA using Lipozyme RM IM. The final product contained 28 % palmitic acid and 33 % palmitic acid at *sn*-2 position.

The interesterification process for HMFS synthesis can be carried out in batch and continuous reactors (*5*). The batch production process is easy to operate and suitable for small scale production. However, at the industrial scales, for an economical production process, continuous operation is preferred rather than batch-wise operation (*52,96*). In continuous reactor system, such as continuous stirred tank reactor (CSTR), plug flow reactor (PFR) or packed bed reactor (PBR), substrate is continuously introduced into the reactor and product is subsequently withdrawn (*97*). PBR is more suitable for industrial-scale production, in contrast to CSTR (*23*).

The advantages of PBR over the batch reactor for production of structured lipids can be seen in the following aspects (*52*), such as: (i) the slow substrate passing through the enzyme column to avoid the enzyme structure being damaged and increasing the stability of the enzyme, (ii) the production can be carried out continuously, and (iii) it reduces the occurrence of acyl migration due to the use of enzyme excessively. To some extent, the continuous process at a high volumetric flow rate is more advantageous than operation at a slow volumetric flow rate. At a high flow rate, possibility of acyl migration reduces, thus raises the productivity (*22*). The acyl migration in a PBR is lower than in a batch stirred reactor (*23*). Nielsen *et al.* (*96*) reported the reaction equilibrium in acidolysis of lard and soybean oil fatty acids in a PBR was reached in < 1.5 h residence time related to minimization of acyl migration (*96*). Zou *et al.* (*52*) reported Lipozyme RM IM could be used for 10 d in a PBR without a significant loss of activity in interesterification between palm stearin and mixed of stearic acid, myristic acid and fatty acids from rapeseed oil, sunflower oil, and palm kernel oil. Wang *et al.* (*39*) also reported that the number of reuses of lipase in a packed reactor increased 2.25-fold compared to that of batch reactor.

FACTORS INFLUENCING HMFS SYNTHESIS

Some aspects considered for HMFS synthesis are biocatalyst concentration, reaction type, substrate composition and mode of operation (*5*). Table 3, Table 4 and Table 5 show selected works on HMFS synthesis using various substrates, enzymes and other relevant parameters. For most of the studies, the reaction conditions are of interest to be optimized to obtain products that resemble HMF.

Effect of lipase concentration

Lipase concentration affects the rate of interesterification reaction. The initial reaction rate increases by the higher lipase concentration due to a higher number of active side pockets available for catalytic activities (*70,92,93*). Lipase concentration also affects amount of DAG and the rate of acyl migration (*50*). A higher lipase concentration upgrades the incorporation of the acyl donors (acyl migration) in acidolysis reaction (*38*). Some published reports that are shown in Table 3, Table 4 and Table 5 are not comparable. The related reacting conditions are not provided (*i.e.,* the enzyme's activity and the amount of substrate). Herein, the discussion presented here merely describing the optimum conditions where the particular study has been carried out and not to compare between these studies. It is worth mentioning that lipase concentration must be optimized. To some extent, the progressive increase of lipase concentration promotes the synthesis of OPO via shortening of the

reaction time and weakening of the acyl migration (*98*). However, the excessive enzyme amount will entail in hydrolytic reaction over esterification.

Zou *et al.* (*50*) reported that after reaction time of 2 h in acidolysis between basa catfish oil and fatty acids from sesame oil using 2 % (*m*/*m* of the total substrate) Lipozyme RM IM, the concentration of *sn*-2 palmitate was 56 %. Meanwhile, at enzyme loading of 8, 11, and 14 %, the percentages of *sn*-2 palmitate were relatively similar of 63.5-65 %. Ilyasoglu *et al.* (*56*) used 10 % Lipozyme TL IM in transesterification between tripalmitin and a mixture of extra virgin olive oil and flaxseed oil to produce HMFS with 25.2 % palmitic acid and 15.9 % α-linolenic acid. Jiménez *et al.* (*36*) used 10 % Novozyme 435 in acidolysis between palm stearin and palmitic acid to produce TAGs containing 70.5 % palmitic acid at *sn*-2 position. In general, the range of enzyme loads for HMFS synthesis by interesterification is 8-10 % (*m*/*m* of the total substrate).

Effect of moisture content

The enzyme inactivation due to dehydration sometimes causes a low operational of interesterification (*32*). Lipase has high activity in the nearly absent-to-micro aqueous system, typically interface activated at the oil-water interface (*70*). The hydrolysis is usually considered the rate limiting reaction in which water acts as a reactant. To some extent, the enhancement of moisture content increases the initial activity of lipase. However, excessive water entails with the formation of by-products (*38*). A small amount of water is important for lipase to maintain its activation (*i.e.,* lubrication of the enzyme's conformation). Herein, the amount of water content must be controlled during especially acidolysis reaction (*50*).

Zheng *et al.* (*70*) reported that OPO content reached a maximum conversion (43.9 %) at 2 % moisture content during the interesterification of tripalmitin and oleic acid. This conversion decreased as the moisture content increased. In other studies, the addition of 1 % moisture content in acidolysis of lard and oleic acid increased OPO yield from 52.8 % to 55.3 %, whereas at 5 % moisture content, the OPO content was gradually declined (*31*). Zou *et al.* (*50*) reported the optimum moisture content in acidolysis between palm stearin and FFAs for HMFS synthesis was about 0.24 %. Thus, the range of water content in HMFS synthesis by enzymatic interesterification is 0.2-2 %.

Effect of solvent

Generally, lipase-catalyzed interesterification for HMFS synthesis can be performed in either solvent system (*i.e.,* organic solvents) or solvent-free system. The solvent increases the solubility of high melting point reactants. Herein, the reaction can be operated at a lower temperature which is

beneficial for the enzyme's stability. However, excessive solvent dilutes the reaction fluid and reduces the random access of substrate to the lipase active sides (*93*). Several factors must be considered when selecting a proper solvent for a particular enzymatic reaction including: (i) compatibility of the solvent with the reaction, (ii) solvent properties (density, viscosity, surface tension, toxicity, flammability), and (iii) cost. Lipase tends to be more active in *n*-hexane than in other solvents such as isooctane, acetone, petroleum ether, toluene, or ethyl acetate. N-hexane plays key roles, such as increasing the solubility of non-polar substrates and shifting the reaction towards esterification rather than hydrolysis (*23*).

Palmitic acid-enriched TAG has a high melting point requires a higher temperature in the solvent-free reaction system in order to keep the substrate liquid during the reaction (*60*). Palm stearin and palmitic acid have high melting points so they are difficult to react without solvent as they require a minimum temperature of 65 °C (*36*). Esteban *et al.* (*60*) reported that the incorporation of oleic acid at the *sn*-1,3 position was slightly lower in the solvent-free system (46.2 %) than in the solvent system (50.4 %) in the interesterification between palmitic acid-enriched TAG from palm stearin and oleic acid. It was caused by a smaller reaction rate due to a lower mass transfer rate when no solvent is available. In addition, Cao *et al.* (*99*) reported that in acidolysis, the rate of acyl migration and the concentration of intermediate or side products (e.g., DAG, MAG) decreased significantly in the anhydrous reaction system.

Effect of substrate ratio

The interesterification reaction rate of HMFS synthesis depends on the substrate ratio (TAG to acyl donor) after the reaction equilibrium has been achieved (*38,70*). Enhancement of the mole ratio of TAG to fatty acids leads to a reaction equilibrium (*31,38,100*), and produces the desired incorporation of fatty acids into TAG (*6*). The presence of excessive TAG substrate reduces lipase active site capabilities. Also, an excessive FFA amount produces environmental acidification, increases the viscosity of the system, inhibits biocatalyst activity, and reduces mass transfer rate (*70*). The high substrate molar ratio may increase the frequency of collisions between the enzyme and substrates (*101*). The increase of palmitic acid content at *sn*-2 position is greater when the molar ratio of TAG to fatty acid is enhanced in the interesterification between palmitic acid-enriched TAG from palm stearin and oleic acid (*60*). The substrate ratio also affects fatty acids at the *sn*-1,3 positions. Increasing the molar ratio decreased the saturated fatty acids at *sn*-1,3 position in acidolysis between a mixture of pam stearin and ARA oil with oleic acid (*46*).

Bryś *et al*. (*87*) reported transesterification between lard and milk thistle oil at a mass ratio of 6:4 and 8:2 at 60 °C using 8 % Lipozyme RM IM. After 4 h, mole ratio of 8:2 obtained HMFS with 21 % palmitic acid and about 75 % palmitic acid at the *sn*-2 position. Meanwhile, at a ratio of 6:4, HMFS contained palmitic acid at the *sn*-2 position lower than 70 %. In addition, Tecelão *et al.* (*85*) reported that the incorporation of oleic acid was increased drastically (from 32 to 51 mol%) by raising the substrate ratio of tripalmitin to ethyl oleate from 1:2 to 1:8. Zou *et al.* (*51*) reported the optimum substrate molar ratio for acidolysis between palm stearin and a mixture of stearic acid, myristic acid, and FFAs from rapeseed oil, sunflower oil, and palm kernel oil of 1:14.6, producing HMFS contained 29.7 % palmitic acid and 62.8 % palmitic acid at *sn*-2 position. Generally, the range of the molar ratio of substrates (*i.e.,* tripalmitin, palm stearin, lard, catfish oil) with fatty acids in the interesterification for HMFS synthesis is 1:2-1:14.

Effect of reaction temperature

The reaction temperature influences the subtle variations in the architecture/ conformation of lipase and leads to thermal inactivation of lipase and reduction in affinity between substrates and biocatalyst (*102*). A higher temperature enhances the mass transfer and to some extent, increases the activity of lipase as well (*93*). In endothermic reactions, higher temperatures provide higher results due to the shift in thermodynamic balance. At high temperatures, the operation of the process is also easy as the solubility of the reactants increases and the viscosity of the solution decreases (*38*). Moderately high temperatures can provide sufficient energy to overcome the reaction barrier, while too high temperatures can cause lipase thermal deactivation (*103*). Therefore, the reaction temperature should be considered as low as possible so that the reaction efficiency and product quality are assured (*50*). The optimal temperature will vary from different lipase sources (*6,104*). The reaction temperature is positively correlated with acyl migration. It also has effect on the acyl incorporation (*105*) in which high temperatures may facilitate acyl migration (*38*).

The OPO content reached a maximum (46.5 %) at a reaction temperature of 50 °C for the interesterification between tripalmitin and oleic acid using *CLL*@mMWCNTs. However, the OPO content decreased with the increase in reaction temperatures, especially above 50 °C (*70*). He *et al.* (*6*) reported that the highest ω-3 PUFAs (13.92-17.12 %) in HMFS obtained by interesterification between TAG from *Nannochloropsis oculata* and fatty acids from *Isochrysis galbana* using Novoyme 435, recombinant CAL-B lipase, Lipozyme TL IM, and Lipozyme RM IM at reaction temperature of 60, 50, 60, 50 °C, respectively. Generally, the range of reaction temperature for HMFS synthesis via enzymatic interesterification is 40-60 °C.

Effect of reaction time

The reaction yield for synthesis of structured lipids is positively affected by an increase in reaction time (*56,65*). Reaction time in the interesterification is governed by the reactor configuration (*i.e.,* batch or continue reactor) (*39*). Wang *et al.* (*39*) reported that the reaction time for HMFS synthesis via interesterification between tripalmitin and PUFAs from microalgae oil in PBR (2.5 h) was faster than that of batch reactor (7 h). Generally, the reaction time in a batch reactor is the most affecting factor leads to increased acyl migration and eventually results in the production of the partial acylglycerols such as DAG and MAG. The acyl migration increases linearly with an increased reaction time (*58*). In addition, the reaction temperature also affects the reaction time. Yang *et al.* (*38*) reported the interesterification between lard and fatty acids from soybean where the reaction time to reach incorporation of 20 % linoleic acid and 3 % linolenic acid declined with the enhancing reaction temperature, from 5 h at 50 °C down to 2.4 h at 90 °C.

Bryś *et al*. (*88*) reported transesterification between lard and milk thistle oil at a mass ratio of 8:2 using 8 % Lipozyme RM IM at 70 °C that yielded HMFS with palmitic acid at the *sn*-2 position above 70 % at 2 and 6 h, while at 4 h only 53.4 %. In addition, Bryś *et al*. (*89*) also reported transesterification between lard and rapeseed oil at a mass ratio of 8:2 using 8 % Lipozyme RM IM at 70 °C for 4 h. The produced HMFS had 24.2 % palmitic acid content and 41.6 % palmitic acid at the *sn*-2 position. Meanwhile, at 8 and 24 h reaction times, HMFS had palmitic acid contents at the *sn*-2 position of 34.9 % and 26.4 %, respectively. The OPO content in the product of interesterification between tripalmitin-rich palm stearin and ethyl oleate in a batch process using Lipozyme TL IM decreased as the reaction time increased by 29.3 % and 18.5 % at 3 and 12 h, respectively (*58*). In addition, Zou *et al.* (*52*) reported the interesterification between palm stearin and a mixture stearic acid, myristic acid, and fatty acids from rapeseed oil, sunflower oil, and palm kernel oil, in PBR with reacting conditions were residence time 2.7 h, temperature 58 °C and substrate molar ratio 1:9.5. Under these conditions, the contents of palmitic acid in TAGs and at *sn*-2 position were 28.8 % and 53.2 %, respectively. Generally, the range of reaction time for HMFS synthesis *via* enzymatic interesterification in a batch process is 2-24 h, while in a continuous process is 1-3 h.

PURIFICATION OF HMFS

The synthesis of structured lipid by the enzymatic interesterification produces TAGs, partial glycerides (DAG, MAG), and FFAs. The acidolysis between TAG and fatty acids produces products with a high FFA content. Product from acidolysis between palm stearin and palmitic acid at a mole ratio of 1:3 contains FFAs of 50 % (*36*). Meanwhile, the transesterification between TAG molecules

produces product having low content of FFAs (0.5-7 %) (*55,57,68*). Thus, in each type of enzymatic interesterification or utilization of different substrates, can result in different complexity in the purification process of HMFS. This complexity as indicated earlier depends on the number of by-products contained in the reaction mixture. Purification after HMFS synthesis is intended to increase a high TAG fraction by removing FFAs and partial glycerides. The removal FFAs can be carried out by neutralization (*56,60,61,64,81*), liquid-liquid extraction (*54,82*), and evaporation using molecular distillation (*44,47,49,50,52,83,84*). Molecular distillation is also applied to remove both FFAs and partial glycerides simultaneously (*47*).

Neutralization is carried out through saponification of FFAs using an alkaline solution such as potassium hydroxide (KOH). The acylglycerol fraction is then extracted using hexane (*56,60,61,64,81*). Ilyasoglu (*56*) reported that the neutralization of the transesterification product between tripalmitin and a mixture of olive oil and flaxseed oil (1:1) (a mole ratio of 1:2.67) using KOH 0.8 N enhanced TAG content up to 78 %. Robles *et al*. (*64*) also reported the neutralization of the acidolysis product between palm stearin rich palmitic acid at the *sn*-2 position and oleic acid (a mole ratio of 1:6) using 0.5 N KOH at 37 °C. The TAG yield was up to 80 %. Esteban *et al*. (*60*) also reported the neutralization of acidolysis product between palm stearin rich palmitic acid at the *sn*-2 position and oleic acid using KOH 0.5 N in the presence and absence of hexane. With (at room temperature) and without solvent at 50 °C, the neutralization can increase TAG purity of 99 % with the yield of 96 %. Using liquid-liquid extraction, Yuan *et al*. (*54*) reported the removal of FFAs from the interesterification product using 85 % ethanol with a mass ratio of 1:1.

Separation using molecular distillation is carried out based on the difference in vaporization temperatures of FFAs, partial glycerides, and TAGs. Using molecular distillation, Qin *et al*. (*44*) reported the purification of the acidolysis product between 34L-leaf lard and camellia fatty acid (a mole ratio of 1:4). By having the evaporation temperature of 180 °C and pressure of 6.7-7.5 Pa, the TAGs were rich in OPO with the purity of 91.39 % and the yield of 40.75 % (*m*/*m* of the total substrate). Zou *et al*. (*49*) also reported the purification of acidolysis product between the solid fraction of basa catfish oil and high oleic sunflower oil fatty acids (a mole ratio of 1:6). At the evaporation temperature of 185 °C and the pressure of 2 Pa, the TAG fraction with the yield of 95.7 % was obtained. A stepwise evaporation process using molecular distillation is also possible for purification of interesterification product. Sørensen *et al*. (*83*) produced TAG fraction of 31.3 % from the acidolysis between butterfat and a mixture of fatty acids from rapeseed oil and soybean oil (a mole ratio of 1:2). The conditions were having pressure of 0.1 Pa and the evaporation temperatures in stage 1 and stage 2 were 90 °C and 185 °C, respectively. The ranges of evaporation temperatures and pressures of molecular

distillation to remove FFAs during HMFS purification are 180-185 °C and 0.1-7.5 Pa. In addition, the separation of TAGs from partial glycerides is carried out at the evaporation temperature of 230 °C and a pressure of 1.0×10^7 Pa (*47*).

In multi-stage process (*i.e.,* two-step acidolysis), purification starts at the first acidolysis to remove FFAs from the reaction mixture. At the second acidolysis, FFAs and DAGs are also removed from the product mixture. A single-step enzymatic process can also produce near-pure HMFS. However, it is challenging to convert all of the intermediate DAGs formed during the reaction. In addition, multiple purification steps are required to remove the by-products (*23*). The concentration of target TAGs containing palmitic acid at the *sn*-2 position in the final product can be increased by separating the other TAGs through fractionated crystallization (*57,80,83*). Lee *et al*. (*80*) reported an increased OPO content from 25.2 % to 53.3 % in the enzymatic transesterification product between palm oil and camellia oil by fractionation at 22 °C for 16 h. Sørensen *et al*. (*83*) reported that HMFS with palmitic acid at the *sn*-2 position of 56 % was produced from fractionation of the acidolysis product between butterfat and mixture of fatty acids from rapeseed oil and soybean oil. Meanwhile, the acidolysis product between solid fractions from fractionation of butterfat and mixture of fatty acids from rapeseed oil and soybean oil produced HMFS with a palmitic acid at the *sn*-2 position of 47 %.

CURRENT DEVELOPMENT OF HMFS PRODUCTION

In the last two decades, HMFS has been developed from a wide variety of substrates, enzymes and reacting conditions. In general, the type of HMFS studied is *sn*-2 palmitate (OPO) because this TAG is the major component of HMF. Thus, the main consideration in HMFS production is to have palmitic acid at the *sn*-2 position (*106*). OPO enriched HMFS is produced from interesterification between palmitic acid-containing source (*i.e.,* lard, tripalmitin, palm oil and its derivatives (palm stearin, palm olein), catfish oil, palmitic acid, or ethyl palmitate) and oleic acid-containing sources (*i.e.,* olive oil, high oleic sunflower oil, oleic acid or ethyl oleate).

A better understanding of the composition and structure of HMF provides detailed information in HMFS investigations (*9*). Recently, Wang *et al.* (*74*) synthesized both OPL and OPO from palm stearin fractionates. OPL synthesis has not received much attention although its content is also abundant in HMF. The OPO to OPL ratios in HMF range from 0.5 to 2.0 (*107,108*). Apart from *sn*-2 palmitate, PUFAs, and MCFAs are also contained in HMF which play an important role during the early human development (*4,109*).

Synthesis of long-chain polyunsaturated fatty acids enriched HMFS can be obtained from fish oil, algae oil, fungal oil, microbial oil, *Silkworm* pupae oil, hazelnut oil, soybean oil, sunflower oil, ALA,

GLA, DHA, and ARA. Ghosh *et al.* (*55*) synthesized HMFS from palm stearin fractionates and fish oil (molar ratio of 2:1) *via* transesterification using Lipozyme TL IM. The final product contained 75.98 % palmitic acid at the *sn*-2 position, 0.27 % ARA, 3.43 % EPA, and 4.25 % DHA. Interesterification between tripalmitin and ARA ethyl ester from ARA-rich single-cell oil from *Mortierella alpine* (a molar ratio of 1:2) using Lipozyme RM IM resulted with 69.57 % ARA incorporation at *sn*-1,3 position and 62.31 % palmitic acid at *sn*-2 position (*42*).

Naturally, MCFAs are present in HMF in the form of MLCTs with the main composition of TAGs is one MCFA and two long-chain fatty acids (MLL type) (*4,110*). MCFAs contained in HMF are a large amount of lauric acid (C12: 0), a small amount of capric acid (C10: 0) and caprylic acid (C8: 0) (*111*). MCT enriched HMFS can also be synthesized from coconut oil, palm kernel oil, and MCFAs containing caprylic acid and capric acid. Recently, Yuan *et al.* (*54*) synthesized HMFS containing TAG with MLL type from catfish oil and coconut oil (a molar ratio of 1.5:1) *via* interesterification using Lipozyme RM IM, Lipozyme TL IM, NS 40086, and DF Amano 15 (DF 15) with an enzyme load of 8 % (*m*/m), temperature of 60 °C, and reaction time of 3 h. The final product contained MLCT and MLL reaching 62.14 % and 39.85 %, respectively, with the main TAGs in HMFS were C12:0/C16:0/C18:1, C12:0/C18:1/C18:1, C12:0/C14:0/C18:1, and C12:0/C12:0/C18:1. Korma *et al.* (*86*) also produced HMFS containing 6.12 % medium-medium-medium TAGs, 53.75 % MLCTs, and 40.13 % long-long-long TAGs from interesterification between fungal oil containing high ARA from *Mortierella alpine* and MCT (a molar ratio of 1:1) catalyzed by recombinant CAL-B of 8 % (*m/m*), temperature of 90 °C, and reaction time of 3 h.

A single step enzymatic transesterification can produce HMFS similar to HMF using the suited molar ratio of substrates. For example, Zou *et al*. (*90*) and Zou *et al*. (*91*) reported a mixture of lard, sunflower oil, canola oil, palm kernel oil, palm oil, algae oil, and microbial oil at a mass ratio of 1.00:0.10:0.50:0.13:0.12:0.02:0.02 for HMFS synthesis. The substrate in the mixture ratio was transesterified at a temperature of 60 °C, moisture content of 3.5 % (*m*/*m* of the lipase), reaction time of 3 h, and using Lipozyme RM IM 11 % (*m*/*m* of the total substrate). The product of HMFS had palmitic acid content of 20.1 % with 38.2 % palmitic acid at *sn*-2 position. The resulting HMFS had a high score degree of similarity with HMF in the composition of total and *sn*-2 position fatty acids, PUFA, and TAG with the values of 92.5, 90.3, 61.5, and 71.9, respectively (*90*). Zou *et al*. (*91*) also used the substrate at that mixture ratio, which was transesterified using Lipozyme RM IM in PBR at 50 °C and a residence time of 1.5 h. The obtained HMFS had 39.2 % palmitic acid at *sn*-2 position, 0.5 % ARA, and 0.3 % DHA. Based on TAG content and purity, the score of the degree of HMFS similarity to HMF was 72.3.

At present, the commercial HMFS for inclusion in infant formulas has been successfully produced from various sources of oils and fats (*4,5,9*). *Sn*-2 palmitate is one of the structured TAGs that is generally supplemented into infant formula (*5,112*). HMFS products that have been commercialized according to the main fatty acid composition they contain are Betapol 60 (C16:0, C18:1, C18:2), InFat™ (C16:0, C18:1), Bonamil (C16:0, C18:1, C18:2, C18:3), Similac Advance (C16:0, C18:1, DHA, ARA), Alsoy (C16:0, C18:1, DHA, ARA), Aptamil 1 (C16:0, C18:1, EPA, DHA), Milu-Milk (C16:0, C18:1, DHA), Bledina Alma (C16:0, C18:1, C18:2), Gallia HA (C16:0, C18:1, DHA, ARA), Cow & Gate Premium (C16:0, C18:1, DHA, GLA, ARA), and Baby Semp 1 (C16:0, C18:1, DHA, ARA) (*23*).

OUTLOOK: CHALLENGES AND OPPORTUNITIES IN HMFS SYNTHESIS

Structured lipids are designed through the modification of oils and fats to have desired nutritional or physicochemical properties suitable for food industries (*9,113,114*). HMFS is one of the ingredients in infant formula that is potentially and continuously developed to support infant growth according to the needs of each stage of baby's age (*i.e.,* infant formula and advanced formula) and baby conditions (normal or premature and low birth weight babies).

The challenge for developing HMFS is the relatively high production cost. To enhance productivity (thus, reducing overall production cost), the synthesis of HMFS is carried out through a careful selection of the substrate, enzyme, reactor configuration, and reaction conditions. Generally, the optimum reacting conditions for HMFS synthesis are at substrate molar ratios between TAGs and FFAs of 1:2-1:14, temperatures of 40-60 °C, enzyme loads of 8-10 % (*m*/*m* of the total substrate), and reaction times of 2-24 h in batch process or 1-3 h in continuous process. Large-scale production of HMFS through a one-stage process using tripalmitin is not attractive because of its high cost and difficulty in producing products resembling HMF (*23*). Meanwhile, the multistep reaction can produce higher yield of HMFS that has properties resembling HMF. However, the increase in reacting system complexity will also tend to increase down streaming costs. It is worth-mentioning that the production of HMFS in a solvent-free system is preferred in terms of food safety and costs (*5*).

One of the potential sources of substrates for HMFS synthesis is palm stearin because of its high palmitic acid content and relatively low price. However, the palmitic acid-rich TAGs at the *sn*-2 position of palm stearin needs to be increased through chemical interesterification (*51,52*), enzymatic interesterification (*36,37*), or fractionation (*46,55,58,115*). It is due to the nature of palm stearin that contains oleic acid abundant at the *sn*-2 position. Herein, the acidolysis between palm stearin and oleic acid using an *sn*-1,3 specific lipase will result in triolein which is not preferred (*74*). Herein, the

HMFS synthetic route using palm stearin has to be started with enhancement of palmitic acid-rich TAGs at the *sn*-2 position (*115*). Then, the fatty acids at the *sn*-1,3 positions from the palmitic acid-rich TAGs are replaced with acyl donors through acidolysis or transesterification. The common acyl donors are single fatty acids (oleic acid, ALA, GLA, EPA, DHA, ARA), FFA mixtures of vegetable oils (such as olive oil, camelina oil, rapeseed oil, sunflower oil, hazelnut oil), sources of omega-3 PUFAs (such as fish oil, microalgae oil) (*5,9,23*), or sources of MCFA (such as coconut oil, palm kernel oil). HMFS that is similar to HMF and has C8: 0, C10: 0, C12: 0, C16: 0, C18: 1, C18: 2, EPA, DHA, GLA, and ARA, is potentially commercialized in the future.

In HMFS synthesis, the high ratio of acyl donors is not attractive due to the difficulties in the separation process (such as deacidification) (*50*). This entails, high costs of post-process separation (*31*). The possibility of producing HMFS with a low ratio acyl donor is very interesting. However, the main limitation in the reaction process is low mass transfer, thus, a lower reaction rate. To overcome this problem, an enzyme that has a higher specificity and stability is needed. Faustino *et al.* (*76*) reported that tripalmitin consumption of 62.7 % *m/m* was achieved at a substrate molar ratio of 1:1.2 at 65 °C using *R. oryzae* lipase immobilized on Lewatit VPOC 1600 during acidolysis between tripalmitin and FFAs from camelina oil. The isolation and genetic engineering of new lipases with better stability during operation at high temperatures are also of interest for future research (*9,18*). The mutagenesis techniques are also promising for creating novel lipases such as an *sn*-2-specific lipase (*21*), which would facilitate the production of OPO. In addition, the use of continuous systems other than PBR, such as enzymatic membrane reactor is also interesting to be developed (*116*). In enzymatic membrane reactor system, a continuous reaction can be facilitated by having immobilized enzyme retained inside the reactor (*116*).

CONCLUSIONS

HMFS is synthesized by the enzymatic interesterification of vegetable oils, animal fats, or blends of oils. The main characteristic of this HMFS is having TAGs with palmitic acid located at the *sn*-2 position and unsaturated fatty acids at the *sn*-1,3 positions. Selection of substrates, enzymes, batch or continuous reactor configuration, and reacting conditions needs to be considered to increase the overall productivity of HMFS production. Lipozyme RM IM, Lipozyme TL IM, and Novozyme 435 are widely used for the synthesis of HMFS. Lipozyme RM IM and Lipozyme TL IM are used as biocatalysts due to their regiospecificity toward *sn*-1,3 positions. Generally, Lipozyme RM IM is used in acidolysis reaction, whereas Lipozyme TL IM is used in transesterification reaction. Novozyme 435 is used due to its regiospecificity toward *sn*-2 position, which is beneficial for incorporating palmitic

acid at *sn*-2 position of the oils and fats, both in acidolysis and transesterification. Generally, the optimum reacting conditions for HMFS synthesis are at substrate molar ratios between TAGs and fatty acids of 1:2-1:14, temperatures of 40-60 °C, enzyme loads of 8-10 % (*m*/*m* of the total substrate), moisture contents of 0.2-2 % and reaction times of 2-24 h in batch process or 1-3 h in continuous process. The separation of interesterification product from FFAs in HMFS synthesis is carried out by neutralization using KOH 0.5 N (1.5 times the number of equivalents of KOH required to neutralize the FFAs) or molecular distillation at the evaporation temperatures of 180-185 °C and pressures of 0.1-7.5 Pa.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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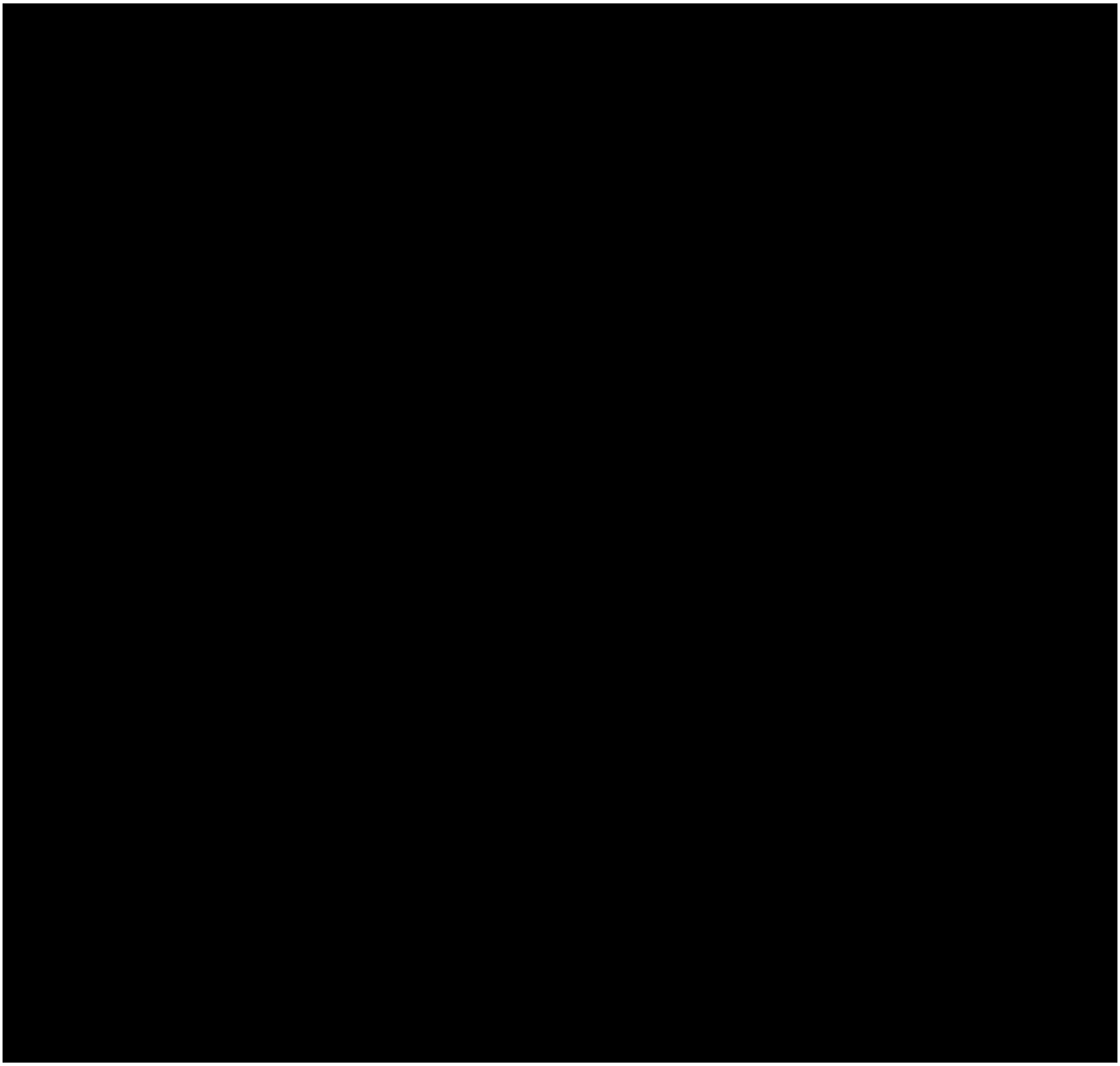

Fig. 1. Schemes of lipase-catalyzed reaction for HMFS synthesis

Table 1. Reusability of lipase for HMFS synthesis

Lipase	Reusability of enzyme	Reacting conditions	Reference
Lipozyme RM IM	23 batch (1 batch for 1 h)	lard and soybean fatty acids (*r*=1:2.4), lipase loading 13.7 %, temperature 61 °C, reaction time 1 h	(*38*)
Lipozyme RM IM	18 batch (1 batch for 2.5 h) using microfluidic packed reactor	tripalmitin and PUFA from microalgae oil (*r*=1:7), lipase loading 90 mg, temperature 60 °C	(*39*)
Lipozyme RM IM, Lipozyme TL IM, Novozyme 435, and *Candida parapsilosis*	System I: Lipozyme RM IM of 10 batch (230 h), half-life times for Lipozyme TL IM for 154 h (6.7 batch), Novozyme 435 for 253 h (11 batch) and *C. parapsilosis* for 276 h (12 batch) System II: half-life times of Novozyme 435 for 322 h (14 batch) and *C. Parapsilosis* for 127 h (5.5 batch)	system I: tripalmitin and oleic acid (*r*=1:2), lipase loading 8.9 % *m/m* tripalmitin, temperature 60 °C system II: tripalmitin and ω-3 PUFA (*r*=1:2), lipase loading 8.9 % *m/m* tripalmitin, temperature 60 °C	(*59*)
Novozyme 435, recombinant CAL-B, Lipozyme TL IM and Lipozyme RM IM	20 batch (Novozyme 435, recombinant CAL-B, Lipozyme RM IM), 4 batch (Lipozyme TL IM) (1 batch for 24 h)	TAG from *Nannochloropsis oculata* and *Isochrysis galbana* fatty acids (*r*=1:3), lipase loading 10 %, temperature 60 °C (Novozyme 435, Lipozyme TL IM), 50 °C (recombinant CAL-B, Lipozyme RM IM)	(*6*)
Novozyme 435	10 batch (1 batch for 24 h)	palm stearin and palmitic acid (*r*=1:3), lipase loading 10 %, temperature 38 °C, solvent system	(*36*)
Candida lipolytica immbolized in magnetic multi-walled carbon nanotubes	20 batch (1 batch for 2 h)	tripalmitin and oleic acid (*r*=1:6), lipase loading 20 g/L, temperature 50 °C	(*70*)
Rhizopus oryzae	10 batch (1 batch for 19 h)	palm stearin containing high palmitic acid at *sn*-2 position and oleic acid (*r*=1:6), lipase loading 3.3 %, temperature 50 °C	(*60*)
Candida sp. 99-125	5 batch (with additive β-cyclodextrin) (1 batch for 16 h)	lard and oleic acid (1:2 m_A/m_B), lipase loading 10 %, temperature 45 °C	(*31*)
Rhizopus oryzae immobilized in Accurel® MP 1000 and Lewatit® VP OC 1600	half-life times for *Rhizopus oryzae* immobilized in Accurel® MP 1000 and in Lewatit® VP OC 1600 for of 34.5 h and 64.0 h, respectively	tripalmitin and oleic acid (*r*=1:2), lipase loading 5 %, temperature 60 °C, in solvent-free system	(*32*)

Table 2. Potential substrates for HMFS synthesis

Source	Fatty acid (%)																Reference
	Total								*sn*-2 position								
	C12:0	C16:0	C18:1	C18:2	C18:3	20:4	22:5	22:6	C12:0	C16:0	C18:1	C18:2	C18:3	20:4	22:5	22:6	
Source of palmitic acid																	
Tripalmitin	-	94.2	1.7	1.0	-	-	-	-	na	Na	na	na	na	na	na	na	(*76*)
Lard	0.7	28.5	35.9	9.4	-	-	-	-	-	78.9	10.0	3.7	-	-	-	-	(*47*)
Palm stearin	0.7	70.1	18.7	-	-	-	-	-	0.1	56.8	30.9	8.3	-	-	-	-	(*51*)
Fractionated palm stearin	-	91.6	2.3	0.4	-	-	-	-	-	92.0	3.7	0.6	-	-	-	-	(*74*)
N. Oculata oil	-	37.9	10.6	2.1	0.7	-	-	-	-	76.2	2.0	1.1	0.4	-	-	-	(*6*)
Basa catfish oil	0.3	32.8	38.9	9.7	0.4	-	0.1	0.1	0.4	49.3	26.3	9.2	0.5	-	0.1	0.1	(*50*)
Solid fraction of Basa catfish oil	0.3	34.6	37.3	9.2	0.4	-	0.1	0.1	0.5	60.4	18.8	8.6	0.4	-	0.1	0.1	(*49*)
Palm oil	-	53.6	34.1	8.7	-	-	-	-	-	18.9	62.3	18.5	-	-	-	-	(*77*)
Palm olein	-	43.6	40.9	9.9		-			-	13.8	66.4	19.0	-	-	-	-	(*67*)
Source of oleic acid																	
High oleic sunflower oil	-	7.1	74.1	9.6	1.7	-	-	-	-	2.8	38.3	43.8	-	-	-	-	(*78*)
Hazelnut oil	-	5.7	82.3	8.5	0.1	-	-	-	-	0.8	86.0	11.7	-	-	-	-	(*65*)
Tea seed oil	-	8.3	76.5	9.0	1.1	-	-	-	-	2.0	83.3	11.2	0.6	-	-	-	(*47*)
Rapeseed oil	0.9	6.7	58.1	22.5	7.8	-	-	-	-	1.1	54.6	33.9	0.5	-	-	-	(*48*)
Extra virgin olive oil	-	16.1	68.3	9.7	1.0	-	-	-	na	Na	na	na	na	na	na	na	(*75*)
Source of linoleic acid																	
Sunflower oil	-	5.7	21.6	65.3	0.1	-	-	-	-	1.0	19.8	78.8	-	-	-	-	(*44*)

Soybean oil	-	10.6	23.4	53.2	5.8	-	-	-	-	1.2	22.9	68.3	5.4	-	-	-	(*44*)
Source of linolenic acid																	
Flaxeed oil	0.0	5.1	18.6	16.4	55.0	-	-	-	-	1.2	22.1	22.6	52.9	-	-	-	(*44*)
Camelina oil	-	6.3	18.1	19.4	35.5	-	-	-	na	na	na	na	na	na	na	na	(*76*)
Source of MCFA																	
Coconut oil	47.1	9.2	7.2	1.9	-	-	-	-	77.6	1.4	5.8	2.1	-	-	-	-	(*44*)
Palm kernel oil	55.7	6.4	10.2	2.1	-	-	-	-	42.3	8.7	25.2	5.4	-	-			(*48*)
Source of EPA and DHA																	
Fish oil	-	19.0	14.3	-	-	-	5.5	50.0	-	5.4	8.4	-	-	-	2.2	82.3	(*79*)
Tuna fish oil	-	22.0	18.7	2.3	-	-	0.6	18.4	-	20.5	2.6	-	-	-	1.5	33.5	(*64*)
Algal oil	-	24.7	1.4	0.4	0.3	-	15.4	42.0	-	14.6	2.4	2.2	0.3	-	13.7	47.8	(*48*)
Microalgae oil (*Schizochytrium* sp.)	-	19.1	16.2	4.1	-	-	16.1	38.6	-	8.3	38.7	1.9	-	-	11.5	34.0	(*39*)
DHA single cell oil	4.5	9.9	22.2	1.0	-	-	-	44.1	na	na	na	na	na	na	na	Na	(*75*)
Source of arachidonic acid																	
Microbial oil	-	10.9	8.6	4.0	2.8	49.0	-	-	-	4.1	13.6	12.8	5.9	45.9	-	-	(*48*)

MCFA=medium chain fatty acid, C12:0=lauric acid, C16:0=palmitic acid, C18:1=oleic acid, C18:2=linoleic acid, C18:3=linolenic acid, C20:4=arachidonic acid, C22:5=eicosapentaenoic acid (EPA), C22:6=docosahexaenoic acid (DHA)

Table 3. Reacting conditions for the production of HMFS through acidolysis

Lipase	Substrate	Solvent system	Operation mode	Enzyme loading/ %	*r* (substrate)	Temperature/° C	Time/ h	Products	Reference
Lipozyme RM IM	lard and soybean oil fatty acids	solvent-free system	batch	13.7	1:2.4	61	1	71.1 % palmitic acid at *sn*-2 position	(*38*)
Lipozyme RM IM	tripalmitin and a mixture of hazelnut oil fatty acids and stearic acid	hexane	batch	10	1:12:1.5 (incorporation of oleic acid) 1:3:0.75 (incorporation of stearic acid)	65 (incorporation of oleic acid) 60 (incorporation of stearic acid)	24	45.3 % palmitic acid, 76.0 % palmitic acid at *sn*-2 position	(*15*)
Lipozyme RM IM	tripalmitin and a mixture of hazelnut oil fatty acids, EPA and DHA	hexane	batch	10	1:12.4	55	24	76.6 % palmitic acid at *sn*-2 position, 5 % total EPA+DHA, 40 % oleic acid	(*45*)
Lipozyme RM IM	lard and a mixture of FFAs from palm kernel oil, tea seed oil and soybean oil	solvent-free system	batch	7	1:2	60	1	fatty acids composition resembles HMF	(*47*)

Lipase	Substrate	Solvent system	Operation mode	Enzyme loading/ %	*r* (substrate)	Temperature/° C	Time/ h	Products	Reference
Lipozyme RM IM	tripalmitin and a mixture of hazelnut oil fatty acids and MCFAs	hexane	batch	19.78	1:3.35	57	24	12.8 % caprylic acid, 10.6 % capric acid, 30 % palmitic acid	(*40*)
Lipozyme RM IM	palm stearin and mixed of stearic acid, myristic acid, and FFAs from rapeseed oil, sunflower oil and palm kernel oil	solvent-free system	batch	10.7	1:14.6	57	3.4	29.7 % palmitic acid, 62.8 % palmitic acid at *sn*-2 position	(*51*)
Lipozyme RM IM	palm stearin and rapeseed oil fatty acids	solvent-free system	batch	8	1:10	60	4	83.7 % at *sn*-2 position	(*48*)
Lipozyme RM IM	palm stearin and mixed of stearic acid, myristic acid, and FFAs from rapeseed oil, sunflower oil and palm kernel oil	solvent-free system	packed bed reactor	-	1:9.5	58	2.7	28.8 % palmitic acid, 53.2 % palmitic acid at *sn*-2 position	(*52*)

Lipase	Substrate	Solvent system	Operation mode	Enzyme loading/ %	*r (*substrate)	Temperature/° C	Time/ h	Products	Reference
Lipozyme RM IM	34L-leaf lard and camellia oil fatty acids	solvent-free system	batch	6	1:4	45	6	51.5 % oleic acid, 84 % palmitic acid at *sn*-2 position	(*44*)
Lipozyme RM IM	tripalmitin and FFAs from *Silkworm* pupae oil	hexane	batch	10	1:12	65	48	97.05 % palmitic acid at *sn*-2 position	(*43*)
Lipozyme RM IM	Tripalmitin and PUFAs from *microalgae oil*	hexane	batch	7	1:7	60	21	89.0 % palmitic acid at *sn*-2 position, 81.3 % PUFAs at *sn*-1,3 position	(*39*)
Lipozyme RM IM	Solid fraction of basa catfish oil and high oleic sunflower oil fatty acids	solvent-free system	batch	12	1:6	50	2	57.8 % palmitic acid at *sn*-2 position, 79.21 % oleic acid at *sn*-1,3 position	(*49*)
Lipozyme RM IM	basa catfish oil and sesame fatty acids	solvent-free system	batch	8	1:3	40	2	23.8 % palmitic acid, 48.3	(*50*)

Lipase	Substrate	Solvent system	Operation mode	Enzyme loading/ %	*r* (substrate)	Temperature/°C	Time/ h	Products	Reference
Lipozyme RM IM	mixture of palm stearin fractionates and fungal oil from *Mortierella alpina* ALK and oleic acid	solvent-free system	batch	8	1:6	60	6	% at *sn*-2 position fatty acids at *sn*-2 position: 68.7 % palmitic acid, 9.8 % ARA, 7.9 % oleic acid	(*46*)
Lipozyme RM IM	palm stearin fractionates, oleic acid and linoleic acid	solvent-free system	batch	8	1:8:4	60	4	OPO and OPL 69.26 %, palmitic acid at *sn*-2 of 87.75 %	(*74*)
Lipozyme TL IM	tripalmitin, extra virgin olive oil fatty acids and DHA	solvent-free system	batch	10	1:3:2, 1:4:2 and 1:5:1	65	24	60 mol% palmitic acid at *sn*-2 position, total DHA content of 7.54 mol% (1:3:2), 6.72 mol% (1:4:2), 5.89 mol% (1:5:1)	(*75*)
Lipozyme TL IM	tripalmitin and a mixture of FFAs from	hexane	batch	10	1:4	60	8	2.0 mol% SDA, 22.9 mol% oleic	(*61*)

Lipase	Substrate	Solvent system	Operation mode	Enzyme loading/ %	*r* (substrate)	Temperature/°C	Time/ h	Products	Reference
	hazelnut oil and *Echium* oil							acid, 46.2 mol% palmitic acid at *sn*-2 position	
Lipzyme IM-20	tripalmitin and oleic acid	isooctane	batch	8.33	1:5	40	12	55.2 % OPO	(*80*)
Novozyme 435	palm olein, DHA and ARA	hexane	batch	10	1:18	60	24	DHA+ARA incorporated at *sn*-2 position of 17.20 g/100 g	(*66*)
Novozyme 435	palm olein and a mixture of 23.23 % DHA, 31.42 % GLA and 15.12 % palmitic acid	hexane	batch	10	1:2	60	22.7	35.11 % palmitic acid at *sn*-2 position, 3.75 % DHA, 5.03 % GLA	(*67*)
Candida sp. 99-125	lard and oleic acid	solvent-free system	batch	10	1:2	45	10	OPO 55.3 % (using additive β-cyclodextrin)	(*31*)
Lipozyme RM IM and Lipozyme TL IM	tripalmitin and a mixture of hazelnut fatty acids and GLA	hexane	batch	10 (Lipozyme RM IM)	1:14.8 (Lipozyme RM IM)	55	24	palmitic acid at *sn*-2 position of 74.9 % (Lipozyme	(*16*)

Lipase	Substrate	Solvent system	Operation mode	Enzyme loading/ %	*r (*substrate)	Temperature/° C	Time/ h	Products	Reference
				6 (Lipozyme TL IM)	1:14 (Lipozyme TL IM)			RM IM), 73.9 % (Lipozyme TL IM) palmitic acid at *sn*-2 position of 79.9 % (Lipozyme RM IM), 75.6 % (Lipozyme TL IM), 61.2 % (Novozyme 435), 87.3 % (*Candida parapsilosis*)	
Lipozyme RM IM, Lipozyme TL IM, Novozyme 435 and *Candida parapsilosis*	system I: 3.9 g tripalmitin and 2.76 g oleic acid system II: 3.9 g tripalmitin and 3.17 g ω-3 PUFA	solvent-free system	batch	8.9 (from tripalmitin)	1:2	60	24		(*59*)
Novozyme 435, recombinant CAL-B, Lipozyme TL IM and Lipozyme RM IM	TAG from *Nannochloropsis oculata* and *Isochrysis galbana* fatty acids	solvent-free system	batch	10	1:3	60 (Novozyme 435, Lipozyme TL IM) 50 (recombinant CAL-B, Lipozyme RM IM)	24	13.92–17.12 % total ω-3 PUFAs, 59.38–68.13 % palmitic acid at *sn*-2 position	(*6*)

Table 4. Reacting conditions for the production of HMFS through transesterification

Lipase	Substrate	Solvent system	Operation mode	Enzyme loading/%	Substrate ratio	Temperature/ °C	Time/ h	Products	Reference
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 ($m_A/m_B/m_C/m_D/m_E$)	60	6	41.5 % palmitic acid at *sn*-2 position	(*57*)
Lipozyme TL IM	palm stearin fractionates and ethyl oleate	solvent-free system	batch	10	1:5.5 (n_A/n_B)	50	3	31.43 % OPO, 80.6 % palmitic acid at *sn*-2 position	(*58*)
Lipozyme TL IM	tripalmitin and mixed of extra virgin olive oil and flaxseed oil	solvent free system	batch	10	1:2.67 (n_A/n_B)	56.7	18	25.2 % palmitic acid, 15.9 % α-linolenic acid	(*56*)
Lipozyme TL IM	palm stearin fractionates and fish oil	solvent-free system	batch	10	2:1 (n_A/n_B)	60	12	75.98 % palmitic acid at *sn*-2 position	(*55*)

Lipase	Substrate	Solvent system	Operation mode	Enzyme loading/%	Substrate ratio	Temperature/ °C	Time/ h	Products	Reference
Lipozyme RM IM	tripalmitin and ARA ethyl ester from fungi *Mortierella alpine*	Hexane	batch	7.58	1:12 (n_A/n_B)	50	13.36	69.57 % ARA at *sn*-1,3 positions, 62.31 % palmitic acid at *sn*-2 position	(*42*)
Novozyme 435	hazelnut oil and ethyl palmitate	hexane and solvent-free system	batch	10	1:6 (n_A/n_B)	65	17	palmitic acid at *sn*-2 position of 35.5 % (mg scale, hexane) and 71.1 % (g scale, solvent-free system)	(*65*)
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 ($m_A/m_B/m_C/m_D/m_E$)	60	4	fatty acid composition resembling HMF	(*68*)

Lipase	Substrate	Solvent system	Operation mode	Enzyme loading/%	Substrate ratio	Temperature/ °C	Time/ h	Products	Reference
Candida parapsilosis	tripalmitin and ethyl oleate	solvent-free system	batch	5 (from tripalmitin)	1:8 (n_A/n_B)	60	24	15 % oleic acid at *sn*-2 position	(*85*)
recombinant CAL-B	fungal oil from *Mortierella alpine* and MCT	solvent-free system	batch	8	1:1 (n_A/n_B)	90	3	6.12 % MMM, 53.75 % MLCT, 40.13 % LLL	(*86*)
Lipozyme RM IM	Basa catfish oil and coconut oil	solvent-free system	batch	8	1.5:1 (n_A/n_B)	60	3	62.14 % MLCT, 39.85 % MLL, 46.14 % palmitic acid in *sn*-2 position	(*54*)
Lipozyme RM IM	Lard and milk thistle oil	solvent-free system	batch	8	8:2 (m_A/m_B)	60	4	21 % palmitic acid and about 75 % palmitic acid in *sn*-2 position	(*87*)
Lipozyme RM IM	Lard and milk thistle oil	solvent-free system	batch	8	8:2 (m_A/m_B)	70	4	above 70 % palmitic acid in *sn*-2 position	(*88*)

Lipase	Substrate	Solvent system	Operation mode	Enzyme loading/%	Substrate ratio	Temperature/ °C	Time/ h	Products	Reference
Lipozyme RM IM	Lard and rapeseed oil	solvent-free system	batch	8	8:2 (m_A/m_B)	70	4	41.6 % palmitic acid in *sn*-2 position	(*89*)
Lipozyme RM IM	Lard, sunflower oil, canola oil, palm kernel oil, palm oil, algae oil, and microbial oil	solvent-free system	batch	11	1.00:0.10:0.50:0.13:0.12:0.02:0.02 ($m_A/m_B/m_C/m_D/m_E/m_F/m_G$)	60	3	20.1 % palmitic acid and 38.2 % palmitic acid in *sn*-2 position	(*90*)
Lipozyme RM IM	Lard, sunflower oil, canola oil, palm kernel oil, palm oil, algae oil, and microbial oil	solvent-free system	packed bed reactor	-	1.00:0.10:0.50:0.13:0.12:0.02:0.02 ($m_A/m_B/m_C/m_D/m_E/m_F/m_G$)	50	1.5	39.2 % palmitic acid in *sn*-2 position	(*91*)

MMM=medium-medium-medium chain fatty acids, MLL=medium-long-long chain fatty acids, LLL=long-long-long chain fatty acids, MLCT=medium-long chain triacylglycerol

Table 5. Reacting conditions for HMFS production through two-step reactions (two-step acidolysis, alcoholysis-esterification)

Lipase	Substrate	Solvent system	Operation mode	Enzyme loading/%	Substrate ratio	Temperature/°C	Time/h	Products	Reference
Rhizopus oryzae, *Mucor miehei*, Lipozyme RM IM, Lipozyme TL IM and *Alcaligenes sp.*	TAG containing high palmitic acid at *sn*-2 position (acidolysis of palm stearin and palmitic acid) and oleic acid	hexane and solvent-free system	batch stirred tank reactor	13.3 (hexane) 3.3 (solvent-free system)	1:6 (n_A/n_B)	37 (hexane) 50 (solvent-free systme)	1 (hexane) 4 or 19 (solvent-free system)	palmitic acid at *sn*-2 position of 67.8 % (hexane) and 66.0 % (solvent-free system)	(*60*)
acidolysis I: Novozyme 435 acidolysis II: *Rhizopus oryzae immobilized* in Accurel MP-1000	acidolysis I: tuna fish oil and palmitic acid acidolysis II: neutralized product acidolysis I and oleic acid	hexane	batch stirred tank reactor	acidolysis I: 10 acidolysis II: 13.3	acidolysis I: 1:1 (m_A/m_B) acidolysis II: 1:6 (n_A/n_B)	37	acidolysis I: 48 acidolysis II: 1	Acidolysis I: 57 mol% palmitic acid at *sn*-2 position Acidolysis II: 52 mol% palmitic acid, 15 mol% DHA, 3 mol% linoleic acid at *sn*-2 position	(*64*)
Lipozyme RM IM	2-monoglyceride from alcoholysis of palm stearin, then esterified	hexane	batch	10	1:3 (m_A/m_B)	50	12	43.86 % MCFAs, 39.37 % lauric acid, 24.18 %	(*87*)

Lipase	Substrate	Solvent system	Operation mode	Enzyme loading/%	Substrate ratio	Temperature/°C	Time/h	Products	Reference
	with *methyl ester* from coconut oil							palmitic acid at *sn*-2 position	
Lipozyme RM IM	2-palmitoyl monoacylglycerol from ethanolysis of lard using Novozyme 435, oleic acid, linoleic acid and lard	hexane	Batch	10	3:5.2:3.5:7 (n_A/n_B)	37	6	fatty acids composition resembles HMF	(*41*)
Candida sp. 99-125	monopalmitin at *sn*-2 positon from alcoholysis of tripalmitin, then esterified with oleic acid	solvent-free system	Batch	9	4.5:18 (n_A/n_B)	38	1.5	yield of OPO 65 %, 75 % palmitic acid at *sn*-2 position	(*88*)