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María Luján Ferreira
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Enzymatic Synthesis of Structured Triglycerides

From Laboratory to Industry

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Preface

This book presents the state of the art on the topic of enzymatic synthesis of structured triglycerides (STs) and diglycerides (SDs). It is focused on special fats and oils that involve a distribution of the fatty acids in specific positions of the glycerol backbone, taking into account the most recent available literature from 2010 through 2016.

The current brief addresses the issue of STs and SDs covering scientific and technological aspects, and considering the production, applications, industrial processes and the market. This contribution is divided into six chapters. Chapter 1 reviews several important aspects of structured tri- (TGs) and diglycerides (DGs. The metabolic fate of TGs, as well as the health benefits and nutritional properties of STs and SDs are presented. Several approaches to the reactions leading to STs and SDs are briefly described including chemical and enzymatic interesterification. Chapter 2 focuses on the enzymatic synthesis of STs and SDs considering single/multiple stages and discussing the use of solvents or solvent-free synthesis. The main substrates are presented and critically reviewed. Chapter 3 describes the batch and continuous reactors used in ST synthesis, and analyzes and discusses the several problems found in the literature and in the practical settings for the production of STs, especially when a scale-up is considered. Chapter 4 evaluates the potential solutions to the several drawbacks related to laboratory testing of lipases in ST and SD synthesis; economic aspects are also considered here. Chapter 5 contemplates the industrial perspectives on the topic focusing on the feasibility of a stronger interaction between industry and academia in order to focus the research on the solution of current problems. Finally, Chap. 6 describes and analyzes the success of some industrial applications of enzymes towards the production of STs and SDs.

The aim of this brief is to conduct a critical survey of the main results and opinions of the involved participants on this particular topic. The limit of the ability to use lipases to produce new and better SDs and STs has not been reached, and the field is waiting for further improvements and breakthroughs. The authors hope that the readers will find the current book interesting and useful.

In closing, the authors would like to thank the Universidad Nacional del Sur (UNS) and the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) for providing the financial support to write this book.

Buenos Aires, Argentina

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Contents

Abbreviations

CBE	Cocoa butter equivalent
ChCl	Choline chloride
CLA	Conjugated linoleic acid
CSTR	Continuous stirred tank reactor
DG	Diglyceride
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
FA	Fatty acid
FBR	Fluidized bed reactor
FFA	Free fatty acid
GLA	Gamma-linolenic acid
HMFT	Human milk fat substitute
LCFA	Long-chain fatty acid
LCT	Long-chain triglyceride
MCFA	Medium-chain fatty acid
MCT	Medium-chain triglyceride
MG	Monoglyceride
MI	Microwave irradiation
MUFA	Monounsaturated fatty acids
PBR	Packed bed reactor
PUFA	Polyunsaturated fatty acid
SCFA	Short-chain fatty acid
SCT	Short-chain triglyceride
SD	Structured diglyceride
ST	Structured triglyceride
STR	Stirred tank reactor
TG	Triglyceride

Chapter 1
What Is The Importance of Structured Triglycerides and Diglycerides?

Contents

1.1 Some Definitions

Lipids are an essential nutrient for human beings and necessary in the daily food intake. Lipids make food creamy and smooth, and enhance flavor (many aromas are oil-based) and provide a concentrated source of energy. From a quantitative point of view, triglycerides are the main constituents of dietary lipids (93–95%). The triglycerides (or triacylglycerols, TGs) are triesters of fatty acids (FAs) with glycerol and comprise the class of lipids known as fats and oils. FAs and their corresponding TGs can be classified according to their carbon chain length, and the number and position of double bonds and isomers, among others (as it is shown in Table 1.1). Short-chain fatty acids (SCFAs) refer to FAs with less than six carbons. Medium-chain fatty acids (MCFAs) refers to FAs of 8–12 carbon atoms and long-chain fatty acids (LCFAs) have 14 or more carbons.

M. Luján Ferreira, G. Marta Tonetto, *Enzymatic synthesis of structured triglycerides*, SpringerBriefs in Molecular Science, DOI 10.1007/978-3-319-51574-8_1

Table 1.1 Classification of fatty acids

Classification	Fatty acids					
FA chain length	SCFA C2–C6	MCFA C8–C12	LCFA C14–C22			
Saturation degree	Saturated	Saturated	Saturated	MUFA	PUFA	
				ω-9	ω-6	ω-3
Examples of FA	Butyric acid 4:0	Lauric acid 12:0	Stearic acid 18:0	Oleic acid 18:1 (n-9)	Linoleic acid 18:2 (n-6)	Alpha-linolenic acid 18:3 (n-3)
Examples of fats and oils containing the FAs		Coconut oil	Cocoa butter	Virgen olive oil	Corn oil	Linseed oil
					Safflower oil	Fish oils
		Palm oil	Palm	High oleic sunflower oil	Soybean oil	
		Kernel oil	Butter		Sunflower oil	
			Lard			
			Tallow			

SCFA short-chain FA, *MCFA* medium-chain FA, *LCFA* long-chain FA, *MUFA* monounsaturated FAs, *PUFA* polyunsaturated FAs

Medium-chain triglycerides (MCTs) are triglycerides containing only MCFAs (caprylic, capric, and lauric) and long-chain triglycerides (LCTs) are composed of LCFAs. They are metabolized by different biochemical pathways.

On other way, FAs can be classified by the degree of saturation of the C chain:

- Saturated FAs do not present double bonds. Single bonds create a straight molecule, which can be easily packed together to form a solid at room temperature (FA with ten or more carbon atoms),
- Unsaturated FAs can have one or more double bonds. The double bonds produce a kink in the C chain, which results in looser packing making them liquid at room temperature. They are classified into:
  - Monounsaturated FAs (MUFAs) contain a single double bond between two C atoms
  - Polyunsaturated FAs (PUFAs) present more than one double bonds

Table 1.2 presents a list of the most common fatty acids, with their chemical and trivial names. It is important to note that when the two primary hydroxyl groups of glycerol are esterified with different FAs, the resulting triacylglycerol can be asymmetric. In this case, glycerol C2 is a chiral or asymmetric carbon, namely a tetrahedral carbon atom bearing four different substituents. Each of the C atoms in the glycerol molecule is identified using the "stereospecific numbering (*sn*) system" as shown in Fig. 1.1.

Structured triglycerides (STs) are triglycerides that are modified chemically or enzymatically in order to change their structure by the modification of the FA composition and/or their positional distribution in the glycerol backbone with the goal of obtaining some required nutritional and/or physicochemical properties [1, 2]. The functionality of the TGs can be adjusted modifying the oxidative stability,

Table 1.2 Chemical and trivial names of more common fatty acids

Chemical name	Common name	Abbreviation
Hexanoic	Octanoic	6:0
Octanoic	Caprylic	8:0
Decanoic	Capric	10:0
Dodecanoic	Lauric	12:0
Tetradecanoic	Myristic	14:0
Hexadecanoic	Palmitic	16:0
Octadecanoic	Stearic	18:0
Eicosanoic	Arachidic	20:0
Docosanoic	Behenic	22:0
9-Hexadecenoic	Palmitoleic	16:1
9-Octadecenoic	Oleic	18:1
9,12-Octadecadienoic	Linoleic	18:2
9,12,15-Octadecatrienoic	a-Linolenic	18:3
6,9,12-Octadecatrienoic	g-Linolenic	18:3
8,11,14-Eicosatrienoic	Dihomo-g-linolenic	20:3
5,8,11,14-Eicosatetraenoic	Arachidonic	20:4
5,8,11,14,17-Eicosapentaenoic	EPA	20:5
4,7,10,13,16-Docosapentaenoic	DPA	22:5
4,7,10,13,16,19-Docosahexaenoic	DHA	22:6
cis 9, *trans* 11-Octadecadienoic[a]	CLA conjugated linoleic acids	*cis*-9, *trans*-11 18:2

[a]The most abundant CLA isomer in milk fat [5]

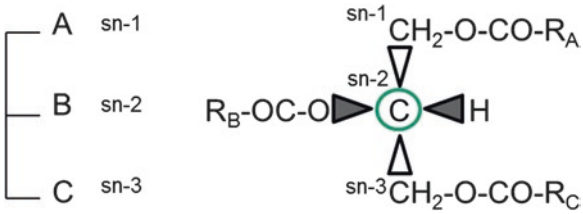

Fig. 1.1 Schematic of triglyceride structure. *A*, *B* and *C* are fatty acids, and © is an asymmetric carbon

melting properties, solid fat content, iodine value, and viscosity. In addition, modification of TGs can be carried out to generate fats with low calorie content. STs can be synthesized by chemical or enzymatic interesterification of fats, oils, or mixtures. One of the most typical STs is the medium-long-chain or MLM-type ST (MLC-ST) having MCFAs at the sn-1 and sn-3 positions and a LCFA at the sn-2 position.

Structured diglycerides (SDs) are defined as diglycerides chemically or enzymatically synthesized in order to have a specific fatty acid composition with a defined FA positional distribution in the glycerol molecule and so obtain some nutritional and/or physicochemical properties (see Fig. 1.2). There are three isomers of DGs: the sn-1,3-DG, the sn-1,2-DG, and the 2,3-DG. The natural ratio of sn-1,3-DG to the sn-1,2/2,3-DGs is 7:3 [3]. 1,2- and 2,3-diglycerides are always chiral, while 1,3-diglcerides are chiral if the FAs are different from each other.

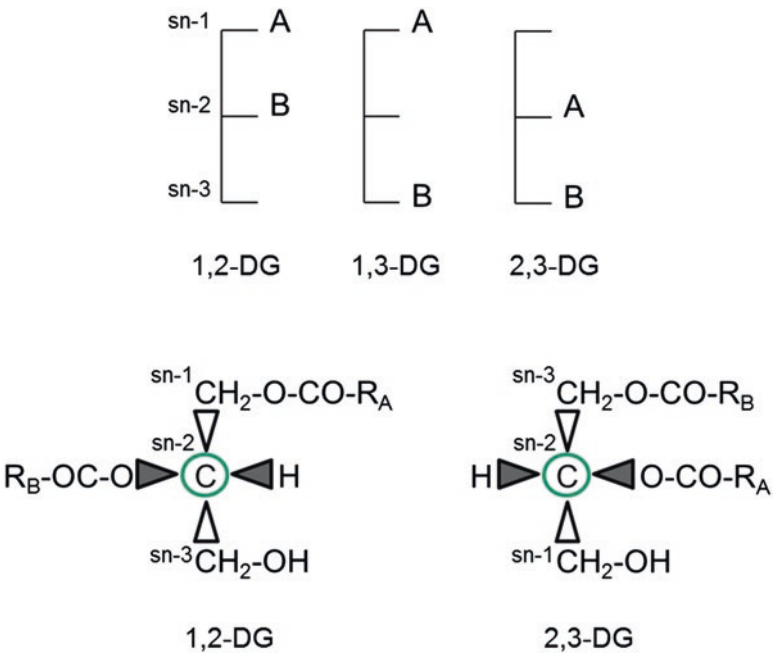

Fig. 1.2 Schematic of diglyceride structure. *A* and *B* are fatty acids, and *C* is an asymmetric carbon

Diglycerides, generally in a mix with monoglycerides, are common food additives largely used as emulsifiers. On the other hand, diglyceride oils do not occur naturally and are synthetically produced. It was found that DG oil aided in reducing body weight or fat accumulation [4].

1.2 Structured Triglycerides and Diglycerides for Nutritive and Therapeutic Purposes

1.2.1 Triglyceride Digestion and Absorption

In order to understand the importance of STs, it is necessary to review the TG digestion process. The digestion of TGs is a complex and stereospecific process requiring the participation of highly specific lipases. The digestion process of TGs begins in the mouth with the lingual lipase activity, which is secreted by the Ebner glands [6, 7]. This lipase acts on the bolus in transit to the stomach and in the stomach cavity. The lingual lipase acts on short- and medium-chain triglycerides and presents high specificity for the sn-3 position, but is much less effective for the sn-1 position [6]. In the stomach, the gastric lipase is secreted by the mucous of this organ [8], and has similar properties to the lingual lipase [9].

Table 1.3 Triglyceride digestion and absorption

	Lipase	Substrate	Product	Absorption
Mouth	Lingual	TG with SCFA and MCFA	FFA + DG	Portal vein
Stomach	Gastric			
Small intestine	Pancreatic + co-lipase + bile salts	TG with SCFA and MCFA		
		TG with LCFA	Unsaturated LCFA + 2-MG	Re-esterification. TG absorption into lymph system as chylomicrons
			Saturated LCFA → insoluble calcium soap	No absorption

The metabolic fate of the generated FFAs depends on the size of the hydrocarbon chain. The SCFAs and MCFAs that are soluble in the gastric contents will be absorbed in the stomach and transported into the portal blood stream. Consequently, they reach the liver directly, where they are used primarily for energy purposes (oxidized by mitochondrial beta oxidation) [10]. This means that the triglycerides containing SCFAs or MCFAs at the sn-3 position constitute an energy intake of very rapid metabolic disposition after the activity of the lingual and gastric lipases [11].

The digestion of TGs and the other hydrolysis products (such as fatty acids with C12:0 or more, sn-2 and sn-1 diglycerides, and a small proportion of sn-2 monoglycerides) continues in the small intestine. The pancreatic lipase with the pancreatic colipase, which has specificity toward ester bonds in positions sn-1,3, completely hydrolyses the TG and forms 2-MG and FFAs [12–14]. FAs which have a chain length of less than 14 carbons (SCFA and MCFA) are absorbed directly through the mucosal cells into the capillaries. They bind with albumin and are carried to the liver via the portal vein. The absorption of 2-MG and LCFA is more complex. They are emulsificated and form micelles as a consequence of the presence of the bile salts. The micelles (with the digested products) diffuse into the intestinal cells. The FAs and 2-MG are absorbed by the enterocytes of the intestinal wall and they are re-esterified to form new TGs and enter the circulation via the lymphatic route as chylomicrons [15, 16]. The chylomicrons also contain other fat digestion products such as cholesterol and fat-soluble vitamins. Saturated FAs with a C chain longer than 16 atoms may form insoluble calcium soaps that precipitate at the intestinal track producing hard feces. This is frequently the cause of intestinal disorders in infants and adults. Table 1.3 presents a schematic review of the triglyceride digestion and absorption presented above.

1.2.2 Structured Triglycerides for Infant Health

Triglycerides provide about 98% of the fats in human breast milk and supply approximately 50% of the energy needed for the development and growth of the newborn infant [17]. Human milk presents a distinctive TG composition: palmitic acid is the

most abundant saturated FA (17–25%) and it is placed at the sn-2 position of the TGs (70%), while oleic acid is the major unsaturated FA and it is mostly esterified at the sn-1,3 positions. [18, 19]. On the other hand, C16:0 is situated at the sn-1 and sn-3 positions in vegetable oil and infant milk formulas which use vegetable oils as a source of fat. As was mentioned above, saturated LCFA and calcium in the intestinal lumen can produce non-soluble Ca soaps, decreasing fat and Ca absorption.

Human milk fat substitute (HMFS) with sn-2 palmitate structured triglycerides mimics a stereospecific TG structure and the FA profile of human milk fat. In these infant formulas, the sn-1 and sn-3 positions of the glycerol backbone are mainly esterified by unsaturated FAs. Recent articles [16, 20, 21] which have reviewed HMFS summarize the current state of understanding of the beneficial effects of ST on infant health:

- Higher palmitic acid absorption (important source of energy) and better mineral balance in term and premature newborn infants, and animals.
- Fecal calcium excretion is significantly lower and stool hardness is reduced compared with standard formula.
- Infants consuming HMFS have a bone matrix development that is comparable to breast-fed infants and better than infants on traditional milk formulas.
- HMFS presents a positive effect on the intestinal microbiome, comparable to human milk-fed infants.
- Improvement of infant comfort in terms of shorter time of crying (and longer sleep time).

The clinical benefits of STs in infant formula have been extensively reviewed by O'Shea et al. [22]. Nevertheless, more studies are needed to confirm these observations.

1.2.3 Medium- and Long-Chain Structured Triglycerides

As previously stated, the metabolism of TGs is related to the chain length of the FAs. The hydrolysis is more rapid for TGs with FAs with less than 12 carbon atoms. In addition, SCFAs and MCFAs are primarily transported via the portal vein, bypassing the lymphatic system. C12:0 can be transported both in lymph and in the portal vein blood [44].

As a result, MCTs can be better tolerated in individuals with severe fat malabsorption, with diseases that impair the secretion of bile (biliary obstruction or liver diseases) or that affect the secretion of lipase from the pancreas (cystic fibrosis) [14, 23]. MCTs provide approximately 10% less calories per gram than LCTs, and they can be used as a source of dietary energy. The use of MCTs in the diet could lead to a decrease in body weight without negatively affecting lipid profiles [24]. In addition, it has been reported that SCFAs and MCFAs have beneficial effects in preventing dental caries as a result of their antimicrobial properties [25].

However, as the MCTs contain exclusively MCFAs, they lack the essential FAs needed for health (such as linoleic acid or α-linoleic acid) and are not suitable to be

used as frying oil due to foam formation [25]. The medium- and long-chain structured triaglycerides (MLCSTs) incorporate LCFAs in the MCT molecule to overcome these weaknesses. MLCSTs are an excellent source of nutrition and energy in intravenous- and enteral-fed patients [26, 27]. Especially, MLM-type STs present the sn-1 and -3 positions esterified by MCFAs (C10 or C12), and LCFAs at the sn-2 position, such as ω-3 LCFAs (e.g., DHA, EPA, or DPA), or ω-6 LCFAs. Picq et al. [28] have produced a very useful review regarding the potential for health benefits of the ST molecules that are rich in DHA and have a well-defined structure.

In a recent review article [29] which sums up the current state of the research in terms of clinical outcomes, pertinent evidence was systematically analyzed. The authors report that, in postsurgical and/or critically ill patients, the administration of MLCSTs versus MCT/LCT physical mixtures was significantly associated with improved protein economy, better liver tolerance and a more efficient TG elimination. With regard to clinical outcomes, a strong trend towards reduced hospital length of stay was observed for MLCST patients.

1.2.4 Reduced-Calorie Fats and Oils

Health problems related to overweight are becoming a major health concern worldwide. Recently, the World Health Organization has estimated that, in 2014, more than 1.9 billion adults, 18 years and older, were overweight [30]. Of these, more than 600 million were obese. Between 1980 and 2014, the worldwide prevalence of obesity doubled.

The consumers' need to decrease the total fat calories has resulted in the development of a number of fat-based fat substitutes. STs can be designed to decrease energy intake. A way to develop reduced-calorie fats is to synthesize fats and oils with conventional functional and physical properties in foods (such as palatability, creaminess, crisp texture, flavor, mouthfeel, etc.) but using one or more energy-modulating approaches [58]. These include the use of:

– SCFAs and MCFAs, with reduced energy content
– Saturated LCFAs, with reduced gastric absorption

SCFAs and MCFAs have a caloric value of ~5 kcal g^{-1} whereas lipids in general have a higher caloric content of ~9 kcal g^{-1} [31]. An example of an ST as a reduced-calorie fat is Salatrim (Benefat) which is an acronym for short- and long-chain acyl TG molecules [32, 33]. It is a family of products based on the use of at least one SCFA (C2:0 and/or C3:0 and/or C4:0) and at least one LCFA (mainly C18:0) with a caloric content of 5 kcal g^{-1}. It can be produced by interesterification of hydrogenated vegetable oils with TGs of C2–C4 acids, with a subsequent separation process. It is a zero-*trans* fat and has the taste and texture of conventional fats. Its functional properties can be adjusted by modifying the relative proportions of SCFAs and LCFAs utilized during the synthesis.

Other commercial low-calorie fats are listed in Sect. 1.3.

1.2.5 *Cocoa Butter Equivalent and* trans-*Free Plastic Fats*

STs are utilized in the production of cocoa butter equivalents (CBEs) [34].

These are fats with physical and chemical properties similar to those of cocoa butter, and useful for the partial or complete replacement of cocoa butter, because they are fully compatible.

The predominant components (>75%) of cocoa butter are TGs with 16:0 and 18:0 FAs at the 1,3-positions and 18:1 at the 2-position. The composition is dominated by three symmetric disaturated-mono-oleoyl TGs: POP, SOS, POS (P: palmitic acid, O: oleic acid, and S: stearic acid, the order of letters indicating the position of the FA in the TG molecule). The melting point of cocoa butter close to body temperature is due to that specific structure [35].

The increasing demand for cocoa beans and their uncertain supply has motivated the development of one of the first enzymatic processes to produce a fat with a similar melting profile to that of cocoa butter. SOS fat was synthesized by acidolysis of high oleic sunflower oil with stearic acid using a 1,3-regiospecific lipase [36].Other enzymatic routes are also being studied. Mohamed [37] restructured olive oil using lipase-catalyzed acidolysis with a mixture of palmitic and stearic acids to produce a ST that is close to cocoa butter in terms of TG structure and melting characteristics. In addition, enzymatic interesterification of soybean oil with methyl stereate [38] and of palm oil mid-fraction and mango seed oil [39] have been reported.

Investigations in the area of special fats have led to the development of STs as healthy substitutes for plastic fats (such as margarine or shortening) [40]. Commercially, plastic fats are produced by the partial hydrogenation of vegetable oils. Hydrogenation increases the melting point and stability but also leads to the formation of *trans*-fatty acids. Via interesterification reactions, it is possible to produce zero-*trans* MCTs-enriched fats, with desirable tenderness, texture, mouthfeel, and extended shelf life. Alternative zero-*trans* MCT-enriched plastic fats have been produced by the enzymatic interesterification of palm stearin with *Cinnamomum camphora* seed oil [41].

1.2.6 *Structured Diglycerides*

Diglycerides (DGs) are used in foods, cosmetics and pharmaceuticals. Mixtures of MGs and DGs are widely used as food emulsifiers. Furthermore, DGs have interesting nutritional properties and dietary effects. Recent studies have indicated that DGs are effective in reducing the deposition of fat in the visceral adipose tissue and decreasing postprandial lipaemia (high blood lipid levels after a meal) and other obesity-related disorders [42–44]. Also, they can be considered as a fatty acid-based alternative for dietary triglyceride fat. DG oil contains ~80–90% DG and can be used as a cooking oil. Since 1,3-DGs or 1,3-DG-rich oils are synthetically produced with a specific FA composition with a defined FA positional distribution, they are SDs.

The mechanism behind the beneficial effects of dietary DGs is still not clear. One hypothesis is that DGs can be hydrolyzed by pancreatic lipase in the same way as TGs, resulting in FAs and glycerol [45]. The re-esterification of glycerol with the FAs would be incomplete in the enterocyte due to the absence of 2-MG, resulting in a fraction of the FAs being absorbed via the portal vein rather than in chylomicrons (similar to MCFAs). However, Kristensen et al. [46] reported that DG bolus feeding (in catheterized pigs) did not increase the portal transport of nonesterified FAs, but did increase the portal transport of glycerol and lower the postprandial lipid concentration in arterial plasma.

1.3 Synthesis Aspects

1.3.1 Production of Structured Triglycerides

STs can be synthetized by different routes, depending on the kind of TG and the nature of the substrate. In the synthesis of STs from natural oils and fats, typically, interesterification is used, which may be followed by fractionation and/or blending. Interesterification is a process that involves the rearranging of the acyl group in a TG. It is performed by acidolysis, alcoholysis (including glycerolysis), and transesterification. Chemical interesterification (usually catalyzed by sodium methoxide) produces TGs with a random distribution of the FAs within the glycerol backbone (Fig. 1.3). In the process, the oil or fat is heated (100–140 °C, under N_2 or vacuum) for a short period, in the presence of a catalyst. This process is simple, inexpensive, and easy to scale-up, but is not capable of modifying specific positions of the glycerol backbone [27]. Moreover, polyunsaturated FAs can be severely altered by oxidation, isomerization, double-bond migrations or polymerization [47]. Nowadays, chemical interesterification is the technology applied in the production of low-calorie fats (such as Salatrim or Benefat from Nabisco). Rousseau and Marangoni presented an excellent review of this area [48].

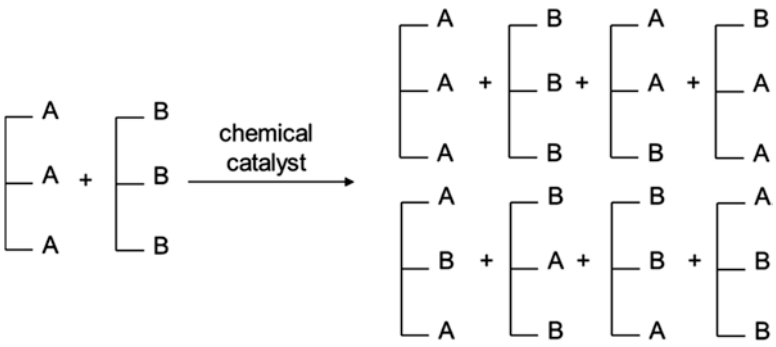

Fig. 1.3 Synthesis of STs by chemical interesterification of two TGs

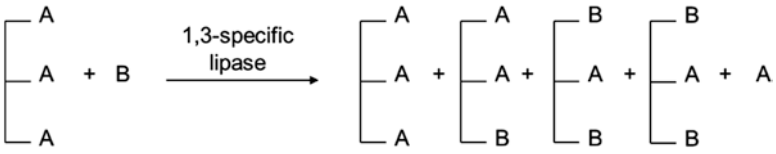

Fig. 1.4 Synthesis of STs by enzymatic acidolysis of a TG with a FA, using a lipase with positional 1,3-specificity. Possible acyl migration or hydrolysis byproducts are not shown

Special fats and oils which involve a distribution of the FAs in specific positions cannot be synthesized using these methods. In such cases, enzymes have an exceptional advantage over traditional methods. Enzymatic interesterification using sn-1,3 specific lipases generates structures in which the FAs are placed in the sn-1,3 positions of the TG. The selectivity of the reaction mainly depends on the substrates and the reactant's molar ratio, the kind of enzyme, and the process technology [49]. Among the various interesterification reactions catalyzed by lipases, one of the most used in the synthesis of STs is acidolysis. This involves an exchange reaction between the acyl moiety of the glyceride and a FA. For MLCT production, the TG came from native oils that contained essential fatty acids with known healthy properties (such as EPA, DHA, or GLA) and for the FA, caprylic acid is usually used. Figure 1.4 shows the synthesis of STs by the enzymatic acidolysis of TGs with FAs, using a 1,3-specific lipase. When a lipase with positional nonspecificity is utilized, the obtained products are those presented in Fig. 1.3.

Glycerol is a low-cost substrate for the production of STs. In this case, direct esterification is the indicated reaction route. Enzymatic methods are preferred when food applications are implied. They avoid toxic solvents and reactants, and the mild conditions of the process are recommended for polyunsaturated FAs. In esterification, the selected FA reacts with glycerol in the presence of the biocatalyst under vacuum pump or N_2 flow in order to remove the water generated in the reaction. MCFAs are usually obtained as byproducts during the processing of coconut oil (or palm kernel oil) via hydrolysis and fractionation of the resultant FAs [44].

The enzymatic synthesis of structured lipids has been well reviewed by Kim and Akoh [50]. Issues such as hydrolysis and acyl migration side reactions, and an alternative synthesis process in two steps will be reviewed in subsequent chapters.

1.3.2 Production of Diglyceride Oils

There are three main reactions to produce DGs using lipase-catalyzed reactions [51]:

- Partial hydrolysis of oils or fats
- Esterification of glycerol with FA
- Glycerolysis

In the first case, a non-specific lipase has to be selected if the desired product is a 1,3-DG, since the acyl moiety in the sn-2 position needs to be hydrolyzed

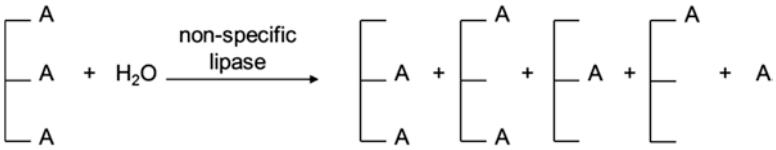

Fig. 1.5 Synthesis of diglycerides by partial hydrolysis of a TG by a non-specific lipase. Possible acyl migration byproducts are not shown

Fig. 1.6 Synthesis of diglycerides by esterification of glycerol catalyzed by a sn-1,3-selective lipase. Possible acyl migration byproducts are not shown

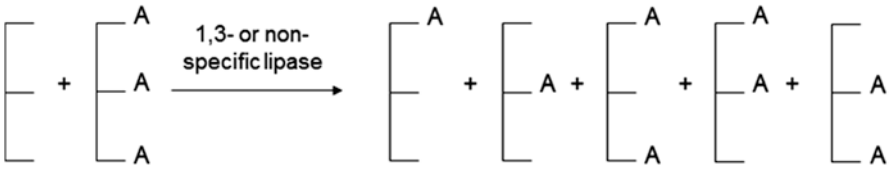

Fig. 1.7 Synthesis of diglycerides by glycerolysis of a TG catalyzed by a sn-1,3- or non-selective lipase. Possible acyl migration byproducts are not shown

(Fig. 1.5) [52, 53]. In the second case, glycerol is esterificated with FAs using a 1,3-specific lipase as the catalyst [54, 55] (Fig. 1.6). Glycerolysis is a further alternative in the enzymatic synthesis of DGs, and can be performed using oils or ethyl esters (Fig. 1.7) [56, 57].

Figures 1.5–1.7 shows that other many products are formed in addition to the desired 1,3-DG. These and other issues related to DG synthesis are discussed in a later section.

1.4 Consumer Market for Structured Di- and Triglycerides

The demand for structured triglycerides may be close to those for functional foods (FFs). STs can be considered as FFs based on the nutritional and biological properties of food lipids (strongly related to the chain length and degree of unsaturation of FAs) and their structural characteristics [58].

Health-conscious consumers, socioeconomic changes, longevity, increased health care costs, and advances in lipid science and technology are responsible for changing consumer attitudes toward functional TGs [59]. A FF is a "food that is similar in appearance to a conventional food , or may be one that is consumed as part of a usual diet, with demonstrated physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions" [60]. The expression FF is ambiguous, and each country has defined the term according to its needs. Japan was the first country to use this term.

Functional foods have a dynamic market: the global FF market revenue for the year 2013 was around USD 168 billion. With an average annual growth rate of about 8.5%, the global market for FF is estimated to go over USD 305.4 billion by 2020 [61]. The development of the FF market could be due to a preventive rather than a reactive trend on health issues. This trend is likely to continue as changing population demographics and the effects of lifestyle diseases create greater demands for food products targeting health and wellness [62].

The market for FF ingredients is projected to reach about USD 2.5 billion by 2020, increasing at a compound annual growth rate of about 6% from 2015 to 2020. In 2014, the United States was the largest market for FF ingredients, while Asia-Pacific is expected to be the fastest-growing market during 2015–2020 [63]. This is motivated by consumer awareness and expectations, and rising incidences of chronic diseases.

STs have been designed to achieve a particular metabolic effect or to change the physical characteristics of fats and oils in order to answer the requirement of health-conscious consumers. This definition covers CBEs, HMFSs, low-calorie fats, health-beneficial FA-rich TGs, DG oils, and margarines or other plastic fats.

Food companies have been manufacturing STs without region-positional requirements using chemical processes. The cost of a specific ST is several-fold higher than one of the randomized STs because of the elevated cost of the enzymes. This has negatively affected the commercialization of this kind of STs.

There are a number of successful STs on the market [64]. Bohenin is a calorie-reduced fat (5 kcal g^{-1}) which contains behenic acid at the sn-1 and sn-3 positions and oleic acid at the sn-2. It is generated by the enzymatic interesterification of TGs derived from oleic acid and ethyl behenate. It was developed by Fuji Oil and can be utilized in chocolates and coatings.

Captrin is the brand name for a MCT manufactured by Stepan. It is a MCT produced by the random esterification of glycerol with mixtures of caprylic and capric fatty acids which have been fractionated from coconut or palm kernel oils. It provides 6.8 kcal g^{-1} and is used in food, nutrition and pharmaceutical applications. It is on the market under the brand of Neobee by Stepan (United States) and Akomed by Karlshamns (Sweden).

Caprenin® was a randomized TG that contained behenic acid and two MCFAs. It was produced by Procter & Gamble and had functional and organoleptic properties comparable to cocoa butter, providing 5 kcal g^{-1}. Caprenin was utilized as a CBS in the United States but it was removed from the market in the mid-1990s.

Benefat® (originally known as Salatrim®) was a development of Nabisco and is commercially produced by Nabisco and Danisco Cultor. It is a reduced-calorie fat substitute (5 kcal g^{-1}) prepared by interesterification of SCTs (such as triacetin or butyrin) with hydrogenated vegetable oils. Salatrim can be used in chocolate chips and other confections.

Betapol® was developed by Loders Croklaan (Unilever) and was the first OPO-type (high sn-2 palmitate content) fat mimicking human milk fat in infant formula. INFAT®, sold by Advanced Lipids, is a fat component used in infant formula which copies the composition and the sn-2 palmitic acid content of human milk fat.

In 1999, Econa-brand diglyceride cooking and salad oil was introduced in Japan by Kao. In 2003, Enova DG was introduced in the United States. It was designated as generally recognized as safe (GRAS) by the US Food and Drug Administration (for use in vegetable oil spreads and home cooking oils). In 2009, Econa products were voluntarily removed from the Japan and USA markets due to the high concentrations of fatty glycidol esters detected in the oils [65].

It is clear that the high cost of the biocatalysts is the central constraint for a larger industrial production of STs and SDs. The production processes of some commercially available STs will be discussed in more detail in a later Chapter.

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Chapter 2
Literature Review: What Has Been Explored About Enzymatic Synthesis of ST and SD?

Contents

2.1 Introduction

Oils and fats are important renewable materials. Research in recent years, and mainly in the last 5 years, has focused on new, cheap routes to obtain high-added value products from low-cost materials in the field. Among the specialty products from oleochemistry, the structured triglycerides (STs) and structured diglycerides (SDs) have a very important place.

In a previous work, we revised the published literature on the topic of STs and published a review [1]. It has been evident that there is a lot of room for improvement at the synthesis step, both then and now.

As the previous chapter shows, the triglycerides (TGs) and diglycerides (DGs) are being revised in terms of their potential for human health, not only in prevention but also in treatment of illnesses.

M. Luján Ferreira, G. Marta Tonetto, *Enzymatic synthesis of structured triglycerides*, SpringerBriefs in Molecular Science, DOI 10.1007/978-3-319-51574-8_2

The questioning about the enzymatic synthesis of STs and SDs has been very much focused on enzyme costs instead on the details of the reactions. We know that the enzymes may be very selective and there are many of them with high reaction rates and selectivity, but we know less about how to control the activities and to change experimental conditions and processes to achieve the products we need, minimizing the products we do not want. In addition the homogeneous versus heterogeneous biocatalysts performance, the development of proper procedures of enzymatic activity comparison and the protocolization of the methodologies are all fields that need improvement. From the enzyme to the reactor design, there are many unexplored questions and points to consider carefully from the academic and industrial points of view. This chapter presents the knowledge for the synthesis of STs and SDs, mainly from 2011 to 2016.

Interesterification and esterification with hydrolysis and glycerolysis are key reactions in the manufacture of glycerol derivatives. The two most desired goals are high substrate conversion together with a competitive selectivity.

2.2 Enzymatic Synthesis of SDs in One Step

DGs present the 1,2 (or 2,3)-DG and 1,3-DG forms in nature. Figure 2.1 shows the different locations of acetyl groups in the three isomers.

Hu et al. [2] reported that chemical/enzymatical glycerolysis of TGs and esterification of fatty acids can produce DGs, with a dehydration system. In this sense, the methodologies of dehydration include the use of a vacuum or a reactor in an open system. In addition, molecular sieves or silica gel have been applied in batch and other reactors. Hydrolysis of TGs produces low amounts of DGs and so is not suitable. Several published studies have used huge amounts of enzyme. The authors

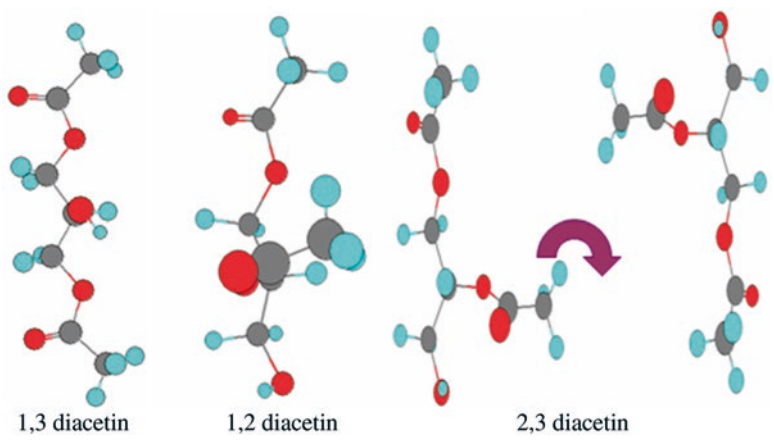

Fig. 2.1 Isomers of diacetin: 1,3 diacetin; 1,2 diacetin and 2,3 diacetin

also mention the use of different relative amount of lipases, different reactors configurations and reaction strategies. The nomenclature and definitions of product purity or conversion/selectivity to DGs was required in 2013.for the improvement of the synthesis of DGs using lipases

The authors carried out a study on the enzymatic ester synthesis and glycerolysis, in different reactor configurations for DG synthesis.

Supercritical fluids and ionic liquids as solvents for lipases have been revised in [3].

2.2.1 Esterification of Glycerol

DGs are prepared with enzymes through the esterification of a fatty acid (FFA) and glycerol. Separation of water is needed. Variable amounts of fatty acid are used and therefore the potential of inhibition at long reaction times is high. Other important drawbacks in the use of water adsorbents in esterification of glycerol are the adsorption of the fatty acid and also of the reaction products, mainly monoglycerides (MGs) and DGs. Comparatively, the reaction of natural fats and oils with glycerol is an interesting process for producing DG products [4].

The literature up to 2012 was focused more on long-chain fatty acids (LCFA-C14:0 and more) for DG synthesis [5] and less on medium chain fatty acids (MCFA C4:0 to C12:0) DG.

von der Harr et al. [6] reported the synthesis of DGs in rapeseed oil. These authors esterified fatty acids with MGs. With Lypozyme RM IM, the authors reported that, with a maximum DG yield of 23.8% (w/w), the lipase-based process could be an interesting alternative for DG synthesis.

Advanced methods of synthesis have been reported [7], e.g., solvent-free, through process intensification and improved operation modes. Among them, the vacuum-driven bubbling operation mode using air produced 95.3% of lauric acid conversion and 80.3% of 1, 3-DG after 3 h at 50 °C, including 5% w/w of Lipozyme RM-IM. Different strategies such as the use of esters of fatty acids (methyl or ethyl), the addition of compounds such as lecithin to the reaction media and the use of silica gel for the glycerol (a known lipase inhibitor) have been reported [8].

Two experimental conditions to produce DGs have been obtained:

- With Novozym 435, unpurified glycerol and the addition of 1% of lecithin, the product composition included 52.8% DG after 1 day. This configuration generated a mix of 1,3-DG (27.1%) and 1,2-DG (25.7%).
- Added Lipozyme RM-IM and glycerol adsorbed on silica gel produced 60.7% DG after 32 h. The content of 1,3-DG (39.8%) in this case was higher compared to the 1,2-DG content (20.9%).

The acyl group source has been a fatty acid or an ester of a fatty acid. Vinyl esters have also been employed. Unfortunately, the esters are more expensive than fatty acids, and vinyl esters may introduce additional interactions with the lipase.

Together with other problems, the solvent seems unavoidable when using glycerol, especially since this polyalcohol seems to be a strong inhibitor for the majority of lipases, CALB being an exception.

In 2004, it was reported that mixing low-vapor pressure compounds with organic hydrogen donors can generate eutectic mixtures through hydrogen bonding. These mixtures are alternatives to ionic liquids. Zeng et al. (2015) [9] reported that, in 1,3-DG synthesis (from glycerol and oleic acid) catalyzed by Novozym 435, 42.9 mol% of 1,3-DG could be obtained if choline chloride (ChCl) was added within 1 h.

2.2.2 Glycerolysis of Vegetable Oils and Synthetic Triglycerides

Reaction of olive oil with glycerol catalyzed with Novozym 435 in a solventless process to produce MGs and DGs has been reported by Singh et al. [10]. This paper summarized the main reports from 1991 to 2012 on the glycerolysis of vegetable oils. Tertiary alcohols as solvents and CALB seem to be the best combination of solvent and lipase to achieve a high final concentration of DGs. Even when the solvent-free media is attractive at first sight, the lipase inhibition, the viscosity of the reaction media and the need to dose the glycerol to minimize secondary reactions makes the industrial scaling of this reaction difficult.

Valerio et al. [11] studied the obtaining of MGs and DGs through the reaction of olive oil with glycerol in *n*-butane at high pressure. High conversion of olive oil was found but MGs were the main product. The authors of reference [3] report that one important problem of reactions of glycerol with oils (using lipases) is that the interaction results in the inhibition of the enzymatic activity.

The esterification of glycerol using caprylic acid (C8:0) in a packed bed reactor produced 82.3% of fatty acid conversion with a DG content of 77.3% with 200 U/g caprylic acid at 65 °C. The esterification in the batch reactor generated 94.4% caprylic acid conversion and a DG amount of 45.4% with 250 U/g caprylic acid at 60 °C and 10 h. Glycerolysis of palm olein in *t*-butanol produced 21.0% MG, 53.2% DG and a TG conversion of 74.2% after 24 h. Process intensification (microwave irradiation, MI) can improve the glycerolysis results and the final DG content was higher with MI than in the case of conventional heating at shorter reaction times. The reaction time and enzyme loading were key parameters in the resulting DG production [2].

For the last 15 years, the role of the solvent in DG synthesis through TG glycerolysis has been studied [12].

Novozym 435 and Lipozyme RM IM (both from Novo) are two of the most used commercial lipases in the glycerolysis of vegetable oils. Wang et al. [13] reported a conversion rate in glycerolysis of soybean oil with Novozym 435 of 98.7% (24 h). In this case, the reaction mixture included nearly 49.8% of DG, and the amount of DG in the final product was 97.9%. The reusability of the immobilized lipase

Novozym 435 achieved 15 batches. In nature, DG is a minor compound in several vegetable oils, reaching a maximum level of 10%.

Kinetic studies of edible oil glycerolysis have been reported. The kinetic modeling uses the sequential ordered Bi Bi mechanism. This mechanism takes into account the reaction with glycerol and with water, besides the esterification steps. A lipase deactivation term due to glycerol is presented. The experiments were carried out at 40–70 °C, 2.5–15% enzyme and glycerol:oil 0.5:1.5 to 6:1 (mol:mol). High conversions of MG (65 wt%) and DG (57%) were obtained with 10% enzyme after 720 min. [14].

Ionic liquids have been reported as improved reaction media for oil glycerolysis. Using 1 mmol oil (885 mg), 0.5 mmol glycerol (45 mg), 88.5 mg Novozym 435, 1 g of ionic liquid, 60 °C, 700 rpm, and 24 h, up to 85% (mol%) of oil conversion and 73% of DG yield were obtained [15].

2.2.3 Alcoholysis of Vegetable Oils and Synthetic Triglycerides

Generally, alcoholysis of vegetable oils is performed to obtain biodiesel. However, there are several publications about the characterization of the final reaction mixture which include reports of glycerols composition. Calero et al. (2014) [16] reported a maximum of 27% DG in an enzymatic reaction with ethanol of sunflower oil using Lipozyme RM-IM lipase with a relative oil/ethanol molar ratio 12–3.5, at 40 °C, using fixed pH and 40 mg of immobilized lipase.

2.2.4 Hydrolysis of Vegetable Oils

Diacylglycerol synthesis through the hydrolysis of vegetable oil under process intensification has been reported [17]. Process intensification is achieved through ultrasound irradiation or microwaves. A method using 11.20 wt% of water content (on the basis of water plus oil mass), 1.36 wt% similar basis, 12 h, 1,2 min and 200 W. Diacylglycerol production was 34.17 wt%.

Lipozyme RM-IM was used for the reaction between rice bran oil (RBO) with elevated acid content and MG for DG-“enhanced” rice bran oil (RBO-DG) [18]. The best conditions were MG/RBO molar ratio = 0.25; 56 °C; lipase = 4.77% w/w and 5.75 h. The best fatty acid and DG contents were 0.28 and 27.98%, respectively. Lipozyme RM IM was reused nine times at the optimum experimental conditions.

Figure 2.2 summarizes the different approaches to improve activity and lipase stability related to DG synthesis.

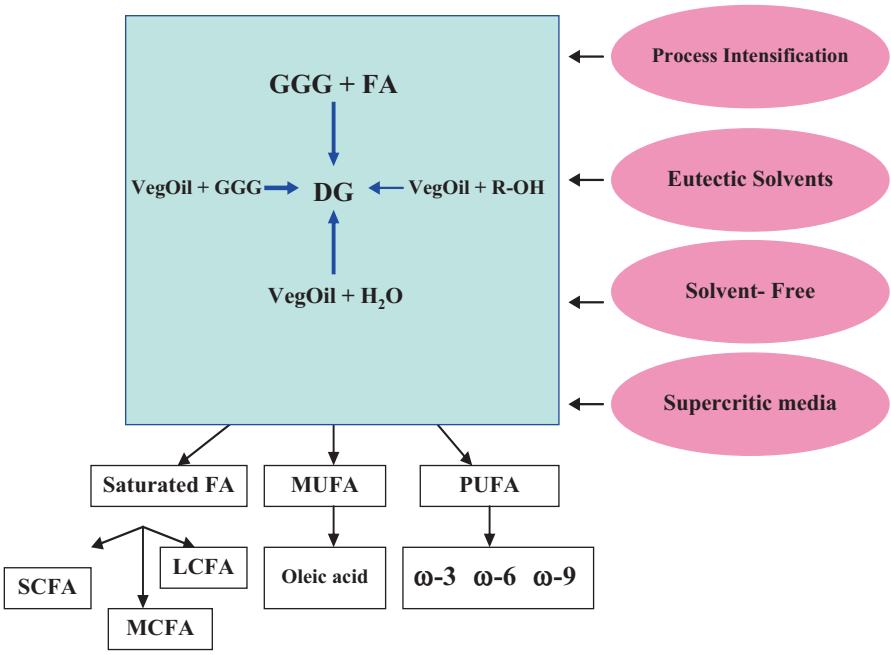

Fig. 2.2 Main routes of enzymatic DG synthesis in one step and process improvements

2.3 Enzymatic Synthesis of STs in One Step

The term "structured triglyceride" is usually restricted to those fats in which the TGs have been engineered to incorporate specific fatty acids. These fatty acids are also esterified to specific positions of the glycerol backbone. STs are synthesized via interesterification (which may be followed by fractionation and/or blending).

Structured TGs (or STs) find applications in nutrition, healthy low-calorie, and confectionery products [19] as summarized in Chap. 1. Among others, walnut, camellia, rice, avocado and microalgae oils have been more and more studied substrates for ST synthesis. The search for low-cost sources of fatty acids is continuous and a 2015 review presented an exhaustive analysis of the potential of lipases in the field [20].

Figure 2.3 shows the main synthesis routes for enzymatic ST (TG) from vegetable oils.

Figure 2.3 shows the main routes of ST synthesis in one step.

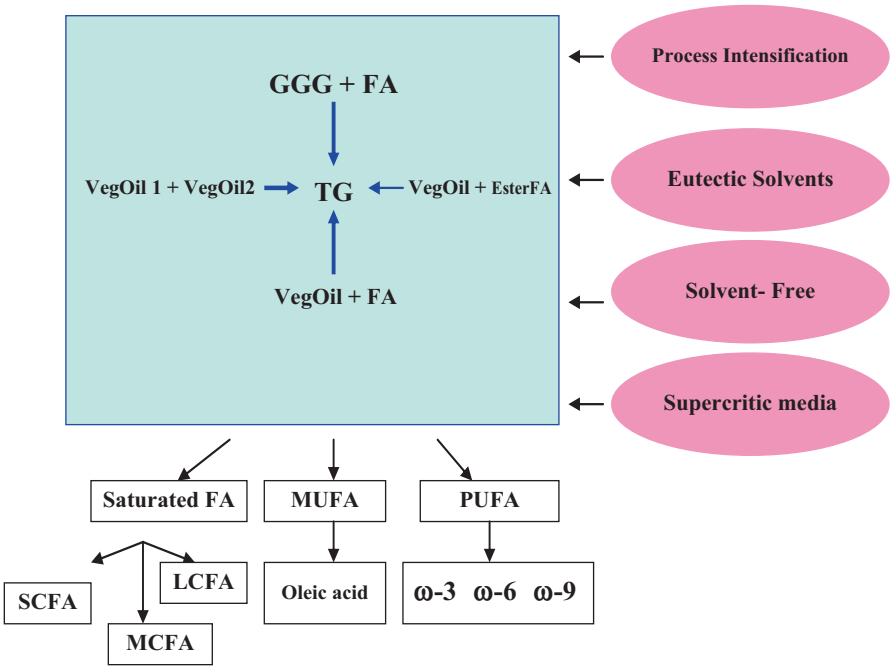

Fig. 2.3 Main routes of ST synthesis in one step

2.3.1 Interesterification of TG

Through interesterification, fatty acids are exchanged between existing esters. Three types of reaction are used:

- Alcoholysis
- Acidolysis that exchanges a free fatty acid with fatty acids on the glycerol, and
- Transesterification (also known as randomization or rearrangement) that exchanges fatty acids between glycerol molecules; this is sometimes reported as interesterification in the literature

In cases of a natural oil, the fatty acids are not distributed equally over the three glycerol positions. In the main vegetable oils, saturated carboxylic acids are present in the extreme hydroxyls of glycerol instead of the central,2 position.

During interesterification of a fat or fat blend, the overall fatty acid composition of the fat remains the same. TG composition is changed and therefore the physico-chemical properties of the fat are modified.

Interesterification has been achieved using a chemical catalyst but the reaction is accomplished now using enzymes (lipases) as catalysts. Following either a chemical or enzymatic process, it is necessary to separate the fatty acids, MGs and DGs. Fatty acids may be neutralized and the MGs removed by distillation. It is important to minimize production of DGs.

2.3.1.1 Chemical Interesterification

Sodium methoxide or a similar material is generally used. The reaction is carried out at close to 90–120 °C under vacuum or under nitrogen. The product is a complex mixture with a complete randomization of the fatty acids across all glycerol positions. A typical reaction is to interesterify a fully hydrogenated oil with a liquid one. The chemical approach is cheaper than the enzymatic process. In addition, although the chemical process can be used to synthesize structured fats such as medium-chain triglycerides (MCTs), it cannot produce a product with control of the TG structure.

2.3.1.2 Enzymatic Interesterification

Reference [12] reports that these days a selection of lipases are readily available commercially. For cost reasons, solvent-free processes are desirable. The problem with this approach is the melting points of reagents, which are so high that the lipase activity decreases when high reaction temperatures are used. Beyond 70 °C, the majority of lipases suffer denaturation.

Some lipases, depending on the reaction conditions, are non-specific, but one of the most important characteristics of some of them is 1,3-positional specificity.

Carboxylic acids at the distal carbons of the glycerol can be exchanged whereas moieties at position 2 can remain unchanged. The number of STs is reduced and limited but very important in several "niches" in industry. The preparation of cocoa butter equivalent (CBE) fats and human milk fat replacers are two of the most obvious examples.

CBE and low calorie fats, together with MCTs, are three of the most important STs produced by different processes.

2.3.2 Acidolysis of Vegetable/Edible Oils or Synthetic Triglycerides

Even though there are many studies and reports of enzymatic acidolysis of vegetable oils (olive, sunflower, safflower, soy) [21, 22], with one or several fatty acids, there are many drawbacks of this route. First, the high cost of using a food as a source of other foods will only be adequate if the product has a high added value; second, the required use of a very expensive immobilized enzyme; third, the low yield of the ST due to the unavoidable hydrolysis and other secondary reactions, even when the total conversion of the oil may be high; and fourth, the expensive downstream separation of byproducts.

A recent article on the synthesis of 1,3 dioleyl-2 palmitoyl glycerol, with or without solvent, reported that carboxylic acid with 16 carbon atoms (C16:0, PA) at the sn-2 carbon of the ST were 92.9% and 86.6% using a solvent or in a solventless

reaction media, whereas OPO content was 32.3% and 40.2%, respectively. Lipozyme TL-IM was not easy to reuse but Lipozyme RM-IM maintained activity for nearly 60 h in this reaction [23]. Process intensification through ultrasound brought about an increase the OPO production to 51.8% after 4 h. OPO content achieved 35.9% after 1 h with previous ultrasonic contact of the reaction media versus 4 h without it. Repeated use of Lipozyme RM-IM for ten times under ultrasonic irradiation did not affect the lipase activity [24].

STs with variable chain length in the acid moieties are "low calorie" fats. TGs including behenic (C22:0), caproic (C10:0) and caprylic (C8:0) acids belong to this group. The incorporation of behenic acid in the animal gut was nearly 28% in animal experiments. Uptake of medium-chain fatty acids and behenic acids into palm olein and palm stearin have been reported. The fatty acids were incorporated following the sequence C22:0 > C10:0 > C8:0 achieving 53%, 42.5% and 35.8%, respectively in kokum fats. A similar trend was found with sal or mango fats as substrates even when the final TGs were different. The products with MCFAs were liquids with no solid compounds, even at 0 °C. They demonstrated a low cloud point due to their higher content of STs with shorter-chain fatty acids.

Wang et al. [25] reported that there are many studies using Lipozyme RM IM to incorporate the C8:0 in vegetable oils, with or without a solvent. STs synthesized with a 1:4 mol canola oil/mol caprylic acid, 10% lipase and 15 h had almost 8% of C8:0, 52.7% C18:1 acid and 28.4% of linoleic acid located at the central position. In addition, the study of "model systems"—such as the OOO reaction (being O oleic acid) with caprylic acid—allowed the development of kinetic models of acidolysis [26]. Even when this is only one of the many publications about kinetics in acidolysis mediated by lipases, it should be pointed out that only a pilot plant data in industrial conditions may give the needed insight to produce a positive outcome. The main issues of reusability, proper fluidodynamics of the reaction media and mixing, and adequate selection of reactive, substrates and reaction media composition have to be studied point by point and close to the real conditions. Enzymatic-catalyzed reactions involving oils, triglycerides and glycerol/fatty acids/alcohols should be properly scaled from laboratory to bench or to a pilot plant to obtain really meaningful data.

Acidolysis of cod liver oil with caprylic acid showed that polyunsaturated fatty acids (PUFA) are difficult to be exchanged by caprylic acid using Lipozyme RM IM. At equilibrium, the ST included: CA 57%, eicosapentenoic acid (EPA) 5.1%, DHA 10.0% and PA 6.3% (taking into account the major fatty acids). EPA and DHA at the sn-2 position of the ST were 13.5%. This percentage was 44% of the complete amount of carboxylic moieties in the central position [27].

2.3.3 Esterification of 1,3 Diglycerides

One simple route to obtain TGs is first to synthesize 1,3 TGs with 1,3 specific lipases and then to esterify the sn-2 position with the desired fatty acid [28].

2.3.4 *Esterification of Glycerol*

Through glycerol esterification up to symmetric TGs of conjugated linolenic acid (CLA), enzymes can be synthesized and applied in a diversity of reaction media, reactor and solvent combinations, with or without solvent at different temperatures, using a vacuum or not. TGs of saturated, monounsaturated fatty acids (MUFA) and PUFA can be obtained. Recent reports report attempts to synthesize TGs of docosahexenoic acid (DHA) through transesterification of ethyl esters of DHA with glycerol under vacuum to remove the ethanol [29].

In laboratory settings, new methods of planning the experimental work permit the reduction of costs and time. The application of response surface methodology in synthesis is found more and more in the literature [30]. However, many aspects are still being studied at the laboratory scale (ionic liquids, for example) even when the scaling to industrial settings would be very expensive, not environmentally friendly, or introduce difficulties in the process operation.

In industrial contexts, the main process to obtain STs is chemical interesterification. Figure 2.4 shows the main problems with the industrial application of enzymes for glycerides synthesis.

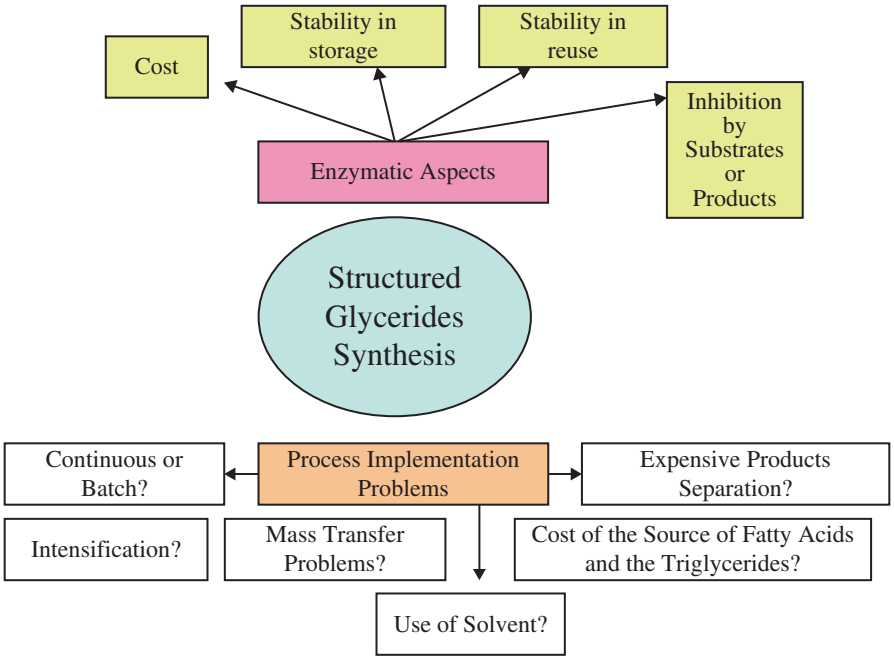

Fig. 2.4 Main difficulties with enzymatic synthesis of structured glycerides

2.4 Enzymatic Synthesis of STs in Two Steps

Different configurations of two-step synthesis of STs have been explored. Among them, the most important are

Approach 1

Step 1—Alcoholysis/hydrolysis/glycerolysis of TGs to obtain sn-2 MGs using a strict 1,3 specific lipase.

Step 2—Esterification of the 1 and 3 positions of 2-MGs using a specific 1,3 lipase with a selected fatty acid (FA).

Approach 2

Step 1—Esterification at 1,3 positions of glycerol with a strict 1,3 specific lipase with a desired FA.

Step 2—Esterification of 1,3 DG at sn-2 position with an active, non-specific lipase with a suitable FA.

The FA are selected considering the final properties to be obtained and the application of the product.

Instead of a purified FA as substrate, esters of FA of different structures have been explored. However, the FA may be an important cost in the final process. In addition, the FA selectivity of the lipases correlates with the hydrocarbon total length of FA in a TG [31]. The use of esters of FA has been published, but in industrial settings the use of esters of FA would be too expensive.

Careful steps of purification between step 1 and step 2 are needed in any approach to obtain a suitable substrate mixture for step 2. Sophisticated methods of separation such as molecular distillation, even if effective, are very expensive. The traditional liquid–liquid extraction with low-cost solvents is one of the pathways to avoid high-cost separation/purification operations.

The group of Molina has published several manuscripts on the two-step synthesis of TGs, rich in PUFA, using Approach 1 [32]. This strategy may be applied to any combination of FA, even when lipase reactivity may be very diverse. The conversion to STs and the incorporation of selected FAs would depend on the length and unsaturation of the FA, the reaction media, the operational mode of the reactor (discontinuous or continuous) and the source of the lipase, among other factors. The book cited in [33] shows a picture of the topic of modifying lipids and its use in foods. The book *Enzymes in Food Processing Fundamentals* [34] and potential applications present the use of enzymes in the fields of milk and cheese industries, baking, beverages, meat, sugar, fragrances and oils, fats and flavors, and in food processing.

Tang et al. [35] reported a new enzymatic approach including two steps to synthesize symmetrical TGs with arachidonic acid at sn-2. The methodology involved the preparation of 2-MGs rich in 2-arachidonoylglycerol (2-AG) by reaction of the TG with ethanol. Symmetrical TGs were obtained by enzymatic reactions between 2-MGs and vinyl palmitate. The desired TGs were synthesized at 89% yield. Vinyl palmitate instead of palmitic acid was selected as a different carboxylic moiety source in the reaction with 2-MGs.

Generally, EPA and DHA are considered very important PUFA. However, lipases do not readily produce esters of these FAs due to steric and electronic factors. Considering fish oil as a source, Chaurasia et al. [36] published the synthesis of a DHA-rich triglyceride through the selective hydrolysis of fish oil with *Candida cylindracea*, the separation of EPA/DHA in the reaction media and finally the transesterification of the FA with ethanol using *Rhizomucor miehei*. A second procedure involved the non-selective hydrolysis of fish oil and later the separation of a mixture of DHA/EPA further esterified with a mixture of CALB and glycerol.

Conjugated linolenic acid or CLA are a group of attractive fatty acids in the field of health. CLA is manufactured by Nisshin Oillio, Lipid Nutrition (Clarinol™) and Cognis (Tonalin). The use of lipases to obtain esters of conjugated linoleic acid (CLA) is increasingly being published. Busch et al. [37] present a very useful summing up of strategies to obtain CLA glycerides. The selection of triacetin (AAA, A being acetic acid) as a glycerol source in a reaction with the esters mentioned above somehow "fixes" the former isomerization path. With continuous separation of alkyl acetate, the goal of a CLA-rich TG is produced with high conversion. The authors reported that a major problem of non-esterified CLA and its esters from short alcohols is their bitter taste. The near-tasteless CLA TG is the chosen derivative in food formulations.

The use of vacuum has been particularly reported in the case of CLA-enriched TG [38]. The best conditions of temperature, lipase amount and vacuum were 60 °C, 10% of the total weight of the substrates of CALB and 0.4 kPa, respectively.

Considering the context, the structured TG synthesis involves several logistic and academic problems from two sides: the enzyme and the process. Even when enzymes are active and selective, high costs may hinder any industrial application. The use of solvents, several steps and an immobilized lipase are other problems.

Figure 2.4 presents some but not all of the detected problems. It is clear why enzymatic interesterification has so far been almost the only industrially developed process using lipases at this point on a large high for obtaining structured glycerides.

Figure 2.4 presents in one frame the main problems using lipases or glycerides synthesis and potential solutions for them.

2.5 Chemo-enzymatic Synthesis of SDs and STs in Two (or More) Steps

Wang et al. [39] reported the obtaining of 1,3-dioleoyl-2-palmitoylglycerol (OPO) through a process in several steps.

First, vinyl oleate was prepared by the reaction between vinyl acetate and oleic acid. Second, the ester was contacted with glycerol in the presence of Novozym 435.

The 1,3-diolein amount present in the non-purified product was 90.8% and was produced at 82.3% (w/w) yield with 98.6% purity. In the third step, purified 1,3-DG was esterified with palmitic acid and 94.8% OPO was produced.

Unfortunately, at the third step, dichloromethane is used and the catalyst to esterify sn-2 position of 1,3 diolein are *N*-ethyl-*N*-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDCI) and 4-dimethylaminopyridine (DMAP).

Haldorsson [40] presents a breakthrough improvement of the approach based on an reaction of glycerol with vinyl esters of MCFA with excellent performance at 0–48 °C. Using these experimental settings and CALB, the lipase showed a perfect regioselectivity by the exclusive attack at the primary hydroxyl of the glycerol. Subsequently, pure EPA and DHA were again introduced into the sn-2 position by EDCI/DMAP as coupling agents with excellent results in the synthesis of structured TGs. Other publications from the same group from 2010 reported the preparation of symmetrically STs with bioactive polyunsaturated fatty acids (EPA or DHA) at the central position of the glycerol and a short-chain fatty acid (C2, C4, C6) esterifying the sn-1/sn-3 hydroxyls of the glycerol backbone.

Complete regiocontrol without acyl migration was achieved in two enzymatic steps using lipase to selectively introduce a carboxylic acid with a low number of carbon atoms into the primary hydroxyl groups of glycerol through activated vinyl esters when a low temperature is used, and an additional coupling reaction involving free EPA and DHA with EDAC as a coupling agent [41]. Finally, another publication from 2015 [42] reported a chemoenzymatic route designed in two steps involving a highly regioselective supported *Candida antarctica* enzyme to insert EPA or DHA as acetoxime esters mainly at the sn-1 and sn-3 of the final ST. The saturated fatty acyl groups were later reacted to the remaining 2-position by EDCI to produce the desired final ST in high yields. This is the first publication on reversed structured TGs including the long-chain PUFAs. Even when these syntheses may be useful for certain particular and specific applications, they are not suitable for the preparation of STs for food, human or animal. The use of toxic solvents, the long reaction times and the use of animated catalysts are serious problems.

It seems that the chemoenzymatic route such as has been proposed in last 5 years would be suitable for specific specialties, but is not to be used in the food industry or in food or health derivatives involving large-scale production.

2.6 Other Enzymatic Syntheses

Different pathways have been explored to synthesize structured triglycerides, sometimes as secondary products.

Using methyl acetate as acyl donor, vegetable oils can be transformed quickly into the fatty acid methyl esters and triacetin. Reference [43] reported conversion of the triglyceride using the supported *Candida antarctica* lipase, and as high as 80% of both fatty acid esters and AAA (glycerol esterified with acetic acid) could be obtained. These results were achieved by controlling the available water and the hydration content of the enzymatic protein.

From the published literature, the main commercial immobilized lipases applied in SDs and STs synthesis are Novozym 435, Lipozyme RM IM and Lipolase100T. In addition, other lipases have also been applied but with less success: lipases from *Candida cylindracea*, *Chromobacterium viscosum*, *Pseudomonas* sp., *Penicillium camemberti*, *Rhizopus arrizhus*, *Rhizopus delemar* and latex from *Carica papaya* and *Yarrowia lipolytica*. *Candida parapsilosis* lipase have demonstrated selectivity to the central position of glycerol. *Rhizopus* sp. lipase was demonstrated to have activity against *cis*-4 unsaturation [44, 45].

The fatty acid selectivities of lipases in the acidolysis reaction have been explored by Karabulul et al. [46]. Lipases acted weakly on saturated fatty acids shorter than 8 carbon atoms and with a chain length longer than 18 carbon atoms. They show a bell-shaped form in the distribution of the fatty acid insertion versus the chain length plot, with a maximum peak around C12–C16.

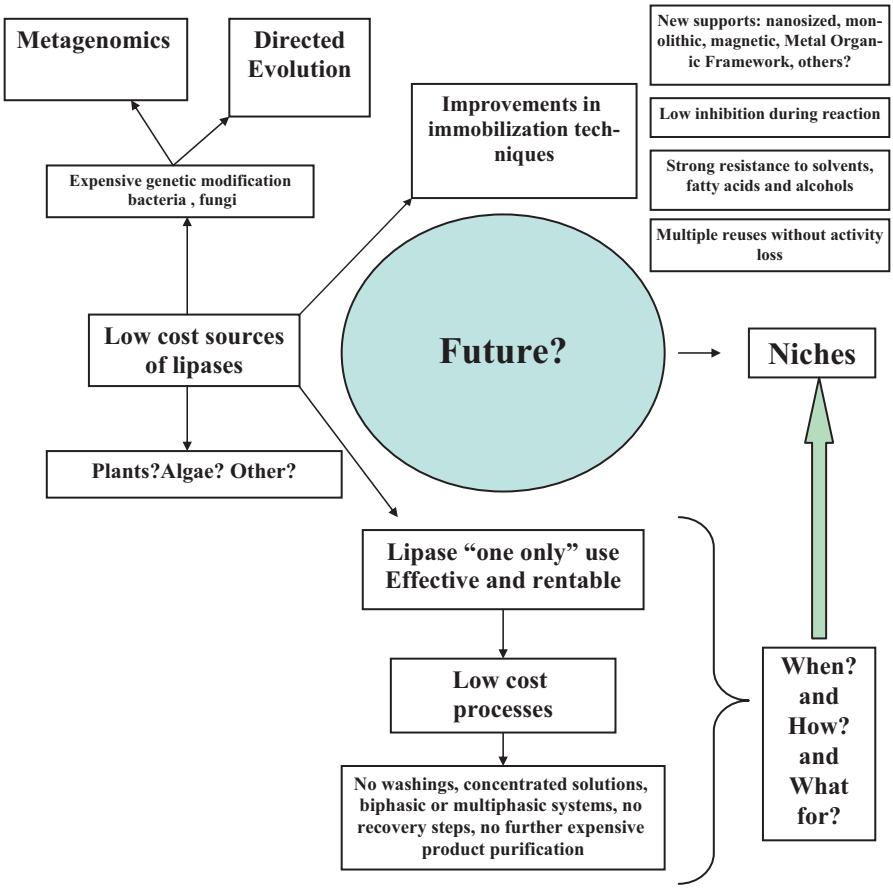

Fig. 2.5 Enzymatic synthesis of glycerides. A view to the future?

Magnetic nanoparticles as part of the supports has been academically explored to immobilize lipases. There are multiple reports on the application of nanotechnology and organic–inorganic composites as advanced supports for lipases [47, 48]. Even though there are reports on the use of membranes [49], liposomes and composites as supports of lipases for ST synthesis and from nano- to microspheres [50, 51], the practical aspects of scaling (mainly costs) have hindered applications at industrial levels. The review of Gupta et al. [50] illustrated advanced enzyme immobilization and stabilization with nano-/micro- and hybrid materials. The selected materials showed sizes from nanoparticles, nanofibers, mesoporous materials and sol–gel silica, to alginate-based microspheres. Other reviews have presented the different lipases and their main fields of application [52, 53]. The less-cited problem of protein production, lipase engineering, enzyme expression and evolution-guided design are other topics to consider [54].

The control of the impact of the polar compound (alcohol or water), the design of the reaction (not only of the primary products but also of the secondary ones) and the search for cheaper sources of lipase and better immobilization procedures (able to apply the biocatalyst in solventless media) are important topics.

Figure 2.5 shows the situation and the challenges that the field of enzymatic synthesis of structured glycerides should face in the next 5–10 years.

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Chapter 3
What Problems Arise When Enzymatic Synthesis of Structured Di- and Triglycerides Is Performed?

Contents

3.1 Introduction

Chemical and enzymatic interesterification (CI and EI, respectively) processes have been compared in many reviews, and their advantages and disadvantages listed for each technology [1, 2]. EI is one of the most efficient approaches available in the food industry to synthesize structured fats (such as cero-*trans* fats, cocoa butter equivalent, or human milk fat substitute, HMFS). The fats manufactured by EI have physical properties comparable to those produced by different technologies [3, 4].

Enzymatic processes are favorable to the environment. EI is a more sustainable alternative, because enzymes act under milder reaction conditions with less water and energy consumption than traditional processes. Furthermore, they are highly

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specific and generate less byproducts and waste than traditional processes. Life cycle assessment has been applied as an estimation tool, to compare EI and CI processes. The results showed that environmental impacts related to EI are smaller compared with chemical interesterification because energy expenditure is lower and production of hard stock increased [5, 6].

However, it is interesting to point out the contrasting opinions [2, 7] about EI that note the need for extensive purification of the raw materials (to prevent the lipase from being inactivated), the lack of flexibility of operation, and the high cost of the process.

As was mentioned in Chap. 1, the interesterification reaction can be catalyzed by alkaline chemicals (usually sodium methanolate) or lipases, and the processes can also be randomized or 1,3-specific. This chapter describes the batch and continuous reactors used in the ST synthesis and analyzes the main drawbacks of the EI process, considering laboratory and large-scale production, and batch and continuous processes.

3.2 Relevant Factors in the Enzymatic Performance During Interesterification

Several factors influence the reaction rate at which EI proceeds, unrelated to the reactor used, and whether on a laboratory scale or in industrial applications [5, 8, 9]:

– The supports for biocatalysts such as Novozym 435 (*Candida antarctica B*) or Lipozyme RM IM (*Rhizomucor miehei*) are expensive and the enzyme immobilization is a complex process. Consequently, biocatalysts are high-cost products and their application is reduced to high-value segments. Lipozyme TL IM (*Thermomyces lanuginosus*), is cheap among the immobilized commercial lipases, and it is an efficient biocatalyst in the fat modification industry.

  On the other hand, considering the performance of the biocatalyst in the production processes (reactor operating and separation step), the mechanical stability of the support is essential. It is critical that biocatalyst supports have high resistance to compression and frictional resistance [10, 11, 18].
– Generally, increasing the temperature increases the reaction rate of the EI; however, elevated temperatures (higher than 70 °C) can decrease the enzymatic activity due to the irreversible denaturation of the lipase. The optimum temperature for EI is 70 °C (long working life for the enzyme and melted fats) and operation at 80 °C for short periods of time is also viable without a severe decrease of enzymatic activity [5]. In fat modification processes for food applications (where organic solvents should be avoided), the reaction temperatures must be high enough to keep the substrates in liquid form.
– Oil blends have to present an appropriate quality. A packed bed reactor can be blocked by soaps and by nickel and phosphorus compounds present in the feed. Thus, they must be kept at levels as low as 1, 0.2, and 3 ppm for soaps, nickel and phosphorus, respectively [28]. Pigments and oxidation compounds have a negative

effect on enzyme stability [12]. A low FFA concentration in the oil is recommended because they are susceptible to oxidation. The peroxide value is required to be lower than 2 meqO_2 kg^{-1} and the anisidine value needs to be less than 5 ppm [13].

– The pH for maximum activity for the majority of lipases lies between 7 and 9, even though they can be catalytically active from about pH 4 to pH 10 [14, 15]. Inorganic acids (from degumming), citric acid (from deodorization) or residual bleaching earth reduce the internal pH in the biocatalyst particle and affect the enzyme working life. The water contained in the oil (<0.2%) can solubilize the citric acid (used as chelating agent) or the mineral acids (sulfuric and phosphoric) remaining in the bleaching earth used in the oil-refining step [31].
– Water is indispensable for the three-dimensional structure of proteins and enzymes and, consequently, the lipase activity. In non-aqueous media, the amount of water necessary will depend on the source of the lipase, the enzyme support and the organic solvent used [16].

  In a reaction system, water plays an essential role in the chemical equilibrium of reversible reactions in which it participates. During the EI reaction, an excess of water contributes to excessive hydrolysis of triglycerides (TGs). In the case of the esterification reaction, the equilibrium can be shifted towards the products by continuous separation of the water generated through the reaction. For EI, the water content when the lipase activity is highest varies between 0.04 and 11 wt% for different commercial lipases. Nevertheless, water contents lower than 1% are needed for selective interesterification [17].
– Substrate steric hindrance. The reaction rate is affected by the conformation of the substrate. The hydrophobic tunnel in the lipase admits hydrocarbon chains and aromatic rings more easily than branched hydrocarbon structures [18]. Sanchez et al. [19] found that TG chain length modified the reaction rate of the ethanolysis of TGs catalyzed by Novozym 435 in a solventless reaction medium. The formation of 2-monoglyceride (2-MG) was favored by saturated TGs with short or medium chain length and limited by long-chain TGs.
– Product accumulation can affect the reaction rate in EI. It has been reported that the presence of high concentrations of non-esterified fatty acids could act as enzyme inhibitors [20, 21]. In addition, glycerol has been identified as a lipase inhibitor [22].
– Two parallel diffusion pathways take place in a heterogeneous reaction [9, 23, 24]:

  External diffusion comprises the transport of substrates from the fluid bulk towards the surface, and product diffusion the transport back out of the fluid layer surrounding the support particle. It is in series with the conversion of substrates occurring at the active site. In order to favor the mass transport in the liquid–solid interphase (for substrate and product molecules), the relative velocity of the biocatalyst particles with respect to the liquid must be the highest possible. The latter can be achieved by increasing the rates of stirring in batch reactors or increasing the flow rate in a packed bed reactor. External mass transfer limitations can be detected if the enzymatic activity remain constant when stirrer rate or flow rate are increased, for batch and continuous reactors, respectively.

Internal diffusion involves the transport of the substrates and products within the pores of immobilized enzyme particles. There can be intraparticle concentration gradients caused by restrictions in the substrate and product transfer within the biocatalyst. The size, depth, and smoothness of the pores of porous biocatalysts affect the substrates and product diffusion. Internal mass transfer limitations can be recognized by measuring the reaction rate at zero time at progressively higher lipase concentrations. If the reaction rate remains constant, the process is mass transfer-limited. When the enzymatic activity increases if the support particles are crushed, the internal diffusion problems can be confirmed.

Some of these aspects will be taken into account in relation to the operation of different types of reactor.

3.3 Production of Structured Di- and Triglycerides in Batch Reactors

Immobilized enzymes can be used in different kind of reactors based on several criteria, such as the stirring rate used during the reaction, mass and heat transfer, the scale of operation, costs (product, substrates, reactor, operation), stability (enzyme, substrate and/or product), etc. [24, 25].

Batch or stirred tank reactors (STRs) are possibly the simplest reactors and are commonly used in the early stages of development of laboratory-scale reaction tests, due to their ease of operation and low cost. They are closed systems that operate under unsteady-state conditions (Fig. 3.1a). The tank is normally equipped with temperature measurement and control devices, mixing elements (e.g., propellers) and fixed baffles

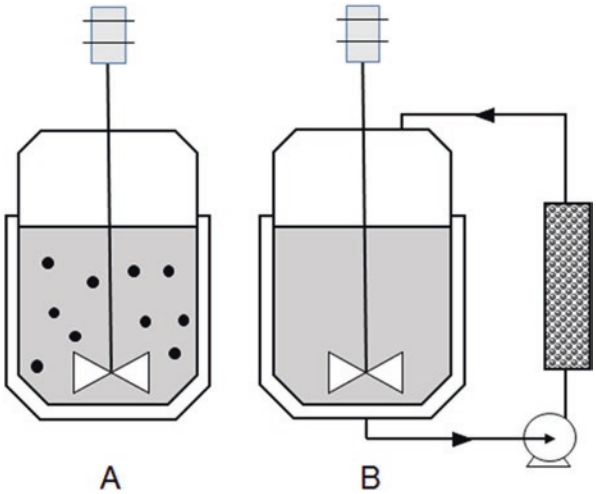

Fig. 3.1 Schematic representation of batch reactors: (**a**) stirred tank reactor; (**b**) recirculating batch reactor

in order to improve efficiency. The reactants and the biocatalyst are placed in the tank and allowed to react. After completion of the reaction, the products and unreacted reagents are removed and the spent immobilized enzyme is separated from the reaction medium by filtration or centrifugation, and recovered to be used in subsequent batches. The biocatalyst is replaced when the enzymatic activity decreases.

These kinds of reactors are versatile and easy to operate (cleaning, maintenance, temperature control). They are indicated for small-scale production of high-value compounds. They are also preferred for viscous reaction mixtures, where continuous operation is difficult. However, for large-scale use, the STR is not the best choice because high-volume tanks or many reuses would be required. The latter generates the attrition of the enzyme particles because of the high shear stresses imposed by the mechanical agitation [5, 18]. In addition, during emptying, cleaning and filling of reactors, there are dead times and there could be loss of enzyme.

In a STR, the reaction rate decreases over time because the substrate concentration is reduced, due to the conversion to the product. In some cases, there may be a need for continuous additions of enzyme and/or substrate (i.e. in batch–fed operation) in order to maintain a constant high degree of conversion. In a recirculating batch reactor (Fig. 3.1b), the biocatalyst is packed in a bed through which the reaction medium is circulated [17]. This configuration allows a smooth operation because the conversion per pass is controllable and operating variables can be regulated in the recirculation chamber [26].

Paula et al. [27] reported the results of the interesterification reaction of milkfat with soybean oil in a STR that included baskets for the biocatalyst, to yield milkfat-based fat more spreadable under low temperature and with lower contents of saturated fatty acids. *Rhizopus oryzae* lipase immobilized on polysiloxane-poly(vinyl alcohol) was used. Non-notable deactivation of the biocatalyst was found during the complete operation period (60 h).

Ilyasoglu et al. [28] studied the production of HMFS with medium-chain fatty acids by Lipozyme® RM IM catalyzed acidolysis, in a laboratory-scale batch reactor. Under the best operating conditions (57 °C, 3.35 mol/mol, and 19.78 wt% biocatalyst), the product contained 12.8, 10.6, and 30 wt% of caprylic acid, capric acid and palmitic acid, respectively.

Saw and Siew [29] studied the interesterification of palm olein catalyzed by Lipozyme® TL IM in a pilot-scale batch reactor. Previously, laboratory-scale interesterification tests had been conducted with the goal of determining the best operating conditions to be applied. The products were analyzed for their TG profile, sn-2 fatty acid composition, and physical properties. This resulted in the production of a new TG with a high melting point (PPP and PPS), at 65 °C and after 8 h (5 wt% biocatalyst, N_2 atmosphere, 300 rpm). However, an important reduction of oleic acid and an increase of palmitic acid at the central position in the products were observed due to acyl migration. The work showed that EI changed the physical properties of the initial substrate from liquid to semi-solid without the modification of the iodine value (IV) or the fatty acid content. The product could be applied for the synthesis of low saturation edible products, such as margarine.

There are reports of DG production in STRs [30, 31]. Watanabe et al. studied the synthesis by the esterification of fatty acids with glycerol in a solventless reaction

media using Lipozyme RM IM in a batch reactor. More recently, monoglyceride (MG) and diglyceride (DG) production was studied in a solventless reaction medium. The reaction tested was glycerolysis of menhaden and olive oil mediated by Novozym 435 [32] and a commercially immobilized lipase B *Candida antarctica* (immobilized on Immobead 150, recombined from *Aspergillus oryzae*) [33].

3.4 Production of Structured Di- and Triglycerides in Continuous Reactors

There are several types of continuous reactor (i.e. stirred tank reactors, packed bed reactors and fluidized-bed reactors) which can be used with immobilized enzymes.

Continuous stirred tank reactors (CSTRs; Fig. 3.2a) is an agitated tank where the reactants and products are introduced and removed at the same rate. The impeller stirs the reagents vigorously to ensure substrate–enzyme contact and good mixing and so that there is a uniform composition and temperature in the vessel. The composition at the outlet stream is the same as in the bulk in the reactor. The immobilized lipase is retained in the tank by means of a filter or a screen [34].

Low costs of construction are normally associated with CSTR, but continuous stirring is strongly related to higher power costs. For large-scale production, a high-volume reactor or a cascade of CSTRs should be used. Another important drawback of the CSTR is the possibility of attrition (breakage) of the particles with the agitation.

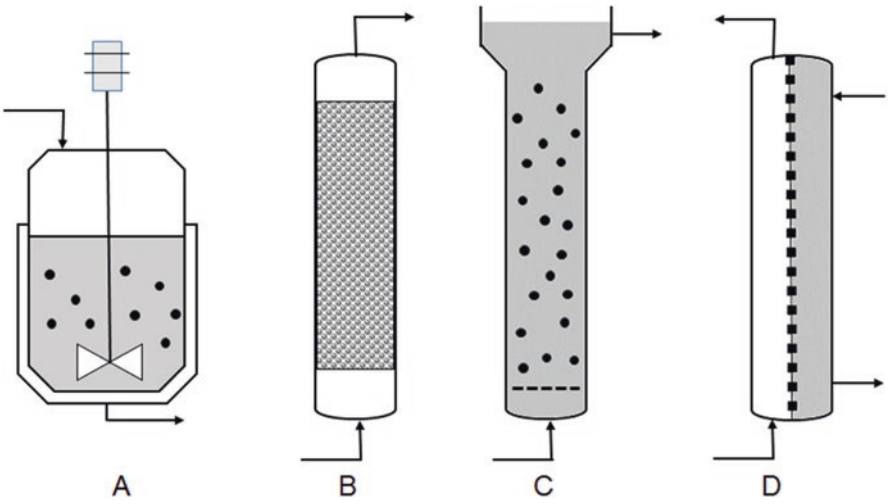

Fig. 3.2 Schematic representation of continuous reactors: (**a**) continuous stirred tank reactor; (**b**) packed bed reactor; (**c**) fluidized bed reactor; (**d**) membrane reactor

A CSTR was successfully used in the selective production of monolauroyl maltose catalyzed by Novozym 435 in acetone [35]. The immobilized lipase was located in a perforated stainless steel basket.

Packed bed reactors (PBRs; Fig. 3.2b), also known as fixed bed reactors, are continuous-flow reactors that consist of a column or cylindrical vessel that contains the immobilized enzyme, and the liquid reactant flows through the biocatalyst. Upward flow is usually preferred because it reduces the possibility of channeling and does not compress the immobilized enzyme beds; however, in EI at industrial scale, downward flow is used [36] because of oil viscosity. PBRs are easy of operate, present high conversion per unit mass of catalyst, and low maintenance cost. Another important advantage of PBRs is the reduction of catalyst loss due to attrition. They are the reactors most commonly used for large-scale enzymatic production. The general possible disadvantages are the poor temperature control, great pressure drops, risk of obstruction of the bed, and difficulty of cleaning the device and of replacing the catalyst [7, 37]. It is convenient that biocatalysts have particle sizes greater than 0.05 mm in order to maintain the pressure drop within acceptable values [18], but increasing particle size increases the diffusion limitation [38]. For EI, in flow-through systems (like PBRs) which are more prone to poisoning and inactivation, the quality of the feed is important to keep the process competitive [7]. The substrate must be refined to remove any poisons or inhibitors that can inactivate the enzyme [2, 12]. Avoiding or decreasing the loss of enzyme activity is essential, since even at a use of only 0.4 kg enzyme/ton of oil, the cost of the biocatalyst accounts for more than 70% of the variable EI costs [1].

When the process is carried out on an industrial scale, oil blend is pumped down through the biocatalyst beds of 4–6 PBRs in series [27]. Every reactor has a water circulation jacket to maintain the reaction temperature at 70 °C and prevent solidification of the fat. The biocatalyst commonly used is Lipozyme® TL IM (*Thermomyces lanuginosus* supported over silica). Each PBR has between 250 and 1000 kg of enzyme, and the bed is supported on a split screen that allows oil to pass (through 150-nm gaps) whereas immobilized enzyme particles are retained (450 nm) [39]. Flow rates are in the range of 1–2 kg oil kg^{-1}_{enzyme} h^{-1}. The first reactor operates as a guard column, and is used to capture compounds that inactivate the enzyme and protect the other reactors. When the enzyme activity of the first reactor is depleted, and the product leaving the reactor cascade does not meet the desired specification, the catalyst in the guard reactor is discarded and the second reactor act as a sacrificial column. Reactor 1 is recharged with fresh biocatalyst and becomes the last reactor in the series. The process is repeated about every 14 days [5, 27].

Paula et al. [40] used a PBR for the production of a more spreadable milkfat under lower temperatures. The interesterification of milkfat with soybean oil was catalyzed by *Rhizopus oryzae* lipase immobilized on SiO_2–PVA, at 45 °C. Interesterification yields varied from 35 to 38 wt%, resulting in a product with 59% lower consistency related to a non-interesterified blend. The system was maintained in steady-state conditions for 12 days. The biocatalyst reported in this manuscript had a half-life time of 34 days.

Da Silva and colleagues [41] performed the interesterification of a blend of 70% lard and 30% soybean oil in a PBR catalyzed by Lipozyme TL IM. The catalytic tests were carried out at 60 °C with the flow rate varying from 0.5 to 4.5 ml min^{-1}. The regiospecific distribution of FAs was modified by flow variations in the reactor. At lower flow rates (higher residence times), there was a higher probability of acyl migration, i.e. saturated FAs migrating from sn-2 to sn-1,3 positions.

The same reaction, under continuous flow regime, was investigated by Soares and coworkers [12]. In this study, the interesterification of a mixture of palm stearin (45 wt%), palm kernel oil (30 wt%) and olive oil (25 wt%) was performed in a PBR using Lipozyme TL IM and Lipozyme RM IM. The reaction tests were performed at 65 °C with a residence time of 8 min. Half-lives of 88 and 60 h were estimated for Lipozyme TL IM and Lipozyme RM IM, respectively. The lower activity presented by the biocatalysts may be assigned to the presence of green pigments and oxidation products in the feed. These compounds are present in virgin olive oil, and they have inhibitory effects on lipases. The distribution of acyl residues at the central position in TGs remained substantially unchanged after the EI, suggesting that acyl migration had not occurred (due to the short residence time).

The application of PBRs in the production of a fatty acid ethyl ester through the lipase-catalyzed transesterification/esterification has been systematically increasing in recent years. Even though the product of interest (biodiesel) is not close to the focus of this book, the involved reactions are the same [22, 42].

Fluidized-bed reactors (FBRs, Fig. 3.2c) are a combination of a PBR and a STR, in which the biocatalyst is kept suspended by the upward flow of the substrate at sufficiently high flow rates [8]. The extensive mixing in all directions generates excellent heat and mass transfer characteristics, but the residence time needed to fluidize the bed may result in low productivity of the desired product [18]. Channeling problems are eliminated, small particles can be used because the pressure drop is not affected and the coalescence of the emulsion droplets is reduced [43].

One of the main drawbacks of FBRs are the pumping and power requirements. Another disadvantage is the difficulty of scaling-up this type of reactor [18].

This is most frequently applied in immobilized enzyme catalysis where viscous, particulate substrates are to be handled.

A FBR was utilized in the esterification of milkfat with soybean oil mediated by Novozym 435 and *Rhizopus oryzae* lipase immobilized over a hybrid of polysiloxane-polyvinyl alcohol [44]. The interesterification yield was 10.5 and 5.7% for Novozym 435 and immobilized *R. oryzae* lipase, respectively. Even though the FFA level was 1.5%, an almost undetected deactivation of Novozym 435 and a half-life of 190 h for *R. oryzae* lipase were observed during the reaction period.

Saponjić and co-workers [45] carried out a comparative study on the synthesis of amyl caprylate in a batch and a packed bed reactor with *Candida rugosa* lipase immobilized on Sepabeads (commercial macroporous polymeric material based on acrylates). The enzyme loading was 37 mg g^{-1} for both reactors. Almost complete conversion of the substrate was observed in the STR at 37 °C and 24 h, when a high initial substrate molar ratio of 2.2 was used. The FBR was operated with total recirculation of the reaction mixture and a 90.2% conversion after 14 h was found

(at 35 °C, residence time: 3.5 min and substrate molar ratio 1:1). It was reported that the residence time was the major factor affecting the reaction rate. The biocatalyst remained stable over five reaction cycles (70 h), after which the activity rapidly dropped. The authors considered that the decrease of enzymatic activity was correlated to the product and/or substrates inhibition.

Recently, three different reactors, STR, PBR, and FBR, were used in the synthesis of butyl butyrate mediated by TLL supported on Immobead 150 [46]. The reaction tests were performed at 40 °C, 3:1 butanol:butyric acid molar ratio, 40 wt% of biocatalyst, and with the selection of hexane as solvent. Using these experimental settings, the STR showed a 84% of conversion after 4 h, with a productivity of 0.27 mmol g^{-1} h^{-1}. The PBR produced the best results, with 85% conversion and 1.1 mmol g^{-1} h^{-1}. The substrates were introduced from the bottom of the reactor at flow rates between 0.02 and 1.3 ml min^{-1}. At a low flow rate, the reaction rate was improved because of the increase of the residence time. An inert column filling (glass beads) was necessary in the reactor bed for improving the flow characteristics by reducing the packing factor throughout the operation. No column clogging or preferential flows were found in the PBR after 30 days of continuous reaction. The FBR produced 60% conversion, with an optimum yield of 0.56 mmol g^{-1} h^{-1} at the lowest flow rate (0.02 ml min^{-1}). The comparison of all three reactors showed that the highest yield of butyl butyrate was obtained when a PBR was used (filled with glass beads to prevent column clogging).

A **membrane reactor (MR)** is an device for simultaneous performing the reaction and a membrane-mediated separation in the same physical vessel [47], where the enzyme is immobilized onto a semipermeable membrane, which may take the form of a flat sheet or hollow fibers [48]. MRs are commonly employed for biphasic liquid systems. Figure 3.2d shows one possible configuration (see [41] and [49] for a comprehensive review). The advantages of MRs are connected with the decrease of the pressure drop and fluid channeling, and the high surface area to volume ratio of the membrane [8]. The disadvantages are related to the pore plugging and the cost of the membrane and its replacement [12].

For esterification and hydrolysis reactions, it is convenient to have the reactants and products separated during the reaction process. Recently, Gupta [50] developed a biphasic enzymatic MR for the hydrolysis of olive, palm and castor oils. *Candida rugosa* lipase was immobilized by an ultrafiltration process on a Polysulfone membrane. In optimal conditions (37 °C, pH 8.0, 24 h, and using iso-octane as solvent), the degree of hydrolysis was 38.5, 35.4 and 21.2% for olive oil, palm oil and castor oil, respectively. The enzymatic activity decreased slowly during the repeated use, and a 22% decrease in activity was observed after five cycles. The deactivation of the enzyme was attributed to the loss of structural conformation.

Immobilized enzymes in continuous reactors are used in the production of DGs. Saberi et al. [51] used a 16-l PBR in the glycerolysis of palm olein catalyzed by Novozym 435. The reaction was carried out at 65 °C, with a flow rate of 850 ml min^{-1}.

A process for preparing a high-purity DG at industrial level was patented by Sugiura et al. [52]. The esterification of FAs with glycerol was performed in a PBR with a residence time not more than 2 min (in order to minimize TG formation).

It used Lipozyme IM, and water removal was accomplished in an external vessel by vacuum. A comprehensive list of DG production process patents has been reported by Lai and Lo [53].

In a recent study [54], a bubble column reactor (BCR) was developed for DG production in a solventless reaction media via esterification of glycerol with FAs using Lipozyme 435. Under selected operation parameters (5 wt% biocatalyst, glycerol/FA mole ratio: 7.5, initial water amount 2.5 wt%, 60 °C, 30 min), the final product contained 91 wt% of DG (non-purified product: 58%). The enzyme retained 95.1% of its former activity during 30 reaction cycles.

3.5 The Use of Adsorbents in ST and SD Synthesis

Water is vital in the preservation of the three-dimensional structure of proteins and for maintaining their catalytic activity in organic media [55]. In esterification, the removal of the generated water is a crucial point, because the equilibrium could shift towards products with a reduction in the available water. The separation of water can be performed by several methods, such as the use of molecular sieves or silica gel as desiccating agents [56–58] or the application of a vacuum pump or a stream of nitrogen [59, 60].

Water can promote the agglomeration of protein particles in an organic solvent, which results in a reduction of the reaction rates. Won and Lee [61] demonstrated that the simple introduction of silica gel to the reaction media could avoid enzyme sticking.

Guo and Sun proposed three different strategies for water removal in solventless production of 1,3-DG of CLA [50]. The esterification of CLA with glycerol, employing Novozym 435 as the catalyst, was performed with magnetic stirring under vacuum, vacuum-driven N_2 bubbling (VNB) and in the presence of molecular sieves. VNB generated an effective interaction of substrate–enzyme in the multiple-phase reaction media with an almost complete water removal and a very high reaction rate (99% conversion of CLA and 92% 1,3-DG yield, after 3 h). The reaction with the molecular sieves gave 80% conversion after 10 h and achieved a maximum yield of 1,3-DG of 44% at 16 h. This inferior performance is believed to have arisen from mass transfer limitations and the inefficiency of water separation due to the high viscosity of the reaction media. In addition, molecular sieve recovery and reuse was difficult and laborious, and so this process could be of less industrial interest.

A similar conclusion was achieved by Ye and Hayes [51]. They studied the solvent-free lipase-mediated production of fructose–oleic acid ester. The esterification reaction was performed in a closed-loop PBR (Fig. 3.1b) at 53 °C, containing Lipozyme RM IM. Water removal was implemented by the application of a molecular sieve packed column, N_2 bubbling, vacuum, or a coupled application of the latter two. This last approach was found to be the most efficient.

When molecular sieves or silica gel are used in the reaction media, it is important to consider the adsorption of reactants or products on the surface of these materials (such as fatty acids, glycerol, etc.) [62]. A strict methodology has to be applied with the samples in order to correctly complete the compound quantification.

3.6 Acyl Migration in SD Synthesis

Figure 3.3 shows the acyl migration in 1,2 DG and 2 MG.

Figure 3.4 shows the mechanism of the intramolecular acyl migration. A proposal is that the mechanism takes place through a SN2 nucleophilic substitution.

There are different transition states. The transition states 2′ and 2″ have higher activation energies than 2. In transition state 2, the charge is dispersed. The increase of water activity favors transition states 2′ and 2″ which have higher activation energies. Therefore, the increase of water activity affects the reaction rate but does not affect the equilibrium .

A very interesting study on the impact of temperature in acyl migration was reported by Yang et al. [63] The reactions considered as examples were the reaction of tripalmitin (PPP) with CLA or with caprylic acid (CA) targeted for HMFS. Acyl migration decreased by 29% (35 h) and 45% (48 h), respectively, in the reaction of PPP with CLA in solvent and solventless reaction media when compared with 37% (35 h) and 61% (48 h), respectively, for the acidolysis of PPP with CA. Acyl migration in the acidolysis of PPP with CA was lower than in the reaction of PPP with CLA. Temperature programming was more important when no solvent was present

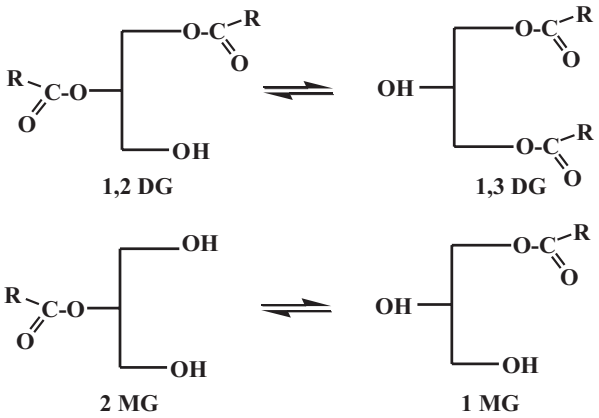

Fig. 3.3 Acyl migration in 1,2 DG and 2 MG

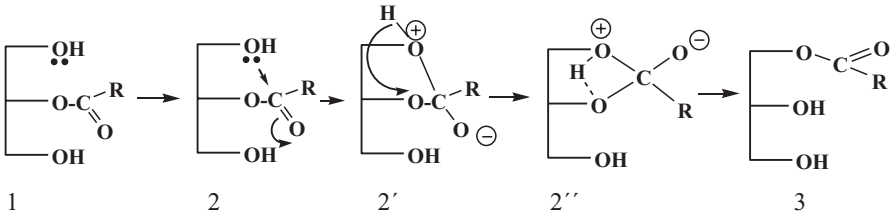

Fig. 3.4 Intramolecular non-catalyzed acyl migration

for the elimination (even partial) of acyl migration. The authors concluded that the solvent (hexane) in the reaction could avoid additional acyl migration.

The acyl migration in partial glycerides was often present during 1,3-lipase-catalyzed reactions from TGs and partial glycerides, including acyl migration of 1,2-DG to 1,3-DG and from 2-MG to 1-MG. Li et al. [64] reported the role of temperature and water activity in acyl migration in the methanolysis of triolein in *tert*-butanol at different water activities. Temperature and water activity were two key factors affecting acyl migration kinetics. The acyl migration reaction rates were favored at high temperatures and reaction rates of 1,2-DG and 2-MG were very different in each case. This finding was related to the different activation energies for these reactions.

Acyl migration was confirmed to be a first-order reversible reaction. The acyl migration rate constant of 2-MG under 30 °C was nearly 7.4-fold that of 1,2-DG. From the Arrhenius plots of k1 (direct reaction constant for 1,2 DG to 1,3 DG) and k3 values (direct reaction constant for 2-MG to 1-MG), the apparent activation energies were 73.8 and 59.4 kJ/mol, respectively, for 1,2 diolein and 2 olein. The equilibrium concentrations of DG and MG were not changed by the water activity present in the medium.

Laszlo et al. [65] reported the reaction kinetics of 1,2-DG to 1,3-DG from 25 to 80 °C, and they studied the resulting compounds using 1H-NMR spectroscopy. Lipase-catalyzed alcoholysis of high-oleic sunflower oil with 1-propanol (solvent-free) generated 1,2-DG using Lipozyme TL IM. The 1,2-DG mole fraction at equilibrium was unaffected by heating. The same authors proposed a ketal intermediary instead of the transition states 2–2′ or 2″ for non-catalyzed acyl migration. They found that the 1,2-DG prepared from sunflower oil with high content of C18:1 resulting in 32% of DG in equilibrium was consistent with literature reports. For long-chain DGs, the average yields were 30–40% 1,2-DG isomer and 2-MG comprises only 9%, being 91% 1-MAG [66]. Activation energy for 2-MG acyl migration was nearly 79 kJ/mol. These authors found that the rate of acyl migration is nearly equal to 1,2-DAG and 2-MG. 2-MG with its two free primary alcohol groups should be more reactive than 1,2-DG with only one. The results of Li et al. are in line with this idea, whereas the results of Laszlo et al. are in disagreement. Probably, these results are not comparable due to different reaction conditions.

The solvent is of paramount importance in the acyl migration in partial glycerides [67].

The majority of the literature has focused on thermodynamically favored reactions. Only recently have topics such as the role of the lipase in the acyl migration, the mechanism of lipase-mediated reactions and the impact of the solvent and adsorbents been studied carefully. There are several aspects to consider in the analysis of the principal literature published on this topic in the last 5 years and the several publications from our group on acyl migration:

(a) Non-catalyzed and the lipase-mediated acyl migration are taking place in parallel; the solvent role should not be minimized.
(b) Each lipase has a different reaction to MG and DG coordination, depending on the tertiary structure, conformation and relative activity, which depend on the FA

from the glycerol backbone. There is a proposal for a key role of 2-MG as an allosteric modulator of the activity and regiospecificity of several lipases [68].

(c) The lipase support may adsorb polar compounds if polar or slightly polar.

(d) There is no clear mechanism of the support as a promotor of acyl migration, especially if not a proton donor or a polar support.

(e) All the compounds added to the reaction to control water or alcohol concentration (silica, zeolites, other adsorbents) should be considered as potential adsorbents for the polar and non-polar compounds from the main reaction (MG, DG, TG and FA).

(f) The reverse reactions in Fig. 3.3 also take place in lipase-mediated reactions. Careful analytical chromatographic methods are required to avoid quantification errors. In this sense, the formation of 2,3 DG and the isomerization of 1,3 DG and 1,2 DG to 2,3 DG should also be considered in the reaction mechanism. The same situation should be analyzed with 3-MG, although in this case the relative proportion is always very low [69]. This is the cause of the presence of TGs in reactions of glycerol esterifications using 1,3 specific lipases.

3.7 Acyl Migration in ST Synthesis and TG Reactions

The recent work of Cao et al. [70] studied reactions without solvents using a 1:1 molar ratio of trilaurilglycerol and 1,3-palmitin-2-olein and three supported commercial lipases. Analysis of TG content and FA distribution showed interesterification in extreme positions and FA exchange in the central position through acyl migration. *Rhizopus oryzae* lipase supported on polypropylene showed preferential exchange in extreme positions of the TGs and almost no reaction in the sn-2. With lipase from *Thermomyces lanuginosus* on silica, completely randomized FA distribution resulted after 1 day. The authors concluded that *T. lanuginosus* lipase (TLL) and silica favored randomization of FA distribution, whereas the *R. oryzae* lipase and polypropylene did not. High water activity favored hydrolysis and thereby increased relative amounts of partial acylglycerols. However, the acyl migration reaction rate of these intermediates was also found to decrease. The main conclusion was that, at a certain degree of interesterification, there was no important impact of water activity on the progress of the reaction at the central position of the STs. Low water content had the great advantage of giving an important production of TG. The authors concluded that, in the production of a TG with a particular composition and FA distribution, it is key to choose adequate lipase, support, water activity and content and reaction time. The TLL was considered responsible for the acyl migration.

In the methanolysis of triglyceride using 1,3 specific lipase, the theoretical methyl ester production is only 67% in molar basis. MGs and DGs should accumulate. The intracellular lipase of *Rhizopus oryzae* (*R. oryzae*) has been demonstrated to be a 1,3 specific lipase but the yield of methyl esters in this reaction could achieve over 80%. The same result was obtained with analog 1,3-specific lipases as biocatalysts for biodiesel synthesis, in which 2 MG and 1,2 DG could be transformed to 1 MG and 1,3 DG, respectively

During the process of *R. oryzae* whole cell-mediated methanolysis of triolein, 1,2-DG and 2-MG were accumulated in the reaction media after the first minutes of the reaction. Even removing the enzyme as a strategy, the content of 1,2-DG and 2-MG still varied considerably with reaction time [71].

Recent publications have expanded the studies of regiospecificity of lipases [72]. Studies of variable amounts of different FAs in TGs and different locations in the glycerol backbone have been performed by enzymatic and chromatographic methods which are time-consuming and involve several chemical manipulations using enormous volumes of organic solvents. Optionally, carbon-13 nuclear magnetic resonance (13C NMR) is a fast and reproducible technique that can be used to determine the saturated and unsaturated FA distribution of the TGs remaining in high-oleic sunflower oil and fully hydrogenated high-oleic sunflower oil mixtures. The enzymatic interesterification was carried out using the supported lipase from *Thermomyces lanuginosus* (Lipozyme TL IM). The results demonstrate that the lipase was not truly regiospecific at sn-1,3 due to the spontaneous acyl migration from position sn-2 to sn-1,3. The interesterification was carried out in mixtures of high-oleic sunflower oil (SO) and fully hydrogenated high-oleic sunflower oil using 60:40, 50:50, 40:60 and 30:70 weight relationships (w/w), respectively. Regiospecificity achieved by EI was compared with the one resulting from C. This paper reported for the first time 13C NMR applied to the study of interesterification reactions.

The effect of the immobilization protocol, pH and use of acetonitrile on the regioselective enzymatic hydrolysis of triacetin to diacetin (using buffers of different pHs) has been published by Hernández et al. using CALB and RML immobilized on Sepabeads modified by glutaraldehyde (covalent immobilization) or octadecyl moieties (adsorption via interfacial activation) [73]. Covalent RML demonstrated the lowest activity towards triacetin. RML and CALB showed a clear 1,3 regioselectivity in this reaction, but using pH 7, the spontaneous acyl migration generated the regioisomeric mix. At pH 5.5, this migration is decreased, and 1,2 diacetin was found as the main product. RML immobilized on octadecyl Sepabeads was recommended as the best preparation.

The main reaction of ST synthesis is interesterification. In the last 5 years, a clear pattern has been emerging

(a) With the use of adsorbed lipases with more stable and thermically and chemically resistant formulations, the support should be considered in the acyl migration and the reaction media should be studied as an additional factor.
(b) The configuration of cost-effective processes to use lipases depending on the reaction acyl migration could be desired (biodiesel synthesis) or avoided (ST synthesis).
(c) The need for a biphasic system if the non-immobilized lipase is used and reused (to optimize activity in short times and minimize or maximize acyl migration if desired).
(d) The need for a special pattern for temperature during the reaction (not fixed but higher at first and then lower).
(e) The minimization of the thermodynamically favored acyl migration.
(f) The use of sophisticated analytical methods to study FA distribution in reaction products (such as 13CRMN and 1H RMN).

3.8 Other Problems

3.8.1 Adsorbent Use and Impact of Water Activity in the Relative Adsorption of Substrates and Products and the Perturbations Introduced by the Adsorbents

The use of zeolites and silica gel to adsorb water and alcohols in different reactions of lipases has been reported several times and has been presented above. However, there is a pattern that it has not been considered so often. MGs, DGs and FAs can be adsorbed on polar adsorbents and this is an important source of errors in the conversion quantification and in the analysis of the product distributions. The mass balance should be a rule in the design of any laboratory- or pilot-scale testing reactor. The adsorption of FAs onto adsorbents in the case of acidolysis or esterification reactions performed in the presence of polar adsorbents may be very important on the laboratory scale.

3.8.2 The Reaction Media Composition and Role of Organic Solvent

The organic solvent and the water activity are key to the promotion of acyl migration, besides temperature. These parameters affect lipase activity through different changes in the conformation of the lipase that may profoundly affect the final product distribution.

3.8.3 Secondary Contamination Due to Protein Leaching in some Immobilized Systems

The immobilized commercial and non-commercial lipases used and tested in lipases reactions involving glycerides should be properly checked in terms of chemical and thermal stability. The support may be (even partially) soluble in the reaction media and the lipase may be desorbed (even with covalent treatments). The lipase desorption to the reaction media should be carefully tested in the analysis of potential lipase applications, in batch or continuous reactors. Strategies to avoid this problem include post-immobilization cross-linking using adequate compounds and the use of additives. However, these strategies introduce their own potential problems (such as the decrease of the lipase activity and mass- transfer limitations).

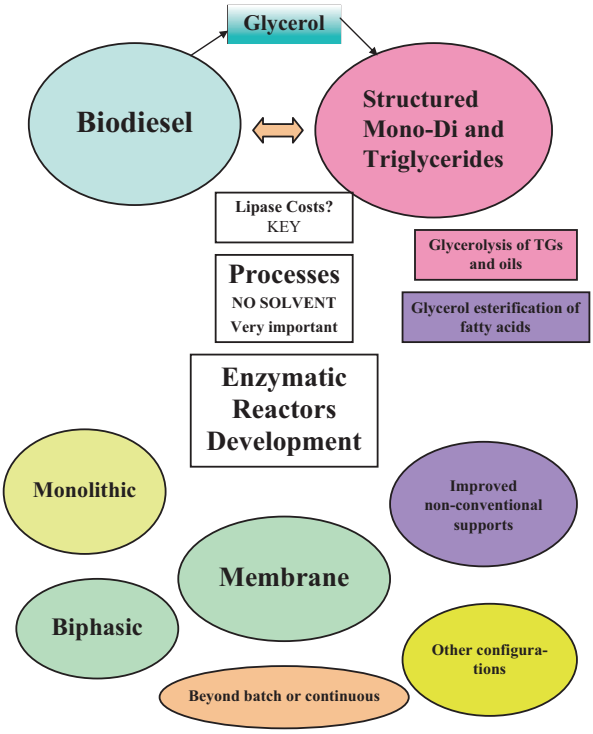

Fig. 3.5 Biodiesel and Glycerides synthesis from glycerol and how these reactions share the excess of glycerol as one of the sources of the renowed interest to go forward in the field. The figure shows also some of the common problems detected in the glycerides synthesis from glycerol and biodiesel obtentio

3.8.4 Enzyme Inhibition or Inactivation (Reversible or Irreversible) Due to Glycerol/Fatty Acids

Glycerol and fatty acids and other polar compounds may inhibit competitively or non-competitively or inactivate reversible or irreversibly the enzyme. Careful analysis of the interactions of the solvents and reactions and the products with the lipase should be carried out.

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Chapter 4
Potential Solutions to Drawbacks What Can Be Done About the Drawbacks in ST and SD Enzymatic Synthesis?

Contents

4.1 Use of Glycerol as Substrate

As has been cited in other chapters, glycerol is an important substrate for ST and SD synthesis in two main reactions: esterification and glycerolysis of triglycerides (TGs).

The use of glycerol as a substrate introduces several problems. First, some enzymes (such as *Rhizomucor miehei* lipase) are very sensitive to glycerol inhibition. In addition, to avoid the covering of the lipase with glycerol and the introduction of mass transfer restrictions to non-polar compounds, the strategy involves the introduction of glycerol adsorbed on a solid. This solid is in general silica due to its low cost, particularly as silica gel [1, 2]. Other options like zeolites have higher costs.

Considering this strategy, the use of solvent is needed. Due to the final application in the food industry, hexane may be an option, but the low normal boiling point and high vapor pressure at low temperatures has to be addressed. Acetone may be used as an alternative, but the problem of lipase inhibition and lower enzymatic activity in acetone has also to be considered. Hexane, petroleum ether and tert-butanol have been used. Tert-butanol is very interesting due to the lack of reaction of tertiary alcohols with fatty acids or glycerides using lipases due to steric hindrance, but an appropriate polar–non polar balance. Process intensification (microwave heating) using lipases and with *t*-butanol has been published for biodiesel synthesis [3].

Considering here the use of an organic, non-toxic, cheap solvent and of silica gel as the glycerol adsorbent, the question of the reactor configuration is not a minor one.

M. Luján Ferreira, G. Marta Tonetto, *Enzymatic synthesis of structured triglycerides*, SpringerBriefs in Molecular Science, DOI 10.1007/978-3-319-51574-8_4

The use of continuous processes and glycerol as the substrate would require, for example, a guardbed with the glycerol adsorbed on silica and a true reactor in which the recirculating stream with the solvent, the desired fatty acids and glycerol at low concentrations interact with the immobilized lipase. An option in this case would be a batch reactor configuration (stirred tank reactor, STR) or, better, a continuous STR. In this reactor configuration, the enzyme is placed in the reactor using membranes located at the reactor outlet. Several combinations are possible. In these cases, high-volume reactors and long reaction times are required. The biocatalysts are prone to deactivation due to the high shear stresses related to the mechanical agitation. Alternatives such as the use of porous baskets, the use of a six-bladed turbine impeller (Rushton turbine) or a packed bed reactor with recirculation have been applied to avoid enzyme inactivation, minimizing internal and external mass transfer problems. Organic solvents should be discarded when possible. Many of them are toxic and flammable, producing damaging effects on the environment, causing additional process steps for solvent removal and reuse, and resulting in increasing production costs. The use of supercritical carbon dioxide (sCO_2) and ionic liquids may be an alternative. In this case, the system glycerol/sCO_2 is biphasic from 40 up to 200 °C and up to 35 MPa. If glycerol is used or produced as in the case of biodiesel synthesis using ionic liquids, glycerol is soluble in the mixture of methanol and ionic liquid. The ionic liquid/alcohol phase with glycerol has to be washed with water 3–4 times to rinse the glycerol. Later, this glycerol is separated and purified to high purity [4].

A recent review of esterification with lipases was very complete with the literature updated to 2013 [5].

4.2 Use of Low Cost Fatty Acids or Fatty Acid Mixtures as Substrates

The use of wasteoils, used edible oils (fried or not), cooking palm oil and other low-cost long- and medium-chain fatty acid sources (such as seed oils, alperujo and waste vegetable oils) has been continuously researched in the last 5 years [6]. As expected, if the goal is to synthesize mono- (MGs) and diglycerides (DGs) with no food applications, the standards of purification and pretreatment are different than if food is involved as a final product,. For non-food applications, grease and more "impurified" oils could be the source of fatty acids.

Considering an enzymatic process as an alternative, lowering the cost of the fatty acids source could be a cost-effective strategy to decrease the cost of the overall processes, even when the specifics of the particular oil have be taken into account for the selection of the reaction conditions and the overall process.

Specific fatty acids have specific oils that may be natural sources of them. In this sense, each vegetable oil has a unique composition that could be tailored depending on the type of the final structure of the SD or ST required, such as in the case of biodiesel synthesis in two steps.

4.3 Other Solutions (The Reactor, The Process, The Lipase, Additives)

Water generated by esterification should be separated to improve the conversion. Different procedures for the separation of this product have been applied. The most efficient procedures are the use of a flow of nitrogen gas or of a vacuum pump. Problems related to adsorbent introduction have been cited in other chapters. A vacuum system would be the best option for the commercial production of DGs. Different low pressures between 1 and 10 mmHg have been applied. The maximum yield of 1,3-DG was found using 1.09 M of glycerol at 1 mmHg. Besides the reaction, other sources of water must be taken into account. It has been reported that nearly 3% of water per gram of overall solid is present in immobilized commercial lipases.

In the commercial step, a vacuum system with a high water rate removal would be suitable for the production of DGs in a SBR.

Figure 1.1 shows a simple method of DG synthesis by esterification using a vacuum to separate the water.

Reactions in a packed bed reactor (PBR) for the esterification of fatty acids and glycerol have not been adequately explored with applications in industrial conditions. Removal of water is required to produce a high amount of product in an

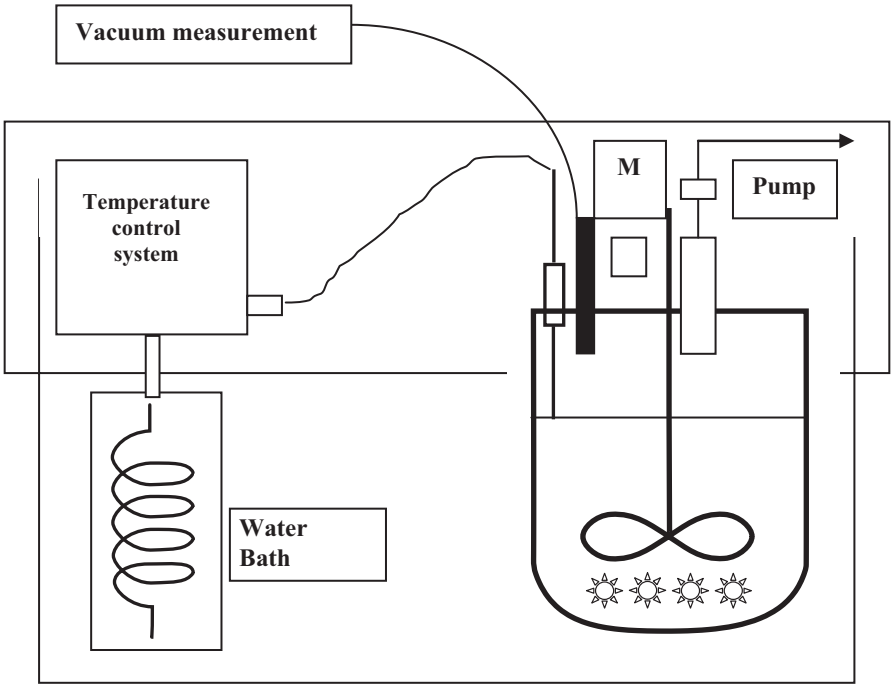

Fig. 1.1 STR with vacuum and enzymatic immobilized catalyst

esterification reaction in this reactor configuration. The accumulation of water in the PBC leads to a decrease in enzyme stability. The methods in this case would be the selection of a polar solvent and the periodic drying of the reaction media by an adequate air flow or by recirculation of pure solvent. A glycerol excess has been used to remove water but the conversion to DG is low and therefore the best configuration is to use a water separation system outside the PBC.

The main data and reviews of enzymatic reactors using lipases can be found for biodiesel enzymatic reactors [7, 8]. The transesterification is defined as a reaction between vegetable oil or animal fat and a primary alcohol in the presence of a catalyst. The final product would be a mix of alkyl esters of fatty acids (biodiesel) and glycerol if all the positions in the TG from the oil or fat react.

In these reactors, a non-polar viscous substrate such as vegetable oil is contacted by a polar substrate, generally considering a solvent as part of the reaction media. The main problems found in enzymatic biodiesel synthesis also apply to enzymatic structured glycerides (STs) synthesis, with the additional problems related to use in the food industry. Another biodiesel synthesis technology is hydroesterification in two steps: first, the hydrolysis of all glycerides (mono-, di- and triglycerides) with the generation of free fatty acids (FAs) and glycerol; and second, the esterification of the FAs using a primary alcohol (methanol, ethanol) to obtain esters (biodiesel) and water. In case of ST or SD synthesis, the second step would include glycerol instead another alcohol.

Four main configurations of enzymatic reactors have been explored for biodiesel: batch stirred tank reactors; continuous stirred tank reactors; fixed bed column reactors (the supported enzyme is maintained as fixed while the reaction media is pumped though the whole system); and fluidized bed column reactors (here, the supported lipase is kept fluidized by means of recycling of the reaction media) [1].

As is the case of structured tri- and diglycerides, in the field of enzymatic biodiesel synthesis there are several strategies to reduce the costs. They include the immobilization of lipases, reuse of the supported enzyme, the correct reactor configuration and the exploration of different, cheaper substrates than usually used. One very important point is the search and the stabilization, immobilization and the reuse of cheaper but very active lipases than those commercially available

Considering the glycerides synthesis, the reaction media evolves to clearly biphasic, especially in esterification, with the production of water. At laboratory scale, these reactions are mainly studied in batch stirred tanks with low volumes and magnetic stirring. At pilot or bench scale, mechanic stirring is used. Then, not only the mass transfer related to different polar/non-polar substrates has to be taken into account but also the stability of the lipase through the selection of the support. An adequate support must have resistance to compression in configurations using very high flow rates in packed bed reactors (PBRs), frictional resistance in STRs. Other requirements involve the increase of resistance towards high velocity in fluidized bed reactors. When applying lipases in non-aqueous medium, the residual water fraction in an immobilized lipase has to be taken into account, because this influences the activity, thermostability, and stereoselectivity. Production of DGs in PBRs and SBRs has been reported [9, 10].

Cheaper and stable supported lipases are required for the synthesis of interesterified low *trans* fats.

The existing industrial processes for the production of margarine and shortening are:

- Blending
- Fractionation
- Hydrogenation of oils
- Chemical interesterification
- Enzymatic interesterification with enzymes

A new 1,3-specific *Thermomyces lanuginose* lipase immobilized in silica, the lipase tested in [11] in enzymatic interesterification, includes a certain amount of water. Non-optimized, former supported enzymes need the continuous addition of water for the lipase to be active.

The process of EI is designed as a continuous operation, whereas chemical interesterification (CE) is carried out as a batch reaction. A 60:40 mix of palm stearin and coconut oil was contacted with Novozymes' Lipozyme TL IM (the immobilized enzyme) or sodium methoxide (the catalyst for CE). The enzymatic process was carried out at 70 °C and compared with the CE in which 0.5% w/w sodium methoxide was used during 30 min at 100 °C. Almost identical solid fat content for the two fats was produced. Color development was decreased compared to chemical interesterification and therefore there are lower requirements for post-interesterification treatments. One group of experiments used mixtures of palm stearin and sunflower oil, and the results were compared for color, total tocopherols and diglycerides contents before and after interesterification (EI or CI). The interesterified blends using the enzymatic process had improved color characteristics and tocopherols content. Diglyceride levels were minor in the case of EI compared with CI. In industrial operations, oil reduction would be lower for an enzymatic process. A presentation from Ballestra [12] included that for chemical interesterification (CIE). Interesterified fat was reported to depress the content of high-density lipoprotein (good cholesterol that is protective against heart attack) more than *trans* fatty acids. In addition, interesterified fat increased blood glucose and decreased the concentration of insulin. This strongly implies that interesterified fat could generate diabetes. Kellens presented that today cost-effective enzymatic interesterification should be the option for commodity fats (shortenings, bakery fats, table spreads), but change is very, very slow. The author questioned "Is it cost, is it flexibility or is it rather a lack of knowledge?" From his analysis, it was none of them, but it seems that enzymatic esterification (EIE) is a technology in ongoing evolution. With improved enzyme performance in activity and productivity, with a rate at which oil can be fed of 1–1.5 kg oil/kg enzyme/h and with an amount of oil that can be interesterified at 2–4 ton oil/kg enzyme, there is room for further research and improvement. The feedstock quality is essential but no predeodorisation would be an important achievement; the continuous system is the best configuration and a minimum of four but better six reactors in series is the best process. The improvement could be given by guardbed effects, optimized enzyme usage and by the use of chromatographic silica pretreatment. The industrial cost reported in this presenta-

tion was US$ 200–300 per kg. However, the same author reported that the cost only increased by US$ 2–5 per ton in EIE versus CIE.

Novozymes has developed reactors of different sizes to test the technology.

First, the testing is being carried out using 600 mL of oil. The reactor is arranged into a frame with a specially designed jacket for easy displacement of the equipment. The required extra parts are a circulating water bath, a stirrer rotor and a vacuum pump.

The required further stage of industrial testing is a continuous column reactor that reproduces the testing to produce larger volumes of modified oils.

A laboratory-scale reactor with usually 5–10 g of enzyme in a column configuration can be implemented to determine the required parametric values. This reactor has two key relative improvements related to other configurations or sizes. First, the amounts of oil are low. In daily industrial operation, 1 g of enzyme will react with 2.5–4.0 kg of oil. Second, the reactor is immersed within the thermostatized bath, and no cold spots can occur. This avoids solidification of the fat and the resulting blocking of the system. These drawbacks can be a huge problem when testing at such a small scale. A larger, semiproduction-scale reactor has also been constructed. These pilot plant--scale configurations can be tested as a direct "plug and play" system for industrial operations in which large quantities of oil must be modified. The enzyme total amount required for this equipment to be operative is about 400 kg, and the start flow rate is 1500 kg/h (see [3]). The investment costs are US$9.1 for CE versus 4.5for EI per ton processed [13].

The review of Itabaiana et al. explored the published literature on continuous flow reactors and lipase-catalyzed reactions, including DG synthesis and other glycerides [14]. Several examples of ST synthesis are presented and discussed. Oils such as Jathropa and waste cooking palm oil have been explored as substrates.

The synthesis of STs including one or several unsaturated fatty acids in the glycerol backbone structure involves the secondary reaction of oxidation of substrates and/or the produced STs. The addition of antioxidants in the acidolysis reaction media when vegetal oils with polyunsaturated fatty acids are modified with capric and caprylic acids in a PBR has no detrimental effect when Lipozyme RMIM is used as biocatalyst.

Different configurations of glycerol addition have been proposed to avoid the enzyme inhibition. Even when the research is active, there emerges a clear pattern of the need for an ad hoc design of the processes, the reactor, the stirring method, the enzyme location and configuration in the reactor, the operational methodology and the niche use of the enzymatic technology when there are clear advantages for this technology to be applied.

The application of surfactants at industrial level is expensive. However, the use of other additives may open opportunities for different configurations of industrial reactors. The additives should be low cost to have industrial potential. Nie et al. reported the use of cyclodextrin to enhance the biodiesel yield in an enzymatic process for biodiesel synthesis carried out in Shanghai, China. This is an example of how, with a free lipase of *Candida* spp. the process became competitive.

Nei et al. reported that, in 2009, the process was carried out on a 5000-l reactor by Luming, Shanghai, China. Waste cooking oil (with an acid value of 120–180 mg KOH/g) from Shanghai was the substrate. The lipase relative amount was 0.4% w/w on a relative basis to the weight of oil. Fatty acid methyl ester (FAMEs) contents were as high as 88% under the most improved experimental conditions. Cost of the enzyme after five times reuse was about 80 CNY/ton biodiesel. The low investment requirements rendered this process competitive in the overall market.

The new process of Novozym for biodiesel based on unrefined oils as substrates, soluble/supported enzyme preparations, and by a mix of phospholipases with a free enzyme for biodiesel synthesis in a "one-step only" is fully competitive. It has been reported that Novozym 435 is very expensive and would need more than 100 reuses to become cost-efficient for high-volume biodiesel production. Free lipases can be produced and sold in the commercial market at a much lower price (30–50 times lower) and can also be re-used several times after recovery from the aqueous phase compared with supported ones.

An innovative liquid lipase (Callera Trans L, Novozymes) was applied for FAMEs production from crude, non-degummed soybean oil. FAMEs at 96% w/w was produced after 1 day's reaction at 35 °C with 200 rpm agitation with 3–15% water. Callera Trans L generated 96.3% and 95.6% FAMEs release, which has never obtained before for non-degummed oils [15]. The concept of sustainability is not minor in the green approach to synthetic fuels.

This example demonstrates that the combination of biphasic systems water–non-polar compounds, low-cost substrates, low-cost free lipases, low-cost lipase sources (neither bacterial nor fungal) and low-cost processes may open up opportunities for the enzymatic esterification of glycerol and the glycerolysis of low-cost fatty acids sources for SD and ST synthesis.

Rachel Burton presented in the Collective Biodiesel Conference—Pitsboro, North Carolina Friday August 15, 2014, the BioFame and Faester Processes, the reasons for the renowned interest in free lipases in biodiesel. A confluence of events is assigned to this new situation:

- The biodiesel industry is evolving, and feedstocks need to be more secure and of lower cost
- High FA virgin oils are available as substrates
- Request for increased fuel quality, fitting quality standards
- Competition for fats/oils from other biomass-based diesel
- Industry awareness of problems with soaps, low quality glycerin and difficulty/expense of esterification with mineral acids

Even when it seems that fitting similar conditions to mono-, di- and triglycerides synthesis for non-food applications would be difficult, the possibilities of niches in the oleochemical industry and high added-value glycerol derivatives through lipase applications to esterification and glycerolysis is giving rise to innovation and creativity.

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Chapter 5
Industrial Perspectives Which Have to Be Taken into Account to Scale from the Laboratory to Industry?

Contents

5.1 Enzyme Stability and Reuse

One of the paramount problems in any enzymatic system is the cost of the enzyme production. In addition, the need for immobilization on suitable supports depending on the particular reaction adds to the costs of obtaining the enzymes.

If the immobilized enzyme is available (considering that the cost may be affordable) the topic of reuse [how many times? what kind of treatment between uses? what are the best conditions (temperature, solvents, time)] is the other main problem to consider. Suitable solvents may be expensive and energetic costs involving long washing steps at higher than room temperature may not be adequate.

The immobilized enzyme (in this case, the lipase) should be able to be used many times with optimized treatment between uses in terms of solvent, temperature and washing time, after recovery from the reactor (for example, by filtration).

The enzymatic stability is also of paramount importance. Loss of activity is expected over time, but the number of uses should be enough to avoid the increase in operating costs. During the last 20 years, the topic of the stability of immobilized enzymes, and lipases in particular, has been more and more explored [1].

The authors of reference [1] differentiate between storage stability and operational stability. They define "storage stability" as being provided by an appropriate

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formulation and several additives that protect the enzyme from denaturation under the recommended storage conditions.

"Operational Stability" is defined as a matter of working conditions and of detrimental effects from the selected reaction conditions, such as pH, temperature, solvents, impurities and other factors that contribute to protein denaturation or modification of functional groups. The authors warned that if actual during industrial operation the active enzyme is deactivated or inhibited, some other formerly "non-working" fraction should to some extent activate and replace the inhibited or deactivated lipase. To avoid misinterpretations of immobilized lipase activity, the assay of the lipase in experimental conditions without diffusional limitations is required. For industrial applications, it is important to consider the enzyme's performance including the measurement of the immobilization process yield (as a percentage of the enzyme immobilized), the efficiency of mass transfer effects, the operational stability in numbers of cycles with stable activity instead of reporting just its half-life. In addition, the performance should be reported as productivity on a per mass basis as well as the enzyme consumption per cycle.

From a practical point of view, the problem of developing adequate methods to quantify protein and enzymatic activity has been of paramount importance. Our group has published improved procedures to test and to compare free and immobilized lipases [2, 3].

Due to the possibility of impurities, the immobilized lipase should be robust in terms of inhibition (competitive or not) from substrates or products and solvents (if used).

Discussions about costs have been centered on solvent-free systems or in reaction systems involving an organic solvent. Both options have drawbacks. The solvent-free systems may introduce additional mass transfer problems, especially for substrates, whereas the organic solvent use adds additional costs to the overall expensive process.

Recently published work has demonstrated that biphasic or multiphasic systems would be an option for several reactions, especially when substrates and products have different polarity. Production of biodiesel using liquid lipase formulations have been reported to be economically feasible. Nielsen et al. [4] reported the application of free lipases that avoided many of the problems reported in the literature.

Solubility of the enzyme can be a challenge due to the potential of lipase desorption from the support and shear stress breaking the carrier into smaller particles than the original ones. The lipase "decay" due to short alcohols (mainly methanol) and glycerol in biodiesel synthesis cannot be ruled out. The partial dissolution of the support of the commercial enzyme (generally polymeric resins) in substrates or solvents has to be taken into account.

The mass transfer should be high without compromising the stability of the lipase support. The type of enzymatic reactors for lipase applications have been extensively reported and they include mixed-batch tanks, air-lifted reactors, packed bed reactors (PBR) and expanded bed reactors. Stirring–mixing using air bubbling has been reported. For the PBR (biodiesel production), the accumulation of glycerin can affect the reaction rate and increase the pressure drop over the column. This problem is

similar to other problems in enzymatic reactions (lipase-mediated) in which a polar, viscous product is produced at the reaction temperature. The expanded bed system with upper flow introduces movement of the supported lipase particles to prevent glycerol sticking to the immobilized enzyme particles.

The publication of Nielsen et al. reported how it is possible to recover the non-immobilized free enzyme in a simple way. The lipase is concentrated in the interface between the water and a non-polar phase. In the case of biodiesel synthesis with a free lipase, this is present in an emulsified phase between a heavier phase and the fatty acid methyl ester organic phase after decantation by gravity. However, it can be difficult and tedious to obtain the purified, clean glycerol phase and recover the emulsion layer. About 95% of the lipase activity is located in this emulsion layer (see [1]). The same publication from 2016 reported that recent improvements have now decreased the lipase cost to a level where the reuse of the enzyme is not needed and a one-time use of the lipase is affordable. The publication also reports that what remains as a still needed improvement is to have a continuous production experience to generate a set of data of sufficient quality to be able to optimize and document the full process economy. The enzyme is discharged after one use. This procedure avoids the risk of accumulating products and renders the complete process more reproducible and simpler.

In the field of biodiesel, but also in other fields such as mono- and diglycerides for food products, the articles that present the results of enzymatic activity are normally not final products fulfilling the specifications (whether in the biodiesel field or food or cosmetics, and so on).

The most recent research has presented a process for enzymatic biodiesel synthesis with a first step of transesterification catalyzed by non-immobilized lipase, and a second step as a "cleaning and refining" process where the biodiesel fulfills the end-product specifications. Polishing can be obtained through two alternatives: esterification of residual fatty acids using enzymes, or the alkaline treatment of fatty acids to generate soaps, and finally centrifugation.

The stability in storage (in terms of up to 6 months) is a parameter that should be taken into account when considering and reporting immobilized lipases. The stability after incubation in different media (such as in water up to 60 °C and in heptane or acetone) are important parameters when evaluating the stability of the immobilized lipase. Unfortunately, reuse, stability in storage and residual activity after incubation are not reported when new supports are being considered as potential improvements to actual commercial supports.

Additional configurations such as the use of cross-linked enzyme aggregates (CLEAs) have been explored. CLEA Technologies is a company that since 2002 has commercialized CLEAs, a new enzyme-supporting technology developed by the biocatalysis department of Prof. Roger Sheldon at the Delft University of Technology (The Netherlands) [5]. This technology avoids high costs, allows reuse and does not involve high cost supports. The combined use of organic solvents is an additional strategy that is being carefully explored [6]. The research suggests that when working with native enzymes each protein must be studied separately even if belonging to the same class. General conclusions and models are hardly applicable when

designing stabilization strategies in biocatalysis. Enzymes to be applied in biocatalysis should ideally be fermented and processed according to tailored and optimized protocols, which enable the full exploitation of the catalytic potential of the enzyme upon immobilization.

Recently, an interesting analysis of the use of lipases has been published [7]. The authors presented the following question "Almost ten years ago, Jaeger and Eggert asked 'What is it that makes lipases so attractive?' Now, we reformulate such question to 'Are lipases still attractive? '" They provided a review of efforts in research and development (patents and publications) over 30 years and analyzed the life cycle of lipase-based technologies in four fields: kinetic resolution, production of detergents, food and feed products and production of biodiesel. In 2014, the food and beverage industry was the second most important market for Novozymes' enzymes (26% of the company's sales). The two most significant contributors were the use of different enzymes for baking purposes and for the production of healthy food.

Nestlé and Kraft Foods are the main patent assignees whereas Novozymes and DSM are also found with 12 and 7 patent families. Universities, institutes and foundations account for only 17% of the total in the patent families search.

In the first 20 years considered in the study of Daha et al. [7], the number of patent documents presented annually were from about 30 to 45 patent groups. The number of scientific publications on the topic was considerably smaller. The use of lipases in the food industry started to capture researchers' interest in the late 1990s. The increase in the patents related to lipases in food and feedstuffs was mainly due to Japan until 2000–2004. After that period, Chinese participation became increasingly important, and China has since evolved to be the main source of the majority of the patents since 2007 (2007–2009). A very important 79% of the patents presented in the tenth triennium (2010–2012) have Chinese priority.

The use of enzymes for biodiesel is not currently widespread in industry due to the higher costs of lipases versus the costs of alkaline catalysts. Novozym 435 and NaOH prices are about US$ 0.14 and US$ 0.006/kg of ester, respectively. This report mentions that Piedmont Biofuels launched the production of biodiesel using the technology FAeSTER, with support from Novozymes in 2012, while in 2013, Viesel Fuel started the production of enzymatic biodiesel also developed in partnership with Novozymes, with the capacity to produce 7 million gallons/year of Blue Sun Biodiesel. In 2014, they established an industrial plant able to produce 30 million gallons/year, again with the help of Novozymes. The immobilization of the lipase appears in 27% of the titles and abstracts of patents analyzed by the authors.

Looking at this field, enzyme engineering (for example, through metagenomics) is a blossoming one. Coupling directed evolution with structure-based rational design appears to bring lipase catalysis closer to the "ideal biocatalyst paradigm," in the search for active, selective, and stable biocatalysts [8]. De novo creation of industrial enzymes at this step still remains difficult, even when the Rosetta method has been applied. Rosetta is a complex computational method and applies quantum mechanics to computational design of new proteins considering existing scaffolds [9].

In [9] it is emphasized that a new trend in the food industry is to develop special foods (called functional), such as "prebiotics, low-calorie sweeteners, and uncommon sugars". The use of esters of fatty acids and the enzymatic synthesis of myristil myristate is particularly mentioned as highly important in the cosmetic industry. Isopropyl myristate (IPM) has been reported as a highly attractive compound because, in many topical and transdermal preparations, it is also used as a co-solvent. The active ingredients of this compound have skin penetration enhancement properties. The esterification reaction was highly dependent on the polarity of the organic solvent used. Isopropyl alcohol was chosen as it mets the requirements of the solvent properties and was also one of the substrate molecules. Optimized molar ratio of the fatty acid to alcohol (1:15) and biocatalyst mass (4% w/w substrate) resulted in >87% conversion after 5 h. The recovery of IPM (97%) by distillation and cold centrifugation ensures the recyclability. This paper is an example of the advantages of using a homogenous single phase reaction medium for immobilized lipase-mediated esterification of fatty acids. [10]

One important aspect to take into account is the impact of the water content on the immobilized lipases. Depending on the nature of the support, the water content can be very different. However, for different applications, the content of water surrounding the lipase or adsorbed onto it and onto the support may be a source of local hydrophilic pockets.

5.2 Configurations That Minimize Acyl Migrations: Are They Feasible?

Looking at the published literature, acyl migration seems an unavoidable reaction due to several reasons: acyl migration from position 2 to position 1/3 of glycerol is a spontaneous reaction that takes place without lipase, if positions 1 or 3 are available as hydroxyl groups; acyl migration from position 1/3 to position 2 of glycerol is a lipase-mediated reaction that takes place with the steric constrictions of the lipase in place. Position 2 should be available with an hydroxyl. This reaction is potentially expected in mono- and diglycerides.

Both catalyzed and non-catalyzed processes take place at once. In series or parallel, esterification is also a competitive reaction in the triglyceride synthesis from glycerol and fatty acids. Mainly, the sn-1/sn-3 positions are esterified enzymatically using lipases.

However, this reaction can be desirable for maximizing production (for example, in obtaining biodiesel using alcoholysis of triglyceride). Taking into account different reports on the topic, several aspects can be mentioned

1. The increase of temperature favors acyl migration
2. Different lipases seems to have different activities for acyl migration from sn-1/sn-3 to sn-2 (in mono- and diglycerides such as 1-MG, 3-MG,1,3 DG)
3. Acyl migration takes place spontaneously from sn-2 to sn-1/sn-3

4. The concentration of lipase in the reaction media is an important parameter that affects the results
5. Long reaction times favor acyl migration including from sn-1/sn-3 to sn-2

This reaction affects the synthesis, alcoholysis, esterification, hydrolysis and more.

One configuration that would potentially minimize acyl migration, for example, in the optimized synthesis of 1,3 diglycerides, is the use of temperature gradients during the reaction. Besides the adequate use of glycerol, this gradient would imply beginning with the reactor at a high enough temperature to achieve a high reaction rate and later to decrease the temperature to control the acyl migration.

Temperature programming was reported 10 years ago as a procedure to control acyl migration [11]. It was of paramount importance in solventless reaction media to avoid acyl migration. Acyl incorporation was unaffected by temperature programming. It is possible to decrease acyl migration by adequate change of the acidolysis reaction temperature without important loss of the reaction yield of the desired products.

A tailor-made approach to study the acyl migration for specific reactions should be the norm.

5.3 Operative and Initial Investment Costs

A recent publication on the market for enzymes was by Li et al. [12] Carbon hydrases will be the leading enzymes in the following decade. A continuous growth in products from the food and beverages market, animal feeds and the detergent industry is expected. The pharmaceutical enzyme applications will see the highest and most important increases. The USA will remain the main consumer of enzymes. Following the report of Li et al., the Asia/Pacific region is expected to win western Europe as the second largest consumer of enzymes, with high demands from China and India. Western Europe will occupy the place of the largest producer of enzymes in the next decade. Japanese and Chinese industrials in the biotechnology arena are having an increasing and important role.

The food industry is not the only main field of application of microbial lipases. Lipases for degreasing in the leather industry, for skin care in cosmetics and for degradation of crude oil hydrocarbons, have also been reported, besides cheese ripening and flavor, dough stability and conditioning, pitch control in the pulp and paper industry, polycondensation in polymers and fat stain elimination in the detergent industry. The interests in other fields for improvements in lipase activity and stability will also affect the potential of applications in structured DG and TG synthesis.

Looking at different reaction media, the solvent-free and organic media are potentially attractive to improve stability and activity. However, the use of surfactants or additives could be required to optimize performance, thus increasing costs.

Lipases are active as very efficient catalysts in organic solvents with almost 0% water. This offer alternatives such as shifting of the thermodynamic equilibrium

promoting synthesis, the application of hydrophobic substrates, the control or modification of lipase characteristics by solvent engineering, etc., including minimizing contamination.

However, the stability and activity of lipases in organic systems have been reported to be low, and these are important drawbacks. Most proteins denature in organic solvents and this is key in the search for lipases that are highly active in non-aqueous media or biphasic media but also maintain this high activity in reuse or storage. Log *P*, a parameter for solvent hydrophobicity, has been selected as a useful tool to explore the activity/stability of lipases in different reaction media. The solvents with log *P* less than 5 attack the cell membrane, but generally the less polar the better for organic solvents in the case of the esterification of fatty acids, The use of lipase avoids expensive kinds of post-reaction purification and separation steps and lateral microbial contamination, lowering the overall operating costs [13].

Recombinant DNA technology and protein engineering are being explored as potential solutions. These approaches might be an adequate pathway to overcome the problems in lipase industrial use and to produce stable enzyme biocatalysts in large amounts [14].

Abdelmoez et al. [15] reported that, in the lipase field, there are two main groups of publications: those on lipase production and optimization (including metagonomics and directed evolution), and the second group focused on the use of commercial immobilized lipases in the different reactions. The authors warned about the need for studies focused on integrated industrial protocols. They presented a "tandem" fermentation and industrial plant for enzymatic hydrolysis to lower costs.

The monoglycerides synthesized from glycerol plus long unsaturated fatty acids are used not only in food industry but also in the cosmetic, pharmaceutical and baking industries as emulsifiers. They have been recognized as safe, while antimicrobial properties have been reported for several of them. The use of enzymatic catalysis avoids discoloration and side-product formation, residual waste and energy consumption. It can produce monoglycerides including unsaturated non-oxidized fatty acids. These compounds are almost impossible to obtain through chemical procedures.

Glycerol esterification has been explored but in general requires additional improved technologies to be affordable. The use of pervaporation, ionic liquids, supercritical CO_2 and acetone has been reported.

Beyond costs, the procedures or processes that can assure high lipase activity inside the process correlated with high productivity using less enzyme are being more and more explored. The main point is then to cover the enzyme cost with the product profit margins.

One of the most studied processes for lipase application is the biodiesel synthesis process. However, the research has been carried out mainly in industry. Several publications in the period 2010–2016 have explored the impact of enzyme cost, product margin profits and the application of enzymes to different configurations of reactors. As such, they can be seen as an approach to similar systems in terms of the impact of polar–non polar substrates and products and the complete desired plus non-desired products, the bioreactor configurations and the management of the polar products.

Enzymatic biodiesel production is mainly complicated by the low tolerance of lipase toward acyl acceptors such as short alcohols [16]. The alternatives to solvents have been solvent-free reaction media or the use of co-solvents, where oil and glycerol are both soluble in the selected co-solvent. Selecting the appropriate feedstock is of key importance in the cost balance of the process, especially if the analysis is coupled with scaling-up and bioreactor design. Potential alternatives to edible oil have been waste oil, non-edible plant oil, oleaginous microorganisms, microalgae, bacteria, yeast, and fungi. Not only short alcohols may be a problem as phospholipids and chlorophylls may also be inhibitory. The use of a co-solvent (generally a tertiary alcohol such as *tert*-butanol), the combination of lipases, the two-step methods and the added-value byproducts may be attempts to increase biodiesel production, but the cost of implementation is high. The use of methyl esters instead of short alcohols and dimethyl carbonate have also been reported as competitive for tailored products.

Biodiesel and polymers need yields of 2000–10,000 kg-product/kg-immobilized enzyme. Productivities of 50–100 kg product/kg-immobilized enzyme are sufficient for pharmaceutical compounds. The optimized best enzymatic process has 74 times higher yield than the alkali-catalyzed process using 1 wt% sodium hydroxide in every reaction (i.e., 100 kg-biodiesel/kg-catalyst). The lipase price should be 22 times lower than the actual one in the market. Economic optimization requires the analysis of not only an increased plant cost due to a long life time of a lipase but also a decreased separation cost especially in downstream processes. Application of a whole-cell biocatalyst is also a promising approach, but it is not easy.

Alternative approaches to enzymatic biodiesel synthesis includes in situ transesterification with solid feedstock. Problems have arisen in the obtaining of residual biomass and the lipase. These problems have been approached using (1) lipase in germinating seed and (2) a catalyst basket made of stainless steel mesh for the separation and recovery of the supported enzyme. Magnetic supports may be a technological improvement for the separation of the immobilized lipase. Lipozyme TL IM has been used in biodiesel production due to its lower cost (US$ 800/kg) compared to Novozym 435 (US$ 9500/kg), and therefore optimized immobilization methods plus recombinant lipase technologies may be the key to further progress [17].

Health and life sciences are leading to the industry convergence of food and pharma production through important modifications. The products resulting of these are nutritional supplements, functional foods, and medical nutrition. The process involves knowledge convergence which needs consolidation (with technological convergence in terms of supply and market convergence in demand, and finally industrial convergence with maturation). The authors reported that, even when medical nutrition can be thought of as an industry in a sophisticated step of convergence, predictions cannot be made about whether the pharma and food industry sectors will fully converge or whether the medical industrial approach will transform into a blooming field [18].

Figure 5.1 summarizes the main problem in industry related to costs in the enzymatic synthesis of structured glycerides.

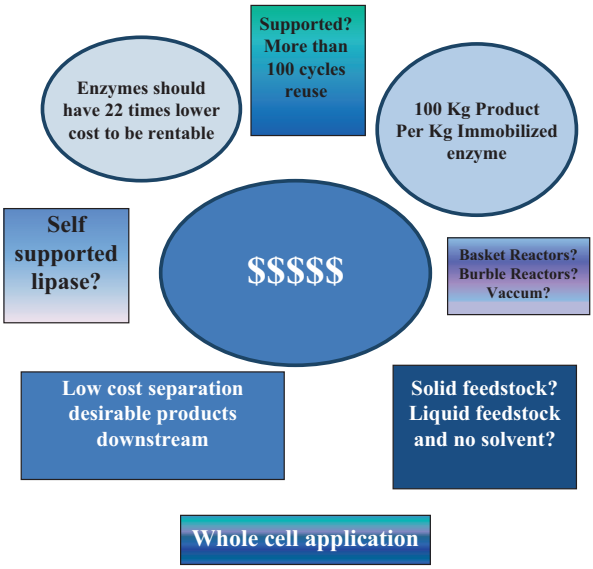

Fig. 5.1 Some costs involved in the potential progress of lipases in structured glycerides synthesis

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Chapter 6
Examples of Successful Industrial Synthesis of Structured Diglycerides and Triglycerides

Contents

6.1 Introduction

As it was mentioned in Chap. 1, there are some structured triglycerides (STs) that have been successfully commercialized. Human milk fat substitutes and cocoa butter equivalent are the STs that mimic fatty acid (FA) composition and structure of the parent fats. In this chapter, the industrial processes for their manufacturing are briefly reviewed. Also it is revised the case of a diglyceride (DG) oil.

6.2 Maternal Milk Fat Substitutes

The human milk presents a distinctive triglyceride (TG) composition: palmitic acid is the most abundant saturated FA (18–25%) and oleic acid is the major unsaturated FA (22–36%) [1]. Some 60–70% of palmitic acid is positioned at the sn-2 position, and stearic, oleic and linoleic acids are placed at the sn-1 or 3 positions of the glycerol backbone [2, 3]. Human milk fat substitutes (HMFSs) mimic this unique composition and position of FAs on the TGs.

Loders Croklaan was the pioneer in the development of a process to produce a 1,3-dioleyl-2-palmitoyl (OPO) TG (Fig. 6.1) that copies the composition and sn-2 palmitic acid content of human milk fat [4]. In 1995, the product obtained European

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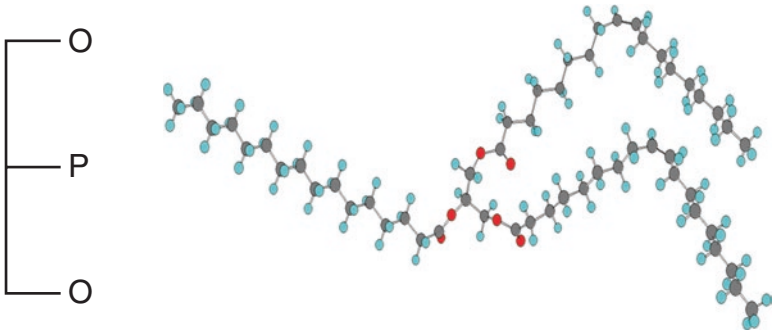

Fig. 6.1 Schematic of OPO triglyceride structure. *P* palmitic acid; *O* oleic acid

approval as an infant formula ingredient, and in following years started to be commercialized as Betapol®.

Figure 6.2 shows a schematic representation of a two-stage process to obtain OPO TG [5, 6]. The process was first patented by in 1987 [7]. The enzymatic acidolysis is performed in two packed bed reactors (PBR) filled with an immobilized 1,3-specific lipase (*Rhizomucor miehei*). The feed is palm tripalmitin and oleic acid. A two-stage process is used in order to increase FA conversion. After the first bioreactor, the intermediate reaction media is distillated to separate the fee fatty acids (FFAs). Then, new oleic acid is added to the purified product, and they enter the second bioreactor. Fatty acids are removed by distillation, and diglycerides and unreacted substrate oil are separated from the final product by fractionation. The resultant fat (OPO) is bleached and deodorized. There are no data regarding the measures taken in order to avoid acyl migration (and the generation of byproducts).

Currently, several commercial HMFS are being manufactured, such as In*Fat*™ (Advanced Lipids—Enzymotec and AAK), Bonamil (Wyeth Ayerst), Cow & Gate Premium (Nutricia), Alsoy (Nestlé), etc. (for more examples, see [4]).

6.3 Cocoa Butter Equivalent

Cocoa butter equivalent (CBE) was mentioned in Chap. 1 as an important ST. The synthesis of CBE involves the specific design of the three dominant TGs of cocoa butter (CB): POP, POS, and SOS (P: palmitic acid, O: oleic acid, S: stearic acid; Fig. 6.3) in order to reach a composition close to that of CB [8]. This composition and distribution at the glycerol backbone give CB a well-defined melting profile.

CBEs are predominantly used for fillings, coatings and supercoatings (CB is replaced by CBE) [8]. A European Union regulation (EU Directive 2000/36) restricts the use of oils enzymatically modified in the production of CBE to be utilized in chocolate-labeled products, and limits the CBE content to no more than 5% in chocolate [9]. The fats that can be used in the vegetable fat part of chocolates are illipe, palm oil, kokum gurgi, sal, shea, and mango kernel. This directive only applies to chocolate marketed within the EU.

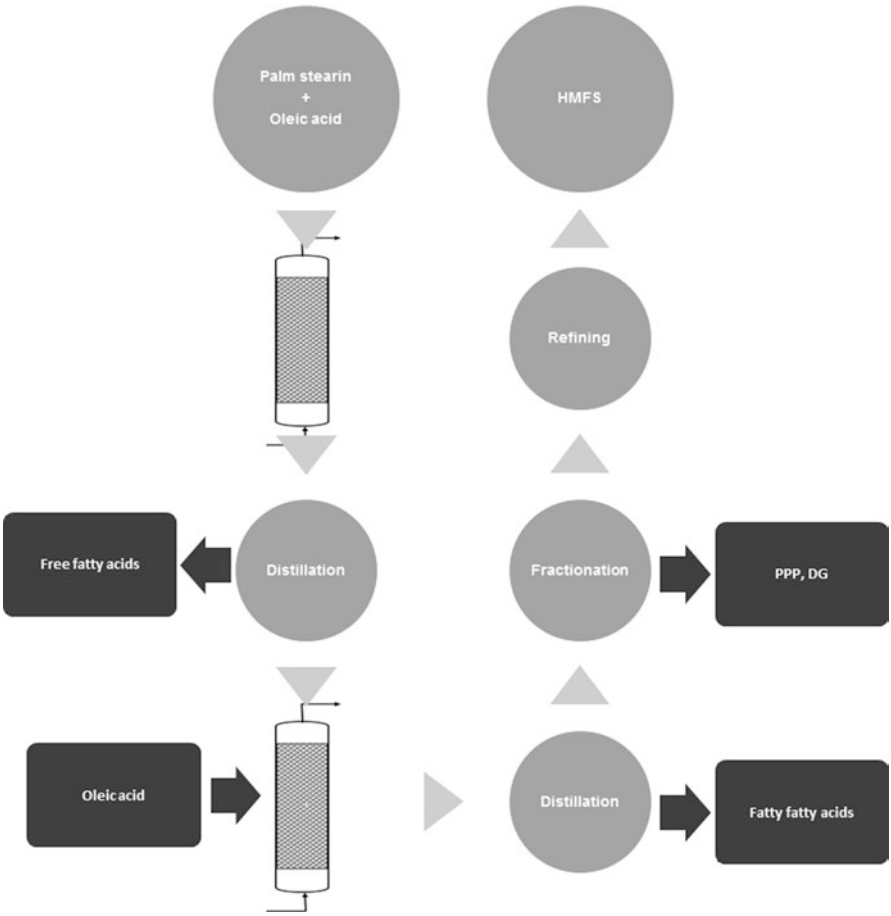

Fig. 6.2 Schematic representation of a two-stage enzymatic process for the production of OPO triglyceride [4, 5]

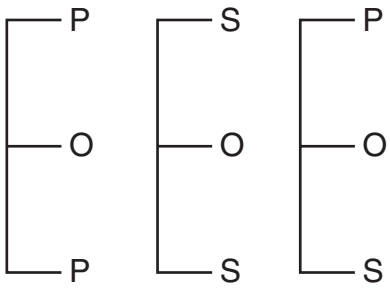

Fig. 6.3 Schematic structure of the three predominant triglycerides in CB. *P* palmitic acid; *O* oleic acid; *S* stearic acid

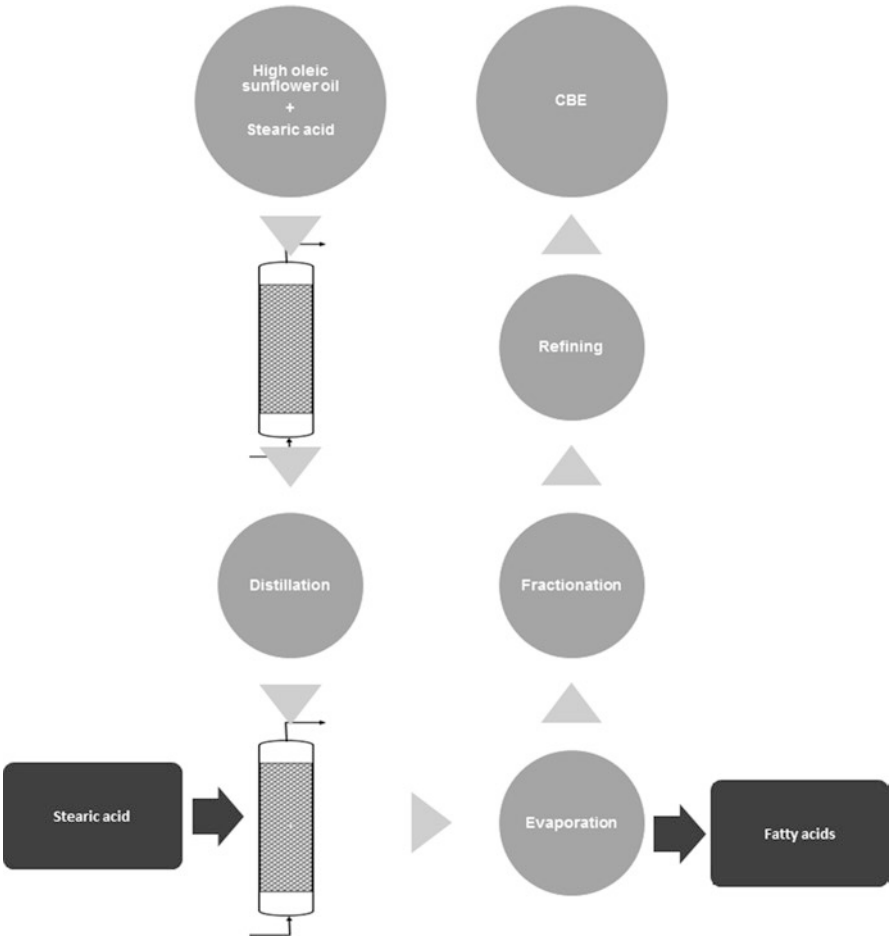

Fig. 6.4 Schematic representation of a two-stage enzymatic process for the production of CBE

In 1980, Fuji Oil [10] and Unilever [11] were pioneers in the enzymatic synthesis of CBE. Figure 6.4 shows a schematic representation of the process applied by Loders Croklaan (Unilever) to obtain CBE by enzymatic acidolysis of high-oleic sunflower oil with stearic acid [5, 12, 13]. The enzymatic acidolysis is performed in two PBR reactors filled with an immobilized 1,3-specific lipase.

Using immobilised *Rhizopus oryzae* lipase, the reaction was performed at 70 °C [14], without solvent in a packed bed reactor. After a reaction time between 50 and 100 h, and a two-stage solvent fractionation process (in acetone), the concentration of SOS was 70.9% (yield = 38%), very close to shea stearin composition.

Fuji oil process utilizes fatty acid esters and TGs for the production of CBEs by enzymatic interesterification [5]. The process can be performed in batch or continuous modes, but the latter is more efficient [15]. A schematic representation of the process is shown in Fig. 6.5, in which palm mild fraction and stearic acid ethyl ester are used as raw materials.

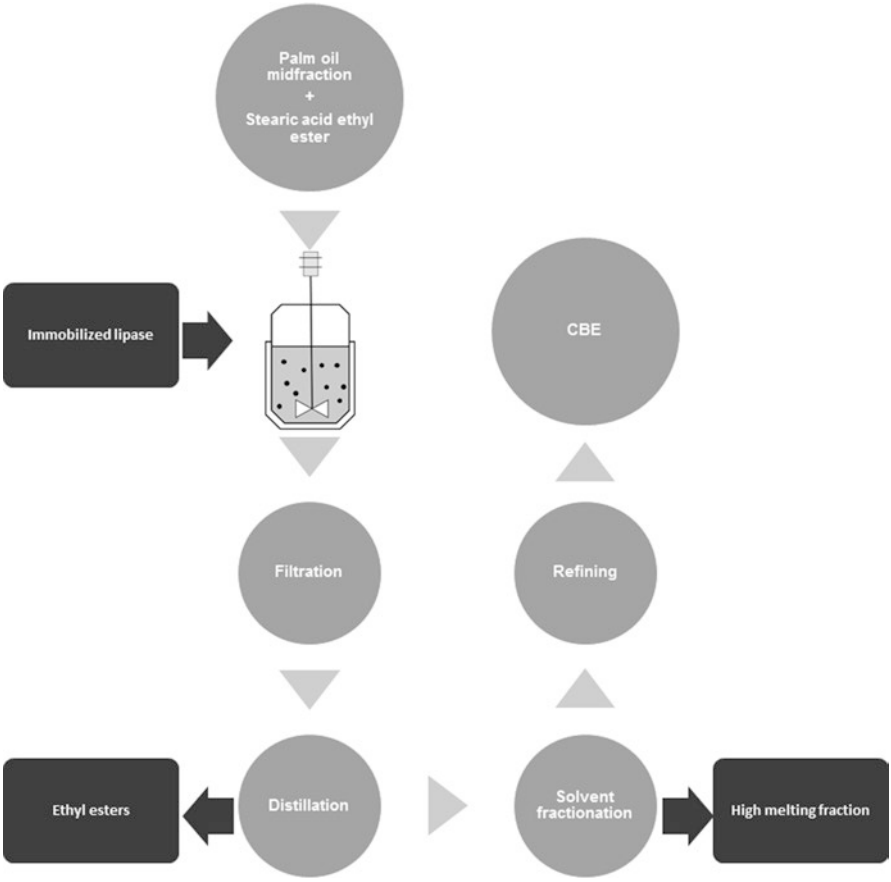

Fig. 6.5 Fuji oil process for the enzymatic production of CBE in one stage [5, 14]

6.4 Diglyceride Oil

"Healthy Econa Cooking Oil" was a cooking oil containing approximately 80 wt% DG, manufactured in Japan by KAO. It was approved by the authorities as a "Food for Specified Health Use" in 1999 [16, 17]. In the USA, it was designated as generally recognized as safe (GRAS) for use in vegetable oil spreads and home cooking oil [18], and was marketed as "Enova Oil" in 1983.

DG oil was produced by the esterification of food-grade fatty acids (prepared from soy and canola) and glycerol (or monoglycerides; MGs) in the presence of an immobilized 1,3-specific lipase (Lipozyme IM) [16, 19]. Figure 6.6 shows a schematic representation of the process applied to produce DG oil. The reaction takes place in a packed bed reactor, with a residence time between 20 and 50 s in order to avoid TG formation. The water content in the reaction fluid is reduced in a dehydration tank under reduced pressure. The reaction fluid is recirculated to the

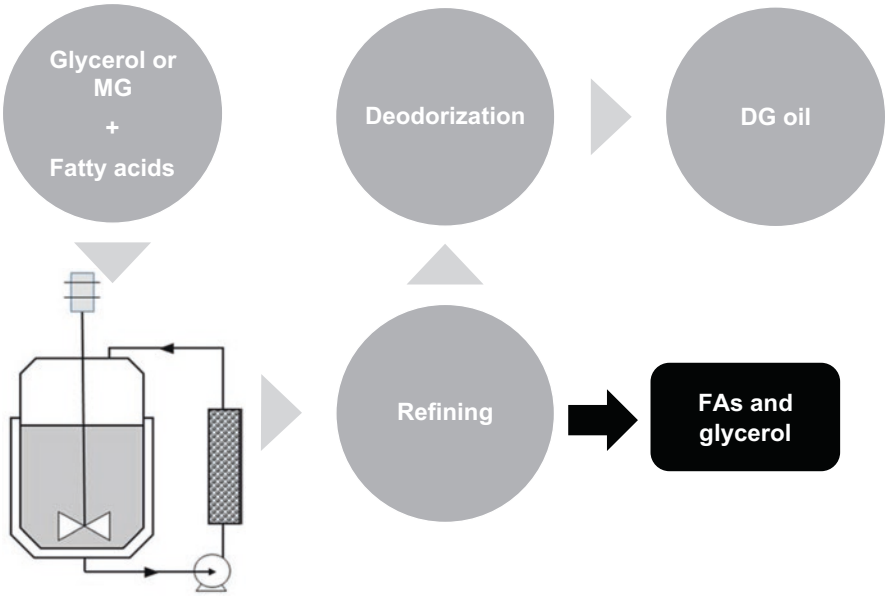

Fig. 6.6 Schematic representation of the manufacturing enzymatic process for the production of DG oil [15, 16]

PBR until the desired DG concentration is achieved. Then, FAs and glycerol are removed from the reaction mixture through molecular distillation. The product is refined and deodorized using techniques commonly employed by fats and oils.

In 2009, Econa products were voluntarily retired from the Japan and USA markets due to the high concentrations of glycidol fatty acid ester (glycidyl esters) detected in the oils. Glycidol (the hydrolyzate of glycidyl ester) has been classified as "probably carcinogenic to humans" [20]. At that time, the total annual sales for the Econa brand were approximately US$ 200 million in Japan, and accounted for 9% of the cooking and salad oil market [21].

Glycidyl ester formation is associated with the fat and oil refining process [22]. The glycidyl ester content in edible oils increased continuously and significantly with increasing deodorization time, and the presence of DGs and MGs could significantly increase its formation.

According to the published information, DG oil has proven safe [17] and there is evidence indicating its beneficial effect for decreasing body weight [23]. As a consequence, a future new opportunity for DG oil in the market could be possible if the glycidyl ester concentration can be reduced to acceptable values.

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Chapter 7
Conclusions

The perspectives of lipases in the food industry related to glycerides were very promising 10 years ago. Today, the market is wide and demanding for high-quality, "healthy" food products. The progress of the research on the value of "healthy" foods with the new findings in the roles of ω3, ω6 and ω9 fatty acids in health and disease are fueling this interest. The importance of structured glycerides as sources of fatty acids is of paramount importance, the metabolism of them being more and more elucidated. Among them, diglycerides seem competitive compared to triglycerides as sources if the processing aspects are improved, especially when high-temperature deodorization should be avoided. The glycerides including polyunsaturated (PUFA) or monounsaturated (MUFA) could be important tools in weight control and hypertension if their benefits are confirmed, but how, when and with what other compounds (such as antioxidants) should be properly clarified. In this sense, even monoglycerides obtained through enzymatic processes could have interesting markets, but the main problem of the high cost of the enzyme is still present as it has always been. Beyond food applications, the field of biolubricants and long alcohol esters of fatty acids could have importance in the renewed interest in lipases at the industrial level in the future.

It seems that the field of lipase application at industrial scales should improve the obtaining and purification of lipases before a breakthrough is expected. The demands for highly qualified personnel in industries with knowledge of fermentation, separation and immobilization or use of lipases in multiphasic systems make the small industries with small assets unavailable due to the high level biotechnology involved. In addition, the application of genetic engineering if needed and the high investments required to obtain results require companies with high levels of resources. It is not surprising that the lipase commercial market is concentrated in 4–5 main companies, which also develop processes with their own technologies and generate many patents in the field. In addition, on times occasions the success or failure of the processes including lipases are not directly related to the lipase activity, stability or costs, but to other aspects of the process or to the physical and chemical aspects of the transformations that the compounds suffer during industrial manufacture.

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The generation of secondary products and their potential toxicity is a very important issue when dealing with PUFA and MUFA, and this aspect should be taken very carefully into account. An example of such situations is the unfortunate result of Enova oil and its withdrawal from the market.

When dealing with the practical aspects, lipases can do many things, but it should be taken into account that they cannot do ***everything***. Not only that, but they cannot do the same as many chemical processes that produce commodities or low-cost products due to unexpected (and sometimes unknown until too late) physical and/or chemical aspects. The consideration of industrial "enzymatic" degumming that it is more a "de-oiling of the gum" (where only the chemical treatment is really operative at the industrial level) is an example of this situation. The process (complete), the reactor, the reaction media, the costs not associated to the lipase but to the separation processes, the desired product, and the desired purity are all key to understanding the (bio) technological aspects. In addition, the regulatory local aspects, the cultural small industry considerations and characteristics, and population demands, needs and tastes should also be taken into account if food is the product.

In general, the situation is such that the main niches are now occupied by the pioneers in the fields, and they have cemented a strong presence in their fields, with the current knowledge ***such as it is***. This is the case of Betapol®, developed by Loders Croklaan (Unilever) and the first OPO-type human milk fat substitutes. There are now others, such as INFAT®, manufactured by Advanced Lipids (joint venture of AAK and Enzymotec). They are the main milk substitutes around the world, and they have much competence in the market. Another clear example is CLEATechnologies in the field of cross-linked enzyme aggregates in Europe.

It seems to us that the field can go forward when local sources of lipases, improved, adapted and modified locally with research and development, can be included almost as an industrial plant into the main industrial plant in the production process. The importation of commercial lipases is very expensive and the obtaining of local sources of lipases and their purification, testing and evaluation at laboratory and industrial scales should be an important goal for the future. Costs become competitive if the company has its own lipase and does not depend on a foreign supplier. In addition, as an alternative, only when low-cost, easy to perform, genetic engineering is developed can it be applied to the production of engineered lipases which could be widespread even for small industries.

Several aspects have been detected during the writing of this book to be critical in the biotechnological aspects of lipase implementation at an industrial level:

(a) **The use of a single media or biphasic reaction media with enhanced activity and stability of the active lipase**

Lipases very active in acetone or low-cost solvents should be available. The use of ionic liquids or eutectic solvents is being studied mainly at the academic level in 2016. Non-toxic solvents or no solvent at all should be selected.

(b) **The use of non-immobilized lipase as a route to minimize costs**

There are several examples of the application of lipases to biphasic systems or "one use only" in the field of industrial biodiesel, but not in the field of structured glycerides (at least, not yet).

(c) **The use of alternative low cost feedstocks to lower additional costs**

Low-cost, local and affordable feedstocks could be part of an overall strategy to decrease the needed inversion, but they are not enough to make an overall production affordable. This low-cost feedstock should be transformed into a high-added value product, easy to separate, using low-cost, cheap and easy to apply lipases.

(d) **The improvements in the lipase support to avoid filtration and to include, for example, magnetic separation, and with focus on nanomagnetic compounds encapsulated in the solid support**

The development of magnetic supports able to be separated, avoiding filtration and support/biocatalyst manipulation, is urgently needed. The leaching of the lipase should be very carefully avoided, such as the partial dissolution of the support and the potential contamination of the product.

(e) **The use of intensification of processes with cavitation or other particular configurations such as pervaporation**

When using process intensification, the increase in productivity should be able to compensate for the additional inversion to allow the intensification to take place.

(f) **The integrated approach of fermentation to produce the source of the lipase and its isolation and purification, plus the application of the purified lipase in the desired reaction at industrial scale**

The processes should avoid very expensive equipment and technology, and there should be more collaboration among universities and industries to achieve the needed goals of high fermentation efficiencies, easy lipase separation, low production of waste, and high activity and stability of the purified lipase.

(g) **The alternative sources of lipases such as plants or whole cells or the presentation of self-supported lipases in nature**

The alternative sources of lipases (such as plants or algae) require the contribution of the fields of botany and or plant biology experts, working with the usual staff required for industrial scaling (such as (bio)chemists, chemical engineers and biotechnologists).

(h) **The application of recombination methods or metagenomics to improve the activity and stability or both**

The techniques of genetic engineering and metagenomics should lower the costs.

(i) **The improvements in processes to avoid operations downstream of the reactor and to optimize productivity and costs**

The processes should be optimized to avoid, for example, high-temperature deodorization or very expensive separations (for example, the use of molecular distillation).

(j) **The configuration of bioreactors using basket or self-immobilized lipases.**

The use of basketed immobilized lipases or self-immobilized lipases or lipases immobilized onto very cheap supports should be properly studied, evaluated and parameterized in such a way that the industrial scaling possibilities increase.

(k) **The analysis of advantages and disadvantages of the use of adsorbents for water uptake (among them, high cost) or the application of vacuum or gas-flow (preferred)**

The literature on the topic demonstrated that the use of adsorbents is not the best to separate water or alcohols generated in lipase-mediated reactions. In addition, water or alcohol, fatty acids, MGs and DGs may also be adsorbed. The best configurations are those where gas-flows are used or vacuum is applied to separate, during the reaction, the polar, non-desirable, low-molecular weight products from the non-polar, desirable and relative higher-molecular weight products.

(l) **The purification of feedstocks to fit a suitable quality for enzymatic inter-esterification and other reactions**

Lipases should be more robust in order to process unpurified feedstocks. The cost of production of robust lipases should be lower than the costs of the required purification steps.

(m) **The reuse of lipases is a key point in applications on the industrial scale**

Unfortunately, there has not been sufficient interest in the reusability of free and immobilized lipases in research and development.

A strong interaction between academia and industry is needed to focus on the industrial aspects that require much more study and improvement.

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