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Synthesis and Characterization of Phenolic Lipids

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<http://dx.doi.org/10.5772/66891>

Abstract

Omega-3 polyunsaturated fatty acids (ω 3 PUFAs) from fish oils promote well-established health and antiaging benefits that justify their use as functional ingredients in dietary supplements, healthy foods, and nutraceutical products. Dietary supply is needed because human metabolism exhibits limited ability to synthesize ω 3 PUFAs. However, the practical use of such lipids as food ingredients is often limited due to their high susceptibility to oxidation, which is responsible for the undesirable off-flavor and odor of rancid oils, associated with the loss of nutritional value. Produced phenolic lipids were a good solution for all these problems. These phenolic lipids are preferentially produced by enzymatic bioprocesses that exhibit high selectivity toward polyfunctional substrates and mild reaction conditions compared with chemical synthesis pathways. This chapter presents the acylation of phenolic compounds and lipids using enzyme under various operating conditions. In conclusion, the acylation of lipids with natural phenolic compounds resulted in the formation of a lipophilic ester that should be able to stabilize the oil, fats, and emulsions against oxidation. Acylation of lipids to phenolic compounds that have antioxidant properties thus protects the lipids from oxidation, and the phenolic lipid derivatives carry the combined health beneficial properties of lipids and the phenolic molecules.

Keywords: phenolic compounds, omega-3, phenolic lipids, enzymatic esterification, oxidative stability, neuroprotection, fish oil

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1. Introduction

In the recent years, interests for natural substances with beneficial activity to human have sharply risen. In fact, there is a significant increase in nutraceuticals and pharmaceutical products, based on natural compounds. The main interest has been observed for natural substances with strong antioxidant activity because oxidative stress induced by multiple factors is the main cause of many pathological conditions such as inflammation, cancer, coronary

heart disease and even skin aging. Also, there has been a significant consumer interest in health enhancing the role of specific foods or physiologically active food components.

Unsaturated lipids have been widely recognized for their role in the maintenance of human health. These lipids, especially those from the omega-3 (ω -3) series, have been linked to inhibitory effects on atherosclerosis cardiovascular and Alzheimer's diseases [1–3]. However, the use of such lipids remains strongly limited due to their high susceptibility to autoxidation. To overcome this difficulty, a lot of researches have been carried out focusing on the development and the use of antioxidants that could delay or even prevent omega-3 lipid oxidative degradation. In this context, natural plant phenols were perceived by many researchers as potential substitutes for controversial synthetic antioxidants; however, the major drawback of these compounds is their low solubility in matrices that strongly restrains their use in food applications [4, 5].

The hydrophilic nature of phenolic compound reduces their effectiveness in oil-based formulae and emulsions [6]. The synthesis of more lipophilic derivatives, especially esters, could help to increase their lipophilicity and then their interactions with lipidic phases that need to be stabilized. To achieve this goal, acylation with fatty acids appears as a promising way (lipophilization) that could extend the scope of application of phenolic antioxidants in lipid-rich food matrices. When applied to polyunsaturated lipids, this approach is expected to provide stable ingredients with high nutritional value and high antioxidant potential. Additional effects could be an increased bioavailability of phenols as well as cumulative and even synergistic biological activities [7, 8].

Many studies reported the enzymatic synthesis of phenolic lipids based on the ability of lipases to catalyze the acylation of phenolic compounds with either fatty acids or triacylglycerols (TAGs) [9–13]. Main advantages of enzyme-catalyzed processes include the use of mild reaction conditions that limit substrate degradation and high selectivity that avoids the production of undesirable compounds and facilitates further purification protocols [14].

2. Fatty acids and phenolic compounds

Dietary fat is an essential component for digestion, absorption, and transport of fat-soluble vitamins and phytochemicals, such as carotenoids and lycopenes. Dietary fat contributes approximately 34% of the energy in the human diet. Because fat is a main source of energy (9 kcal/g), humans are able to obtain adequate energy with a reasonable daily composition of fat-containing food item products.

2.1. Fatty acids

Fatty acids are classified as saturated fatty acid (SFA), monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA). The essential fatty acids (EFAs) refer to those polyunsaturated fatty acids (PUFAs) that must be provided in our food because these EFAs cannot be synthesized in our body, and they are necessary for a good health. The main two families of EFAs are omega-3 (ω -3) and omega-6 (ω -6). ω -3 and ω -6 structures are based on the position of the double bond from the methyl (omega) terminal of the aliphatic carbon chain [1, 15]. The

parent fatty acid of the ω -6 series is linoleic acid (18:2n-6) and the parent fatty acid of the ω -3 series is linolenic acid (18:3n-3). ω -3 includes alpha-linolenic acid (ALA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) (**Figure 1**).

Human body can synthesized omega-3 and omega-6 from linoleic acid and linolenic acid, respectively, through a series of desaturation (addition of a double bond) and elongation (addition of two carbon atoms) reactions [16]. Unlike linolenic and linoleic acid, oleic acid (18:1n-9) is consumed in substantial amounts in the Western diet and is not an essential fatty acid. There is a little eicosatrienoic acid (ETA, 20:3n-9) in cell membranes, however, probably because of the overwhelming competition from dietary linoleic acid for the relevant desaturase and elongase enzymes. The pathways for desaturation and elongation of ω -3 and ω -6 fatty acids are given in **Figure 2**.

2.2. Health benefits of omega-3 fatty acids

The ω -3 fatty acids provide a wide range of benefits from general improvements in health to protect against inflammation and disease. Several studies have indicated that the consumption of ω -3 fatty acids provides benefits in reducing the risk of cardiovascular diseases [1, 2].

Omega-3 fatty acids

Omega-6 fatty acids

Figure 1. Omega-3 and omega-6 fatty acids.

Figure 2. Desaturation and elongation pathway of ω -3 and ω -6 fatty acids.

DHA and EPA have been used in a number of small clinical trials to understand their efficacy and shown to possess immunomodulatory properties depending on their localization in different cell types. DHA is selectively incorporated into retinal cell membranes and postsynaptic neuronal cell membranes, suggesting that it plays important roles in vision and nervous system function [17–19]. DHA content in the brain may be particularly important, since animal studies have shown that depletion of DHA in the brain can be resulted in learning deficits. It is not clear how DHA affects brain function, but changes in DHA content of neuronal cell membranes could alter the function of ion channels or membrane-associated receptors, as well as the availability of neurotransmitters [20, 21]. Increasing ω -3 fatty acid intake enhances the DHA content of cell membranes, resulting in higher proportions of DHA in the body (Figure 3).

The ω -3 fatty acids are reported to associate with the brain development; also, it is important for the vision and the functions of the reproductive system. This may be due to the fact that DHA is a component of brain nerve synapses, in the eye's retina, in the testes, and in sperms and plays a vital role in the development and functions of these organs and systems [20]. The nervous system contains approximately 35% PUFAs as its lipid content; most of which are long-chain (LC) PUFAs. In addition, higher prenatal intake of DHA has been shown to be associated with improved visual, cognitive, and motor development in offspring. Children given ω -3 PUFA-supplemented formula demonstrated enhanced visual and mental capabilities [19],

Figure 3. Beneficial effects of omega-3 oil for human body.

while in human adults, clinical studies have suggested a low intake or inadequate. The ω -3 fatty acids possess antithrombotic properties, which in combination with their anti-inflammatory effect is likely to positively aid cardiovascular disease treatment. DHA and EPA also appear to possess anticancer and antiapoptotic effects. Additionally, these PUFAs suppress gene expression of lipogenic genes in the liver and trigger adipose fatty acid oxidation, suggesting a potential role against obesity [15, 22].

2.3. Phenolic compounds

Phenolic compound is chemically defined as a substance that contains an aromatic ring containing one or more hydroxyl substitute including functional derivatives [23]. In general, phenolic compounds are present in a wide variety of food plants as esters or glycosides

conjugated with other compounds, such as flavonoids, alcohols, hydroxyl fatty acids, sterols and glucosides. Phenolic compounds found in foods may be categorized accordingly to three groups, simple phenols and phenolic acids, hydroxycinnamic acid derivatives and flavonoids. The simple phenols include the monophenols, such as p-cresol found in berry fruits (e.g., raspberry, blackberry) and diphenols, such as hydroquinone found commonly in vanilla [5, 24].

Phenolic compounds play a major role in the protection against oxidation processes. The antioxidant properties of phenolic compounds can act as free radical scavengers, hydrogen donors, metal chelators and singlet oxygen quenchers [25, 26].

2.3.1. Nutritional and antioxidant properties

Phenolic compounds are natural antioxidants that are present in food or in the body, to delay or stop the oxidation of that substance. The main advantages of these natural antioxidant are (1) they are readily acceptable by the consumers; (2) they are considered to be safe; (3) no safety tests are required by legislation; and (4) this natural antioxidant is identical to the food which people have taken over a hundred years or have been mixing with food. Phenolic compounds are associated with nutritional and organoleptic qualities of foods from plant origin [24, 26]. Phenolic compounds at low concentration protect foods from autoxidation, but at high concentration, they can cause undesirable discoloration as a result of their interaction with the carbohydrate or protein components.

Among naturally found phenolic compounds, phenolic acids are of high interest due to their potential biological properties [27, 28]. Many phenolic acids are known to be potent antioxidants through their radical scavenging activity, and due to their chemical structure, the reactivity of phenolic acids increases as the number of hydroxyl and methoxyl groups increases [29]. The consumption of fruits, vegetables, and soft drinks such as tea and coffee, which contain phenolic compounds, has been linked to lower risk of some diseases, such as cancer and CVD [30, 31]. However, the use of phenolic acids as natural antioxidants in foods and nutraceutical supplements has the limitation of low solubility in oil-based media. Nevertheless, lipase-catalyzed reactions of lipids with phenolic acids could produce structured lipids with phenolic moieties, which would have health benefits and improved solubility characteristics [32–35].

2.4. Synthesis of phenolic lipid (PL) compounds

Phenolic lipids (PL) are types of fats and oils modified to improved nutritional or physical properties by incorporating phenol compound on the glycerol backbone. Phenolic lipids play an important role as antioxidant and biological active compounds, but their contents in the nature are minor, and the procedures for separation and purification are not easy, very expensive and take a long time, which makes their applications in the food or cosmetic industry very inconvenient. Consequently, the synthesis of PL has attracted more attention in recent years because it is a good way to improve the hydrophobic nature of phenolic compounds, which could be achieved by chemical or enzymatic synthesis.

2.4.1. Chemical synthesis of phenolic lipids

Chemical synthesis is a traditional method that is used for PL preparation. Synthesis of PL through chemical synthesis could be done by using Friedel-Crafts acylation reaction or Fisher acid catalysis esterification. These processes are generally carried out at relatively high temperatures and pressures under anhydrous conditions, using rather unspecific alkali metal or alkali catalysts. Some related works have been provided in this topic, one of them is the work of Qianchun et al. [36] about the chemical synthesis of phytosterol esters of polyunsaturated fatty acids (PUFAs), which could be used in different formulations of functional foods. Direct esterification of phytosterols with PUFA was catalyzed by sodium bisulfate to produce sterol esters of PUFA without organic solvent. The modeling of sodium bisulfate with superfluous fatty acids as solvents to synthesize phytosterol esters of PUFA was successfully performed with degree of esterification up to 96% and less oxidative products in the reaction process [36].

The chemical esterification of flavonoids with some fatty acids was provided by [37] and its product exhibited lipophilic, antiradical and antioxidant properties. In works reported by Zhong and Shahidi [38, 39] on epigallocatechin gallate (EGCG), the predominant catechin in tea was structurally modified by esterification with fatty acids, including stearic acid (SA), docosapentaenoic acid (DPA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The esterification of EGCG with these fatty acids using acylating agents, namely, the acyl chlorides, resulted in yields of 65.9, 42.7 and 30.7 for SA, EPA and DHA, respectively [39]. This esterification leads to produce various compounds that have anti-inflammatory effect and also shows higher inhibition effect against hydroxyl and peroxy radical-induced DNA scission [38]. Phenolic lipid (PL) chemical synthesis meets some partial needs; this pattern possesses a low degree of regioselectivity and is generally accompanied by drastic reaction conditions, many intermediary stages and purification steps to remove by-products and catalyst residues. The main drawbacks to chemical transesterification are (1) non-selectivity leading to random distribution of FAs, (2) isomerization of sensitive PUFAs by the alkali catalyst, (3) production of fatty acid soaps and unwanted by-products and (4) requiring substantial post-treatment and downstream processes, especially when food applications are concerned.

2.4.2. Enzymatic synthesis of phenolic lipids (PLs)

The application of enzymes is widely in different fields such as pharmaceutical, cosmetic and food industry. Enzymatic synthesis of PL from fats and oils is receiving a lot of attention as a method for their modification because of the advantages of milder reaction conditions, minimization of side reactions and by-product formation, a selective specificity, a wider variety of pure synthetic substrates, fewer purification steps and a more environmentally friendly process [40]. Even if enzymes may be more expensive than chemical reagents, the enzyme-catalyzed acylation is a well-mastered technique for synthesis of selectively modification of PL at present. A high degree of conversion to the desired products could be achieved under the optimal reaction conditions. The enzymatic processes can be used in the production of fats and oils containing beneficial fatty acids and phenolic compounds. Some reviews have given a comprehensive understanding and shown a whole outline on the enzymatic synthesis of PL [41–46].

In particular, enzymes appear to be very effective for the synthesis of molecules involving the grafting of a lipophilic moiety or a hydrophilic one. This review will be described and discussed in some of the recent works in the field of enzyme-assisted acylation of fatty acids with phenolic compounds in order to modify the hydrophilic/lipophilic properties of the initial molecules to obtain new products with multifunctional properties combining, for example, antimicrobial, antioxidant and emulsifying properties. The enzymatic synthesis of phenolic lipids has been reported previously in Refs. [10, 45, 47–49]. A lot of enzymes can be used in the synthesis of PL and selectivity is the most important characteristics of enzymes used in phenolic lipid synthesis. Lipase is the most enzymes used in this type of process because of high selectivity, lower overall reaction time and fewer side reactions when compared with chemical methods [50]. An example of a synthesis reaction catalyzed by the lipase is shown in **Figure 4**. This overwhelming interest is based largely on consumers' desire to maintain overall well-being with minimal effort and an industries' ability to respond to this need. Furthermore, with the consumption of manufactured foods continually on the rise, there is a distinct advantage to providing more healthful choices for consumers. The concept of a natural phenolic lipid composed of a long-chain aliphatic and phenolic moiety readily fits this mold, particularly since the inclusion of unsaturated lipids into these compounds could result in additional nutritional benefits. Lipases constitute the most important group of biocatalysts for biotechnological applications.

Figure 4. Enzymatic synthesis reaction capsates (fatty vanillyl alcohol acid ester) catalyzed by lipase Novozym 435® [51].

2.5. Lipases

2.5.1. Definition, sources, and applications

Lipase enzymes are defined as glycerol ester hydrolases that can hydrolyze tri-, di-, and monoacylglycerols [52, 53]. Lipases are soluble in water as a result of their protein nature, but it could act on lipids, which are water insoluble, at the interface between oil and water [53, 54] and catalyze esterification and transesterification in addition to the hydrolytic activity on TAG [55–57]. Lipases are originated from a wide variety of sources including animals, plants and microorganisms. Animal lipases include pregastric esterase, pancreatic lipase, and lingual lipases [53]. Plants such as wheat germ and castor beans also contain lipases [58, 59]. Finally, microbial sources including yeast (*Candida* and *Geotrichum*), molds (*Rhizopus*, *Aspergillus*), and bacteria (*Bacillus*, *Pseudomonas*) [60]. Lipases are widely used because of their ready availability, low cost of production, utility in food, biotechnology, and pharmacology [61]. Novel biotechnological applications have been successfully established using lipases for the synthesis phenolic lipids, the production of pharmaceuticals, agrochemicals, and flavor compounds [52, 62, 63]. Moreover, the use of lipases in the food industry is increasing due to the need for the production of esters, biodegradable polyesters, and specific FAs [64].

2.5.2. Mechanism of action

Lipase-catalyzed reactions have been gained a lot of interest over the last years; the major reason for this is that lipase can promote either ester formation or ester hydrolysis. Moreover, lipase can control the acylation and deacylation to produce specific fatty acids and triacylglycerols (i.e., phenolic lipids). Lipase-catalyzed reactions can be classified into three groups which are hydrolysis, esterification, and transesterification [65].

2.5.2.1. Hydrolysis

Hydrolysis of lipids by lipases refers to the splitting of fat into its constituent acids and alcohols in the presence of water. Lipase-catalyzed hydrolysis can be used for the preparation of fatty acids from oils, especially for the selective hydrolysis and concentrations of PUFAs from edible oils [10]. Furthermore, lipase catalyzed hydrolysis reactions only in the presence of amount of water. This is due to the fact that water molecules participate in the breaking of covalent bond in the substrate as well as subsequent incorporation of their elements into these bonds to form reaction products [66].

Different products are determined during the extent of hydrolysis reaction as shown in **Figure 5**. Mixtures of monoacylglycerols, diacylglycerols and free fatty acids are produced; the more complete the hydrolysis, the higher the concentration of free fatty acids in the final reaction medium. In the end of lipase- hydrolysis reactions glycerol esters-enriched in ω -3 fatty acids were produced from fish oil. Reactions are ideal for removal of fatty acids from unstable oils, including conjugated or highly unsaturated fatty acids, which effectively reduce unwanted oxidation reactions [67]. Lipase catalyzed hydrolysis reactions produce glycerol esters enriched in ω -3 fatty acids from fish oil [68, 69]. Because natural fish oils do not contain more than about one-third of their fatty acids from the ω -3 family, hydrolysis reactions are particularly helpful for the purpose of concentration.

Figure 5. Enzymatic hydrolysis of triacylglycerol molecule. Reverse reaction corresponds to synthesis by esterification. R1, R2 and R3 are different acyl groups.

2.5.2.2. Esterification

Esterification is the reverse reaction of hydrolysis and is used to synthesize selected products under appropriate reaction conditions [70]. The products of an esterification reaction are usually an ester and water. The water content of esterification reaction system strongly effects on lipase activity. Low water content shifts the equilibrium of the reaction to favor the synthesis of lipids. So that additional techniques were used to drive synthesis reaction including removal of water that formed during the process by evaporation under reduced pressure [71] or by adding molecular sieves to adsorb the water. Direct enzymatic esterification of some primary alcohols and selected carboxylic acids was catalyzed by the *Candida antarctica* and *Rhizomucor miehei* lipases. The reactions were performed in solvent-free medium with the removal of water [72].

2.5.2.3. Transesterification

Transesterification is a process of acyl exchange between two molecules. This process normally takes place between an ester and alcohol (alcoholysis), an ester and an acid (acidolysis), or an ester with another ester (interesterification), and no water is involved in the reaction. Acidolysis is one of the most frequently used reactions to incorporate novel fatty acids into TAG in several researches [13, 73, 74]. Interesterification involving hydrolysis and esterification, firstly hydrolysis of the TAG molecule, then followed by re-synthesis of the liberated fatty acids onto the glycerol molecule. Interesterification is another main strategy to incorporate PUFAs into TAGs. The literature reported extensive research work on the interesterification reaction [75, 76]. Lipase-catalyzed alcoholysis, acidolysis, and interesterification reactions are described clearly in **Figure 6**.

2.5.3. Selectivity and specificity of lipase

It is beneficial to have knowledge about lipase selectivity/specificity to guide research to the best choice of lipase for particular fatty acid or for synthesis of PL containing ester of a specific fatty acid. Specificity generally refers to the ability of enzyme to differentiate between several substrates. Lipases can be divided according to their specificity into three groups: (i) nonspecific lipases, (ii) acyl-group specific lipases, and (iii) positional specific lipases. Nonspecific lipase can catalyze the release of FA from any position on the glycerol molecule. Acyl-group specific lipases catalyze the release of a particular type of FA from the TAG molecules, while positional lipases attack sn-1,3 positions on the TAG molecule. The use of positional specific lipases has led to the production of useful TAG mixtures whose composition could not be produced by simple chemical transesterification. In recent years, positional specific lipases have been intensively used in research purposes and food industry sectors [77–79].

2.6. Enzyme reactions in organic solvent media (OSM)

Enzymes in organic solvents have manifested good selectivity and stability; however, catalytic activities in this environment are generally lower than in aqueous solutions. This could be partly explained by the fact that in low water environments, enzymes are less flexible. On

Alcoholysis

Acidolysis

Interesterification

Figure 6. Lipase-catalyzed transesterification reactions. R1, R2, and R3 are different acyl groups.

the other hand, the activities of enzymes also depend on the type of organic solvent, since some are known to inactivate or denature biocatalysts. Meanwhile, the advantages of using organic solvent media are increased solubility of hydrophobic compounds that permits for greater interactions between substrates and enzymes as well as advantageous, partitioning of substrates and products; specifically, this is because partitioning of products away from the enzyme can decrease the possibility of inhibition due to excess product around the biocatalyst [51, 72].

When enzymes are placed in OSM, they exhibit novel characteristics such as altered chemo- and stereoselectivity, enhanced stability, increased rigidity, insolubility, and high thermal stability [80]. It has also been reported that the thermal stability of lipases can be improved in organic solvent systems since the lack of water prevents the unfolding of the lipase at high temperatures [81]. The activity of lipase in OSM depends on the nature and concentration of the substrate and source of the enzyme. Moreover, the organic solvent used can dramatically affect the activity of the lipase. Lipases are more active in n-hexane, n-heptane, and isooctane as compared to other solvents, such as toluene, ethyl acetate, and acetonitrile [82, 83]. It has been reported that the hydrophobicity of the solvent can affect the degree of acyl migration during transesterification using a 1,3-specific lipase [84]. Since the choice of organic solvents based on minimization of acyl migration may conflict with maximization of transesterification, acyl migration is usually minimized by reducing reaction times [85]. With increasing concern for the environment, synthesis of PL in solvent-free systems [86–88] and ionic liquids systems [89] has been extensively studied.

The enzymatic synthesis of vanillyl-PUFA esters from fish oil and vanillyl alcohol in acetone solvent medium was studied by [10]. Lipase-catalyzed esterification of vanillyl alcohol with different fatty acids was carried out by [51] to the synthesis of capsiate analogs. Equimolar concentration of vanillyl alcohol and fatty acid was solubilized in *tert*-butanol and esterified using *C. antarctica* lipase (Novozym 435) at 55°C for 4 h.

2.7. Enzyme reactions in solvent-free medium (SFM)

Enzymatic catalysis in solvent-free medium (SFM) has attracted considerable interest in the recent years [90]. It used as an efficient approach to the synthesis of natural products, pharmaceuticals and food ingredients. Under nonaqueous conditions, the industrial utility of enzymes can be improved, recovery of product and enzyme is eased, and the ability to catalyze reactions that are not favorable in aqueous solutions [91]. However, it would be technically beneficial if the enzymatic reactions were performed in mixtures of substrates in the absence of solvents. Lipase-catalyzed PL has been extensively studied in systems using organic solvents; however, if such a process is intended to be used in the food industry, it is preferred to develop solvent-free systems. The downside of organic solvents is that they are expensive, toxic and flammable and their use involves higher investment costs to meet safety requirements [80]. On the other hand, solvent-free systems, which are a simple mixture of reactants and the biocatalyst, present the advantages of using nearly nonaqueous organic solvents while offering greater safety, reduction in solvent extraction costs, increased reactant concentrations and consequently higher volumetric productivity defined as kg product per unit of reactor volume [53, 80].

Phenolic lipids have been received increasing attention in the food area, since they are a good way for providing nutraceutical FA to consumers. Hong et al. [47] studied the esterification of vanillyl alcohol with conjugated linoleic acid under vacuum in solvent-free system. Further studies on the enzymatic synthesis of structured phenolic lipids in SFM have also been conducted by [34, 44, 92]. In these studies, phenolic acids were esterified with fatty acids resulted in the formation of more lipophilic constituents that can be used as a nutraceutical product. In addition, feruloylated mono- and diacylglycerols were synthesized in SFM using *C. antarctica* lipase, and the yield was 96% [92].

Lipase-catalyzed synthesis in SFM has a number of advantages as compared to that in OSM, including the use of a smaller reaction volume, maximization of substrate concentration and with no additional solvent recovery. In addition, downstream processing is easier as fewer purification steps are required providing significant cost savings, as well as toxic organic solvents are completely avoided (clean conversions), and an increase in the volumetric productivity can be achieved [80]. However, there are some problems with the use of SFM, mainly, the high viscosity of the medium as well as the production of high amounts of glycerol, free FAs as by-products. These by-products affect the reaction equilibrium and limit the mass transfer rate [93]. Thus, the development of a bioprocess for the lipase-catalyzed synthesis in SFM is of major interest but with great challenge.

2.8. Parameters affecting the enzyme activity and conversion yield of phenolic lipids

Grafting of phenolic compound substrates with lipids is the major difficulty to overcome in such lipase-catalyzed reactions. Several parameters must be considered in order to achieve

the reaction in satisfactory kinetics and yields and to overcome the fact that the two substrates greatly differ in terms of polarity and solvent affinity.

2.8.1. Effect of solvent

The interesting strategy is to carry out the synthesis reaction without using solvent. However, when it is not possible, the choice of an adequate solvent is important. The type of organic solvent employed can dramatically affect the reaction kinetics and catalytic efficiency of lipases [94]. Two factors must be considered when solvent is selected; solvent affects the enzyme activity and solvent effect on the equilibrium position of the desired reaction. Polarity of the solvents is an important characteristic which determine the effect of solvents on enzymatic catalysis reactions. Log P value, the partition coefficient between water and octanol, is used as the indicator of solvent polarity. Laane et al. [95] reported that solvents with $\log P < 2$ are not suitable for enzyme-catalyzed systems, since they strip off the essential water from the enzyme and therefore inactivate them. However, solvents with $\log P$ values in the range of 2–4 were weak water distorters, in which enzymes display medium activity and solvents with $\log P > 4$ are ideal media for enzyme-catalyzed systems since they do not distort the essential water from the enzyme. Therefore, intermediate polarity media are often chosen. Other factors that must be taken into account in determining the most appropriate solvent for given reaction include solubility of reactants, solvent inertness, density, viscosity, surface tension, toxicity, flammability, waste disposal, and cost [96]. A good contact between the substrates must be obtained, and the selected solvent must be solubilizing them at least partially.

Various authors have tried to find original strategies to improve enzyme activity in organic solvent [71, 93, 94]. The effect of solvent concentration on the conversion yield of phenolic lipids synthesized from flaxseed oil and phenolic acids was demonstrated by [41]. Solvent concentration of 7% was the best concentration with 61.1% of conversion yield.

2.8.2. Lipase conditioning

Another important parameter in the synthesis reactions of phenolic lipids (PLs) is concerning with the enzyme itself and especially its conditioning. Various techniques for lipase conditioning have greatly improved during the last 10 years in the field of enzyme immobilization, chemical modification, or molecular engineering [97, 98]. Lipases are used after immobilization on a support. Different carrier materials are employed, and the resulting immobilized enzyme usually exhibits an improved thermostability compared to its free form. Moreover, the use of immobilized enzymes allows an easy removal and recovery of the biocatalyst once the reaction is over [99]. Lipase from *Candida rugosa* was immobilized onto montmorillonite via two techniques, i.e., adsorption and covalent-binding montmorillonite [100].

2.8.3. Influence of water activity

Water content refers to the total amount of water present in the reaction system. Controlling of water activity is very important in lipid modification processes. Water content in the reaction system is a determining factor in whether the reaction equilibrium will progress toward

hydrolysis or ester synthesis [101]. While ester synthesis depends on low water content, too low water activity prevents all reaction from occurring. The monolayer of water on the surface of enzyme is required to maintain the three-dimensional structure of the enzyme, which is essential to enzymatic activity [102]. This layer acts as a buffer between the enzyme surface and the bulk reaction medium. However, too much water can cause hydrolysis of the TAG [14]. The activity of lipases at different water activities is dependent on the source of the enzyme and the type of solvent and immobilization support used [103]. Lipases from molds have shown to be more tolerant to low water activity than bacterial lipases. The optimal water content for most interesterification reactions by different lipases has been reported to be in the range of 0.04 to 11% (w/v) [104].

However, the amount of water in the system should be minimized in order to decrease the by-products. Lipases tend to retain the greatest degree of original activity, when immobilized on hydrophobic supports. Therefore, when the immobilized lipase contacts with oil in water emulsion, the oil phase tends to associate with and permeate the support, which can be assumed that an ordered hydrophobic network of lipid molecules will surround the support. Any water that reaches the enzyme for participation in the reaction must diffuse from the bulk emulsion. Thus, to avoid diffusional limitations, the oil phase must be well saturated with water [105].

Zhao et al. [106] investigated the effect of different reaction parameters on the enzymatic acidolysis of lard with capric acid catalyzed by Lipozyme TL-IM. They achieved the highest incorporation of capric acid (35.56 mol%) without added water. The amount of incorporation was almost constant up to 10% added water but decreased significantly above this amount. The current research work shows that Lipozyme TL-IM-catalyzed interesterification can easily be moved to the industrial sector for commercial exploitation. Both stirred tank reactors [107] and PBR [108, 109] can be used for the production of plastic fats, and the control of water activity in the system presents no particular difficulty, as is often the case in other lipase-application systems, in which the lipase activity was not affected by the reduction of water content in the system [107, 110].

2.8.4. Molecular sieve

In order to promote the synthesis of phenolic lipids by shifting the reaction toward synthesis rather than hydrolysis, a reduction of water content in the reaction mixture can be accomplished through the addition of molecular sieve pellets as dehydrating agents. Li et al. [111] reported that the addition of molecular sieves increased the rate and conversion yield; this is due to the effect of the molecular sieves to sequester the water layer from the enzyme molecule which is essential for the water-enzyme interaction. Mellou et al. [112] found that the conversion yield of rutin during esterification reaction with oleic acid catalyzed by immobilized *C. antarctica* lipase B in different solvents was varied from 37 to 71% under the use of molecular sieves (100 mg/ml). However, Karboune et al. [113] observed 28 and 35% decrease in the maximum conversion yield upon the addition of 10 mg/ml of molecular sieves to the lipase-catalyzed biosynthesis of cinnamoylated lipids. This could be explained by the fact that molecular sieves promote the lipase-catalyzed synthesis reactions by dehydrating; however, excess of molecular sieves will capture the necessary water of enzyme, which may inhibit the enzyme activity.

2.8.5. Substrate composition and concentration (molar ratio)

Chemical structures of the phenolic compounds have an effect on the conversion yield of the end products. Different studies presented the effect of chemical structure of phenolic compounds which are hydroxylated or methoxylated derivatives of cinnamic, phenyl acetic and benzoic acids on the conversion yield [34, 35, 42, 44]. The presence of a hydroxyl group in the sn-2 position has a negative inductive effect. Thus, TAG is hydrolyzed at a faster rate as compared to DAGs, which are hydrolyzed faster than MAGs. Substrate conformation can also affect the reaction rate, since the hydrophobic tunnel in the lipase accepts aliphatic chains and aromatic rings easier than branched structures. Moreover, oxidation of substrates, such as PUFAs, could cause inhibition and decrease in lipase activity due to the production of hydroperoxides and their consequent breakdown to free radicals.

Substrate concentration has an effect on the rate of enzyme hydrolysis and transesterification. So, the selection of a suitable substrate molar ratio in terms of reaction efficiency (incorporation level of acyl donors per unit time) and productivity (product quantity per unit time) in a reaction system is very important. The choice of the proper substrate molar ratio is also related to the downstream processing expenses and associated difficulties of separating FFAs or acyl donors by evaporation and/or distillation. Previous studies have shown that high substrate molar ratio would require a shorter reaction time, move the reaction equilibrium to the product formation, and improve the acyl incorporation [114, 115]. Yang et al. [114] reported the positive effect of substrate molar ratio on the interesterification reaction between EPA and DHA ethyl esters and tripalmitin. They indicated that the optimization results suggested a molar ratio of 6 along with an enzyme load of 20% (Lipozyme TL-IM) and a 17.9 h reaction time would provide the highest incorporation. However, due to the downstream purification expenses, they decided to select the optimal conditions to be a molar ratio of 5 along with a 20% enzyme load and 20 h reaction time. Lee et al. [115] investigated the synthesis of 1,3-dioleoyl-2-palmitoyl glycerol-rich HMFS from tripalmitin-rich fraction and ethyl oleate by lipase-catalyzed interesterification. Similarly, these authors reported an increase of OPO content (25.7%) with an increase of substrate molar ratio up to a ratio of 1:6 of tripalmitin-rich fraction to ethyl oleate.

The study of Sabally et al. [32] investigated the enzymatic transesterification of selected PAs with TAGs, including trilinolein and trilinolenin in organic solvent media (OSM), and reported that the affinity of Novozym 435 was found to be greater for DHCA than that for ferulic acid; these authors suggested that the presence of both the methoxyl substituent and the double bond on the side of the aromatic ring of the ferulic acid could explain its lower affinity for the transesterification reactions with TAG.

Karboune [42] study the effect of PA structure on the bioconversion yield (BY) of phenolic lipids (PLs) obtained by acidolysis of FSO with selected PAs, including hydroxylated and/or methoxylated derivatives of cinnamic, phenyl acetic and benzoic acids in OSM, using Novozym 435 as biocatalyst. The overall findings showed that the BY of PL was dependent on the structural characteristics of PAs. The highest BY was obtained with cinnamic acid (74%). In addition, Karboune et al. [42] concluded that the presence of p-OH groups on the benzene cycle of cinnamic acid derivatives may have an inhibitory effect on the lipase activity, since

the BY decreased to 45 and 11%, respectively, when *p*-coumaric and caffeic acids were used as substrates. The inhibitory effect of *p*-OH substituent was most likely due to their electronic donating effect rather than to their steric hindrance in the enzyme-active site as the inhibition was much less significant (56%) in the presence of a double bond on the side chain conjugated with the aromatic ring of DHPA.

2.8.6. Reaction temperature

Temperature changes effect on different parameters including enzyme stability, affinity, and preponderance of the competing reactions [71]. Temperature normally affects lipase activity, and high temperatures usually increase the initial transesterification rate. However, high reaction temperatures deactivate the enzyme due to its protein nature [35]. The optimal temperature used in transesterification reactions is mainly based on considering properties of feedstock, such as melting behavior at different temperatures as well as the reaction system that is with or without solvent. In a solvent-free system, the temperature is maintained high enough to keep the substrates in liquid state [40].

The optimal temperature for the most immobilized lipases ranges from 30–60°C, while it tends to be lower for free lipases. Heat stability of lipase also depends on whether a substrate is present. This is because substrates remove excess water from the immediate vicinity of the enzyme, hence limiting its overall conformational mobility. Ishihara et al. [116] studied the effect of temperature on vanillyl alcohol acylation with nonanoic acid to give vanillyl nonanoate in *n*-hexane solvent medium. The authors found that the optimum temperature for enzymatic acylation was 70°C. Higher temperatures than 70°C lead to decrease the conversion yield due to the deactivation of enzyme at high temperature. The effect of temperature on the synthesis of capsate analog by lipase-catalyzed esterification of vanillyl alcohol and conjugated linoleic acid (CLA) was presented [47]. The range of temperature tested was from 30 to 60°C. The results demonstrated that the yield increased when the temperature increased from 30 to 50°C. However, when temperature increased to 60°C, there is no increase effect on the yield.

2.8.7. Enzyme concentration

Normally, as the enzyme concentration increases, the reaction equilibrium will be shifted quickly toward the synthesis [117]. However, for economic reasons, it is important to reduce the enzyme loading and the reaction time. In addition, the presence of high enzyme concentration in the reaction medium may increase the probability of its collision with the substrate subsequently enhancing the reaction rate [118]; however, after reaching certain enzyme concentration, the conversion yield was constant. Carrin et al. [117] reported that during the Lipozyme TL-IM-catalyzed acidolysis of sunflower oil with palmitic acid and stearic acid mixture, the extent of palmitic and stearic acids incorporation was enhanced by increasing the amount of enzyme in the reaction; however, when the enzyme concentration was greater than 8% by weight of substrates, there was no significant increase in the esterification yield. The effects of lipase concentration on the synthesis of capsate analog were depicted in the work of [47].

2.8.8. Agitation speed

In a heterogeneous enzymatic system, it is important to ensure that the rate of substrate diffusion will not limit the rate of the synthesis reaction. The increase in agitation speed may decrease the boundary liquid layer surrounding the porous support, leading to lower diffusion limitations. Lue et al. [102] reported an increase of the enzymatic activity from 108.6 to 156.5 nmol/g/min, when the agitation speed of the system was increased from 0 to 200 rpm. The increase in the enzymatic activity indicated that external diffusion limitations of substrates did occur within the range of agitation applied. Kumari et al. [118] reported that carrying the reaction at the optimum agitation speed can limit the external mass transfer limitations, in the case of immobilized enzymes, where the reactants need to diffuse from the bulk oil to the external surface of the enzyme particles and from there, subsequently to the interior pores of the catalyst. In addition [44] investigated the effect of agitation speed on the conversion yield of phenolic lipids synthesized from flaxseed oil and DHCA; the results have shown that the conversion yield increased significantly from 39 to 62.5% when the agitation speed was increased from 50 to 150 rpm, before it was decreased to 44.8% at agitation speed of 250 rpm. The low conversion yield could be attributed to insufficient agitation rate, a condition in which a hydrophilic layer of glycerol may be formed around the enzyme, limiting hence the mass transfer rate of the oil to the surface of the lipase.

2.8.9. Carbon chain length

The effect of carbon chain length of fatty alcohols on the reaction rate was examined by [119]; the esterification of C4–C18 straight-chain fatty alcohol with dihydrocaffeic acid (DHCA), as a model of phenolic acid, was systematically evaluated. The results indicated that the conversion of DHCA was significantly affected by the number of carbon chain of fatty alcohols. Conversion yield of 95% was achieved within 3 days when hexanol was used as an acyl acceptor, while only 56 and 44% conversions were achieved when 1-butanol and octadecanol were employed, respectively. The conversions of ferulic and caffeic acids under the same conditions were much lower than was that of DHCA. In another by [120], various alkyl cinnamates were formed in high to moderate yield by lipase-catalyzed esterification of cinnamic acid and its analogs with fatty alcohols in vacuo at moderate temperatures in the absence of drying agents and solvents.

Several carboxylic acids of different chain lengths from acetic, propionic, butyric, caproic, and caprylic acids were tested via an enzymatic esterification reaction to produce hexyl ester in *n*-hexane and supercritical carbon dioxide (SCCO₂). The reactions were carried out at 40°C, and the amount of enzyme used was 13.8 g/mol alcohol. Substrates were added at equimolar concentrations, with sufficient stirring to avoid external diffusion control. The results in both solvents show that the reaction rate increases with the chain length of the acid, but the final yields were similar.

2.9. Analysis and characterization of phenolic lipids

The structural analyses of phenolic lipids have been carried out using a wide range of various techniques. These mainly include thin-layer chromatography (TLC), high-performance liquid

chromatography (HPLC), gas-liquid chromatography (GLC), and liquid chromatography-mass spectrometry (LC-MS). Thin-layer chromatography has been used for initial qualitative analyses of substrates by employing a wide range of organic solvent mixtures. Products from the esterification reactions are characterized and analyzed by TLC using silica gel G-25 plates [10, 47]. The elution solvents used depend on the nature of synthesized compounds. In the study of [10], the elution solvent used was chloroform/methanol mixture (80:20, v/v) and pure chloroform; the plates were visualized under UV light (254 nm), meanwhile, in the work of Hong et al. [47], the elution solvent was n-hexane/diethyl ether/formic acid (160:40:5.5, v/v/v), and the plate was visualized with 0.2% (w/v) 2,7 dichlorofluorescein in methanol solution under UV light.

High-performance liquid chromatography (HPLC) has often been used over other instrumentations and has shown scientifically to be the overall preferred method of choice for quantification and separation of phenolic lipids following synthesis reactions. Phenolic lipids were separated on C18 reverse-phase column using a gradient elution system with UV detection at 280 nm [10]. Gas-liquid chromatography (GLC) analysis has been conducted for determining the fatty acid composition of the synthesized phenolic lipids. REF has reported on the GC analysis of phenolic lipid esters through the use of a CP-Sil CB-MS column linked to an FID detector.

Recent research on phenolic lipids has also made using liquid chromatography-mass spectrometry (LC-MS) that is considered being one of the most powerful techniques used for the characterization of biomolecules due to its high sensitivity and specificity. Generally, its application is oriented toward the specific detection and potential identification of chemicals in the presence of other chemicals (in a complex mixture). LC-MS has been used for the structural characterization of lipids and phenolic lipids [10, 116].

Many lipid systems have been studied by Fourier transform infrared spectroscopy (FTIR) in order to determine several aspects including the degree and the form of unsaturation of the acyl groups as well as their length [121]. The infrared region of the electromagnetic spectrum extends from 14,000 to 50 cm^{-1} and is divided into three areas: the far infrared from 400 to 50 cm^{-1} , the mid infrared region from 4,000 to 400 cm^{-1} , and the near infrared from 14,000 to 4,000 cm^{-1} [122].

2.10. Application of phenolic lipids

Phenolic lipids, compounds which have been known for a century, are more recently being extensively studied not only from the biological but also from the chemical point of view. Phenolic lipids used as novel antioxidants that synthesized enzymatically. These natural antioxidants increased the antioxidant capacity and the oxidative stability of the edible oils [123]. These products can be used as nutraceuticals for their nutritional value and antioxidant capacity as well as natural ingredients for their physicochemical characteristics [39]. Enzymatic esterification of omega-3 PUFAs with vanillyl alcohol leads to protect these compounds from oxidation, and the PUFA-phenolic derivatives prepared confer the combined health beneficial properties of PUFA and the phenolic molecules [10]. Studies of Zhong and Shahidi [38, 39] indicated that antioxidant activity of esters produced from the esterification of EGCG with

PUFA (EPA and DHA) was superior to that of parent compound in retarded of the oxidation of bulk oil and emulsion. The results suggest that these lipophilic derivatives of EGCG could be considered for use in food preservation and health promotion [38].

Recently, most of the observed activities of phenolic lipids were rather nonspecific and resulted from their amphiphilic and phenolic nature. Further investigation on various aspects of biology may open new opportunities to exploit their properties, as, for example, chemopreventive and antitumor agents, and to develop pharmaceuticals based on phenolic lipid compounds.

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