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Solvent-free esterifications mediated by immobilized lipases: a review from thermodynamic and kinetic perspectives

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Esters are a highly relevant class of compounds in the industrial context, and biocatalysis applied to ester syntheses is already a reality for some chemical companies. Their syntheses in solvent-free systems using immobilized lipases show many economic and environmental advantages. However, considering the complexity and the variety of the simultaneous phenomena involved, the optimization of reactions in these systems is challenging. In a solvent-free system, the molar ratio of the reagents is of utmost importance, defining the behavior of the reaction medium in terms of polarity, mutual solubility, and water activity. Furthermore, the molar ratio of reagents determines the environment in which the immobilized lipase will act, and the intensity of its influence depends on the biocatalyst loading. The variation of the molar ratio and biocatalyst loading, essential parameters to be determined in an optimization study, will significantly impact the thermodynamics and kinetics of the synthesis. In this context, this review intends to show the most relevant aspects for solvent-free enzymatic esterification from thermodynamic and kinetic perspectives.

Introduction

Esters have a wide range of applications in day-by-day products. Esters of short-chain carboxylic acids, aliphatic esters, are applied as fragrance and flavors in food, cosmetic, and pharmaceutical products; esters of mid or long-chain aliphatic carboxylic acids are primarily used as emollients, surfactants, and emulsifiers in many kinds of products; long-chain esters find application as biofuels, biolubricants, and bioplastics.[1–7] Their relevance lies not only in the diversity of structures and applications but also in the current world market as well as the projections of their growth.[3,5,8,9]

Currently, at the industrial level, esters are generally produced using chemical catalysts, like mineral acids or metals that lack good selectivity, requiring harsh reaction conditions and complex downstream operations for purification of the product.[3,8,10] Biocatalysis emerges as an alternative to improve the product quality due to superior selectivity and specificity and mild reaction conditions where they can perform their function.[8,11] For ester synthesis, a particular class of hydrolytic enzymes, lipases (triacylglycerol ester hydrolases EC 3.1.1.3), is usually adopted.[2,3,5,12–14] Considering their stability, broad specificity, and versatility, lipases (in many instances in an immobilized form) are among the most studied biocatalysts in applications for esterification reactions.[12,14–16] However, the high cost of biocatalysts and their low activity compared to chemical catalysts are the major drawbacks to be solved for a broad application of biocatalysis in these processes.[17] In this sense, reaction conditions that promote high yield and reaction rate with a minimum quantity of biocatalyst need to be set for the industrial use of biocatalysts.

Despite the high price of commercial enzymes, important chemical companies such as BASF, Evonik, Croda, or Eastman have been offering products obtained by enzymatic catalysis in their portfolio.[5,15] It is expected that the growing market for esters and the search for more sustainable forms of production in agreement with the need for safer and higher quality products will increase ester industrial production by enzymatic catalysis in the following years.[15] The global ester market was valued at USD 1 billion in 2019 and is estimated to reach USD 3 billion by 2027.[18] In 2018, the global market of industrial enzymes was estimated at USD 5.5 billion with a compound annual growth rate (CAGR) of 4.9%,[8,19] indicating a convergence between the growth of both markets.

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Esterification reactions mediated by immobilized lipases: general aspects

Enzymatic esterification involves complex phenomena: the reaction is a thermodynamically controlled process; lipases are susceptible to inhibition or inactivation by reagents, products, or temperature; also, some water content is necessary for catalytic activity, but water in the medium favors hydrolysis, reducing the yield of esterification.[2,20–25]

An overall description of an esterification reaction thermodynamics includes the determination of Gibbs free energy, enthalpy and entropy variations, and thermodynamic activity coefficients. These parameters may be obtained successfully by mathematic modeling and computational simulation. These parameters help to check, in general terms, that: i) esterification reactions are spontaneous processes; ii) esterification reactions are frequently endothermic processes, with a positive variation of enthalpy; iii) the variation of entropy is positive, considering the formation of water and its partitioning in the reaction medium.[21,26–30]

The kinetics descriptions of the esterification reaction provide the reaction rate and the determination of apparent kinetic constants. For enzymatic esterification, the use of immobilized lipases in solid supports has some evident advantages related to the separation of the catalyst from the medium and the possibility of its reuse.[2,16] Consequently, other phenomena are introduced in this system, considering the interactions between the support of the lipase and the reaction medium, the resistance of the support under intense stirring and heating, and mass transfer aspects.[2,14,31–37]

It has been shown that one of the problems using immobilized enzymes during ester production is the accumulation of water and acids on the solid particles, as water formation may be quicker than water elimination.[38,39] The impact of this problem can be solved using very hydrophobic supports or ultrasound.[40–43] Novozym 435, the most widely studied lipase for synthetic applications, presents notable deficiencies related to operational stability, as recently reviewed by Ortiz and co-workers (2019),[14] due to its lack of mechanical strength and dissolution of its immobilization support in certain media. Mass transfer limitations may arise when highly viscous reagents are present, which may require intense mixing or heating, affecting the operational stability of lipases and the overall yield of the reaction.[6,44–46]

The use of solvents in enzymatic esterification may attenuate the magnitude of these issues in the reaction medium.[20,46–48] Solvents have an essential role in the solubility of substrates, generating a great impact on the reaction yield when substrates have poor mutual solubility.[5,6,20,46,49,50] However, the use of solvents brings additional costs and environmental concerns.[46,51,52] To overcome these drawbacks, the adoption of solvent-free systems for enzymatic esterification has received increased interest in the last few decades.[6,45,53–63] Many studies have obtained, with different degrees of yields, esters by enzymatic routes in solvent-free systems for applications in cosmetics, personal care products, and biofuels, as reviewed by Khan & Rathod (2015, 2018),[5,64] Pourzolfaghar *et al.* (2016),[65] Sá and co-workers (2017),[8] and Antczak and co-workers (2009).[48] More recently, high conversions using solvent-free systems (SFS) for enzymatic syntheses of important esters were described in the literature. Aguieiras and co-workers (2019) obtained conversions of 80% in the syntheses of wax esters using agroindustrial wastes as a source of fatty acids and raw material for obtaining biocatalysts;[66] babassu residues were also successfully used by Moreira *et al.* (2020)[67] as a source of fatty acids in SFS enzymatic esterification mediated by *R. miehei* immobilized on magnetic particles for biodiesel production. Flavor esters have also been successfully synthesized (conversions close to 90% or higher) in SFS esterification using different immobilized lipases – pentyl valerate,[68] methyl and ethyl butyrate,[69] propyl benzoate,[70] and *n*-butyl acetate.[71] Mid and long-chain esters, useful as emollients, surfactants, or plasticizers, were also obtained in SFSs with high conversions, as shown by Silva *et al.* (2020, 2021)[72,73] in the synthesis of 2-ethylhexyl oleate and isopropyl palmitate, Musa *et al.* (2019) in the syntheses of different butyl esters,[74] and Lee *et al.* (2019) in the synthesis of diisononyl adipate.[75]

The optimization of solvent-free esterifications deals with obtaining high conversion while avoiding excessive quantities of reagents and catalysts, with energy savings. Solvent-free reactions, nevertheless, present specific challenges considering the drastic changes that may occur in the reaction medium during the reaction.[27] Specific studies are required to establish the optimum quantities of reagents and catalyst under these conditions, as well as the optimum temperature, in which the thermodynamic and kinetic aspects have a convergence towards high conversions.

The optimization of enzymatic esterification demands intense experimental work. Several studies aimed to obtain optimized conditions in solvent-free ester syntheses, using response surface methodology (RSM)[46,76] or the classical approach of studying variables one-by-one with different detailing levels. Some authors dealt with predictions of the equilibrium position of esterification reactions in solvents[21,25,77,78] or in solvent-free systems[27,79] using sophisticated mathematical tools or software. As the determination of the equilibrium position is independent of the amount of (bio)catalyst in the systems, these tools do not address one of the crucial issues for optimizing enzymatic esterification: biocatalyst loading. Although kinetic studies deal with the behavior of the enzyme in a given medium, these studies, by themselves, do not provide a complete picture of the reaction in terms of the molar ratio of reagents, biocatalyst loading, and other physical–chemical aspects required to design a process. A simple mathematical tool, named SER (substrate–enzyme relation), was recently proposed by Sousa *et al.* (2020)[58] for predicting the performance of enzymatic esterification, which evaluated the coupled effects of the molar ratio of reagents and the biocatalyst loading to obtain high yields. SER was

successfully applied on octyl caprylate synthesis from *n*-octanol and caprylic acid, but the extension of its applicability has yet to be proved for the synthesis of other short and mid-chain esters.

Some aspects of enzymatic solvent-free esterification are briefly mentioned in recent literature. Dhake and co-workers (2013)[80] dedicated a specific topic for solvent-free systems in a review of enzymatic syntheses of flavor and fragrance esters. In recent work, Kaabel and co-workers (2020)[81] compiled recent advances in mechanochemical enzymatic reactions, an emerging topic that may overcome the constraints of mutual solubility between reagents (and consequently the need for solvents in syntheses) by using operations such as grinding, extrusion, and milling, among others. This review will focus on the practical aspects of solvent-free enzymatic esterifications using immobilized lipases, aiming to provide a comprehensive theoretical basis for optimizing these reactions in a broad sense, independent of the product application or reaction system. An adequate domain about the aspects related to the chemical equilibrium and enzymatic inhibition in solvent-free systems enables realistic conditions to be obtained in optimization work for scaling-up of biocatalytic processes.

Critical variables for optimizing enzymatic esterification reactions

Most studies of enzymatic esterification consider the molar ratio of reagents, biocatalyst loading (frequently described as enzyme loading in the literature), and temperature as the main variables that determine the reaction yield. The phenomena involved in esterification are directly or indirectly studied when these variables are considered, as shown in Table 1.

There are two possible strategies to shift the equilibrium in esterification: i) using an excess of one of the reagents (improving the yield regarding the limiting reagent, but decreasing it regarding the exceeding reagent) or ii) removing one of the products from the reaction medium (preferably water).[21] When a stoichiometric excess of one of the reagents is used, alterations in the reaction medium may occur, related to the polarity, viscosity, mutual solubility, and pH in the enzyme environment. The magnitude of these changes depends on the nature of the exceeding reagent and its quantity, being critical in solvent-free systems, where the reagents and the products are the reaction medium.[21,27,82] Moreover, changes in the enzyme performance – denaturation, inactivation, or inhibition – may occur if some reagents present adverse effects on the biocatalyst.[21,45,83] The variation of the reagent concentration also affects the reaction rate, as described by the Michaelis–Menten equation,[84] with different effects. Their effects, however, are not the same – a beneficial kinetic effect may be found if using an excess of the limiting substrate.

Biocatalyst loading is a critical variable due to the high cost of biocatalysts[17,85] and impacts the reaction time. The amount of biocatalyst in the reactor is also limited by the stirring system's dispersion capacity and the system's filtration capacity. Furthermore, both the support and enzymes may capture some of the water molecules, changing the optimal initial water activity of the system.[2,86–88] The quantity of enzymes in the system does not affect the ester molar fraction in equilibrium (*i.e.*, the reaction yield) but affects the reaction rate.[2,84] In theory, the minimal biocatalyst loading necessary to convert all the substrate at a given time can be predicted using the esterification activity data (U g^{-1}). However, inhibitions caused by the product(s) may affect this

Table 1 The relation between the reaction variables, their practical implications, and the phenomena involved in a high-performance ester synthesis mediated by immobilized lipases

Reaction variable under evaluation	Required/desired condition	Practical implications	Associated phenomenon
Molar ratio of reagents	Excess of one of the reagents Removal of one of the products	Complexity of the downstream operations Increasing operational costs and capital investment	Shift of the chemical equilibrium
	High concentrations of reagents (in the case of using solvent) or solvent-free reactions	Optimized volumetric productivity	Enzymatic kinetics Enzymatic inhibition Diffusion limitations Partitioning of the reagents Viscosity of the media
Biocatalyst loading	As low as possible	Reduced operational costs Increase of biocatalytic process productivity	Enzymatic kinetics Enzymatic inhibition Dispersion of the biocatalyst's particles Water demand
Temperature	As close to room temperature as possible[a]	Reduced operational costs	Enzymatic kinetics Equilibrium position Viscosity of the media Mass transfer aspects

[a] Limited by the melting point of substrates, mutual solubility of reagents, a drastic increase in viscosity of the media, or a significant impact on reaction time.

prediction. The accuracy of this prediction depends on the nature of the reagents involved and their capacity to affect the enzyme preparation, whose evaluation requires deep experimental work. At a specific situation in the reaction, inhibition of lipases caused by the reaction products may occur.[10,21] This problematic product concentration is not absolute but relative to the biocatalyst loading. The derivation of the Michaelis–Menten equation assumes that the substrate concentration exceeds the enzyme concentration at a level in which the enzyme concentration becomes mathematically negligible.[84] This situation will always be present in ester production. Some studies evaluate different biocatalyst loadings using a given molar ratio of reagents within a constant time interval.[6,40,56,58,89–91] In these cases, the observed differences in the reaction conversion may be associated with insufficient time to consume all the substrate. Three reasons may cause this situation: i) the reaction is slow and does not reach the thermodynamic yield in the studied reaction period; ii) product inhibition becomes so significant that the reaction stops far from the thermodynamic equilibrium; iii) the enzyme becomes inactivated.[27,58,61,90] In practical terms, enzymes may be more susceptible to inhibition in reduced biocatalyst loadings than in large ones, as observed from experimental data,[27,91] considering that a lower enzyme amount will concentrate the inhibitor molecules in its vicinity. Summarizing, the determination of the optimum biocatalyst loading and the molar ratio for esterification, considering these effects, demands great experimental effort.

Temperature positively affects the energy of the reagents, favoring the number of effective collisions that lead to the formation of the product.[6,45,92] However, a collateral effect may occur – high temperatures may induce conformational changes in enzymes that lead to the reduction (or the loss) of the enzyme catalytic activity.[40,45,84,93] Therefore, the reaction kinetics increment due to the temperature increase may be offset by the decrease in the lipase catalytic activity.[94] Several studies indicated the range of temperatures at which enzymes remain stable and active,[5,12,93,95–97] but these conditions must be studied under actual operation conditions (reaction medium, substrates, and products). Temperature affects directly the thermodynamic aspects of the esterification reaction. The free energy of the system (ΔG) tends to decrease with increasing temperature in conventional esterification, which means that the reaction is thermodynamically favored (endothermic reaction, with a positive enthalpy of reaction ΔH).[26,28,29,98,99] However, a recent publication by Khan & Rathod (2020)[30] found that although a positive variation of enthalpy is observed in enzymatic esterification (*n*-butyl palmitate synthesis) under conventional stirring, the opposite situation occurs under ultrasound stirring or microwave irradiation, considering the ability of these techniques to enhance the effective collisions in the reaction. Another interesting observation is that only marginal differences were observed in calculated values of ΔH, ΔG, and ΔS when the temperature varied from 40 to 70 °C under conventional stirring, ultrasound, or microwave irradiation.[30] Temperature also affects the reagents' solubility, which implies considerable changes in the availability of the reactants for the enzyme. These changes affect the apparent equilibrium position, as only solubilized reactants will participate in the process thermodynamics,[77] as well as only solubilized substrates will be accessible to enzymes. However, in some cases, observed variations in conversion results due to changes in the temperature may be associated with an insufficient reaction time to achieve the equilibrium at sub-optimal temperatures and not directly influenced by the chemical equilibrium.[92] Some experimental data showed only discrete variations of equilibrium constant when the temperature was varied.[79,98] The viscosity reduction is another expected effect of increasing the temperature, diminishing potential mass transfer limitations.[5,6,45,46] This factor might become relevant mainly when viscous reagents are involved, like long-chain fatty acids or alcohols. In general terms, an optimum temperature for enzymatic esterification should facilitate a proper diffusion of the reagents in the medium, preserving the biocatalyst performance.

The discussion about the influences of the molar ratio of reagents, biocatalyst loading, and temperature in enzymatic esterification can be split into two major groups – thermodynamic and kinetic aspects. But in terms of optimization of a reaction, the aspects directly related to the chemical equilibrium of the reaction, reaction rate, and enzymatic inhibition are the most crucial due to their direct association with reaction performance – conversion and time of the reaction. Studies that include additional evaluation of thermodynamic parameters (ΔH, ΔG, ΔS) or kinetic parameters (K_m, V_{max}, K_i, *etc.*) are generally carried out after obtaining optimized conditions of molar ratio, biocatalyst loading, and temperature or separately.[30,45,90,98,100,101] Thus, relevant aspects of chemical equilibrium and enzyme kinetics directly associated with optimization of a reaction will be further discussed, considering the particularities of solvent-free systems.

Chemical equilibrium on esterification reactions

A comprehensive study of the chemical equilibrium in biocatalytic esterification is not trivial, and several studies deal with the aspects involved in this issue. Flores and co-workers (2000)[79] presented a brief revision of chemical equilibrium studies on esterification reactions, observing that predictions or measures of the equilibrium position may be associated with the partition coefficients of the reagents in the medium, activity coefficients, or solvent properties.[25,77,78,102,103] An interesting finding is that the chain-length of the fatty acid affects the molar fraction of the ester in equilibrium (*i.e.*, the yield of the reaction), and this effect may be attributed to the fact that different fatty acids can solvate the formed ester differently in a solvent-free reaction.[79]

Increasing the ester mole fraction is required to decrease the thermodynamic activity of the products of the reaction

(ester and water) and increase the thermodynamic activity of reactants (carboxylic acid and alcohol).[21] This condition can be reached using a suitable solvent. As the polarity of the solvent decreases (more hydrophobic medium), the solvation of the functional groups of alcohols and acids decreases, which implies an increase in its thermodynamic activity.[27,77] Likewise, the reduced polarity of the solvent will favor the solvation of the ester, reducing its activity.[21,77,79] Hydrophobic solvents (or a hydrophobic reaction medium) also increase the pK_a of organic acids, which means that the non-ionized form of acids that contribute to the chemical equilibrium will predominate in the system.[104–106] In this context, therefore, hydrophobic media tend to favor esterification.

The chemical equilibrium of esterification is strongly dependent on water due to its role as a reaction product and its distribution in the medium.[21,107] To exemplify this effect, when water is formed in a highly hydrophobic medium, a reduced quantity of water will be available in the organic phase, which implies that hydrolysis does not tend to occur.[107] Moreover, Halling (1990)[77] indicated the possibility of using the solubility of water in solvents as a parameter to choose suitable solvents for enzymatic esterification, considering the water partitioning in the medium. Although enzymes, as any catalyst, do not affect the chemical equilibrium, some materials used as immobilization supports of lipases may alter the water and reactant partitioning in some cases, and, thus, affect the apparent equilibrium position and the respective yield of the ester.[2,27,108] The effects of water on the chemical equilibrium of enzymatic esterification will be further detailed in the following topics.

The enzymatic inhibition of immobilized lipases

Product inhibition is a reduction (reversible or irreversible) in the enzyme activity that occurs by the enzyme's interaction (directly or indirectly) with substrates or products. Although inhibition of enzymes caused by the product acts as a regulatory mechanism in the metabolism of living organisms,[84,109] it is not common to observe inhibition by the esters on lipases.[10,110] The ping-pong bi–bi mechanism – where a reaction between two reagents forms two products – is widely accepted to describe the overall kinetics of esterification reactions mediated by lipases, following Michaelis–Menten kinetics.[10,45,94,100,111] This mechanism involves the formation of an acyl-intermediate in which an alcohol molecule is incorporated, with subsequent release of ester and water. The general kinetic equation (from a second-order reversible kinetic model) is expressed considering the possibility of inhibition by both substrates that form dead-end complexes with the enzyme (alcohol) or with the acyl-intermediate complex (acid).[26,28,46,99,112–115] The determination of the kinetic parameters – K_m (Michaelis–Menten constant), K_i (inhibition constant), and V_{max} (maximal reaction rate) – can be made by Lineweaver–Burk linearization or by numerical methods. The magnitude of differences in K_m and K_i values expresses, respectively, the differences in biocatalyst affinity by substrates and substrate inhibitory effects on enzymes. These parameters are essential to scaling-up studies and design of reactors.[10,94,111]

Different types of enzymatic inhibition are described in textbooks of biochemistry and enzymology. Nevertheless, considering the ping-pong bi–bi mechanism, competitive inhibition may occur.[116,117] Competitive inhibition takes place when both substrates (or a substrate and a solvent) can bind (reversibly) to the active site of the enzyme but not simultaneously; increasing the concentration of the non-inhibitor substrate reduces the probability of this kind of inhibition.[84,99] Some authors reported enzyme inhibition by both carboxylic acid and alcohol;[10,46,100,113,118,119] contrarily, other authors established enzyme inhibition only by acid[46,83,91,98,120] or by alcohol.[27,52,90,101] These observations are intrinsically related to the nature of reagents studied (short-chain or long-chain acids and alcohols), the presence or absence of solvents, the intrinsic properties of lipases, and the features of the immobilization support, indicating that inhibition effects are associated with the partitioning of reactants in the medium.[91] As lipases are commonly studied for the synthesis of organic molecules, and the kinetic aspects show a strong dependence on the support used for the immobilization of the enzymes,[121–131] the literature describes efforts in understanding the inhibition of immobilized lipases under different conditions.

In general, inhibition studies use a specific biocatalyst loading sufficient to convert the substrate at a constant temperature.[10,83,98,118] The inhibitor substrate concentration is varied until a certain level, and the reaction rate reduction (inhibition) is observed at increasing concentration of the substrate. In optimization studies (at lab-scale context), it is not mandatory to determine the kinetic parameters neither make a complete kinetic description of the reaction, because the evaluation of different molar ratios, biocatalyst loadings, and temperatures of the reaction provides an overview about the kinetics or inhibition occurrence that is enough for establishing suitable reaction conditions. The literature describes a variety of optimization studies carried out without an in-depth discussion of kinetics,[6,17,40,56,76,132–135] although a qualitative understanding of this issue has crucial importance for the optimization steps.

Inhibition, *per se*, is related to the interaction of an inhibitor and the enzyme (in its active center or another enzyme site), whose effect is the reduction of the catalytic activity. However, reducing this activity may also occur due to environmental conditions not favorable to the enzyme's optimum operation, for example, acid or water accumulation in the enzyme environment.[40–42,45,108,136] The presence of water in the microenvironment of the enzyme is mandatory to retain its catalytic activity.[20,47] Water promotes flexibility on proteins, and in the complete absence of water, enzymes become very rigid, with reduced capacity to achieve the conformational changes necessary to catalysis.[2,20,25,77,86–88,107,137,138] Thus, if a hydrophilic substance substitutes the water in the vicinity of

enzymes, distortions in the enzyme conformation will occur with a consequent decrease in enzyme activity.[46,107,139] However, the positive correlation between water activity and enzyme activity is limited, and the effects are not the same for lipases from different sources[2,25,140] or subjected to different immobilization protocols. In anhydrous media, the enzyme activity will be initially slow but will increase along with the esterification due to water formation. Contrarily, the use of water-saturated reagents in the medium may decrease the enzyme activity if a water phase may be formed inside the biocatalysts, reducing the diffusion of the hydrophobic substrates, favoring the hydrolytic action of the enzyme, or even inactivating the enzyme if in this water phase the acid molecules become accumulated.[82,86] The formation of an excessive hydration layer is also favored when the water is formed more rapidly than its diffusion to the bulk phase, the material of the support used for enzyme immobilization is hydrophilic, or in a highly hydrophobic reaction medium.[87] If a short-chain acid is present, this microenvironment around the enzyme will have low pH, leading to enzyme inactivation.[14,76] Moreover, it is noteworthy that high water content in the medium will favor the reaction equilibrium toward hydrolysis.[2,14,27] In this sense, a balance in the water content is required to obtain the optimum activity of enzymes.

Both situations – inhibition by the action of a potential inhibitor substrate or the reduction in enzymatic activity due to an unfavorable environment – are widely described in the literature and may co-occur. However, it is not possible to determine which one is more probable to occur considering the number of variables involved in the issue. The adoption of a favorable reaction medium is required to obtain an optimized performance of immobilized lipases, which includes not only their maximum activity in the reaction but also their operational stability. High operational stability of immobilized lipase will guarantee the achievement of adequate productivity for the biocatalytic process.

Thermodynamic and kinetic aspects in solvent-free enzymatic esterifications

The behavior of the reaction medium in solvent-free enzymatic esterifications

Up to here, we discussed commons aspects for esterifications with solvents and without solvents. The use of solvents in enzymatic esterifications may be required to promote the solubility of reagents and their diffusion in the medium and reduce the inhibition potential of some substrates.[10,46,83] However, solvent use is undesirable from an economic and environmental perspective, as it reduces volumetric productivity, increases the complexity of downstream processes, and may generate hazardous liquid wastes that should be treated or recycled.[27,82,111] The use of solvent reduces the variations of physicochemical parameters of the reaction medium during the reaction caused by reducing substrate concentrations and increasing product concentration. Contrary to the reactions in the presence of solvents, the adoption of solvent-free systems impacts both kinetic and thermodynamic aspects of esterification reactions,[21,46,141] resulting in several changes in the reaction medium in the course of solvent-free esterification, which includes changes in partition coefficients (as log P), water content, pH, mutual solubility, and viscosity of the medium.[21,27,82] These changes have significant impacts on chemical equilibrium and enzyme kinetics (particularly enzymatic inhibition). A discussion of these parameters will be made in the following paragraphs.

Log P (water–octanol partition coefficient), as any partition coefficient, is related to the availability of a substance in the different phases of a mixture.[142–144] Hence, this parameter may be correlated with the equilibrium constant and equilibrium position, although the literature already described a poor quantitative correlation of log P with thermodynamic parameters.[21,25,27,79,144] Log P is an indirect parameter of hydrophobicity of a given substance readily available in the literature for carboxylic acids, alcohols, and esters,[77,145] which also can be calculated for a mixture of two components, being proportional to their molar fractions.[144,145] For this reason, it is easy to say (or predict) something useful about a solvent-free reaction medium using the log P of the reagents. If resultant log P is <2, the medium is considered hydrophilic (polar); between 2 and 4 is considered mid-polar, and log P higher than 4 indicates that the medium is hydrophobic (non-polar). To a certain degree, it may be possible to obtain some predictions about the behavior of the water and solvation of the reagents and products in the medium using log P for a solvent-free reaction medium.

The water content in the medium is a crucial parameter in enzymatic esterifications, with impacts on thermodynamic, enzyme stability, and kinetic aspects. Water content refers generically to the water concentration in the medium. However, for thermodynamic purposes, water activity is a more specific and useful concept that refers to the molecules of water that can interact directly with other chemical species.[24,77,138,143] In an esterification reaction, water will be present in the enzymes' hydration layer, dissolved in the reagents, absorbed from the humidity of the environment, or formed as a by-product during the progress of the reaction. Obviously, as the reaction produces water, the water activity will change in the medium during the reaction with potential undesirable effects, such as reducing enzymatic activity due to excessive hydration[2] or favoring hydrolysis. For this reason, different kinds of water activity control – continuous removal using vacuum pressure, molecular sieves, or the strict control of water activity using salt solutions – are commonly adopted in esterification reactions.[2,86–88] However, in the case of reactions with highly polar substrates, it is helpful to add some water into the medium due to the capacity of this kind of reagent to strip off the essential water of the enzymes[27,47,140,146] with a consequent decrease in the enzymatic action.[2,20]

Only non-ionized carboxylic acid participates in chemical equilibrium, but acid dissociation will occur differently with different alcohols and water content, affecting the pH of the enzyme nano environment. It is desirable to have a solvent (or a reaction medium) that increases the pK_a of the acid, reducing its ionization. Short-chain alcohols like ethanol or methanol have a limited capacity to stabilize the non-ionic forms of carboxylic acids.[104–106] Mid or long-chain alcohols are less polar, showing an increased capacity to do the same; however, as they are less miscible with water, consequently, the dissociation of an acid in a medium like that will be less intense.[105] As the by-product of an esterification reaction is water, if an aqueous phase is formed in the enzyme environment (which occurs if the support is not very hydrophobic), the non-reacted acid in the aqueous phase may drastically decrease the enzyme nano environment pH, affecting the lipase activity/stability and the reaction completion, resulting in a high residual acidity index of the product, which is an important parameter for industrial production.[3,5] Considering the biodiesel production, unreacted FFA results in both the decrease of the global yield of the reaction and the high acidity of the final product, requiring additional steps of sequential reactions, purification, or neutralization complying with the biodiesel specifications and national standards.[147,148] The final acidity, which is a consequence of enzyme capacity to consume all the acid in the medium, and the strategies to overcome this issue have been reported in many studies, mainly considering the importance to replace the refined vegetable oils as feedstocks for biodiesel production with side-streams of refining.[148,149] In the cosmetics sector, the quality of esters is also associated with the residual acidity index.[150] As an advantage, the solvent-free systems with an optimized molar ratio of reagents and continuous water removal by vacuum allows the production of the target molecules without further purification steps.[3,5,151]

The mutual solubility of reagents is required for a proper operation in solvent-free systems, which will depend on the nature of the substances involved and the temperature.[152] Reagents with similar polarities will show good mutual solubility, and contrarily, reagents with very different polarities may generate a macroscopic separation of phases. The mutual solubility facilitates the diffusion inside the biocatalyst particle, increasing the contact between both substrates and the catalytic phase, promoting the formation of products.[2,46,152] On the other hand, mutual solubility favors the solvation of one reagent on another and, depending on the solvation extent, the thermodynamics of the reaction towards the synthesis may be unfavored.[27,77,153] If the bulk phase is hydrophobic, a hydrophobic reagent will be very well solvated, implying that its thermodynamic activity will decrease.[27,77,153] This condition is not desired, as it is necessary to increase the thermodynamic activity of both substrates to increase the conversion.[21] The thermodynamic activity is strongly favored by the desolvation of the substrate (from the solvents or the exceeding reagent under solvent-free conditions) into the active site of the enzyme, which does not occur if the substrate is very well solvated by the medium.[153,154] In a solvent-free system, the polarity of the surplus reagent will strongly influence the global polarity of the medium and, consequently, affect the solvation of the limiting reagent and its respective conversion.

The lack of a solvent turns the viscosity of the medium into a relevant factor to be considered[27] in the esterification of long fatty acids or long-chain alcohols, which are viscous liquids or even solids at room temperature. In some cases, the adoption of a temperature high enough to melt the solid substrate is required,[27,155] but too high temperatures may affect the operational stability of enzymes, as previously mentioned.[45,94,152] The viscosity of the medium may change drastically when different molar ratios of the substrates are evaluated, for example, in the esterification of a long-chain fatty acid and short-chain alcohol. In these cases, high biocatalyst loadings (required when using lipases with low esterification activity) may bring some additional challenges for stirring and catalyst recovery. Although questions like that are relevant, there is a lack of experimental information in the literature of mass transfer limitations related to viscosity.[6,27,45,90] In terms of biocatalyst performance, the viscosity of the medium may affect the diffusion of reagents and enzyme conformational changes, with impacts on kinetics. These issues may be overcome by the adoption of a suitable temperature.[156]

In the context of reaction rates, the presence or absence of solvents has a strong impact on esterification reactions. Sandoval and co-workers (2002)[27] found that esterification between oleic acid and ethanol has high reaction rates in the solvent-free system than in hexane until a specific stoichiometric excess of ethanol. Kuperkar and co-workers (2014)[45] found the same when comparing the synthesis of isopropyl palmitate in a solvent-free system with the study of Varma & Madras[157] who synthesized the ester in the presence of supercritical CO_2. Aljawish *et al.* (2019)[136] found a comparable yield between the enzymatic synthesis of butyl and octyl formate in acetonitrile and under solvent-free conditions, although using different molar ratios of acid and alcohol in both cases. It has been shown how the role of the cosolvents may be different depending on the immobilization support. For example, the use of very hydrophobic supports shows that the addition of a cosolvent significantly reduced the enzyme activity, while using a hydrophobic one, the effect was positive.[158–160]

In solvent-free systems, the reaction medium is a dynamic environment susceptible to intense physical–chemical variations: initially, the medium is just a mixture of substrates; at the end, the medium will be formed by the product, the reagent utilized in excess, and the traces of the limiting reagent. A set of optimization experiments that includes evaluation of different molar ratios and biocatalyst loadings means to deal, in some circumstances, with entirely different reaction media under each condition. We will explore briefly in the following sections the practical aspects

of studying the molar ratio and biocatalyst loading in solvent-free systems.

The effects of the molar ratio of reagents on a solvent-free esterification reaction

Solvents influence the equilibrium position due to changes in the reaction partitioning[25,77–79,102,103] and, in solvent-free esterification, the exceeding reagent, in general alcohol, may act as the solvent. Thus, the exceeding alcohol will not only shift the equilibrium towards synthesis by thermodynamic reasons but may affect the behavior of the reaction and its yield due to the changes in thermodynamic activities of reagents and products, water activity, log *P* of the medium, pH, mutual solubility of the reagents, and viscosity, as well as the enzyme performance. Therefore, the extent of these effects will depend on the quantities and the nature of the surplus reagent in the medium.

A myriad of situations may occur and, although log *P* is not an accurate parameter for thermodynamic predictions, we can use log *P* to briefly illustrate the effects of the surplus reagent in solvent-free enzymatic esterification. In a solvent-free reaction between a long-chain fatty acid (as oleic acid, C18:1) and short-chain alcohol (as *n*-pentanol, C5), there is a great difference between their polarities – oleic acid log *P* is 7.64 and *n*-pentanol log *P* is 1.51. The resultant log *P* of an equimolar mixture of both reagents will be 4.51, and up to a molar ratio acid : alcohol of approximately 1 : 1.2, the resultant log *P* will be higher than 4, which means that the reaction medium will be a hydrophobic environment even with some stoichiometric excess of hydrophilic alcohol. Within this range of molar ratio conditions, all the implications of hydrophobic medium will be present for the enzymes. This simple kind of analysis may help to avoid extensive experimental assays and find the optimum conditions faster.

Another interesting hypothetical example is a solvent-free esterification reaction involving mid-chain substrates. If we react hexanoic acid (C6, log *P* equals 1.9) with *n*-hexanol (C6, log *P* equals 2.03), there is just a small difference between the polarities of both reagents. An equimolar mixture of hexanoic acid and *n*-hexanol has a resultant log *P* equal to 1.96, which means a mid-polar environment at the beginning of the reaction. However, if we consider that during the reaction, the medium is mainly composed of hexyl hexanoate and the water is removed by molecular sieves, the reaction medium gradually becomes a non-polar environment due to the high log *P* of the formed ester (4.68). This implies that the enzymatic activity may not be favored at the beginning of the reaction, considering the potential of reagents to affect the enzymes' hydration layer, but this effect may be decreased along with the reaction. On the other hand, questions related to the solvation of one reagent on another become highly relevant due to the mutual solubility, which will impact the equilibrium position.[2] Sandoval and co-workers (2002)[27] used the variations of log *P* in the beginning and at the end of the reaction as a tool to predict relevant information about thermodynamics and kinetics; the authors considered every different molar ratio as a different solvent in their modeling, which corroborates how vital the molar ratio of reagents in the solvent-free system is.

Considering this kind of approach, it is possible to hypothesize that in esterification between a short-chain acid and mid or long-chain alcohol it could be interesting to adopt a large stoichiometric excess of alcohol. This strategy would help reduce the inhibition potential of short-chain acids on lipases by reducing their concentration and influence on the pH of the enzyme's hydration layer and reducing the polarity of the reaction medium. Aljawish and co-authors (2019)[136] used this strategy successfully to synthesize esters of formic acid, a short-chain acid with high inhibitor potential. Similarly, in the esterification of long-chain fatty acids and short-chain alcohols, the large stoichiometric excess of alcohol may also be useful, not due to inhibition or inactivation risks but to improve the solubility of fatty acids (which are solid at room temperature in many cases) and facilitate the diffusion of substrates in the medium. We emphasize, however, that the exceeding quantity of a reagent should be evaluated considering the feasibility of post-reaction operations, which include separation of products and recovery of the biocatalyst.

The effects of biocatalyst loading on a solvent-free esterification reaction

The molar ratio of reagents in solvent-free systems may affect directly or indirectly the activity of enzymes, and the biocatalyst loading in the medium will modulate the magnitude of these effects. In solvent-free systems, the concentration of reagents is very high, as well as the influence of the reagents' properties on the enzymes. It is intuitive to think that if a small amount of immobilized lipase is present in a reaction medium with high concentration levels of a potential inhibitor, inhibition certainly will occur, mainly if the inhibitor is concentrated in the biocatalyst particle. However, if the biocatalyst loading increases, the availability of a higher amount of biocatalyst in the system will increase the reaction rate and may reduce the inhibition effects. This effect can be seen in many different studies[6,17,27,45,52,58,76,136,161] that observed a positive correlation between conversion results and biocatalyst loadings in a given molar ratio within a limited interval of time. As already discussed, biocatalyst loading (the quantity of immobilized lipase in the system) does not affect the equilibrium position, but these observations are related to kinetics in two different ways – the occurrence of some level of enzymatic inhibition and/or insufficient time to reach the equilibrium. By confirming the occurrence of inhibition using low biocatalyst loading and the non-occurrence when using higher dosages under the same reaction conditions, it is possible to conclude that inhibition is dependent on the biocatalyst loading. Thus, as biocatalyst loading is a critical parameter for optimizing the synthesis considering the high

cost of immobilized lipases, obtaining high yields using low biocatalyst loading in a solvent-free system is challenging.

The probability of enzyme inhibition due to an exceeding reagent is higher than that produced by the limiting reagent. When an exceeding reagent with potential to damage the hydration layer (*i.e.*, a hydrophilic one) is present, and the biocatalyst loading in the system is relatively low, the probability of a deleterious effect on the hydration layer of enzymes is high. In other words, only high loadings will cope with the excessive quantities of alcohol or acid in the system. On the other hand, a large stoichiometric excess of a reagent may improve the solvation of the second reagent (if both reagents are hydrophilic, for example), reducing its inhibition potential.[83,118] This is not the case for highly hydrophobic reagents with minimal solubility in the catalytic phase (aqueous), which implies that inhibition will be low in this case.[91] As already discussed, inhibition may also occur due to the excessive accumulation of water in the vicinity of enzymes, forming a macroscopic water layer, and this effect can be diminished by the adoption of high biocatalyst loadings.[87]

The effects of reagent interactions with the support used for immobilization of lipases are also relevant in solvent-free systems. Physical adsorption is a widely adopted protocol for the immobilization of lipases. However, the presence of some reagents with detergent properties (as monoglycerides, diglycerides, and long-chain fatty acids) or some solvents may cause undesirable desorption of the enzyme from the support during the reaction.[2,14,162–164]

We can adopt the same rationale that these effects are dependent on the biocatalyst loading in the system, although the literature rarely describes this kind of approach, using a constant quantity of immobilized lipase in studies of operational stability.[34,36,40,165,166]

The water activity has a strong influence on the thermodynamics and kinetics of the esterification, and in the particular case of solvent-free esterifications using immobilized lipases, water will be partitioned between the support and the reaction medium.[108,116,167] The affinity of the immobilization support for water and the reaction rate (*i.e.*, how fast the water is formed in esterification) will determine if the water can accumulate in the vicinity of the enzyme. As high biocatalyst loading in the support promotes a rapid formation of water, water may accumulate, leading to a reduction in enzymatic activity.[40] This phenomenon also has a thermodynamic implication due to changes in water availability in the organic phase, reducing the possibility of hydrolysis.[2,27,108] Colombié and co-workers (1998)[87] studied the continuous esterification of oleic acid and ethanol, reporting that the adoption of high biocatalyst loadings may diminish the water accumulation on the immobilization support when using *n*-hexane and acetone as solvents. Although obtained in the presence of solvents, these results reinforce that not only the concentration of reagents in the reaction medium will drive the extension of the effects on the immobilized lipases but also the quantity of lipases in the system.

Conclusions

This review shows that solvent-free esterification reactions using immobilized lipases present some intrinsic optimization challenges, besides the issues involved in any enzymatic esterification. In a solvent-free reaction, the evaluation of the molar ratio of the reagents should consider the changes that will occur in the reaction medium and their impact on the enzyme performance and the reaction yield. The molar ratio of the reagents gains additional importance in solvent-free esterifications because the surplus reagent may act as the solvent of the reaction besides its action on the shift of chemical equilibrium. The nature of the surplus reagent, its quantity, and the biocatalyst loading adopted are crucial to estimate the magnitude of these effects. A critical point to be considered is the change in the medium features when the reaction progresses, moving from a medium formed only by the substrates, with defined physical properties, to a medium mainly composed of the surplus reagent and the product, which is more hydrophobic than substrates. To fully obtain the advantages of a solvent-free system, it is necessary to evaluate the reaction parameters from a thermodynamic and kinetic perspective alongside the optimization process.

Conflicts of interest

There are no conflicts to declare.

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