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Medium-engineering: a useful tool for modulating lipase activity and selectivity

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Abstract: The design of a specific reaction medium capable to enhance activity, stability, and productivity of biocatalysts has been a recurring topic of study during the last three decades. The remarkable properties and valuable applications of enzymes, especially lipases, have inspiried different strategies for improving their performance in near-anhydrous media. As lipases are the most frequently used enzymes in organic synthesis, understanding the influence of reaction media on their activity and selectivity is crucial. In this paper, we review the key features of lipases and demonstrate how medium-engineering is a useful tool to modulate the activity and selectivity of lipase-catalyzed reactions.

Keywords: Lipase, non-aqueous media, medium-engineering, selectivity

1 Introduction

Lipases represent 5% of the world enzyme demand, and the market is projected to reach US $345 million in 2017 [1]. Lipases have a variety of applications: in detergents and the cleaning industry; in the synthesis of fine chemicals; in the pharmaceutical, food, pulp, paper, and energy industry; and in bioremediation and environmental processes, among others [2]. Lipases are ubiquitous in nature, being produced by animals, plants, and microorganisms [3]. From an industrial point of view, microbial lipases are the most interesting because they are easy to produce and have fewer impurities. Several important characteristics of lipases include their stability in organic solvents, their broad range of substrates, their selectivity and specificity, and their capacity to catalyze reactions without the presence of cofactors [2]. The design of a specific reaction medium capable of enhancing the activity, stability, and productivity of biocatalysts has been a popular area of study over the last three decades. "Medium-engineering" is a term defined in 1987 by Laane [4] and was applied to the design of reaction environments that produced the best enzyme performance. Indeed, the remarkable properties and applications of enzymes, particularly lipases, have initated the development of different strategies for improving enzyme performance in near-anhydrous media. Medium-engineering involves the manipulation of interactions between the enzyme matrix, reactants, products, and the biocatalyst, and stabilizing the essential water layer around the biocatalyst. In this paper, we review the key features of lipases and how medium-engineering is a useful tool to modulate their activity and selectivity in synthesis reactions.

2 Characteristics of lipases

2.1 Structural characteristics of lipases

Lipases are classified as triacylglycerol acylhydrolases (E.C. 3.1.1.3), and they form part of the group of serine hydrolases enzymes [5]. However, according to their structural characteristics, 38 superfamilies and 112 homologous families are identified [6]. Structurally, lipases have a common α/β hydrolase fold and a well-conserved catalytic triad; in addition, lipases have the consensus motif G-X1-S-X2-G [2]. The components of the common α/β hydrolase fold include a central, parallel β-sheet with eight beta strands, where only the second strand (β2) is antiparallel, along with six α helices on the sides of the central β-sheet that connect the strands β3 to β8 [2]. The catalytic triad is formed by a histidine, a nucleophile, and an acidic residue, where the most conserved nucleophile is serine and aspartate or glutamate is used as the acidic residue

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[2,7]. Another important structural component of lipases is the oxyanion hole, which stabilizes the tetrahedral intermediate formed during the catalytic mechanism [2]. From the sequence surrounding the oxyanion hole, three types have been identified: G**X**, GG**G**X and Y [8,9].

Analysis of the three-dimensional structures of lipases has revealed a structural component, known as a "lid," which covers the active site [2]. The basic component of the lid is one or more α helices linked to the main structure of the lipase by a flexible structure that covers the active site. In the inactive or closed conformation of lipases, the lid blocks the active site. In the presence of a lipid-water interface (or in organic solvent), the lid uncovers the active site and the lipase becomes active. This behavior is known as "interfacial activation" and is responsible for the non Michaelis-Menten behavior of lipases [10,11]. Fig. 1 depicts the structural conformations of the lipase from *R. miehei*.

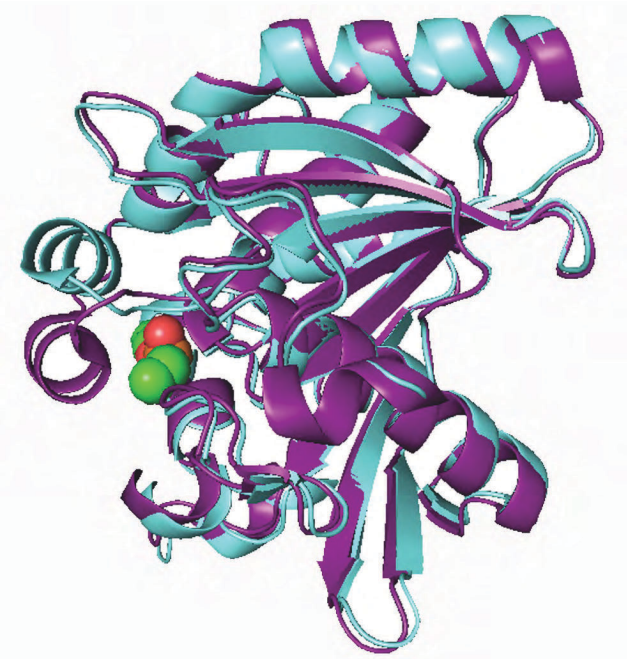


Figure 1. Opened and closed structure of Rhizomucor miehei **lipase.** Open conformation (cyan) with diethyl phosphonate as substrate (green/red); closed conformation (purple). PDB used for open form: 4TGL [12]; PDB used for closed form: 3TGL [13].

2.2 Reactions catalyzed by lipases

The natural reaction catalyzed by lipases is the hydrolysis of the ester bond found in tri-, di- and mono-glycerides to produce free fatty acids and glycerol. However, one of the most important characteristics of lipases is their ability to catalyze reactions on a wide range of substrates (Fig. 2).

Synthesis reactions can be carried out in two ways: at the interface of a biphasic system formed by an immiscible organic phase, containing the hydrophobic substrate and water [2]; or in a system with favorable thermodynamic conditions, such as low water activity. Lipases are capable of catalyzing esterification and transesterification reactions (Fig. 2) [2,11]. In esterification, the lipase links a fatty acid to an alcohol by a covalent bond, synthetizing an ester and releasing a molecule of water. Mechanistically equivalent reactions are thio-esterifiaction and amidation, where the alcohol is substituted by a thiol or an amide. Transesterification reactions catalyzed by lipases include alcoholysis, acidolysis, aminolysis, and interesterification.

More recently, novel and useful applications have been reported for different lipases, namely in lipase-catalyzed Michael addition reactions. Among the different types of bonds that can be formed through this reaction, the most significant are C–C, C–N, C–O, or C–S bonds [14]. Lipases use most of the catalytic site to accomplish the reaction, including the Thr and Gln in the oxyanion hole and the His and Asp of the catalytic triad [15]. Some lipases are also capable of catalyzing the perhydrolysis reaction, depending on the structure of the enzyme and the amino acids close to the active site [16].

2.3 Catalytic mechanism

Reactions catalyzed by lipases take place in the active site, located inside a pocket on the top of the central β-sheet [2]. The binding sites of lipases have different shapes, sizes, and depth, and are classified according to their geometry as crevice-like, funnel-like, or tunnel-like [17]. The catalytic mechanism of lipases is shown in Fig. 3. The mechanism begins with an acylation step, where a proton is transferred between the serine, the histidine, and the aspartate residues of the catalytic triad, activating the hydroxyl group of the catalytic serine [2]. This activation increases the nucleophilicity of the serine hydroxyl group, which then attacks the carbonyl group of the substrate in order to form the acyl-enzyme intermediate. The tetrahedral intermediate formed is stabilized by the oxyanion hole (Gln, Thr). In the deacylation step, a nucleophile (either water or an alcohol) attacks the acyl-enzyme complex via hydrolysis or alcoholysis and releases the product, regenerating the enzyme [2].

A different catalytic mechanism is proposed for lipase-catalyzed Michael addition reactions, which involve the addition of a nucleophile to an α, β-unsaturated carbonyl compound activated as an electrophile. Priego *et al.* (2009) proposed a mechanism for the addition of benzylamine to methyl crotonate, catalyzed by the *Candida antarctica* lipase B. They proposed that the Thr40 and Gln106 forming the oxyanion hole stabilize the negative charge of the

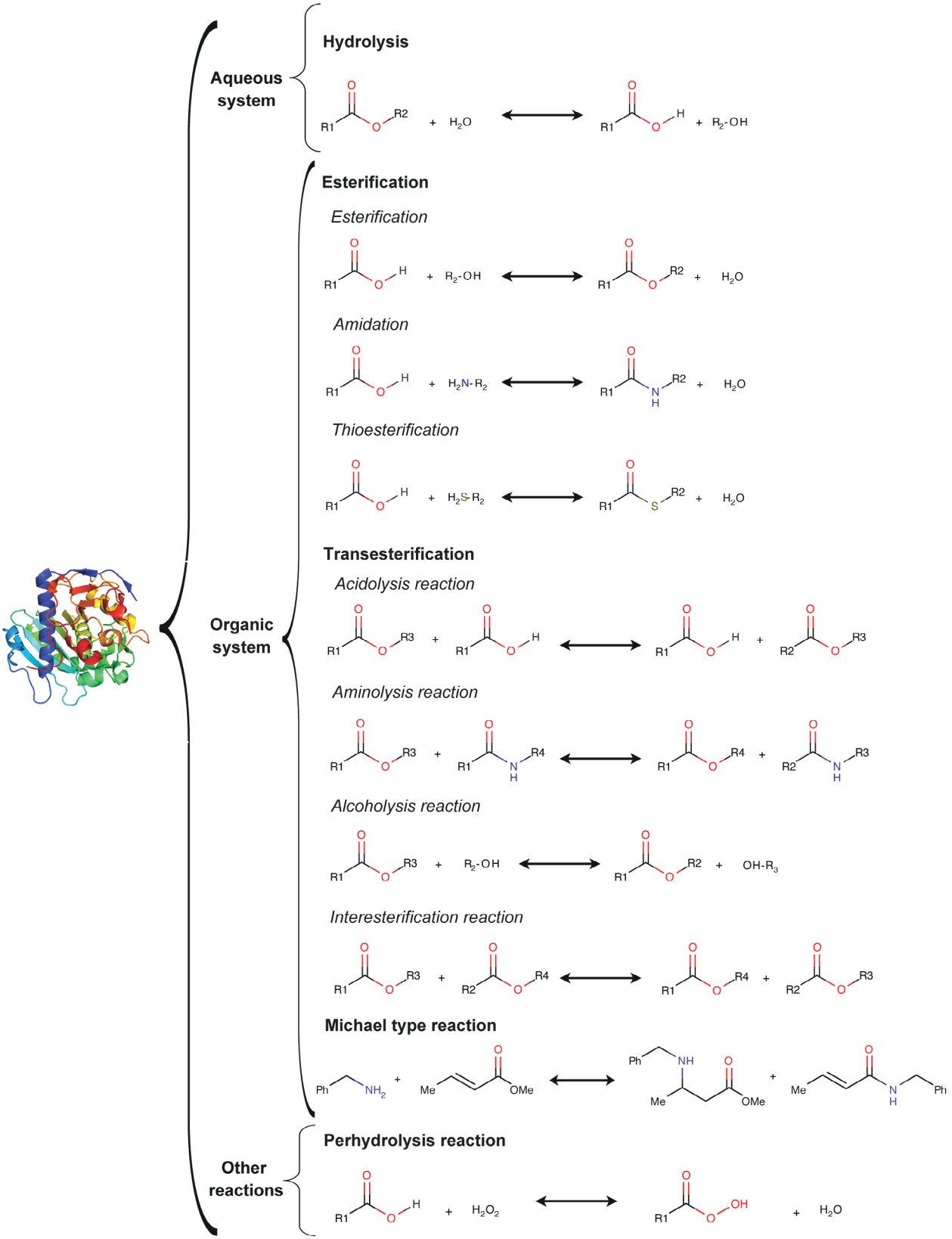


Figure 2. Reactions catalyzed by lipase in aqueous and organic systems.

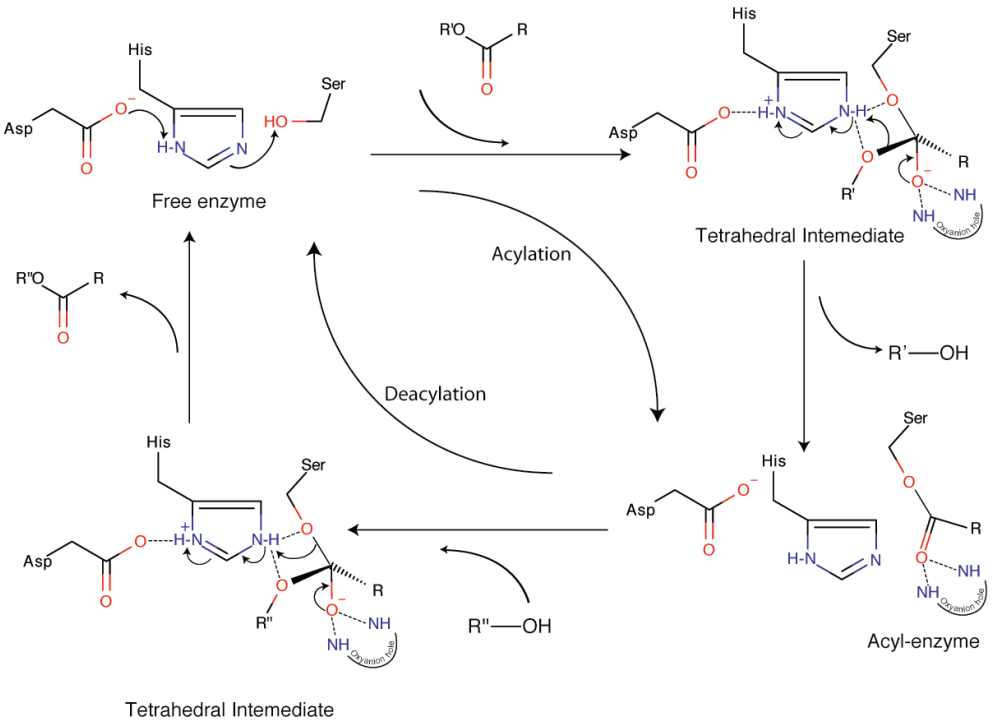


Figure 3. Catalytic mechanism of lipases, adapted from [2].

transition state, favoring the formation of an electrophilic carbocation. On its side, the His224-Asp187 pair of the catalytic triad favors deprotonation of the amine to form an activated nucleophile and facilitates proton transfer during the catalysis (Fig. 4).

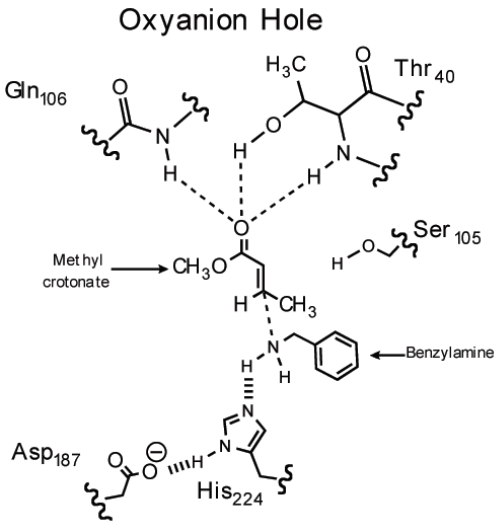


Figure 4. Proposed catalytic mechanism for the addition of benzylamine to methyl crotonate catalyzed by the Candida antarctica **lipase B.** The oxyanion hole (Thr40 and Gln106) stabilizes the negative charge of the transition state, favoring the formation of an electrophilic carbocation. The His224-Asp187 pair activates the benzylamine nucleophile [15].

2.4 Selectivity

Lipase selectivity in a reaction system includes (i) substrate selectivity, (ii) regioselectivity, and (iii) enantioselectivity (Fig. 4). Substrate selectivity is related to the lipase's preference of fatty acid chain length (*i.e.*, short, medium, long), the degree of unsaturation or substitution of the fatty acid, and the nature of the nucleophilic substrate [2]. The size of the acyl group and the type of binding site in the lipase determine the acyl group preference. Regioselectivity refers to the preference of lipases toward a particular position of the ester bond in a molecule. In the glycerol backbone of triglycerides, regioselectivity is defined as *sn*-1,3 or *sn*-2, where *sn*-1,3 selectivity is the prevalent among lipases (Fig. 5). Enantioselectivity is the selectivity of a lipase for the *R* or *S* enantiomer of a chiral molecule in a racemic mixture. Resolution of racemic mixtures by lipases is one of the most important applications of lipases in the pharmaceutical industry.

3 Lipases in organic solvents.

Aqueous systems are optimal for enzymes because water is the primary solvent in nature, and they allow the enzyme to be in an active conformation for catalysis. However, in order to catalyze synthesis reactions, a reaction medium with low water content is often required. The effect of

solvents on lipases must be considered in selecting the optimal reaction conditions for a system. An organic system increases the solubility of hydrophobic substrates, improves the thermostability of the enzyme, and shifts the thermodynamic equilibrium to synthesis over hydrolysis. However, reaction media can also impact enzyme and reactor stability (section 3.1), as well as enzyme activity, via structure modifications (section 3.2). This has an effect on the water activity (section 4), the thermodynamic activity coefficients, and the availability of substrates and products; therefore, the conversion at equilibrium (section 4.1), reaction rate (section 4.2) and selectivity (section 4.3) are all affected by the choice of reaction medium.

3.1 Lipase and reactor stability in non-aqueous media

The first requirement when choosing a reaction medium is enzyme stability; the solvent must not denature or inhibit the enzyme. Although lipases are more stable in organic solvents than other enzymes [18], some solvents can act as inhibitors. Studies on the kinetics of lipase-catalyzed esterification have revealed that primary alcohols are competitive inhibitors [19]. Indeed, following a ping-pong bi-bi mechanism, the primary alcohol either binds the acyl-enzyme intermediate (see Figure 2) or enters the active site to form an inactive lipase-alcohol complex. This phenomenon becomes more critical when the primary alcohol is also used as a solvent in "solvent-free" esterification [19] or transesterification, for example, to produce biodiesel [20].

Solvent-competitive inhibition has been studied using *Candida antarctica* lipase B (CALB) for the reaction between methyl propanoate and 1-propanol, using six organic solvents: 2-pentanone, 3-pentanone, 2-methyl-2-pentanol, 3-methyl-3-pentanol, 2-methylpentane and 3-methylpentane [21]. Results showed that ketones inhibited the activity of the enzyme, while the presence of tertiary alcohols and hydrocarbons had no negative effect. This competitive inhibition partially explains the effect of solvents on lipases.

Besides enzyme stability, reactor stability is very important for industrial applications and economic feasibility of lipase-catalyzed reactions. A particular solvent could be highly efficient in a one-time batch but inadequate for repeated batches or use in a continuous reactor. In fact, reaction system instability has been demonstrated by Colombié and co-workers for continuous lipase-catalyzed esterification in a packed-bed reactor (PBR) [22]. Hexane was the most effective solvent in batch conditions, but in a PBR the hydrophobicity of hexane

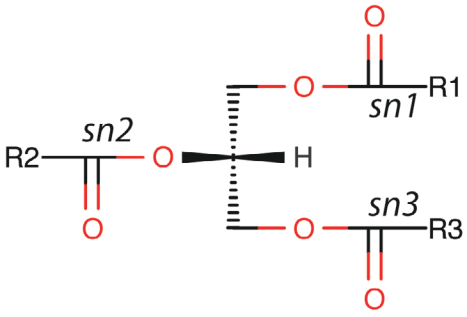


Figure 5. Regioselectivity of lipases. Ester bonds potentially hydrolyzed by lipases in a triglyceride.

caused water accumulation in the reactor (therefore decreasing the conversion). The use of acetone or 2-methyl-2-propanol as co-solvents in the PBR allowed the conversion to be maintained at higher values than those obtained in pure *n*-hexane because polar co-solvents dissolve and evacuate water. The replacement of hexane by more polar solvents or solvent-free systems with 2-methyl-2-propanol as a polar additive allowed a steady-state conversion of about 80% [22].

3.2 Structure and flexibility of lipases in organic and biphasic media

As explained in section 2.4, most lipases undergo a marked interfacial activation, where this phenomenon is strongly influenced by the surrounding environment. Therefore, the structure and flexibility of lipases in organic and biphasic media has been studied to better understand the mechanism under these conditions. The closed conformation predominates in water (as demonstrated by the low activity of lipases in water), whereas the open conformation is expected in biphasic or organic media after interfacial activation. For *Burkholderia cepacia* lipase (BCL), molecular dynamics simulations have shown lid closure in an aqueous environment and spontaneous interconversion from closed to open form within 20 ns of simulation in octane and a biphasic octane/water system [23]. Barbe and co-workers also performed experiments with increasing concentrations of detergent, observing that the presence of detergent facilitates the opening of the lid in biphasic media. The lipase from *R. miehei* (RML) was modeled in hydrophobic and hydrophilic environments to analyze the dynamic of the enzyme [24]. The solvents studied were water, methyl hexanoate, and cyclohexane. In nonpolar solvents, the charged side chains of RML folded onto the surface of the protein and more hydrogen bonds were formed internally. In addition, the loop that links the N-terminus to a β-sheet folds to the protein

surface, displacing the loop Trp55-Asn63 and pushing the active site region, causing a slight opening of the lid. In the simulation with methyl hexanoate, the solvent molecules around the RML moved more slowly than the water molecules [25]. In organic solvents the size of the protein is similar to its structure in water, but the lipase in more rigid and the electrostatic interactions in the surface of the protein are stronger [25]. The structure and flexibility of CALB was studied by Trodler and Pleiss [26] in water, methanol, chloroform, isopentane, toluene, and cyclohexane, and the simulations were compared to the crystal structure. The simulations showed that there were no significant structural changes in the lipase for all the solvents studied, and the enzyme showed high stability. However, they observed changes in the binding sites of water molecules at the surface of the enzyme. A spanning water network (SWN) consists of water molecules bound to polar side chains or to the backbone of the enzyme, with water molecules bridging these bound waters. In organic solvents, an SWN covered the surface of CALB, and SWN coverage decreased with decreasing partition coefficient (logP) of the solvent. In water and methanol, SWN was not observed. Fig. 6 shows less mobile water molecules at the surface of CALB for simulations in water (A) and cyclohexane (B), where an SWN occurs for cyclohexane.

In organic solvents, CALB was observed to be less flexible in the core and the active site, with increasing flexibility at the surface. Flexibility decreased in the following order: methanol, isopentane, chloroform, toluene, and cyclohexane [26].

It has also been reported that the hydrophobic areas on the surface of CALB in solvent are larger than in the crystal structure, and the hydrophilic areas decrease with increasing polarity of the solvent [27]. In nonpolar solvents, the polar side chains usually accessible in the crystal structure move to the interior of the enzyme, while the nonpolar side chains are exposed to solvent molecules [27]. The funnel-like binding site of CALB remains stable in the presence of organic solvents, but polar solvents cause a conformational change in the active site, reducing the activity of this enzyme [27].

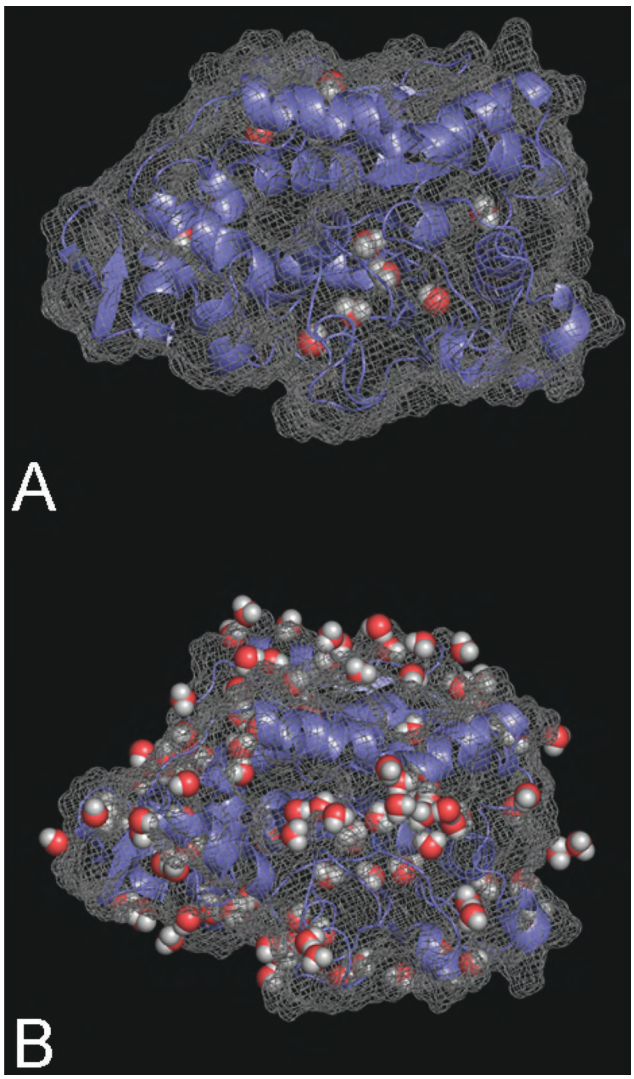


Figure 6. Less mobile water molecules at the surface of CALB for simulations in water (A) and cyclohexane (B). Water is represented by red and white spheres. Figure reproduced from [26] under the terms of the Creative Commons Attribution License.

4 hermodynamic Strategies

In constrast to the behaviour of enzymes in aqueous media, the behaviour of biocatalysts in non-aqueous media (NAM) depends on different parameters that take into account the molecular interactions between substrates and products, and enzyme preparation in each reaction medium. Enzyme stability is a main consideration in the choice of NAM. In fact, enzymes require a certain amount of water in order to maintain their catalytic properties and to avoid distorting the essential water-biocatalyst interactions [4]. As a rule of thumb, non-polar solvents are recommended as reaction media because they do not strip essential water from biocatalysts [28]. On the contrary, polar solvents are not suitable reaction media because they promote the decay of enzyme activity by weakening the water-biocatalyst interactions. Nowadays, these considerations are still applied to lipase-catalyzed reactions carried out in solvent mixtures, neoteric solvents such as ionic liquids, and free solvents [29–32]. It is noteworthy that the total water content in NAM may give limited information about the water requirements of the biocatalyst. Although the total water content may be the same in different

reaction media, the unique chemical properties of each medium give rise to different interactions with water; therefore, the availability of water or interactions with the enzyme will differ [33]. One of the most useful parameters for describing the relationship of water with the reaction medium and enzyme is the thermodynamic water activity (a_w, Eq. 1). This thermodynamic parameter is a measure of the effective amount of water that is able to chemically interact with the system. The a_w is commonly expressed as

$$a_w = \gamma_w X_w \quad \text{Eq. 1}$$

where X_w accounts for the total moles of water in a mixture and g_w is the activity coefficient that describes the deviation of water in a mixture from ideal behavior [34]. Therefore, for the numerous solvents and solvent mixtures available for an enzymatic process, water activity should be individually estimated.

4.1 Manipulating yields and productivities in lipase-catalyzed reactions

In addition to the stability of biocatalysts, attention has been given to enhancing reaction yields, catalytic activity, and selectivity in lipase-catalyzed reactions. For lipase-catalyzed reactions in NAM, the yield is dependent on the final equilibrium position, which is strongly influenced by the mild conditions under which lipases function and also several thermodynamic properties of solvents [32,35]. In particular, manipulating the equilibrium position has been applied to controlling regio- and chemoselectivity as well as the degree of acylation in many lipase-catalyzed reactions [36-46]. In fact, experiments show that certain thermodynamic parameters of solvents, such as partition coefficient (logP) and dielectric constant, are correlated to the equilibrium position in lipase-catalyzed esterifications performed in organic solvents [43-45]. Early attempts to rationalize the effect of solvents on the thermodynamic position of equilibrium involved the lipase-catalyzed acylation of polyols in aqueous-organic two-phase systems. Jenssen and co-workers [40,41] proposed that the yield of products at equilibrium is strongly influenced by the solvent-solute interactions generated by substrates, products, and solvents as the main components of the system. The thermodynamic activities of substrates and products (a_i) were calculated as a reliable estimation of these interactions; therefore, the position of thermodynamic equilibrium was determined on this basis. From these calculations, it was proposed that polar products are favored in the presence of polar solvents, while non-polar products are preferentially obtained in non-polar solvents. In order to enhance product yield and to avoid undesirable byproducts, this principle was later applied to multi-step, reversible reactions carried out in monophasic systems [36,37]. Accordingly, manipulation of the thermodynamic equilibrium positions on multi-step, reversible reactions was carried out under reliable estimation of the thermodynamic activities of substrates and products.

The thermodynamic equilibrium constant (*Keq*) of any lipase-catalyzed esterification is determined from the equilibrium expression (Eq. 2):

$$R - OH_{(alcohol)} + R1 - COOH_{(acid)} \leftrightharpoons R1 - COOR_{(ester)} + H_2O_{(water)} \quad \text{Eq. 2}$$

The constant *Keq* or *KXeq* may be defined in terms of concentrations (Eq. 3) or mole fractions (Eq. 4) of water, ester, alcohol, and acid:

$$K_{eq} = \frac{[water][ester]}{[alcohol][acid]} \quad \text{Eq. 3}$$

or

$$K_{Xeq} = \frac{X_{water} * X_{ester}}{X_{alcohol} * X_{acid}} \quad \text{Eq. 4}$$

Both expressions are considered to be suitable for ideal solutions but are not accurate for real solvents. One of the parameters used to represent the deviation from ideal behavior is the thermodynamic activity coefficient (*yi*). This parameter accounts for most of the interactions occurring between chemical species in a solution and allows for more accurate estimations of *Keq*. The combination of *yi* and the effective concentration of a substance in a solution (*Xi*) determine the thermodynamic activity of a substance, *ai* (Eq. 5). This parameter is a measure of the effective amount of a substance needed to carry out a chemical process.

$$a_i = \gamma_i X_i \quad \text{Eq. 5}$$

Therefore, **Keq** may be adjusted for deviations of ideal behavior as follows:

$$K\gamma = \frac{\gamma_{water} * \gamma_{ester}}{\gamma_{alcohol} * \gamma_{acid}} \quad \text{Eq. 6}$$

$$Ka_{eq} = K_{Xeq} * K_\gamma = \left[\frac{X_{water} * X_{ester}}{X_{alcohol} * X_{acid}}\right] * \left[\frac{\gamma_{water} * \gamma_{ester}}{\gamma_{alcohol} * \gamma_{acid}}\right] \quad \text{Eq. 7}$$

$$Ka_{eq} = \frac{a_{water} * a_{ester}}{a_{alcohol} * a_{acid}} \quad \text{Eq. 8}$$

The mole fraction of a desired product at equilibrium increases in the presence of solvents that decrease the activity coefficient (Eqns. 7 and 8). Solvents that interact strongly with the desired product decrease the value of γi, therefore increasing the effective concentration of the product in solution (*Xi*). On the other hand, in order

to avoid undesirable byproducts at equilibrium, weakly interacting solvents should be used, which increase the γ*i* value. Therefore, a need arose for a reliable method for calculating γ*i* values in complex mixtures. Using the UNIversal Functional Activity Coefficient (UNIFAC) group contribution method, Janssen and co-workers estimated γ*i* values of substrates and products and predicted *Kaeq* for different lipase-catalyzed reactions [40,41]. This method was also used for the consecutive esterification of polyols carried out in monophasic mixtures of organic solvents [36,37]. Calculations of *yi* using UNIFAC allowed the prediction of thermodynamic equilibrium position as a function of solvent composition. Some deviations of the model were observed in all approaches and were attributed to deficiencies in the semi-empirical UNIFAC calculation method. The lack of precision in the classical UNIFAC method for some applications has been attributed to its limited ability to address complex molecular interactions, such as molecular orientations, molecular conformations, and even intramolecular interactions [34]. Recently, Klamt and co-workers [47] proposed an alternative theoretical method for predicting thermophysical properties of pure and mixed fluids: the COnductor-like Screening MOdel for Realistic Solvation (COSMO-RS). This method, based on quantum chemical calculations and statistical thermodynamics, has proven to be a powerful predictive tool for estimating thermodynamic properties of solutes and solvents. The COSMO-RS model has been used to calculate activity coefficients for chemical species present in complex mixtures of solvents, and the model has been validated by its correct prediction of the thermodynamic equilibrium position of different biocatalytic esterification processes [35,38]. It is important to emphasize that an understanding of molecular interactions among substrates and products with reaction media contribute to predicting the behavior of enzymatic processes. For non-aqueous phases, this understanding is essential for describing the enzyme kinetics and predicting thermodynamic equilibria.

4.2 Thermodynamic strategy for understanding Kinetics of Lipase-Catalyzed Reactions

The purpose of using NAM in biocatalysis is not only to shift the thermodynamic equilibrium towards synthesis. In fact, the choice of solvent will also affect the kinetics of the enzymatic processes. As previously mentioned, the catalytic properties of enzymes are clearly affected by the solvent properties. Indeed, much information has been published concerning the structural modifications of enzymes promoted by solvents [48,49]. However, limited information is available concerning the effects of solvents on enzyme kinetics, bearing in mind that solvents affect the thermodynamic activity of substrates and products. In fact, it is important to note that the kinetic behavior of enzymes according to the Michaelis-Menten model is strongly dependent on the substrate and product concentrations. For example, in the case of substrate inhibition, the choice of solvent becomes crucial in order to maintain the optimal substrate availability and therefore attain the maximum reaction rate. In their seminal work, Sandoval and co-workers report the kinetics of lipase-catalyzed esterification between oleic acid and ethanol. They propose a modified Michaelis-Menten equation where the thermodynamic activities of the reactants are used (Eq. 9) instead of their concentrations (Eq. 10) [50].

$$V_i = \frac{V_m a_{\text{Oleic}} a_{\text{Eth}}}{Km_{\text{Oleic}} a_{\text{Eth}} \left(1 + \frac{a_{\text{Eth}}}{K_i}\right) + Km_{\text{Eth}} a_{\text{Oleic}} + a_{\text{Oleic}} a_{\text{Eth}}} \quad \text{Eq. 9}$$

$$V_i = \frac{V_m [\text{Oleic}][\text{Eth}]}{Km_{\text{Oleic}} [\text{Eth}] \left(1 + \frac{[\text{Eth}]}{K_i}\right) + Km_{\text{Eth}} [\text{Oleic}] + [\text{Oleic}][\text{Eth}]} \quad \text{Eq. 10}$$

Where *Vi* is the initial reaction rate; *Vm* is the maximum rate; [Oleic] and [Eth] are the concentrations of oleic acid and ethanol, respectively, and a_{Oleic} and a_{Eth} are their corresponding thermodynamic activities; Km_{Oleic} and Km_{Eth}, are the affinity constants for oleic acid and ethanol, respectively; and *Ki* is the competitive inhibition constant of ethanol. Although in the original paper the activity coefficients were calculated using the classic UNIFAC group contribution method, the COSMO-RS method also yielded accurate results (data not published). This thermodynamic kinetic strategy, known as TABEK (Thermodynamic Activity-Based Enzyme Kinetics), was used to calculate the kinetic parameters of different lipase-catalyzed esterification and transesterification reactions carried out in a reference solvent. Taking into consideration that the activity-based kinetic parameters were identical in all solvents, the kinetic behavior of the lipase was predicted for different solvents. Experimental results showed a high correlation with the prediction, particularly when reactions were carried out in hydrophobic solvents. When reactions were carried out in polar solvents, an adjustment of the *Vm* kinetic parameter was made in order to account for possible solvent-enzyme interactions. Until now, this thermodynamic strategy has been one of the most consistent in predicting the effects of solvents on enzyme kinetics.

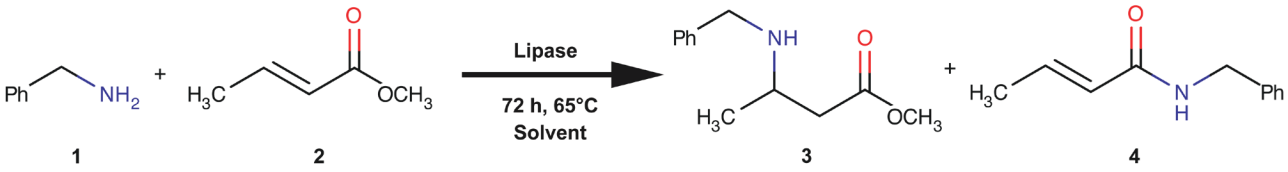
4.3 Thermodynamic Control of Selectivity in Lipase-Catalyzed Reactions

Enzymes are recognized to be highly selective and moderately stable in aqueous media. However, in NAM, selectivity may be improved or even reversed, and operational stability significantly enhanced. Although an important part of this phenomenon is the inherent chemo-, enantio-, prochiral- or regioselectivity of the enzyme, numerous reports suggest there is a powerful influence of reaction media on enzyme selectivity.

Concerning the lipase-catalyzed resolution of enantiomers, it has been concluded that it is very difficult to correlate the properties of solvents (*e.g.*, logP, solvent size, electron pair acceptance index, or dielectric constant) to changes in selectivity [51,52]. For example, Rivera and co-workers [53] found high enantioselectivities in solvents with logP values ranging from 3.2 to 5.6 for the *Carica papaya* lipase (CPL)-catalyzed racemic resolution of (*R*,*S*)-2-bromophenylacetic acid octyl ester, although differences in initial reaction rate were observed (Table 1). As Wolf and co-workers stated when performing a thermodynamic quantitative evaluation of interactions between solvents and substrates, "there is no rule on the changes induced by the solvents over the enzyme enantioselectivity but rather an individual effect of solvents on enantioselectivity" [54]. Indeed, the overall thermodynamics of the system has an effect on the initial reaction rate [50] and therefore on enantioselectivity (defined as the ratio of initial velocity of transformation or formation of each enantiomer).

The role of the physicochemical properties of organic solvents on enzyme chemoselectivity in lipase-catalyzed reactions has also been considered, particularly in different lipase-catalyzed *aza*-Michael reactions [15,55,56]. Novel and valuable applications of this type of reaction have recently been reported, namely *aza*-Michael addition type reactions involving the addition of amines to α,β-unsaturated systems. When α,β-unsaturated systems involves a carboxylic acid, the chemoselectivity of lipase-catalyzed *aza*-Michael additions is mainly hampered by the two electrophilic sites present on these compounds (*i.e.*, the α,β-unsaturation and the carboxylic or ester functional group). Based on thermodynamic considerations, a solvent engineering strategy was proposed as a tool for controlling chemoselectivity in a lipase-catalyzed reaction between benzylamine (1) and methyl crotonate (2) to produce a Michael adduct (3) over the aminolysis product (4) (Scheme 1).

The chemoselectivity of this process was explained in terms of the polarity of the medium. Thus, while Michael adduct (3) was preferentially formed in hydrophobic reaction media, amminolysis product (4) was favored in the presence of polar solvents. Recently, Steunenberg and co-workers [56] reported that the lipase-catalyzed chemoselective addition of primary and secondary amines to acrylate and alkyl acrylate esters was influenced by the polarity of the solvent. In hydrophobic solvents, the synthesis of Michael adducts was favored, while in polar solvents, aminolysis products were preferentially accumulated. In this context, Rivera-Ramírez and



1 2 3 4

Scheme 1. Lipase-catalyzed reaction between benzylamine (1) and methyl crotonate (2). Michael adduct product (3) and aminolysis product (4).

Table 1. **Solvent effects on the racemic resolution of** (R,S)**-2-bromophenylacetic acid octyl ester catalyzed by CPL.** Reprinted from [53], with permission from Elsevier.

Solvent (LogP)[a]	**Initial reaction rate** *S*- **enantiomer,** r_s **(μmol.h^{-1}.g enzyme^{-1})**[b]	*E* (r_s/r_R)
Cyclohexane (3.2)	1.2 ± 0.31	>200
Octane (4.0)	1.1 ± 0.23	>200
Heptane (4.6)	1.6 ± 0.15	>200
Decane (5.6)	2.4 ± 0.10	>200

[a] LogP (Solvent partition coefficient) values taken from Chemspider (www.chemspider.com).
[b] Mean and standard deviation of two independent experiments are presented.

co-workers obtained different *aza*-Michael adducts from benzylamine and different acrylates with high chemoselectivity [55]. In agreement with previous reports, Michael adducts were exclusively synthesized in highly hydrophobic solvents, while the synthesis of aminolysis products was more favorable in the polar solvent 2-methyl-2-butanol. Interestingly, a thermodynamic analysis of these processes using the COSMO-RS method estimated the thermodynamic interactions of solutes and solvents (γi) and led to an appropriate selection of a chemoselective reaction medium. It is worth mentioning that in these processes enzyme promoted chemoselectivity was in agreement to the IUPAC definition, "chemoselectivity is the preferential reaction of a chemical reagent with one of two or more different functional groups."

5 Conclusions

We have shown in this review that attempts in the past three decades to explain and modulate lipase selectivity and activity in organic media follow three main strategies of medium-engineering: 1) solvent polarity choice according to substrates, products, and reactor characteristics; 2) structural analysis of changes induced by the solvent; and 3) thermodynamic control of the overall reaction system. In addition to these strategies, other useful tools, such as immobilization and protein engineering, can be used to enhance the performance of lipases.

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