Biotechnology Advances 31 (2013) 1846–1859

Contents lists available at ScienceDirect

Biotechnology Advances

journal homepage: www.elsevier.com/locate/biotechadv

Research review paper

Advances in lipase-catalyzed esterification reactions

Panagiota-Yiolanda Stergiou [a], Athanasios Foukis [a], Michalis Filippou [b], Maria Koukouritaki [b], Maria Parapouli [b], Leonidas G Theodorou [a], Efstathios Hatziloukas [b], Amalia Afendra [b], Ashok Pandey [c], Emmanuel M Papamichael [a,*]

[a] University of Ioannina, Department of Chemistry, Group of Enzyme Biotechnology and Genetic Engineering, Ioannina 45110, Greece
[b] University of Ioannina, Department of Biological Applications & Technologies, Group of Enzyme Biotechnology and Genetic Engineering, Ioannina 45110, Greece
[c] CSIR-National Institute for Interdisciplinary Science & Technology (NIIST), Head, Centre for Biofuels & Biotechnology Division, Trivandrum 695 019, India

ARTICLE INFO

Article history:
Received 22 May 2013
Received in revised form 2 August 2013
Accepted 5 August 2013
Available online 15 August 2013

Keywords:
Esterification
Lipases
Biofuel
Influence of pH
Lipase immobilization
Water activity
Ester yield
Whole cell biocatalysis

ABSTRACT

Lipase-catalyzed esterification reactions are among the most significant chemical and biochemical processes of industrial relevance. Lipases catalyze hydrolysis as well as esterification reactions. Enzyme-catalyzed esterification has acquired increasing attention in many applications, due to the significance of the derived products. More specifically, the lipase-catalyzed esterification reactions attracted research interest during the past decade, due to an increased use of organic esters in biotechnology and the chemical industry. Lipases, as hydrolyzing agents are active in environments, which contain a minimum of two distinct phases, where all reactants are partitioned between these phases, although their distribution is not fixed and changes as the reaction proceeds. The kinetics of the lipase-catalyzed reactions is governed by a number of factors. This article presents a thorough and descriptive evaluation of the applied trends and perspectives concerning the enzymatic esterification, mainly for biofuel production; an emphasis is given on essential factors, which affect the lipase-catalyzed esterification reaction. Moreover, the art of using bacterial and/or fungal strains for whole cell biocatalysis purposes, as well as carrying out catalysis by various forms of purified lipases from bacterial and fungal sources is also reviewed.

Contents

* Corresponding author. Tel.: +30 2651008395; fax: +30 2651008799.
E-mail addresses: pstergiu@cc.uoi.gr (P.-Y. Stergiou), afoukis@cc.uoi.gr (A. Foukis), Michalis.Ph@hotmail.com (M. Filippou), maria_koukou7@hotmail.com (M. Koukouritaki), me00762@cc.uoi.gr (M. Parapouli), me00217@cc.uoi.gr (L.G. Theodorou), ehatzilu@cc.uoi.gr (E. Hatziloukas), aafendra@cc.uoi.gr (A. Afendra), pandey@niist.res.in, ashokpandey56@yahoo.co.in (A. Pandey), epapamic@cc.uoi.gr (E.M. Papamichael).

0734-9750/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.biotechadv.2013.08.006

1. Introduction

Lipases are highly stable enzymes, which remain active even under unfavorable conditions. They are obtained in satisfactory yields from animals, plants, and natural or recombinant microorganisms, and have found a plethora of applications in food and pharmaceutical industries and technologies, as significant biocatalysts (Pandey et al., 1999). The physiological role of lipases (water-soluble triacylglycerol acylhydrolases, EC 3.1.1._) is the catalytic conversion of tri-glycerides into di-, or mono-glycerides, fatty acids and glycerol. A number of lipases are unable to hydrolyze ester bonds at secondary positions, as most of microbial lipases do, while another group of these enzymes hydrolyzes both primary and secondary esters. A third group of lipases exhibits fatty acid selectivity, and cleaves ester bonds of particular types of fatty acids (Krishna and Karanth, 2002). Lipases, which are serine hydrolases, are of considerable industrial potential, and catalyze esterification, interesterification and transesterification reactions in non-aqueous media (organic solvents and supercritical fluids), usually for biofuel production. Lipases catalyze also alcoholysis, acidolysis and aminolysis reactions, as well as hydrolyze organic carbonates (Pandey et al., 1999). In any case, the course followed by a lipase depends strongly on the aqueous content of the reaction medium, as absence of water eliminates the competing hydrolysis reaction. Conversely, there is a variety of reports on lipases catalyzing synthetic reactions in non-aqueous, or in low water content systems (Knežević et al., 2004; Krishna and Karanth, 2002; Hasan et al., 2009).

Lipases perform catalysis via a motif comprising three residues (serine, histidine and aspartate or glutamate); however, existing evidence indicates a convergence of the catalytic motifs of serine proteases and lipases (Kokkinou et al., 2012; Matsumura et al., 2008). An essential catalytic feature of lipases is a surface loop, the lid domain, which covers their active site (Aloulou et al., 2006; Meier et al., 2007), although a few lipases do not display a lid structure (Krishna and Karanth, 2002). Different reaction mechanisms describe lipase-catalyzed hydrolysis, esterification, and transesterification reactions, depending also on the specific used medium; thus, mostly non-Michaelis–Menten kinetic models have been suggested, which are applied in non-isotropic media and comprise steps leading to lipase activation and the formation of the corresponding enzyme–substrate complexes (Aloulou et al., 2006; Papamichael et al., 2012). Nevertheless, it should be emphasized that any lipase-catalyzed process (including synthesis) is influenced by the lipase stability, selectivity, mass transfer and other factors (Tufvesson et al., 2011), and one might choose from a variety of lipase-forms in order to use it as a biocatalyst, i.e. as: (a) whole-cell catalysis (lipases kept inside the host cell), in either a free or immobilized form, concomitantly taking into account the cost of side reactions, (b) liquid formulated lipases (lipases dissolved in aqueous solutions), and (c) immobilized lipases (lipases immobilized in solid matrices) either by cross-linking, or encapsulation, or adsorbing and/or covalent linking onto a matrix. In addition it should be taken into account that lipases should be able to retain water, since these enzymes may need the interface to work (Nielsen et al., 2008).

Finally, as most of the articles dealing with the lipase-catalyzed esterification focus mainly on the developed techniques and the yield optimization, herein, we not only report on the available mechanistic principles of the esterification reaction and its inherent difficulties due to fact that it is performed in organic solvents and/or in biphasic media (Kvittingen, 1994 and references therein), but also in contrast deal with the key factors and courses affecting the lipase-catalyzed esterification for biofuel production, their trends, challenges and future perspectives.

2. The main reactions

2.1. Hydrolysis

In general, enzymatic hydrolysis is considered important in science, technology and industry. Specific hydrolases, e.g. lipases, degrade lipids and other esters in a variety of scientific and industrial processes (Papamichael et al., 2012). The hydrolysis of natural and artificial esters is an unusual reaction, more likely due to the opposite polarities of hydrophobic substrates, and hydrophilic catalysts and products; this reaction is mainly occurring at the aqueous/organic solvent interface, although the interfacial composition is a matter of further research concerning the reaction microenvironment. Since many years ago, an array of works has been published reporting that the feedback mechanism of ester hydrolysis, including the digestion of triglycerides, could offer important information concerning the understanding and control of this reaction course (Aloulou et al., 2006; Reis et al., 2009).

2.2. Esterification

Enzyme-catalyzed esterification acquired increasing attention in many applications, due to the significance of the derived products. More specifically, the lipase-catalyzed esterification reactions attracted research interest during the past decade, due to an increased use of organic esters in biotechnology and the chemical industry (Torres and Castro, 2004). For this reason, esterification by lipases was developed a few decades ago (Okumura et al., 1979) and various microbial lipases have been employed in experiments using either primary or secondary alcohols, or both, free-solvent systems, or organic solvents. Among the important factors which influence the ester yield are the concentrations of enzyme and substrates, their molar ratio, the reaction pH-value and temperature, the mixing rates, and the water content (Zaks and Klibanov, 1988 and references therein).

3. Lipases as biocatalysts — the kinetics

Lipases, as hydrolyzing agents are active in environments, which contain a minimum of two distinct phases, where all reactants are partitioned between these phases, although their distribution is not fixed and changes as the reaction proceeds. Furthermore, the complications of phase heterogeneity and its temporal changes should be also considered. As a consequence, the level of understanding of the regulation of the catalysis by lipases has lagged behind that for the homogeneous one (Pandey et al., 1999; Reis et al., 2009).

In recent reports, numerous enzymes were classified according to their folding pattern. Lipases from different sources are normally categorized in the α/β-hydrolase folding group, sharing the same or at least similar folding patterns with esterases. Lipases, as hydrolyzing enzymes possess specific sequences of α-helices and β-strands. Detailed information on these structures has been reported previously (Jaeger et al., 1999, and references therein; Joseph et al., 2008 and references therein). In addition, a tool has been described to differentiate between lipases and esterases based on their protein surface electrostatic

distribution, and amino acid sequence and composition. However, lipases exhibit a statistically significant characteristic, i.e. the occurrence of non-polar residues clustered around the active site at high solvent accessibility values. Furthermore, any information on the three-dimensional structures, as well as on the factors, which influence the regiospecificity and enantiospecificity of lipases, is essential for its future applications, even though these properties were found distinctly different among lipases, despite their high amino acid sequence homology (Fojan et al., 2000 and references therein).

In order to improve both yield and quality of the lipase-catalyzed ester synthesis, efforts were made in studying the kinetics (i.e. the development of rate expressions, and the reaction mechanisms), the rate of product formation, the effects of changes in system conditions, and the design of the appropriate reactors (Yahya et al., 1998). Most of the mechanistic models reported up till today are based on several applications e.g. Michaelis–Menten kinetics, ping-pong bi-bi mechanism, etc. (Krishna and Karanth, 2002).

Although, new, irreversible lipase-catalyzed methods have been developed for carboxylic acid esterification in non-aqueous media based on the chemical destruction of the water produced during the process (Jeromin and Zoor, 2008), most of the problems of these type of reactions could be overcome by immobilizing lipases on water insoluble carriers, which augment their use and contributes to the development of economical continuous bioprocesses (Knežević et al., 2004). Different types of immobilization can be attained, either by attachment to a carrier in various ways (covalently, by hydrophobic binding, by ion exchange, by cross-linking), and by holding in a barrier (i.e. reversed micelles, polymeric matrices, hollow fibers, etc), or by means of precipitation in organic solvents. However, adsorption is commonly considered as easy, and less expensive and/or less harmful for the lipase activity (Paiva et al., 2000). Nevertheless, it should be taken into account that an immobilized enzyme is normally localized in a defined inert material, which although allows its physical separation from the bulk reaction medium, it is permeable to all reactant molecular species (Balcão et al., 1996a).

Furthermore, a reaction scheme needs to be established along with the identification of substrates, products, and key-characteristics, as well as the process constraints (phase behavior and kinetics during the reaction). Additionally, if immobilized lipases need to be selected the task of the project should be fulfilled (phase behavior and reaction hydrodynamics), as well as the reactor and its characteristics (reaction hydrodynamics and operation mode). Moreover, an enzyme kinetic study (activity and stability), and the study of other characteristics (flow pattern, solid suspension, mass transfer, etc.) in laboratory scale are also fundamentals before an industrial scaling up of any proposed organic acid esterification process.

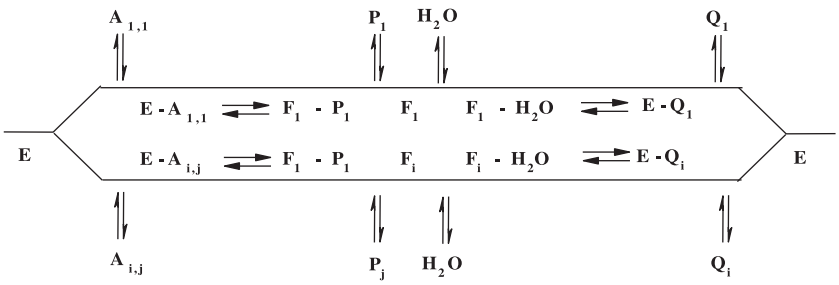
Finally, we should refer to what is well known among enzymologists as kinetics and mechanisms. Generally, in the case of immobilized lipase-catalyzed reactions, only awkward equations can be formulated for the reaction rate expressions, whose simplified forms are still complicated; anyway, it is understood that there is no reason to refer herein to kinetics and mechanisms of non-immobilized lipases.

It is widely accepted that lipase-catalyzed reactions (hydrolysis, esterification, etc.) can be described by a ping-pong bi-bi mechanism, which proceeds through the formation of an acyl-enzyme, as it is expressed in reaction Scheme 1 (as modified from Cleland, 1970). In Scheme 1 E = lipase, A = ester moiety, P = alcohol moiety, F = Acyl-lipase, Q = acid moiety, and $i = 1, 2, \ldots, I$ and $j = 1, 2, \ldots, J$, are types of acid and alcohol moieties, respectively. In these cases, the equilibrium is converted between hydrolysis and synthesis, depending highly on the water content of the reaction medium, as hydrolysis and ester synthesis are promoted by macro- and micro-aqueous systems, respectively. Therefore, and in order to describe the system under consideration in mathematical terms, suitable assumptions need to be made on the occurring elementary reaction steps, i.e. to suggest a mechanism, and estimate the values of rate expressions and kinetic parameters using appropriate experimental data, and equations.

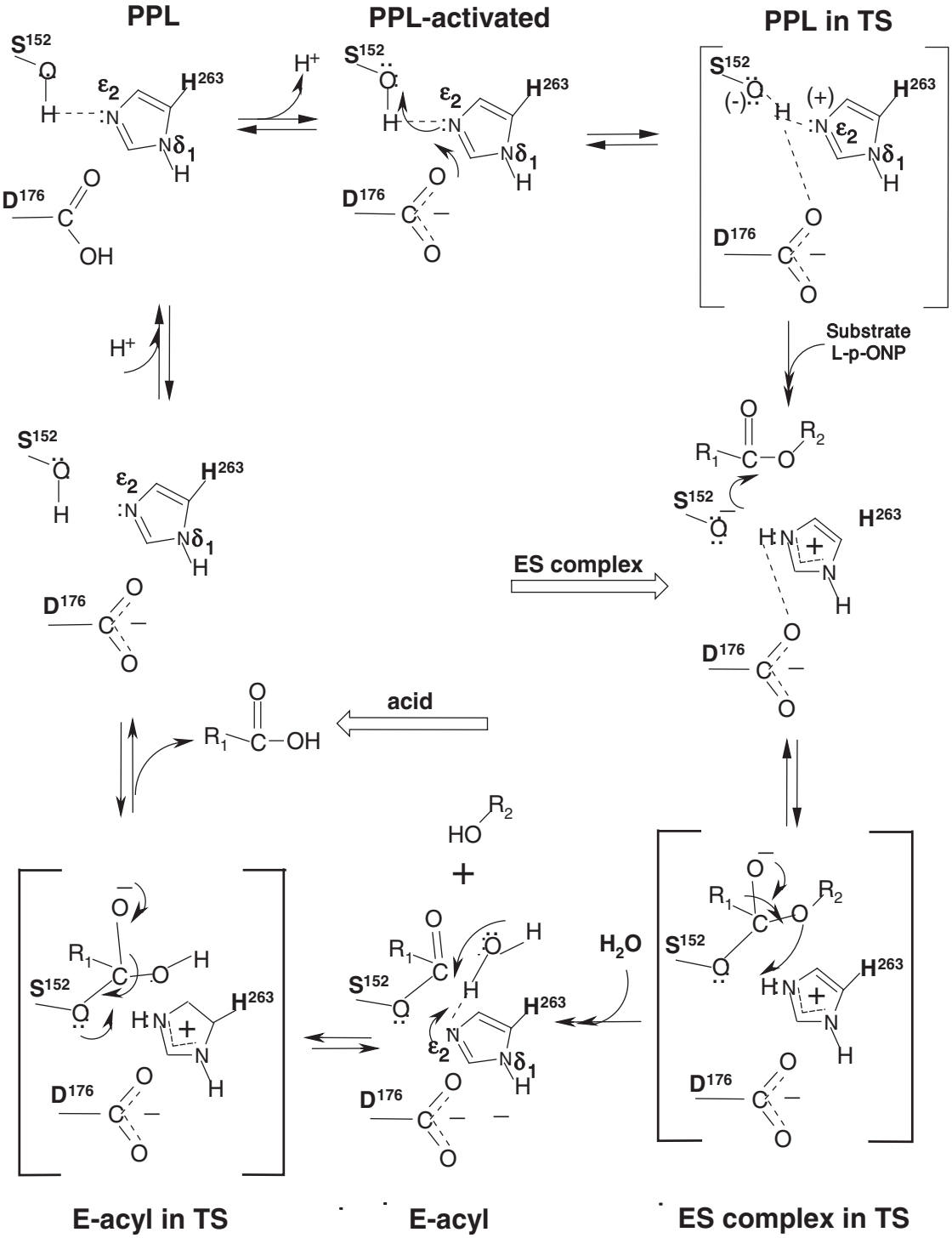
As already mentioned, a catalytic triad consisting of three residues (serine, histidine and aspartate or glutamate) carries out the catalysis by lipases, however there is little homology among the known sequences of these enzymes, whose catalytic motifs are evidently of convergent nature to those of serine proteases. In the amino acid sequence of lipases their conserved catalytic motif, i.e. serine (the nucleophile), aspartate or glutamate and histidine are found always in this order, and it differs significantly from that observed in other hydrolases equipped with catalytic triads. In several lipases, a cysteine or an aspartate plays the role of the nucleophilic serine. In the α/β hydrolase folding, the catalytic aspartate (or glutamate) is hydrogen-bonded to the catalytic histidine, which is located in a loop with variable length and conformation (Joseph et al., 2008 and references therein).

As lipases and serine proteases perform catalysis via similar catalytic triads, it is expected to proceed along similar mechanisms in aqueous media; however, and as it has been reported previously, the development of the oxyanion hole differs in lipases due to their structural particularities. In this way, a full mechanism of action has been reported for the bovine pancreas lipase in its hydrolysis of p-nitrophenyl laurate (reaction Scheme 2, as modified from Kokkinou et al., 2012), which is analogous to this, reported for serine proteases (Kokkinou et al., 2012; Papamichael et al., 2012).

However, when lipases perform catalysis in multi-phase systems (e.g. in non-aqueous media or as immobilized enzymes on water insoluble carriers, etc.) and since these are non-isotropic media, they should be described by alternative kinetic models, which could be based on the Michaelis–Menten approach. Different models of this kind have been suggested, comprising a physical adsorption of lipase at the aqueous/lipid interface, leading to the formation of a lipase–substrate complex, followed by a chemical step, which gives the products and the adsorbed free enzyme (Aloulou et al., 2006 and references therein). In a typical two-dimensional Michaelis–Menten catalytic approach (e.g. hydrolysis), the soluble Lipase (E) is adsorbed on an aqueous/organic interface (E*), which binds the substrate molecule and forms the E*S complex. Then, after several necessary reaction sub steps, the soluble products P_1^* and P_2^* are diffused in the water layer (P_1 and P_2), as illustrated in reaction Scheme 3 (as modified from Aloulou et al., 2006, and



Scheme 1. The ping pong bi-bi reaction scheme.



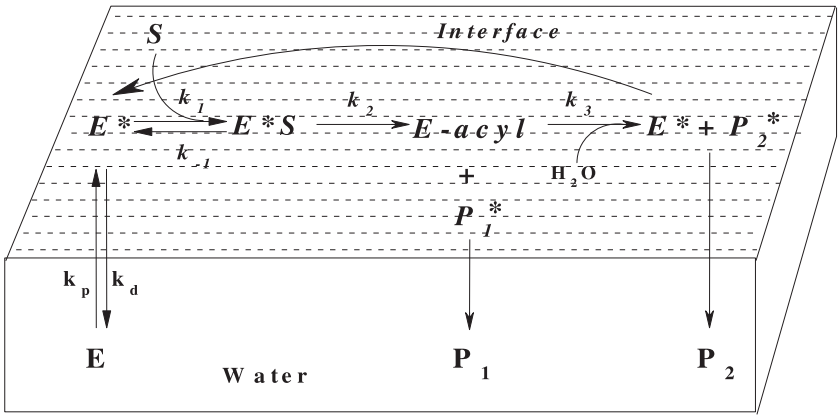
Scheme 2. The full mechanism of action of bovine pancreas lipase hydrolyzing the synthetic substrate p-nitrophenyl laurate. As modified from Kokkinou et al. (2012).

Papamichael et al., 2012), where the rate constants k_p and k_d are associated with the adsorption/desorption of enzymes between the aqueous and lipid/water interface. This function of the catalytic motif of lipases does not constitute a steady, but rather a dynamic ontologic entity, showing in general, that considering enzymatic mechanisms should not be focused only on the markedly referred catalytic residues.

As far as it concerns the mechanism of lipase-catalyzed esterification, it has been shown that a fatty acid forms an acyl-enzyme intermediate complex with lipase, releasing a water molecule, followed by the binding of alcohol onto this acyl-enzyme; the latter is then transformed into a lipase–ester complex releasing the ester and the free enzyme. This concept takes into account that both acid and alcohol were assumed as competitive inhibitors of the esterification process; the corresponding mechanism could be represented by reaction Scheme 4 (as modified from Gandhi et al., 2000; Kokkinou et al., 2012).

3.1. Esterification with lipases

Biocatalysis is an advantageous synthetic means, when it is performed in organic solvents due to both the switching thermodynamic equilibrium towards synthesis (esterification, etc.) and by increasing the



Scheme 3. Example of a typical two-dimensional four-step reaction scheme of action accepted for lipases catalyzing the hydrolysis of a substrate at the aqueous/lipid interface. As modified from Aloulou et al. (2006), and Papamichael et al. (2012).

solubility of non-polar substrates and products. A number of attempts have been reported dealing with the improvement of activity and stability of lipases in non-aqueous media by means of: (a) non-covalent interaction with surfactants, (b) entrapment in water-in-oil micro-emulsions, (c) reverse micelles, (d) immobilization on appropriate insoluble supports (e.g. matrices, porous inorganic carriers, polymers, etc.) and/or utilization of lyophilized enzyme powders suspended in organic solvents, as well as (e) protein engineering (Torres and Castro, 2004 and references therein).

Furthermore, few studies have been published on enzymatic esterification reactions in order to simplify and improve biofuel production and yield (Larroche and Pandey, 2005). More recent attempts focused on studying immobilized lipase-catalyzed reactions, i.e. less viscous multi-phase systems, which can dissolve larger amounts of the used alcohol; in these cases, water is the major by-product of esterification, and therefore, both mass transfer and inhibitory effects of un-dissolved alcohol are strongly reduced. Therein, the major advantage is something like pushing all available organic acids to react towards increasing the ester yield, as an alternative of removing the produced water, whose levels should be maintained as low as possible (>500 ppm). Another choice could be the use of large amounts of alcohol in order to push the reaction in favor of ester formation. However, a balanced choice of lipase and a type of an anhydrous alcohol are a prerequisite before the initiation of the esterification reaction (Xu, 2012). Moreover, water needs to be removed during the reaction course, either by performing multi-step reactions (removing water between steps), or by removing it instantaneously, *in situ* by using a variety of methods (Wang et al., 2006 and references therein; Jeromin and Zoor, 2008); the latter constitutes the major difficulty of scaling up an esterification method for industrial application, along with the operational and mechanical stability of the immobilized biocatalyst.

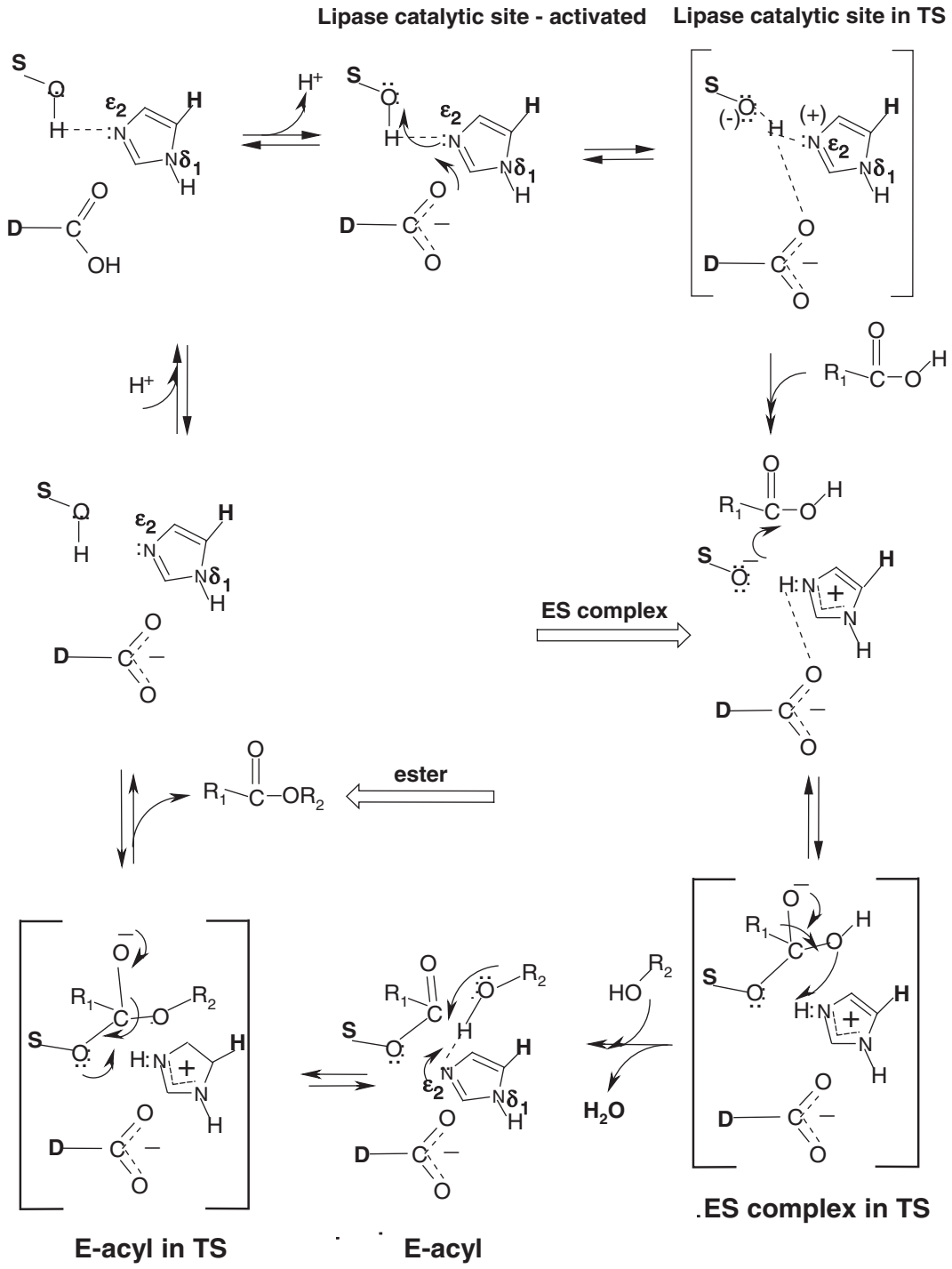
3.2. Factors affecting lipase-catalyzed esterification and ester yield

The activity of lipases seems more likely not to be directly correlated with their substrate concentration, but rather with the total substrate area, and the thermodynamic activity of water in the medium (Krishna and Karanth, 2002; Papamichael et al., 2012). However, relatively recent studies revealed the effects of the interfacial microenvironment in the catalysis by lipases, and showed that their activity is more attributed to substrate inaccessibility, rather than to enzyme denaturation as a function of the interfacial composition (Reis et al., 2009). Moreover, the enzymatic activity of lipases strongly depends upon the method of their preparation, as these enzymes are not soluble in most organic solvents. In general, carboxylic acids can undergo reversible acidic catalysis, via an oxonium ion to become esters after an exchange reaction with an alcohol, whose presence in a large excess pushes the equilibrium towards the esterification. The occurrence of water, which is a stronger nucleophile than alcohols are, does not favor esterification (Pandey et al., 1999; Reis et al., 2009). Additionally, the effects of organic acid and/or the chain length of alcohol substrate(s) are significant in lipase-catalyzed esterification reactions, which proceed through enzyme-substrate binding; undoubtedly, any factor, among many, which affects substrate binding, influences the esterification rate (Vaysse et al., 2002 and references therein).

After all, temperature and pH are two more factors, which generally affect the lipase-catalyzed esterification, as in most homogeneous and heterogeneous reactions. The thermo-stability of lipases has been explained in terms of their substrate binding, as owed to both its structure and the removal of excess water molecules from the immediate vicinity of the enzyme molecule; the latter restricts the overall conformational mobility of lipases. Moreover, the pH-value of the reaction medium and whatever it is as a physical entity, certainly plays a major role in lipase-catalyzed esterification reactions. Accordingly, various mammalian lysosomal lipases exhibit pH optima on the acid side, while lipases from microbial sources exhibit alkaline pH optima, however these latter values have been recorded in aqueous media. These pH optima may be shifted to more acidic values due to the presence (and/or absence) of several important factors (e.g. salts, emulsifiers, substrates, etc.) (Krishna and Karanth, 2002).

3.2.1. Effects of the reaction media

The catalytically active 3D-structure of enzymes depends on many hydrogen bonds, hydrophobic effects, van der Waals forces, and dipole interactions, which occur within the protein molecule and between the protein and the solvent. Thus, prerequisites for enzymatic non-aqueous homogeneous catalysis are the solubility and the stability of biocatalysts in the used organic solvent, moreover, because some of them (e.g. DMSO, DMF, etc.) supplement the enzyme inactivation. Neat non-aqueous organic solvents, where lipases are more soluble and stable, seem to suggest solutions to the problem (Costas et al., 2008, and references therein). Generally, an enzyme's solubility in organic solvents varies as it depends, mainly, on the properties of both the enzyme and the solvent, i.e. according to the physicochemical conditions at the protein–solvent boundary, on the temperature and on the ionic strength of the solvent. In the cases of homogeneous aqueous-organic reaction media, the organic solvent distorts the enzyme's micro-structure, which in addition unfolds in the presence of water due to its conformational flexibility, which may lead to a reversible or irreversible denaturation of the enzyme molecule (Torres and Castro, 2004). Polar solvents usually affect the side chains of lipases, which are more flexible in water than in organic solvents. These are straightforwardly applied in the dynamics of the lid, which is less flexible in organic solvents and affects the catalytic activity of the used lipase;



Scheme 4. Example mechanism of action of lipase-catalyzed esterification. As modified from Gandhi et al. (2000) and Kokkinou et al. (2012).

however, in this environment the side chains of hydrophobic substrates tend to become more flexible (Tejo et al., 2004).

Another important factor, which affects the lipase-catalyzed synthesis, is the nature of the used organic solvent, and its interaction with the contained water, in the reaction medium (Costas et al., 2008, and references therein). Organic solvents may extent specific effects on the flexibility of solvent-exposed side chains of lipase, as in the case of the lid, which fluctuates as a rigid body about its average position. It is more likely that organic solvents reduce the flexibility of the lid, but they increase the flexibility of side chains in the hydrophobic substrate binding site (Tejo et al., 2004). The function of the lid has been modulated in three different recombinant lipases i.e. *Candida rugosa* lipase isoform-1, *Pseudomonas fragi* lipase, and *Bacillus subtilis* lipase A (Secundo et al., 2006) and it was found that the replacement of the lid seems to affect the specific activity of the lipase, but not its specificity towards acyl donors in synthetic reactions (Dave and Madamwar, 2010).

Lipases have been employed in synthetic reactions performed in organic solvents, and offer relatively greater selectivity and higher rates. On the other hand, and as far as it concerns the lipase-catalyzed esterification, opinions and experimental evidences contradict each other on whether or not, completely anhydrous organic media are suitable or not, as enzymes mostly retain their native structure in media containing some amounts of water. It seems more likely that the answer is based on the enzyme-bound water, whose role is crucial for the enzyme

structure rather than the role of bulk water (Zaks and Klibanov, 1988 and references therein; Jeromin and Zoor, 2008), Nevertheless, for biotechnological purposes there are some advantages in performing lipase-catalyzed reactions in organic media: enhanced lipase thermostability, thermodynamic equilibrium shifted towards ester synthesis, recovery of the immobilized lipase by simple filtration, elimination of microbial contamination, and minimization of lipase inhibition (Zaks and Klibanov, 1988 and references therein).

Monophasic organic media, where the bulk water has been removed and exists only in traces on the lipase surface, are usually used and proved to be extremely reliable, versatile, simple, and easy to use, due to the fact that lipases remain catalytically active. Furthermore, proper selection of the organic solvent influences the effectiveness of lipases and thus several physical and chemical properties have been considered (polarity, boiling and freezing points, molar mass, viscosity, heat capacity, acidity, basicity, etc.) in order to determine a solvent's suitability and specific parameters, which have been suggested to quantify the aforementioned properties. Such parameters may be the relative permittivity ε_r, which is a measure of the solvent's polarity, the Hildebrandt solubility parameter, the dielectric constant, the partition coefficient, and the dipole moment (Laane et al., 1987; Zaks and Klibanov, 1988). In anhydrous reaction media ($[H_2O] \ll 0.01\%$), which are comprised as bulk organic phases, the enzymes have been dehydrated and free water has been removed. A measure of the solvent's polarity is the log(P), i.e. the partition coefficient of the employed solvent between water and octanol in a two-phase system. Values of log P > 4.0 forward the reaction in favor of esterification, more likely due to both, the better dissociation of weak organic acids, and the removal of the micro-aqueous layer around the lipase, which in turn sustains essentially the structure and the catalytically active conformation of lipases (Krishna and Karanth, 2002).

Both, the type of organic solvent, as well as the status of the enzyme (immobilized or not) affect the product yield of lipase-catalyzed esterification; ester yields are increased when reactions are performed in non-polar solvents, while polar and less hydrophobic solvents may distort the micro-aqueous layer around the lipase, possibly causing denaturation of the enzyme (Soumanou and Bornscheuer, 2003). However, the increased use of enzymatic solvent-free systems showed their robustness versus the organic solvent systems, concomitantly effecting a cost reduction and improved control of the process (Köse et al., 2002 and references therein). Alternatively, it seems that solvent-free systems consisting only of substrates (alcohol and acid) are advantageous, as compared to the previously described non-aqueous and anhydrous ones; herein, fewer components participate in the reaction, and thus minimize the production cost, permitting also the use of high substrate concentrations, when they are both in a liquid phase (Jeromin and Zoor, 2008).

3.2.2. The influence of reactors and the scaling-up process

Multiphasic reaction media are typically used, when catalysis is performed by immobilized lipases and thus the choice of a suitable reactor is an important task. An experimenter could choose from a variety of reactors, e.g. a well-mixed reactor, which can be operated in both batch and continuous modes, although the immobilized lipase is usually damaged (Buchholz et al., 2005). Other types of reactors could be also used such as classic plug flow reactors, which are preferred in cases of reversible reactions, although they show large mass transfer limitations, etc. (Nielsen et al., 2008).

Reactors harboring immobilized lipases as biocatalysts can be classified into two main types: (a) the ones containing an aqueous phase and where the lipase restricted by a surfactant membrane into an organic solvent and (b) those, where the lipase is immobilized in a solid matrix dispersed within either an organic solvent, or rarely within an aqueous phase. A variety of reactor systems are available including batch stirred and continuous tank reactors, as well as bed or membrane (diaphragm) reactors (Balcão et al., 1996a).

It is always an effective task to scale-up experimental procedures performed in the laboratory, and to achieve equivalent results in specific reactors (Fjerbaek et al., 2009). To succeed in such an effort, it is significant to appreciate proper objectives and methodologies, as well as the chances and risks of failure, due to the inherent complication of the systems under study and the lack of knowledge on important parameters of the reactor (e.g. fluid flow performance, fortuitous environmental variations, etc.). It seems more likely, that the key difficulty of the aforementioned approach is based on the multiphasic nature of the lipase-catalyzed hydrolyses and syntheses, which do not allow the experimenter to attain comparable mass transfer conditions and reaction rates (Xu, 2012 and references therein).

3.2.3. The crucial role of water activity

A direct relationship between water content and lipase stability and activity has been reported in previous works, since the hydration of an enzyme molecule can be described using a lot of different approaches (Krishna and Karanth, 2002). Few water molecules still remain bound to enzymes' molecules even after careful drying, contributing much more than the total water concentration in keeping their active structure and increasing their activity (Zaks and Klibanov, 1988). The trace quantities of water contained in organic solvents can be controlled over a narrow range only, having a profound effect on the lipase activity and in addition, water acts as a competing nucleophile. Unlike other hydrolases, lipases are well activated, when adsorbed onto oil/water interfaces, and on the other hand, their substrate specificity, regioselectivity, and stereoselectivity can be controlled by varying the reaction media (Knežević et al., 2004; Krishna and Karanth, 2002).

Water is produced during esterification reactions, and if it is accumulated in the reaction medium decreases both the reaction rate and the ester yield, independently of the type of reaction, enzyme-catalyzed, or not. The presence of water in the medium, during an esterification reaction, can be quantified by its thermodynamic activity $a_w = p/p_o$, where p and p_o represent the water vapor pressure over a substance to that over pure water (p_o). This quantity (a_w) governs the hydration of enzymes, giving also a direct indication of the mass action of water (Scott, 1957). Various techniques have been used in order to deal with the effect of a_w during an esterification reaction, as its value changes due to the continual production of water, and it may causes unfavorable results (Kvittingen, 1994 and references therein; Villeneuve, 2007). Conventionally, either molecular sieves (Akoh et al., 1992), or chemical destruction of the produced water (Jeromin and Zoor, 2008) has been used. Likewise, many methods have been reported in estimating a_w of a medium during a reaction course, using media of various origins (Resnik et al., 1984 and references therein). Furthermore, the estimation of a_w has been also elaborated by using mathematical methods (Yamane et al., 1989). However some of them are not recommended for estimation of a_w in organic solvents (Halling, 1984).

3.2.4. Nature and influence of pH-value, of the reaction media

The concept of measuring the concentrations of reactants, and estimating conventional parameters, such as the pH-value, is a difficult task in multiphase and/or organic solvent systems. Thus, despite the numerous reported attempts, the collection of any information necessary for analyzing an enzymatic mechanism from the microenvironment around the hindered lipase is limited. For example, pH-optima of lipase-catalyzed esterification have not yet been reported and it seems more likely, that relative assumptions were not based on concrete experimental data. Moreover, the use of protonated or deprotonated acid as a substrate was not taken into account in previous reports.

Diluted aqueous solutions are the only media where pH-value maintains its well known meaning, while in non-aqueous media this scale is not valid. Therefore, where it is necessary, specific methods and procedures should be used to both understand and surmount the existing problems, e.g. low conductivity of some organic solvents, presence of high $[Na^+]$, etc. Furthermore, the pH-value of any reaction medium affects the important functional groups of an enzyme, whose ionization influences the reaction rate. Enzymes are influenced similarly in organic

solvents, where ionization of their functional groups is still important, although the concept of pH is different. However, it should be mentioned, that there is experimental evidence on a peculiar property; ionized compounds when they were lyophilized (including enzymes and of course lipases), conserved their ionic status according to the pH-value of the aqueous solution, where they last existed. This phenomenon was termed pH memory (Constantino et al., 1997), while some publications report exceptions of this phenomenon, when referring to several oxidoreductases (Skrika-Alexopoulos and Freedman, 1993).

The contribution of substrates, i.e. of acids and/or alcohols, in lipase-catalyzed reactions is considerable in configuring the pH-value, and accordingly, also to their protonation status; moreover, reaction time course and temperature also contribute significantly in the same direction. Especially the pH-value cannot be determined according to common protocols and needs a profound elucidation, where the influence of the protonation degree of the acid, as well as the catalytic mode of action of the used lipase, has to be taken into account (i.e. the nucleophilic status of the catalytic serine residue) (Buthe et al., 2005; Krishna and Karanth, 2002).

Acid and/or base properties of aqueous solutions are measured by using the well known Brønsted pH scale, i.e. $pH = -\log_{10}[a(H^+, aq)]$, where $a(H^+, aq)$ in mol L^{-1}, symbolizes the activity of the proton in the aqueous phase (solution). Any change of the pH-value in homogenous media corresponds to some electrochemical potential variation, which is directly connected to thermodynamics (Bates, 1973). On the other hand, the determined pH-values, in non-aqueous media, do not follow the conventional concept and thus are not directly comparable. Besides, the pH-value in organic solvents of high permittivity, as well as in mixtures of water and organic solvents, has been defined by the IUPAC (Commission of Electroanalytical Chemistry) as described by Eq. (1), where m is the molality and γ_m the activity coefficient of protons in the liquid phase under consideration (Mussini et al., 1985; Rondinini et al., 1987); however, this equation is somewhat awkward to be managed in order to be applied in all cases.

$$\mathbf{pH} = -\mathbf{log_{10}}\left[a\left(\mathbf{H^+}\right)\right] = -\mathbf{log_{10}}\left[m\left(\mathbf{H^+}\right)\mathbf{x}g_{\mathbf{m}}\left(\mathbf{H^+}\right)\right]. \quad (1)$$

Likewise, Mandel pioneered in putting forward a unified scale for quantitative comparison of acidities throughout different media (Mandel, 1955); additionally, a unified Brønsted acidity scale has been proposed, which is based on the absolute chemical potential $\mu_{abs}(H^+, solv)$ of the proton in liquid phase independently of the medium. In the latter concept $\mu^{\ddagger}_{abs}(H^+)$ and $\alpha(H^+)$ are the standard chemical potential, and the activity of proton, respectively; nevertheless, the concept of acidity among different systems can be validated based on the chemical potential of the diluted protons, and by using as reference the chemical standard potential of the gaseous proton as the zero point, which was set axiomatically to 0 kJ mol^{-1}, i.e. (Eq. (2)):

$$\mu_{\mathbf{abs}}\left(\mathbf{H^+}\right) = m\left(\mathbf{H^+}\right) - \mu^{\ddagger}\left(\mathbf{H^+}, \mathbf{g\ at\ 298.15\ K}\right) = \mathbf{0\ kJ\ mol^{-1}}. \quad (2)$$

Therefore, an absolute thermodynamic acidity scale has been constructed and leads to $\mu_{abs}(H^+)$ values related to the chemical standard potential of the proton in the gas phase, which in turn underlines, that interactions between reaction media lead to a lower chemical potential of the protons according to the relation $\Delta_{solv}G^{\ddagger}(H^+,g) = \mu^{\ddagger}_{abs}(H^+, solv)$. In fact, in any liquid phase, the chemical standard potential of the gaseous proton is lowered by its solvation standard Gibbs energy $\Delta_{solv}G^{\ddagger}(H^+, g)$, due to proton transfer from the ideal gas phase (at 1 atm) to an ideal 1 M solution of protons, i.e. for pH = 0, and where the activities of the included protonated species, as well as the absolute chemical potential of proton can be determined by using the rCCC – relaxed COSMO Cluster Continuum model. Therefore, it was found that $\Delta_{solv}G^{\ddagger}(H^+, H_2O) = -(1105 \pm 8)$ kJ mol^{-1}, which implies that for two distinct solutions a difference of one pH unit equals to a change of the chemical potential by 5.71 kJ mol^{-1} (i.e. for $\Delta pH = -\Delta\log_{10}[a(H^+)] = 1$, and at T = 298.15 K, it holds true that $\Delta\mu_{abs}(H^+, solv) = RT\ \Delta\log_{10}[a(H^+)] = 2.303\ RT\ \log_{10}[0.1] = -5.71$ kJ mol^{-1}). By taking into account also a general definition of chemical potential (Laidler and Meiser, 1982), in the case of protons and under constant pressure, temperature and activities of all other species of the solution, Eq. (3) could be written; in dilute solutions, the acidity, which correlates with the activity of the solvated protons, is described by the pH-value:

$$\mu\left(\mathrm{H^+}\right) = \left(\frac{\partial G}{\partial n(H^+)}\right)_{P,T,n(X\neq H^+)} = \mu^{\ddagger}\left(\mathrm{H^+}\right) + \mathrm{RT}\ln\left[\alpha\left(\mathbf{H^+}\right)\right] = \mu^{\ddagger}\left(\mathbf{H^+}\right) + \mathbf{2.303} \times \mathbf{RT}\ \mathbf{log_{10}}\left[\alpha\left(\mathbf{H^+}\right)\right] = \mu^{\ddagger}\left(\mathbf{H^+}\right) - \mathbf{5.71\ kJ\ mol^{-1}} \times \mathbf{pH}. \quad (3)$$

Therefore, it is possible to estimate acidities to H^+(solv) at all activities according to Eq. (4), independently of the solvent, for which, only the value of $\Delta_{solv}G^{\ddagger}(H^+) = \mu^{\ddagger}_{abs}(H^+, solv)$ is necessary. Values of $\Delta_{solv}G^{\ddagger}(H^+)$ are accessible from the literature (Himmel et al., 2010). However, we should point out, that standard conditions correspond to an activity of H^+(solv), of 1.0 mol L^{-1} at 298.15 K and 1 atm, which means that pH = 0, as well as that the absolute acidities of solutions are comparable due to Eq. (4).

$$\mu_{\mathbf{abs}}\left(\mathbf{H^+}, \mathbf{solv}\right) = \Delta_{\mathbf{solv}}G^{\ddagger}\left(\mathbf{H^+}\right) - \left[\mathbf{pH\ x}\ \left(\mathbf{5.71\ kJ\ mol^{-1}}\right)\right] \quad (4)$$

Furthermore, it is allowed to divide $\Delta_{solv}G^{\ddagger}(H^+) = \mu^{\ddagger}_{abs}(H^+, solv)$ by −5.71 kJ mol^{-1} and produce Eq. (5), to assign absolute (not conventional) pH_{abs}-values (Himmel et al., 2010).

$$\mathbf{pH_{abs}} = \frac{\mu_{abs}(H^+, solv)}{-5.71\ kJ\ mol^{-1}} \quad (5)$$

3.2.5. Influences of reaction factors

3.2.5.1. Lipase concentration. In biocatalysis, unlike an ordinary chemical process, the total quantity of the used enzyme is important, as it affects both, the reaction time and the product yield (Kim et al., 2004). However, in most cases, it seems as preferable to use immobilized lipases in esterification reactions, where in first place a small loss of the percentage activity of the entrapped enzymes is observed. On the other hand, the remaining enzymatic activity usually shows improved esterification capabilities versus. free lipases, probably due to a more favorable conformation. A loss of enzymatic activities amounting to about 50% was observed in cases of non-immobilized lipases (Adlercreutz, 2008). However, the yield of an esterification reaction should be higher, as the concentrations of substrates (acid and alcohol) are higher and lesser is the total amount of immobilized lipase (Lozano et al., 2004). Additionally, all factors which may commonly affect the lipase-catalyzed esterification activity (i.e. the overall lipase active mass, the molar ratio of the used acid to alcohol, the fatty acid chain length, the type of the reaction medium, its acidity and temperature), should be considered as well (Villeneuve, 2007).

3.2.5.2. Temperature. Experimental results using a porcine pancreas lipase showed the thermal stability of this enzyme at relatively high temperatures, approaching 80 °C, during an esterification reaction (Kokkinou et al., 2012). It is generally accepted, that enzymes found in hydrophobic environments (e.g. organic solvents) become generally more stable, as the temperature rises and up to a limit characteristic for each individual enzyme (Garcia et al., 2002). Thermal stability has been explained by taking into account the conformational stability of these enzymes (Kiran et al., 2001 and references therein). The effect of temperature on the esterification reactions is partitioned into three

components i.e. (i) in a direct influence on the reaction rate, (ii) in the enzyme's stability, and (iii) in the substrate's solubility, which is improved by reducing mass transfer limitations (Facioli and Barrera, 2001). As in most cases, at higher temperatures the lipase activity could be decreased significantly due to its denaturation. In addition, a lipase-catalyzed esterification reaction rate could be decreased gradually with increasing temperature, probably due to the effect of a synergic inhibition between temperature and ethanol on the lipase (Z. Li et al., 2011, W.N. Li et al., 2011).

3.2.5.3. Chain length and molar ratio of the reacting acid and/or alcohol. An important factor, which affects the yield of lipase-catalyzed esterification reactions, is the chain lengths of both substrates (acid and alcohol). Primary alcohols augment the ester yield with an increasing chain length and up to a limit, which depends among others, also on the used lipase. Several works have reported reasonable explanations for these phenomena in terms of the released energy, due to binding of substrates at the lipase active site. This energy seems more likely to be sufficient to support conformational changes of the used lipase to a more catalytically efficient structure. It is well known, that a lot of lipases are equipped with two types of active sites: one for smaller substrates and another for larger substrates (Varma and Madras, 2010). Nevertheless, small molecules diffuse at the lipase active site more easily, though the nucleophilic character of alcohol decreases as the length of its aliphatic chain increases. However, it has been reported, that methanol inhibits the lipase catalyzed esterification (Chaibakhsh et al., 2009). A proven strategy to eliminate the negative effect of methanol is its stepwise addition (Shimada et al., 1999). Another way to solve the problem of lipase inactivation due to methanol's insolubility is the selection of an appropriate solvent such as *tert*-butanol. *tert*-butanol solubilizes the excessive methanol which significantly increases the methyl ester content (Wang et al., 2006).

Furthermore, several researchers investigated various aspects of immobilized lipase-catalyzed synthesis of short and long-chain esters of fatty acids with short-chain alcohols in *n*-hexane. Based on experimental data concerning the lipase-catalyzed esterification process, the yield of the produced ester increases, as the ratio of alcohol:acid is increased up to a critical value. Beyond this value, the ester yield decreases, more likely, due to the distortion of the water layer by the polar compound alcohol. This distortion causes modifications of the protein's tertiary structure and therefore, it promotes inhibition phenomena. The critical value of this alcohol/acid ratio varies according to the used enzyme, as well as according to the type and the composition of the reaction medium (Guncheva and Zhirkoya, 2008).

3.3. Courses improving the lipase-catalyzed esterification and the ester yield

As pointed out in the Section 1, immobilized lipases carry out the most and more efficient synthetic reactions, versus the non-immobilized ones, as far as it concerns the product yield (e.g. esterification). Consequently, immobilization makes the separation of products easy, and enhances the lipase's stability towards the temperature, pH-value and other features of the reaction media, mainly through more flexible lipase/substrate binding modes. Lipase-catalyzed reactions, which take place at the aqueous − organic solvent interfaces, i.e. in emulsions, are technically and economically awkward, as opposed, mainly, to membrane-immobilized lipases in two-phase media. Lipase inactivation is often observed in the former case, due to intensive stirring and/or contamination of the products with surfactants. Moreover, the membrane-immobilized enzymes are advantageous in continuous systems. In this course, organic synthetic membranes, usually hydrophobic versus hydrophilic, have been extensively used as lipase carriers, where both of them have advantages and disadvantages. An investigator has to design efficient lipase immobilization systems, based on different criteria, techniques and carriers, which are largely dependent on the individual lipase used, as well as, on the nature of the reaction medium (aqueous, organic, two-phase, etc.), and the conditions (temperature, pH-value, etc.) (Knežević et al., 2004).

3.3.1. Immobilized lipases

Various immobilization techniques have been used to enhance the biocatalysis in organic solvents such as, adsorption or covalent attachment to solid materials, and/or entrapment in organic or inorganic polymers or microemulsions (Costas et al., 2008, and references therein). These techniques are important in terms of stability, cost, recovery, and denaturation of purified lipases after completion of the catalytic reaction. Thus, immobilization in solid matrices makes lipases insoluble in aqueous media (Kenedy and Cabral, 1987). Moreover, immobilized enzymes are thermostable and can be stored for longer periods of time, than non-immobilized ones. The materials used for the immobilization of lipases are larger in size compared to the enzyme molecule, regardless whether they are liquid (e.g. reversed micelles) or solid, however, in all cases, the activity of lipases is influenced by the properties of the immobilization matrix. When porous materials are used, then lipases can be immobilized without conformational changes (Balcão et al., 1996a).

When immobilization is performed by adsorption of lipases, where the aqueous phase is brought into close contact with the adsorbing surface, then it is not uncommon to assume a reversible process $L_{bulk} \rightleftarrows^{k_1}_{k_{-1}} L_{lqbd}$ (Balcão et al., 1996b), where L_{bulk} and L_{lqbd} denote lipase molecules in the bulk solution and the liquid boundary layer, respectively, as well as k_1 and k_{-1} which denote the rate constants corresponding to adsorption and desorption, respectively. It was assumed, that $k_1 >> k_{-1}$ due to the unique hydrophobicity of lipases. In general, an immobilized lipase-catalyzed reaction may be described by reaction Scheme 5 (see also Scheme 1). Likewise, expressions like $v = \frac{V^{app}_{max}[Es]}{K^{app}_m+[Es]}$ and/or $v = \frac{\alpha_0+\alpha_1[Es]+\alpha_2[Es]^2-\ldots}{1+\beta_1[Es]+\beta_2[Es]^2-\ldots}$ can be produced, whose terms are easily explained.

In the presence of a large excess of water in the reaction medium, hydrolysis is promoted and a common ping-pong bi-bi simplified rate expression similar to the Michaelis − Menten one can be written, as Eq. (6), where $V^{app}_{max} = \frac{V^f_{max}[H_2O]}{K^{H_2O}_m+[H_2O]}$, denotes an apparent maximum rate in the forward direction (f) and $K^{app}_m = \frac{K^{Ester}_m[H_2O]}{K^{H_2O}_m+[H_2O]}$, denotes an apparent Michaelis − Menten constant (Paiva et al., 2000).

$$v = \frac{V^{app}_{max}[Es]}{K^{app}_m + [Es]} \tag{6}$$

However, the exact form of the appropriate equation depends on the rate controlling step (acylation and/or deacylation of lipase) (Garcia et al., 1992, 1999). When a single type of ester (i.e. $I = J = 1$ − notations in Scheme 1) is involved in the reaction, then the general equation takes the form of Eq. (7) (Bates and Watts, 1988).

$$v = \frac{\alpha_0 + \alpha_1[Es] + \alpha_2[Es]^2-\ldots}{1 + \beta_1[Es] + \beta_2[Es]^2-\ldots} \tag{7}$$

The immobilized lipase molecules will eventually interact in the hydrophobic boundary layer with an increasing number of adsorption points on the carrier surface, according to the reaction Scheme 6, where a rate constant k governs the transformation of a non-adsorbed lipase molecule into an adsorbed molecule of lipase on the membrane through

$$Ester_{i,j} + H_2O \rightleftharpoons Acid_i + Alcohol_j$$

Scheme 5. Outline of an immobilized lipase-catalyzed reaction following the ping pong bi-bi mechanism (see also Scheme 1).

multipoint attachment. n^* is one of n available adsorption sites on the membrane.

3.3.2. Chemically modified lipases

Different methods have been reported regarding immobilization of lipases. In most cases, glutaraldehyde has been used as the cross-linking branch for applications in organic solvents. This is a facile and inexpensive method and it results in enhanced lipase thermostability and tolerance. However, the extent and exact locations of the modifications are often obscure. In addition, under heterogeneous reaction conditions, the number of enzyme active sites, after the modification, were unknown and thus the obtained experimental results were mostly not assessed, and in an ambiguous manner, when they were compared among different publications. Alternatively, chemically modified lipases, i.e. covalently modified with the amphiphilic polymer PEG, have been successfully functioning when applied in organic solvents (Krishna and Karanth, 2002). Similarly, several experiments have been performed with modified amino acids, lipids, surfactants, and hydrophobic polymers of various origins (poly-vinyl, poly-methyl, methyl-acrylate, etc.). Such modified enzymes were found stable and active in a variety of polar and non-polar solvents. The achievement of successful modifications with solubilized enzymes in a preparative level, in order to surmount internal diffusion limitations, is nevertheless an ambitious task. On the other hand, the use of solubilized enzymes in organic media in continuous processes was discouraging, due to the enzymes' recovery problems (Dave and Madamwar, 2010; Krishna and Karanth, 2002).

3.3.3. Genetically engineering of lipases

Organic synthesis by means of enzymatic catalysis was found advantageous, due to its mild reaction conditions, higher reaction rates and larger industrial applications, and it has influenced researchers and industries, especially in the field of biotechnology. Lipases followed the fate of all hydrolases in expanding their applications in industrial and biotechnological applications. Thus, certain genetic engineering techniques were developed as opposed to possible chemical modification of native lipase molecules, and specific databases have been designed offering all necessary important information to the researchers (http://www.led.uni-stuttgart.de, n/d).

Different approaches to lipase engineering have been applied, i.e. rational design (continuous improvement of structure to function relation through engineering of particular lipase properties), and directed evolution (effective mutations of the lipase molecule followed by varying the reaction conditions in order to improve its stability, or activity, and/or selectivity, etc.) (Stemmer, 1994 and references therein). Several examples may contribute to appreciate these methodologies. At first, random mutagenesis procedures based on a trial-and-error logic, improved the thermostability of certain lipases (Shinkai et al., 1996 and references therein). In other attempts, activity, enantioselectivity and regioselectivity of recombinant lipases from different microorganisms, were enhanced (Reetz et al., 1997), and/or well organized hydrolytic enzyme mutant libraries with improved substrate specificity and stability were assembled using known host bacteria and/or fungi (e.g. *Escherichia coli*, *Saccharomyces cerevisiae*, etc.) (Crameri et al., 1998; Giver et al., 1998).

4. Lipases as biocatalysts for biofuel production

Increased energy demands have turned scientists' interest in search of alternative fuels from renewable sources. During the last years, biofuel has attracted a lot of attention, as one of the most potential fuels against fuels produced from petrol, since it is biodegradable and non-toxic. Biofuel can be synthesized from fats and oils via transesterification and/or esterification, in which the substrate is triglycerides or fatty acids, respectively and an alcohol. The reactions were carried out in the presence of an inorganic catalyst or enzyme (Leung et al., 2010 and references therein). The lipase-catalyzed esterification and/or transesterification have been considered as friendly and thus "green" for the environment.

$$L_{\text{non-adsorbed}} + n^* \xrightarrow{k} L_{\text{multipoint-adsorbed}}$$

Scheme 6. Outline of the interactions of immobilized lipase molecules onto the hydrophobic boundary layer with an increasing number of adsorption points on the carrier surface.

Vegetable oils are potential sources for production of biofuel as they are of high quality. However, they increase significantly the cost of the preparation, a fact that subsequently raises social and ethical issues for the use of edible oils in fuel production during a period of food crisis. For this reason, many scientists have focused their research in using waste fats and non-edible oils, e.g. jatropha (*Jatropha curcas*), as a substrate for biofuel production (Koh, 2011). The majority of non-edible vegetable oils contain high levels of free fatty acids (FFAs), which can be converted to biofuel by esterification. When this reaction is catalyzed enzymatically by a lipase, it gives the best results, because it does not form soaps as in the case of basic catalysis, and proceeds in one step without an intermediate washing procedure. Moreover, it does not need a large amount of alcohol, as is the case with acid catalysis (Fan et al., 2012). The most promising lipases for biofuel production are derived from yeasts; however, a number of lipases isolated from bacterial species have been applied with remarkable results and will be described below.

4.1. Bacterial lipases

In general, a broad spectrum of bacteria and archaea synthesize hydrolases. Lipases have been isolated from a large number of bacterial hosts and their properties, and the structures of several of them, have been extensively analyzed revealing their catalytic action in detail. Many convenient methods for their isolation and overproduction have been developed. Today, they represent one of the most widely used enzyme categories in biotechnological applications (Carrasco-López et al., 2009).

Bacterial lipases are efficient catalysts possessing valuable properties such as a wide variety of substrates that they use, selectivity, stability in organic solvents, and activity in various conditions. Therefore, they are used in a wide spectrum of industrial applications including production of fats and oils, detergents, fine chemicals, pharmaceutical drugs, pulp and paper, as well as use in bioremediation, waste treatment, oil biodegradation and medical application.

A large number of lipases have been isolated and characterized from a great variety of bacteria and their properties have been reviewed in detail (Pandey et al., 1999). Representatives of them are *Bacillus* species including among others *Bacillus subtilis*, *Bacillus pumulus*, *Bacillus stearothermophilus*, and *Bacillus licheniformis*. Furthermore, other genera were also used, such as *Burkholderia cepacia*, *Burkholderia glumae*, *Chromobacterium violaceum*, *Clostridium*, *Colwellia psychrerythraea*, *Corynebacterium*, *Dehalococcoides*, *Geobacillus*, *Propionibacterium*, *Pseudoalteromonas haloplanktis*, *Pseudomonas* (*Pseudomonas aerugiosa*, *Pseudomonas fluorescens*, etc.), *Rhodoferax*, *Serratia*, *Staphylococcus* (*Staphylococcus aureus*, *Staphylococcus xylosus*, etc.), *Streptomyces*, *Vibrio*, etc. Some significant examples of lipases originating from extremophiles should be also mentioned, such as LipA and LipB from the hyperthermophilic anaerobe *Thermosyntropha lipolytica* (Salameh and Wiegel, 2007) and the first true lipase from the hyperthermophilic archaeon *Archaeoglobus fulgidus* (Levisson et al., 2009), the ones of the psychrophiles *Psychrobacter immobilis* (Joseph et al., 2008 and references therein), *Psychrobacter okhotskensis* (Yumoto et al., 2003) and *Acinetobacter calcoaceticus* (Pratuangdejkul and Dharmsthiti, 2000), as well as from the halophilic archaeon *Haloarcula marismortui* (Müller-Santos et al., 2009).

More bacterial lipases isolated from *Burkholderia*, *Pseudomonas*, *Enterobacter* and *Chromobacterium* have been applied for synthetic purposes with remarkable results. A lipase from *P. fluorescens* has been used for biofuel production by soybean oil and methanol (3:1 methanol-to-oil molar ratio) under mild conditions (Luo et al.,

2006). In a later study *P. fluorescens* MTCC 103 cells were immobilized on sodium alginate during a batch process (Devanesan et al., 2007). *Pseudomonas cepacia* lipase was immobilized within a chemically inert, hydrophobic sol-gel support prepared by polycondensation of hydrolyzed tetramethoxysilane and iso-butyltrimethoxysilane, and exhibited higher activity compared to the free enzyme. It exhibited a small activity loss after repeated uses (Noureddini et al., 2005) and was used in modified biocatalyst formulation CLEAs (cross-linked enzyme aggregates) and PCMCs (protein-coated microcrystals) to convert the oil of *Madhuca indica* into ethyl esters in a solvent-free system. In later studies, *P. cepacia* lipase was immobilized on a silica–PVA composite and used for biofuel production against babassu oil and ethanol in solvent free systems, as well as for the conversion of jatropha oil via ethanol after its immobilization on celite, and high yields were achieved (Shah and Gupta, 2007 and references therein). The immobilized lipase was used four times without any loss of its catalytic activity.

Recently, a *B. cepacia* lipase immobilized in an n-butyl-substituted hydrophobic silica monolith was used for the conversion of crude *Jatropha* oil and methanol (3:1 methanol-to-oil molar ratio) to biofuel in a continuous process reaching a yield of 90%. The continuous production of biofuel at similar high yields was also obtained using a lipase-immobilized silica monolith bioreactor (Kawakami et al., 2011). At parallel time, a *Burkholderia cenocepacia* lipase has been immobilized in a macroporous resin NKA showing similar catalytic efficiency in biofuel production (Liu et al., 2011). Moreover, an *Enterobacter aerogenes* immobilized lipase was used for biofuel production from *Jatropha* oil with *t*-butanol as a solvent and a 4:1 methanol-to-oil molar ratio, reaching a maximum conversion of 94% (Kumari et al., 2009); *Chromobacterium viscosum* lipase was used as both a free and as an immobilized enzyme on Celite-545 for biofuel production from *Jatropha* oil (Shah and Gupta, 2007 and references therein).

The last decade metagenomic approaches have been applied in order to isolate lipase genes from microorganisms that are practically uncultivable. Therefore, DNA was isolated directly from environmental samples and screened either by constructing a DNA library and introducing it usually in *E. coli* followed by a lipase assay of the recombinant clones (Zuo et al., 2010), or in a PCR-based approach with degenerate primers specially designed from conserved regions of lipase genes (Bell et al., 2002).

4.2. Fungal lipases

In recent years much attention was given to fungal lipases, which have been widely used in free and/or immobilized forms, or even as whole cell biocatalysts for biofuel production. The direct esterification of fatty acids and alcohols, catalyzed by fungal lipases for the production of biofuel, is an advantageous alternative to other methods due to the versatility of the properties of these lipases, their substrate specificity and ease of mass production. Many lipases, which have been considered as important due to their stability and esterification potential originate from fungi of the genera *Mucor*, *Rhizopus*, *Geotrichum*, *Rhizomucor*, *Aspergillus*, *Humicola*, *Candida*, and *Penicillium* (Benjamin and Pandey, 1998 and references therein; Ellaiah et al., 2004; Larios et al., 2004; Tan et al., 2003).

In general, the application of fungal lipases for synthetic purposes is well documented in a number of publications which have reported on esterification reactions of free fatty acids and alcohols, catalyzed by commercial fungal lipases, in non-aqueous or micro-aqueous media; the fungal lipases Lipozyme RM-IM (from *Rhizomucor miehei*), Lipozyme TL-IM (from *Thermomyces lanuginosus*), and Novozym 435 (from *Candida antarctica*) were used in these works. Lipases from *Aspergillus niger*, *Rhizopus delemar*, *Geotrichum candidum*, and *Penicillium cyclopium* were employed in the synthesis of esters of oleic acid with various primary alcohols, whereas lipases from the species *Rhizopus niveus* and *Aspergillus terreus* have been used for the esterification of free fatty acids (FFAs) in solvent free systems with promising results (Russell et al., 1998).

At this point, it should also be addressed that changes in mycelial morphology of filamentous fungi can have a profound effect on lipase production and vice versa. Specifically, it has been proposed (Haack et al., 2006) that in *Aspergillus oryzae*, the swelling of the hyphal tip observed, could be a result of high lipase productivity. In the case of *A. niger* MYA 135, the highest extracellular lipase production was observed in filamentous growth (Colin et al., 2010). Furthermore, it has been suggested (Nakashima et al., 1990) that cell immobilization (aggregation) of several *Rhizopus* sp. cells on biomass support particles (BSPs) enhanced lipase production whereas aggregated mycelia of *Rhizopus chinensis* were also suggested to achieve high lipase production (Teng et al., 2009).

4.3. Whole cell biocatalysis

As a matter of fact, the use of whole cells was the sole method of biocatalysis for thousands of years, in preparing and processing of dairy products and other kinds of food, of vegetables, handicrafts, etc. (Stergiou et al., 2012; Theodorou et al., 2006). Much later, purified free and/or immobilized enzymes possessing specific properties were employed in the industrial and biotechnological biocatalysis processes. Nowadays, the well known whole cell biocatalysis was proposed recently as a cost-effective alternative method. Thus, instead of using various forms of purified enzymes, the activity of the cell produced enzymes is exploited for industrial and other applications (Fukuda et al., 2008). In this way, various methodologies have been reported on biofuel production in relative high yields, which in turn are also useful in respect of stability of the cells, which is a crucial factor for industrial applications. Such an example is the glutaraldehyde-treated cells of *Rhizopus oryzae* IFO 4697, which were immobilized on cuboidal polyurethane foam biomass support particles, and maintained their lipase activity during six batch cycles without a significant decrease, probably due to stabilization provided by the glutaraldehyde-treatment (Ban et al., 2002; Hama et al., 2007; Oda et al., 2004 and references therein). Additional information has been reported concerning the whole-cell biocatalysis, for example, that *R. oryzae* IFO 4697 cells showed higher durability in repeated use, when obtained from air-lift bioreactor cultivation, than from shake-flask cultivation due to the lower degree of saturation of the membrane of air-lift cultivated cells (Oda et al., 2004). Later, more microorganisms were used in whole cell biocatalysis for biofuel production as *R. chinensis* CCTCC M201021, *A. oryzae*, etc (Adachi et al., 2011; Qin et al., 2008).

Moreover, different approaches have been developed concerning the yeast whole cell biocatalysis systems (e.g. *R. oryzae* lipase was overexpressed in *S. cerevisiae* MT8-1) and it has been demonstrated that these systems can be efficient in solvent-free reaction media for biofuel production (Matsumoto et al., 2001). Contrary to *R. oryzae* lipase for which whole cell biocatalysis methodology is quite common, to our knowledge, there is only one reference available concerning the display of *T. lanuginosus* lipase along with *C. antarctica* lipase on the surface of *Pichia pastoris* cells providing relatively high yields of methyl ester for singly displayed *C. antarctica* lipase, *T. lanuginosus* lipase and co-displayed enzymes (Yan et al., 2012). Further reports are referred in the use of recombinant *R. oryzae* lipase LY6, a mutant from *R. oryzae* DSM853 which was expressed in *Pichia pastoris*, and where the crude pro-lipase, i.e. the mature form of the enzyme plus a pro-sequence, was used for biofuel production in high yields (Hama et al., 2007 and references therein; Fukuda et al., 2009; Z. Li et al., 2011, W.N. Li et al., 2011).

5. Conclusions

The production of biofuel by direct esterification of fatty acids and alcohols catalyzed by lipases is an advantageous alternative versus both the conventional chemical processes and the lipase-catalyzed transesterification reactions. However, in order for the enzymatic

esterification reaction in both laboratory and industrial scales to become competitive, there are several issues that should be resolved. Herein, essential answers to this query are reviewed by considering also the new perspectives in the industrial application of the lipase-catalyzed synthesis of esters for biofuel production.

Esterification by lipases was developed a few decades ago and various microbial lipases have been used for this purpose. Perquisites for their use regardless of their synthesis scale, should be both, the high ester yield and the reduction of the reaction time. Therefore, a better understanding, appreciation, and knowledge of various reaction parameters affecting the esterification reactions could save cost and time for future industrial biotechnology applications. In this review, commented on are key factors and courses affecting generally the lipase-catalyzed esterification reaction and in regard to their experimental sequence, the following points are considered:

(a) The significance of the concentrations of lipase and its substrates, as well as the molar ratio of the reacting acid and alcohol, depends partly on the thermodynamic activity of water in the reaction medium, the substrate accessibility towards the lipase molecules, and the interfacial composition. As it has been reported these phenomena were explained in terms of the released energy due to the binding of substrates on the active site of the used lipase and by taking into account also the conformational changes of lipase to a more catalytically efficient structure.

(b) The organic acid-to-alcohol molar ratio and the mixing rate of the reaction medium, as both of these substrates may induce inhibitory effects and moreover lipases are active in at least biphasic media, should obviously be well mixed during the esterification reaction.

(c) The nature, the influence, and the measuring of the pH-value in multiphase and/or organic solvent media are difficult tasks, and thus the absolute thermodynamic acidity scale, which has been constructed, offered the possibility to comprehend and estimate acidity values (as equivalent to pH-values) independently of the reaction medium, concluding, that for this purpose, only the value of $\Delta_{solv}G^{\ddagger}(H^+) = \mu^{\ddagger}_{abs}(H^+, solv)$ is necessary.

(d) The temperature of the reaction mixture affects homogeneous and heterogeneous esterification reactions, while the observed thermo-stability is more likely due to the binding of substrates on the lipases and the removal of excess water molecules from the immediate vicinity of the enzyme molecule.

(e) The crucial role of the water content in the reaction medium, which is a stronger nucleophile than alcohols and whose absence eliminates the competing hydrolysis reaction, is well documented. Although several ambiguities have been brought up regarding the use of completely anhydrous media, the answer seems to be based on the importance of enzyme-bound water rather than of bulk water. These uncertainties can be avoided by using various kinds of immobilized lipases on water insoluble carriers (covalently, by adsorption, hydrophobic binding, ion exchange, cross-linking, in reversed micelles, polymeric matrices, hollow fibers, by precipitation in organic solvents, etc.) in less viscous multi-phase systems where larger amounts of the esterification substrates (organic acid and alcohols) can be dissolved and both mass transfer and inhibitory effects are strongly reduced.

(f) The mechanisms of the lipase-catalyzed esterification which have been studied and valuable reaction models and rate equations were reported based on suitable assumptions concerning the elementary reaction steps and comprising both the lipase activation and the formation of the corresponding enzyme–substrate complexes. These reactions are performed in non-isotropic media (organic solvents and/or in biphasic media), present inherent difficulties and thus can be described only by awkward equations; however they offer a lot of information which is regularly used in the improvement of the esterification process.

(g) Genetic engineering has been developed as an alternative to chemical modification of native lipases from different microorganisms by applying various techniques such as rational design, directed evolution, etc., and it contributed to the improvement of their activity, stability, regioselectivity and enantioselectivity. Furthermore, the increased applicability of engineered lipases, which possess novel properties and is used in the food and pharmaceutical industries and technologies as significant biocatalysts, has emerged based on optimized growth-media.

(h) Whole cell biocatalysis, which offers low-cost and effective alternatives to the use of lipases for industrial, biotechnological and other applications, tends to substitute the various forms of the purified enzymes. Moreover, various novel reactor designs are equipped with immobilized whole cell biocatalysts offering effective and efficient bioconversion processes.

Unambiguously, the art of the lipase-catalyzed esterification of fatty acids and alcohols should take into serious consideration the aforementioned key factors and courses, whose improved use, handling and exploitation, could be the means for future trends, challenges and perspectives in this area of biocatalysis.

Acknowledgments

This article is in line of a research project co-financed by the European Union (European Regional Development Fund — ERDF), through the operational programs for "competitiveness and entrepreneurship" and regions in transition "Cooperation 2011 — *Partnerships of Production and Research Institutions in Focused Research and Technology Sectors*", of the National Strategic Reference Framework (NSRF) 2007–2013, and the Hellenic Ministry of Education, Lifelong Learning and Religious Affairs — General Secretariat for Research and Technology.

References

Adachi D, Hama S, Numata T, Nakashima K, Ogino C, Fukuda H, et al. Development of an *Aspergillus oryzae* whole-cell biocatalyst coexpressing triglyceride and partial glyceride lipases for biodiesel production. Bioresour Technol 2011;102:6723–9.

Adlercreutz P. Fundamentals of biocatalysis in neat organic solvents. In: Carrera G, Riva S, editors. Organic synthesis with enzymes in non-aqueous media. Weinheim: Wiley-VCH Verlag GMBH; 2008. p. 3–24.

Akoh CC, Cooper C, Nwosu CV. Lipase G-catalyzed synthesis of monoglycerides in organic solvent and analysis by HPLC. JAOCS 1992;69:257–60.

Aloulou A, Rodriguez JA, Frédéric C. Exploring the specific features of interfacial enzymology based on lipase studies. BBA (Mol Cell Biol Lipids) 2006;1761:995–1013.

Balcão VM, Paiva AL, Malcata FX. Bioreactors with immobilized lipases: state-of-the-art. Enzyme Microb Technol 1996a;18:392–416.

Balcão VM, Vieira MC, Malcata FX. Adsorption of protein from several commercial lipase preparations onto a hollow-fiber membrane module. Biotechnol Prog 1996b;12:164–72.

Ban K, Hama S, Nishizuka K, Kaieda M, Matsumoto T, Kondo A, et al. Repeated use of whole-cell biocatalysts immobilized within biomass support particles for biodiesel fuel production. J Mol Catal B: Enzym 2002;17:157–65.

Bates RG. Determination of pH. Theory and practice. 2nd ed. New York: Wiley; 1973.

Bates DM, Watts DG. Nonlinear regression analysis and its applications. New York: Wiley Interscience; 1988.

Bell PJL, Sunna A, Gibbs MD, Curach NC, Nevalainen H, Bergquist PL. Prospecting for novel lipase genes using PCR. Microbiology (UK) 2002;148:2283–91.

Benjamin S, Pandey A. Review: *Candida rugosa* lipases: molecular biology and versatility in biotechnology. Yeast 1998;14:1069–87.

Buchholz K, Kasche V, Bornscheuer UT. Biocatalysts and enzyme technology2nd ed. Weinheim: Wiley-Blackwell; 2005.

Buthe A, Recker T, Heinemann M, Hartmeier W, Buchs J, Ansorge-Schumacher MB. pH-optima in lipase-catalysed esterification. Biocatal Biotransform 2005;23:307–14.

Carrasco-López C, Godoy C, de las Rivas B, Fernández-Lorente G, Palomo JM, Guisán JM, et al. Activation of bacterial thermoalkalophilic lipases is spurred by dramatic structural rearrangements. J Biol Chem 2009:4365–72.

Chaibakhsh N, Rahman MBA, Basri M, Salleh AB, Rahman RNZRA. Effect of alcohol chain length on the optimum conditions for lipase-catalyzed synthesis of adipate esters. Biocatal Biotransform 2009;27:303–8.

Cleland WW. Steady state kinetics. In: Boyer P, editor. The enzymes, 3rd ed., vol. 2. Academic Press; 1970. p. 1–65.

Colin VL, Baigori MD, Pera LM. Effect of environmental conditions on extracellular lipases production and fungal morphology from *Aspergillus niger* MYA 135. J Basic Microbiol 2010;50:52–8.

Constantino HR, Griebenow K, Langer R, Klibanov AM. On the pH memory of lyophilized compounds containing protein functional groups. Biotechnol Bioeng 1997;53:345–8.

Costas L, Bosio VE, Pandey A, Castro GR. Effects of organic solvents on immobilized lipase in pectin microspheres. Appl Biochem Biotechnol 2008;151:578–86.

Crameri A, Raillard SA, Bermudez E, Stemmer WPC. DNA shuffling of a family of genes from diverse species accelerates directed evolution. Nature 1998;391:288–91.

Dave R, Madamwar D. Preparations for the use of *Candida rugosa* lipase in non-conventional solvents. Biocatal Biotransform 2010;28:157–66.

Devanesan MG, Viruthagiri T, Sugumar N. Transesterification of *Jatropha* oil using immobilized *Pseudomonas fluorescens*. Afr J Biotechnol 2007;6:2497–501.

Ellaiah P, Prabhakar T, Ramakrishna B, Taleb AT, Adinarayana K. Production of lipase by immobilized cells of *Aspergillus niger*. Process Biochem 2004;39:525–8.

Facioli NL, Barrera AD. Optimization of enzymatic esterification of soybean oil deoderiser distillate. J Sci Food Agric 2001;12:1193–8.

Fan X, Niehus X, Sandoval G. Lipases as biocatalyst for biodiesel production. In: Sandoval, editor. Methods in molecular biology, lipases and phospholipases: methods and protocols, vol. 861. New York Heidelberg Dordrecht London: Humana Press, Springer Protocols; 2012. p. 471–83.

Fjerbaek L, Christensen KV, Norddahl B. A review of the current state of biodiesel production using enzymatic transesterification. Biotechnol Bioeng 2009;102:1298–315.

Fojan P, Jonson PH, Petersen MTN, Petersen SB. What distinguishes an esterase from a lipase: A novel structural approach. Biochimie 2000;82:1033–41.

Fukuda H, Hama S, Tamalampudi S, Noda H. Whole-cell biocatalysts for biodiesel fuel production. Trends Biotechnol 2008;26:668–73.

Fukuda H, Kondo A, Tamalampudi S. Bioenergy: sustainable fuels from biomass by yeast and fungal whole-cell biocatalysts. Biochem Eng J 2009;44:2–12.

Gandhi NN, Patil NS, Sawant SB, Joshi JB. Lipase-catalyzed esterification. Catal Rev Sci Eng 2000;42:439–80.

Garcia HS, Malcata FX, Hill CG, Amundson CH. Use of *Candida rugosa* lipase immobilized in a spiral wound membrane reactor for the hydrolysis of milkfat. Enzyme Microb Technol 1992;14:535–45.

Garcia T, Sanchez N, Martinez J, Aracil J. Enzymatic synthesis of fatty acid esters. Part I kinetic approach. Enzyme Microb Technol 1999;25:584–90.

Garcia R, Martinez M, Aracil J. Enzymatic esterification of an acid with an epoxide using an immobilized lipase from *Mucor miehei* as catalyst: optimization of the yield and isomeric excess of ester by statistical analysis. J Ind Microbiol Biotechnol 2002;28:173–9.

Giver L, Gershenson A, Freskgard PO, Arnold FH. Directed evolution of a thermostable esterase. Proc Natl Acad Sci U S A 1998;95:12809–13.

Guncheva MH, Zhirkoya D. High yield synthesis of wax esters catalysed by modified *Candida rugosa* lipase. Biotechnol Lett 2008;30:509–12.

Haack MB, Olsson L, Hansen K, Lantz AE. Change in hyphal morphology of *Aspergillus oryzae* during fed-batch cultivation. Appl Microbiol Biotechnol 2006;70:482–7.

Halling PJ. Effects of water on equilibria catalysed by hydrolytic enzymes in biphasic reaction systems. Enzyme Microb Technol 1984;6:513–6.

Hama S, Yamaji H, Fukumizu T, Numata T, Tamalampudi S, Kondo A, et al. Biodiesel-fuel production in a packed-bed reactor using lipase-producing *Rhizopus oryzae* cells immobilized within biomass support particles. Biochem Eng J 2007;34:273–8.

Hasan F, Shah AA, Hameed A. Methods for detection and characterization of lipases: a comprehensive review. Biotechnol Adv 2009;27:782–98.

Himmel D, Goll SK, Leito I, Ingo Krossing I. A unified pH Scale for all phases. Angew Chem Int Ed 2010;49:6885–8.

http://www.led.uni-stuttgart.de.

Jaeger KE, Dijkstra BW, Reetz T. Bacterial biocatalysts: molecular biology, three-dimensional structures, and biotechnological applications of lipases. Annu Rev Microbiol 1999;53:315–51.

Jeromin GE, Zoor A. A new irreversible enzyme-aided esterification method in organic solvents. Biotechnol Lett 2008;30:925–8.

Joseph B, Ramteke PW, Thomas G. Cold active microbial lipases: some hot issues and recent developments. Biotechnol Adv 2008;26:457–70.

Kawakami K, Oda Y, Takahashi R. Application of a *Burkholderia cepacia* lipase-immobilized silica monolith to batch and continuous biodiesel production with a stoichiometric mixture of methanol and crude *Jatropha* oil. Biotechnol Biofuels 2011;4:42–53.

Kenedy JF, Cabral JMS. Enzyme immobilization. In: Kennedy JF, editor. Biotechnology. Enzyme technologyNew York: VCH Publishers; 1987. p. 247–404.

Kim J, Haam S, Park DW, Ahn IS, Lee TG, Kim HS, et al. Biocatalytic esterification of β-methylglucoside for synthesis of biocompatible sugar-containing vinyl esters. Chem Eng J 2004;99:15–22.

Kiran KR, Sureshbabu CV, Divakar S. Thermostability of porcine pancreas lipase in nonaqueous media. Process Biochem 2001;36:885–92.

Knežević ZD, Šiler-Marinković SIS, Mojović LV. Immobilized lipases as practical catalysts — review. Acta Period Technol-FF 2004;35:151–64.

Koh MY, Mohd Ghazi TI. A review of biodiesel production from *Jatropha curcas* L. oil. Renew Sustain Energy Rev 2011;15:2240–51.

Kokkinou M, Theodorou LG, Papamichael EM. Aspects on the catalysis of lipase from porcine pancreas (type VI-s) in aqueous media: development of ion-pairs. Braz Arch Biol Technol 2012;55:231–6.

Köse O, Tüter M, Ayşe Aksoy H. Immobilized *Candida antarctica* lipase-catalyzed alcoholysis of cotton seed oil in a solvent-free medium. Bioresour Technol 2002;83:125–9.

Krishna HS, Karanth NG. Lipases and lipase-catalyzed esterification reactions in nonaqueous media. Catal Rev 2002;44:499–591.

Kumari A, Mahapatra P, Garlapti VK, Banerjee R. Enzymatic transesterification of *Jatropha* oil. Biotechnol Biofuels 2009;2:1–7.

Kvittingen L. Some aspects of biocatalysis in organic solvents. Tetrahedron 1994;50:8253–74.

Laane C, Boeren S, Vos K, Veeger C. Rules for optimization of biocatalysis in organic solvents. Biotechnol Bioeng 1987;30:81–7.

Laidler KJ, Meiser JH. Physical chemistry. London: The Benjamin/Cummings Publishing Company Inc.; 1982141–4 [187–190].

Larios A, Garcia HS, Oliart RM, Valerio-Alfaro G. Synthesis of flavor and fragrance esters using *Candida antarctica* lipase. Appl Microbiol Biotechnol 2004;65:373–6.

Larroche C, Pandey A. Preface, special issue on biofuels. J Sci Ind Res 2005;64:801.

Leung DYC, Wu X, Leung MKH. A review on biodiesel production using catalyzed transesterification. Appl Energy 2010;87:1083–95.

Levisson M, van der Oost J, Kengen SW. Carboxylic ester hydrolases from hyperthermophiles. Extremophiles 2009;13:567–81.

Li Z, Li X, Wang Y, Wang Y, Wang F, Jiang J. Expression and characterization of recombinant *Rhizopus oryzae* lipase for enzymatic biodiesel production. Bioresour Technol 2011a;102:9810–3.

Li WN, Chen BQ, Tan TW. Esterification synthesis of ethyl oleate in solvent-free system catalyzed by lipase membrane from fermentation broth. Appl Biochem Biotechnol 2011b;163:102–11.

Liu Y, Liu T, Wang X, Xu L, Yan Y. Biodiesel synthesis catalyzed by *Burkholderia cenocepacia* lipase supported on macroporous resin NKA in solvent-free and isooctane systems. Energy Fuel 2011;25:1206–12.

Lozano P, Villora G, Gomez D, Gayo AB, Conesa SJA, Rubio M, et al. Membrane reactor with immobilized *Candida antartica* lipase B for ester synthesis in supercritical carbon dioxide. J Supercrit Fluids 2004;29:121–8.

Luo Y, Zheng Y, Jiang Z, Ma Y. A novel psychrophilic lipase from *Pseudomonas fluorescens* with unique property in chiral resolution and biodiesel production via transesterification. Appl Microbiol Biotechnol 2006;73:349–55.

Mandel MA. A generalization of the concept of acid-strength and acidity. Nature 1955;176:792–3.

Matsumoto T, Takahashi S, Kaieda M, Ueda M, Tanaka A, Fukuda H, et al. Yeast whole-cell biocatalyst constructed by intracellular overproduction of *Rhyzopus oryzae* lipase is applicable to biodiesel fuel production. Appl Microbiol Biotechnol 2001;57:515–20.

Matsumura H, Yamamoto T, Leow TC, Mori T, Salleh AB, Basri M, et al. Novel cation–pi interaction revealed by crystal structure of thermoalkalophilic lipase. Proteins 2008;70:592–8.

Meier R, Drepper T, Svensson V, Jaeger KE, Baumann U. A calcium-gated lid and a large beta-roll sand-wich are revealed by the crystal structure of extracellular lipase from *Serratia marcescens*. J Biol Chem 2007;282:31477–83.

Müller-Santos M, de Souza E, Pedrosa F, Mitchell DA, Longhi S, Carrière F, et al. First evidence for the salt-dependent folding and activity of an esterase from the halophilic archaea *Haloarcula marismortui*. Biochim Biophys Acta (Mol Cell Biol Lipids) 2009;1791:719–29.

Mussini T, Covington AK, Longhi P, Rondinini S. Criteria for standardization of pH measurements in organic solvents and water plus organic solvent mixtures of moderate to high permittivities. Pure Appl Chem 1985;57:865–76.

Nakashima T, Kyotani S, Izumoto E, Fukuda H. Cell aggregation as a trigger for enhancement of intracellular lipase production by a *Rhizopus* species. J Ferment Bioeng 1990;70:85–9.

Nielsen PM, Brask J, Fjerbaek L. Enzymatic biodiesel production: technical and economical considerations. Eur J Lipid Sci Technol 2008;110:692–700.

Noureddini H, Gao X, Philkana RS. Immobilized *Pseudomonas cepacia* lipase for biodiesel fuel production from soybean oil. Bioresour Technol 2005;96:769–77.

Oda M, Kaieda M, Hama S, Yamaji H, Kondo A, Izumoto E, et al. Facilitatory effect of immobilized lipase-producing *Rhizopus oryzae* cells on acyl migration in biodiesel-fuel production. Biochem Eng J 2004;23:45–51.

Okumura S, Iwai M, Tsujisaka Y. Synthesis of various kinds of esters by four microbial lipases. BBA-Mol Cell Biol Lipids 1979;575:156–65.

Paiva AL, Balcão VM, Malcata FX. Review — kinetics and mechanisms of reactions catalyzed by immobilized lipases. Enzyme Microb Technol 2000;27:187–204.

Pandey A, Benjamin S, Soccol CR, Nigam P, Krieger N, Soccol VT. Review: the realm of microbial lipases in biotechnology. Biotechnol Appl Biochem 1999;29:119–31.

Papamichael EM, Stergiou PY, Foukis A, Kokkinou M, Theodorou LG. Effective kinetic methods and tools in investigating the mechanism of action of specific hydrolases. In: Ekinci D, editor. Medicinal chemistry and drug design. Croatia: INTECH open science; 2012. p. 235–74.

Pratuangdejkul J, Dharmsthiti S. Purification and characterization of lipase from psychrophilic *Acinetobacter calcoaceticus* LP009. Microbiol Res 2000;155:95–100.

Qin HE, Yan X, Yun T, Dong W. Biodiesel production catalyzed by whole-cell lipase from *Rhizopus chinensis*. Chin J Catal 2008;29:41–6.

Reetz MT, Zonta A, Schimossek K, Liebeton K, Jaeger KE. Creation of enantioselective biocatalysts for organic chemistry by in vitro evolution. Angew Chem Int Ed Engl 1997;36:2830–2.

Reis P, Holmberg K, Watzke H, Leser ME, Miller R. Lipases at interfaces: a review. Adv Colloid Interf Sci 2009;147–148:237–50.

Resnik SL, Favetto G, Chirife J, Ferro-Fontan CJ. A world survey of water activity of selected saturated salt solutions used as standards at 25 °C. J Food Sci 1984;49:510–3.

Rondinini S, Mussini PR, Mussini T. Reference value standards and primary standards for pH measurements in organic-solvents and water and organic-solvent mixtures of moderate to high permittivities. Pure Appl Chem 1987;59:1549–60.

Russell J, Tweddell R, Kermasha S, Combes D, Marty A. Esterification and interesterification activities of lipases from *Rhizopus niveus* and *Mucor miehei* in

three different types of organic media: a comparative study. Enzyme Microb Technol 1998;22:439–45.
Salameh M, Wiegel J. Purification and characterization of two highly thermophilic alkaline lipases from *Thermosyntropha lipolytica*. Appl Environ Microbiol 2007;73:7725–31.
Scott WJ. Water relations of food spoilage microorganisms. Adv Food Res 1957;7:83–127.
Secundo F, Carrea G, Tarabiono C, Gatti-Lafranconi P, Brocca S, Lotti M, et al. The lid is the structural and functional determinant of lipase activity and selectivity. J Mol Catal B: Enzym 2006;39:166–70.
Shah S, Gupta MN. Lipase catalyzed preparation of biodiesel from *Jatropha* oil in a solvent free system. Process Biochem 2007;42:409–14.
Shimada Y, Watanabe Y, Samukawa T, Sugihara A, Fukuda H, Tominaga Y. Conversion of vegetable oil to biodiesel using immobilized *Candida antarctica* lipase. JAOCS 1999;76:789–93.
Shinkai A, Hirano A, Aisaka K. Substitutions of Ser for Asn-163 and Pro for Leu-264 are important for stabilization of lipase from *Pseudomonas aeruginosa*. J Biochem 1996;120: 915–21.
Skrika-Alexopoulos E, Freedman RB. Factors affecting enzyme characteristics of bilirubin oxidase suspensions in organic solvents. Biotechnol Bioeng 1993;41:887–93.
Soumanou MM, Bornscheuer UT. Lipase-catalyzed alcoholysis of vegetable oils. Eur J Lipid Sci Technol 2003;105:656–60.
Stemmer WPC. Rapid evolution of a protein *in-vitro* by DNA shuffling. Nature 1994;370: 389–91.
Stergiou PY, Foukis A, Sklivaniti H, Zacharaki P, Theodorou LG, Papagianni M, et al. Experimental investigation and optimization of process variables affecting the production of extracellular hydrolases with protease, lipase and α-amylase activities by *Kluyveromyces marxianus* IF0 0288. IFIB-2012, 5th international conference on industrial bioprocesses. Taipei, Taiwan; 2012.
Tan T, Zhang M, Wang B, Ying C, Deng L. Screening of high lipase producing *Candida* sp. and production of lipase by fermentation. Process Biochem 2003;39:459–65.
Tejo BA, Salleh AB, Pleiss J. Structure and dynamics of *Candida rugosa* lipase: the role of organic solvent. J Mol Model 2004;10:358–66.
Teng Y, Xu Y, Wang D. Changes in morphology of *Rhizopus chinensis* in submerged fermentation and their effect on production of mycelium-bound lipase. Bioprocess Biosyst Eng 2009;32:397–405.
Theodorou LG, Lymperopoulos K, Bieth JG, Papamichael EM. Elucidation of the catalytic mechanism of cysteine proteinases: possible applications in cheese and other dairy products processing. In: Larroche C, Pandey A, Dussap CG, editors. Current topics on bioprocesses in food industry. New Delhi, India: Asiatech Publishers, Inc.; 2006. p. 223–34.
Torres S, Castro GR. Non-aqueous biocatalysis in homogeneous solvent systems. Food Technol Biotechnol 2004;42:271–7.
Tufvesson P, Lima-Ramos J, Nordblad M, Woodley JM. Guidelines and cost analysis for catalyst production in biocatalytic processes. Org Process Res Dev 2011;15:266–74.
Varma MN, Madras G. Effect of chain length of alcohol on the lipase-catalyzed esterification of propionic acid in supercritical carbon dioxide. Appl Biochem Biotechnol 2010;160: 2342–54.
Vaysse L, Ly A, Moulin G, Dubreucq E. Chain-length selectivity of various lipases during hydrolysis, esterification and alcoholysis in biphasic aqueous medium. Enzyme Microb Technol 2002;31:648–55.
Villeneuve P. Lipases in lipophilization reactions. Biotechnol Adv 2007;25:515–36.
Wang L, Du W, Liu D, Li L, Dai N. Lipase-catalyzed biodiesel production from soybean oil deodorizer distillate with absorbent present in tert-butanol system. J Mol Catal B: Enzym 2006;43:29–32.
Xu Y. Process technology for immobilized lipase-catalyzed reactions. PhD ThesisDTU-Technical University of Denmark, Department of Chemical and Biochemical Engineering, Center for Process Engineering and Technology, Søltofts Plads, 2800, Kgs. Lyngby, DTU-Chemical Engineering; 201217–26 [54–81].
Yahya AHM, Anderson WA, Moo-Young M. Ester synthesis in lipase-catalyzed reactions. Enzyme Microb Technol 1998;23:438–50.
Yamane T, Kojima Y, Ichiryu T, Nagata M, Shimizu S. Intramolecular esterification by lipase powder in microaqueous benzene: effect of moisture content. Biotechnol Bioeng 1989;34:838–43.
Yan Y, Xu L, Dai M. A synergetic whole-cell biocatalyst for biodiesel production. RSC Advances 2012;2:6170–3.
Yumoto I, Hirota K, Sogabe Y, Nodasaka Y, Yokota Y, Hoshino T. *Psychrobacter okhotskensis* sp. nov., a lipase-producing facultative psychrophile isolated from the coast of the Okhotsk Sea. Int J Syst Evol Microbiol 2003;53:1985–9.
Zaks A, Klibanov AM. The effect of water on enzyme action in organic media. J Biol Chem 1988;263:8017–21.
Zuo K, Zhang L, Yao H, Wang J. Isolation and functional expression of a novel lipase gene isolated directly from oil-contaminated soil. Acta Biochim Pol 2010;57: 305–11.