Universitat Autònoma de Barcelona

Alcoholysis reaction study of biodiesel synthesis by recombinant Rhizopus oryzae lipase

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Alcoholysis reaction study of biodiesel synthesis by recombinant *Rhizopus oryzae* lipase

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Part of the work of this thesis was planned and performed in collaboration with the Division of Biotechnology at Lunds Universitet, under the supervision of Professor Patrick Adlercreutz.

Na **Maria Dolors Benaiges Massa**, Professora titular, i en **Francisco Valero Barranco**, Catedràtic, ambdós membres del Grup d'Enginyeria de Bioprocessos i Biocatàlisi Aplicada del Departament d'Enginyeria Química, Biològica i Ambiental de la Universitat Autònoma de Barcelona,

CERTIFIQUEN: que l'enginyer químic Albert Canet Morral va dur a terme sota la seva direcció al Departament d'Enginyeria Química, Biològica i Ambiental de la Universitat Autònoma de Barcelona, el treball que amb el títol "Alcoholysis reaction study of biodiesel synthesis by recombinant *Rhizopus oryzae* lipase" es presenta en aquesta memòria, la qual constitueix la seva Tesi per optar al grau de Doctor per la Universitat Autònoma de Barcelona dins del programa de doctorat en Biotecnologia.

I per tal que se'n prengui coneixement i consti als efectes oportuns, signen la present a Bellaterra, a maig de 2016.

Dra. Maria Dolors Benaiges Massa

Dr. Francisco Valero Barranco

Abstract

Global warming and environmental pollution have fostered scientific research and encouraged governmental policies to develop and implement greener production processes, a more rational energy usage and recycling life cycles. In that sense, fatty acid alkyl esters – commonly known as biodiesel – have emerged as a green and renewable alternative to conventional diesel and currently it is commercially used in blends with diesel. Biodiesel is obtained by transesterification of vegetable oils or animal fats with an alcohol – most often methanol. After its combustion, carbon dioxide is fixed and converted to new biomass by photosynthesis, from which biodiesel is again produced. Thus, the main advantage of biodiesel over diesel is that its production and utilisation is a closed carbon cycle, which does not contribute to increasing atmospheric carbon levels, and overcomes the future depletion of fossil fuels.

Moreover, the employment of enzymes has become widespread within the industrial sector, especially because enzymatic procedures are greener and have less energy demand compared to traditional chemical ones. However, the high price of enzymes still limits their utilisation and further scientific research is needed. The use of lipases to produce biodiesel constitutes one example of the employment of enzymes to replace an existing chemical production process. In nature, lipases are found in living organisms, which use them to catalyse the hydrolysis of triacylglycerols – *i.e.* lipids –, obtaining free fatty acids after breaking ester bonds. In recent years, their employment in the synthesis of biodiesel has been explored as a suitable alternative to the present chemical catalysis processes, due to its lower energy consumption and also because lipases can produce biodiesel from a larger variety of initial raw materials, compared to conventional process.

This dissertation has been focused on the study of the *Rhizopus oryzae* lipase, expressed recombinantly by *Pichia pastoris*, as biocatalyst to synthesise fatty acid alkyl esters – *Rhizopus oryzae* lipase and recombinant *Rhizopus oryzae* lipase are referred to in the present booklet as ROL and rROL, respectively. Initially, methanol inactivation of the lipase, which remains the main drawback for the employment of lipases in biodiesel production, and the effect of water on this inactivation were investigated, concluding that water can buffer methanol's negative effect on lipase, although it increases the hydrolysis of triacylglycerols.

The presence of free fatty acids in the initial raw material is a major obstacle in conventional chemical biodiesel production, whereas lipases can handle perfectly free fatty acids, esterifying

them to biodiesel. Thus, in the second part of the thesis the effect of free fatty acids on the lipase was also studied, concluding that not only do they not represent a problem for enzymatic biodiesel production, but also their presence reduces lipase inactivation by methanol.

Due to the variety of compounds present throughout the lipase catalysis – tri-, di-, monoacylglycerols, free fatty acids, alcohol, water and biodiesel –, experiments to elucidate the whole transesterification reaction pathway were carried out. It was found that biodiesel was obtained by a combination of reactions, namely, direct triacylglycerol alcoholysis, triacylglycerol hydrolysis and free fatty acids esterification. It was also determined that rROL does not need the so-called interfacial activation, widely attributed to lipases.

Finally, a comparative study of alcohol type and lipase specificity towards acylglycerols was also performed, demonstrating that rROL exhibits better tolerance to ethanol compared to methanol, and higher specificity towards 1-monoolein than triolein.

This thesis project is the first study done by the research group “Grup d’Enginyeria de Bioprocessos i Biocatàlisi Aplicada” in the Department of Chemical, Biological and Environmental Engineering at Universitat Autònoma de Barcelona focused on this subject. Before this work, the biochemical properties of the rROL were explored and compared to the ones of a commercial ROL in a study done by the research group. A first catalysis work was also carried out exploring the use of the lipase in the synthesis of ethyl butyrate. Therefore, during this doctoral period several analytical techniques had to be developed and implemented – especially gas chromatographic methods to detect and quantify biodiesel as well as other compounds – as new experiments on biodiesel synthesis were planned and performed. These analytical techniques can be found in the *Materials and Methods* sections of the journal articles presented in this dissertation booklet.

Resum

L'escalfament global i la contaminació medioambiental han promogut la recerca científica i polítiques governamentals per tal de desenvolupar i aplicar processos de producció més sostenibles, un ús més racional de l'energia i la implenetació de sistemes de reciclatge. En aquest sentit, els alquil èsters d'àcids grassos – coneguts com a biodièsel – suposen una alterntaiva sostenible i renovable al dièsel convencional, i en l'actualitat ja s'usa mesclat amb dièsel. El biodièsel s'obté per transesterificació d'olis vegetals o grasses animals amb un alcohol – normalment metanol. Després de la seva combustió, el diòxid de carboni resultant és fixat per les plantes mitajançant la seva fotosíntesi, i convertit a biomassa a partir de la qual es torna a obtenir oli per fabricar-ne biodièsel. Així, l'avantatge principal del biodièsel respecte el dièsel convencional és que el seu ús és un cicle tancat de carboni, fet que evita l'augment del carboni ambiental, i a més a més no presenta els futurs problemes d'esgotament dels combustibles fòssils.

En un altre ordre de coses, l'ús d'enzims s'ha estès dins la indústria, principalment perquè els processos enzimàtics són més sostenibles i tenen menys consum energètic que els processos convencionals. Malgrat tot, l'elevat preu dels enzims limita encara el seu ús i per tant és necessària més recerca. La utilització de lipases per generar biodièsel és un exemple de l'ús d'enzims per substituir mètodes convencionals. A la natura, les lipases es troben als éssers vius i la seva funció és la hidròlisi dels greixos – és a dir, triacilglicerols –, obtenint àcids grassos lliures. Recentment, l'ús de les lipases s'ha explorat com a alternativa a l'actual mètode d'obtenció de biodièsel, degut a que té un consum d'energia inferior i també perquè les lipases poden sintetitzar biodièsel a partir d'un rang de matèries primeres més ampli.

Aquesta tesi s'ha focalitzat a l'estudi de la lipasa de *Rhizopus oryzae*, expressada recombinantment a partir de *Pichia pastoris*, com a biocatalitzador d'alquil èsters d'àcids grassos. En primer lloc es va estudiar la inactivació de la lipasa causada pel metanol, fet el qual és un dels principals inconvenients en l'ús de les lipases, així com també l'efecte de l'aigua en aquesta inactivació; d'aquest primer estudi, se'n va concloure que l'aigua contrarresta l'efecte negatiu del metanol, encara que la seva presència incrementa la hidròlisi dels greixos en lloc de la seva transesterificació.

La presència d'àcids grassos lliures a les matèries primeres és un inconvenient important pels mètodes químics actuals d'obtenció de biodièsel, mentre que les lipases poden dur a terme

sense problemes la transesterificació amb presència d'àcids lliures, convertint-los també a biodièsel. Així, la segona part de la tesi va ser centrada en estudiar si aquests àcids grassos lliures poden tenir algun efecte sobre les lipases, concloent que no només no perjudiquen l'enzim, sinó que la seva presència redeix l'efecte negatiu del metanol.

Degut a la varietat de components implicats a la transesterificació enzimàtica – tri-, di-, monoacilglicerols, àcids grassos lliures, alcohol, aigua i biodièsel –, es varen realitzar experiments per determinar el mecanisme de reacció de la transesterificació. Es va concloure, que el biodièsel s'obté enzimàticament per combinació de l'alcohòlisi directa dels greixos i l'hidròlisi dels greixos i la posterior esterificació dels àcids grassos alliberats. També és va determinar que la lipasa de *Rhizopus oryzae* no necessita l'activació interfacial, fenomen característic de les lipases.

Finalment, es va fer un estudi comparatiu de l'especificitat de la lipasa respecte acilglicerols i alcohols, demostrant que l'enzim és molt més específic per 1-monooleïna que per trioleïna, i presenta més tolerància a l'etanol que el metanol.

Agraïments

Encara que faré servir poques paraules, vull agrair a tots els qui varen participar a aquesta tesi i hi varen ser d'una forma o altra. A tots vosaltres, moltes mercès!

Albert

Catalunya, a maig de 2016

Summary – List of publications

This thesis is presented as a compilation of journal articles. A short outline of each article and the author's contribution to them are briefly given below. A more expanded conclusions chapter and the papers themselves are provided later in this booklet.

Publication I. Albert Canet, M. Dolors Benaiges, Francisco Valero. Biodiesel Synthesis in a Solvent-Free System by Recombinant *Rhizopus oryzae* Lipase. Study of the Catalytic Reaction Progress. Journal of the American Oil Chemists's Society, 2014, 91:1499-1506.

This publication was focused on determining the effect of important parameters in biodiesel synthesis catalysed by the lipase: methanol to oil molar ratio, stepwise addition of methanol and the amount of water present in the reaction medium. Response surface methodology was used to study both the individual and combined effect of these parameters on the final fatty acid alkyl esters synthesis. Experiments were carried out by transesterification of olive oil with methanol as acyl acceptor in solvent-free medium and using the enzyme immobilised by adsorption. Principally it was concluded that the methanol stepwise addition strategy depends on the water amount in reaction medium in order to avoid lipase inactivation caused by the alcohol.

The author of the thesis planned and performed the experimental work and wrote this present manuscript under the supervision of Professors Francisco Valero and M. Dolors Benaiges, in the Department of Chemical, Biological and Environmental Engineering at Universitat Autònoma de Barcelona.

Publication II. Albert Canet, Kírian Bonet-Ragel, M. Dolors Benaiges, Francisco Valero. Lipase-catalysed transesterification: Viewpoint of the mechanism and influence of free fatty acids. Biomass and Bioenergy, 2016, 85:94-99.

The second publication examined the influence of free fatty acid on lipase performance, through the initial reaction rates and the stability of the biocatalyst obtained in the transesterification of different mixtures of oil and free oleic acid. As in the case of water in

Publication I, the presence of free fatty acids was concluded to avoid lipase inactivation by methanol.

Furthermore, this manuscript also elucidated the lipase transesterification reaction pathway. Apart from acylglycerols, biodiesel and methanol, transesterification involves water – seen in Publication I – and free fatty acids – seen in this second publication. It was determined that the enzymatic synthesis of alkyl esters is a combination of two processes or pathways: a one-step reaction, consisting of a direct alcoholysis of tryacylglycerols, and a two-step reaction which includes a first hydrolysis of tryacylglycerols and the subsequent esterification of the liberated fatty acids. In order to make analysis easier, these experiments were carried out with triolein and linoleic acid.

All the experiments of Publication II were done with the lipase immobilised by adsorption, with methanol as acyl acceptor and without organic solvents – like in Publication I.

The author of the thesis planned and performed the experimental work and wrote this present manuscript with the collaboration of Ph.D. student Kírian Bonet-Ragel, under the supervision of Professors Francisco Valero and M. Dolors Benaiges, in the Department of Chemical, Biological and Environmental Engineering at Universitat Autònoma de Barcelona.

Publication III. Albert Canet, M. Dolors Benaiges, Francisco Valero, Patrick Adlercreutz. Alcoholysis reactions catalysed by free or immobilised *Rhizopus oryzae* lipase for biodiesel production. Submitted to Biochemical Engineering Journal.

This third manuscript exposes the experimental results and conclusions obtained comparing four aspects regarding transesterification catalysed by rROL: acyl acceptors – ethanol and methanol –, biocatalyst preparation – immobilised or free soluble lipase –, reaction system – one or two phases – and acylglycerols specificity – 1, 2-monoolein and triolein. rROL was also compared to a commercial one. Analysis of the different compounds was done, in particular including 1- and 2-monoolein, 1,2- and 1,3-diolein and triolein. This detailed analysis allowed a better understanding of the evolution of the compounds throughout the reaction and especially of the acyl migration phenomenon.

Reactions of the experimental work exposed in this third manuscript were done with triolein as substrate and using heptane as solvent.

The author of the thesis planned and performed the experimental work and wrote this present manuscript in collaboration with the Division of Biotechnology at Lunds Universitet, under the supervision of Professor Patrick Adlercreutz. The work was also supervised by Professors Francisco Valero and M. Dolors Benaiges from Universitat Autònoma de Barcelona.

Contents

1. Introduction

While many chemical reactions occur spontaneously, in others *catalysts* are required to carry out these reactions at a significant rate. The energy barrier required to overcome for a substance – *i.e.* substrate – to be converted chemically to a different one – *i.e.* product – is decreased by catalysts. Although catalysts are molecules that are not consumed during the reaction, in practice, their chemical activity is altered and reduced through time thus they cannot be used indefinitely.

Special types of catalyst are *enzymes* or *biocatalysts*, responsible for carrying out biochemical reactions. Enzymes have evolved throughout time nearby life evolution since its appearance, performing efficiently, specifically and at a sufficient rate the wide range of reactions that sustains cell metabolism.

Enzymes are proteins or polypeptides composed of *n* amino acid residues; bearing in mind that there are 20 different amino acid residues, the possible primary structures of an enzyme are 20^n, which means that there is a huge variety of different enzymes. The *active site* is where catalysis is carried out and it is made up of very few amino acid residues of the total forming the enzyme. Usually this active site is formed by three amino acids, referred to as *catalytic triad*. *Candida antarctica* lipase B, for instance, consists of 317 amino acids and its active site is formed only by three residues – Ser-His-Asp – [1], or elegaxobin II from the venom of *Trimeresurus elegans*, with an active site made up of His-Asp-Ser from a total of 233 residues [2]. The vast number of biochemical reactions that enzymes perform is reflected by an enzyme classification system developed by the Enzyme Commission, EC – a commission of IUPAC, the International Union of Pure and Applied Chemistry – that classifies enzymes into six families, based on the reaction type that can be catalysed [3,4]. These families are listed in Table 1.

Table 1. Enzyme classification developed by Enzyme Commission.

Enzyme class	Function	Example
1 **Oxidoreductases**	Oxidation/reduction involving electrons, hydrogen or oxygen transfer	Dehydrogenases
2 **Transferases**	Functional group transfer from a donor to an acceptor	Transaminases
3 **Hydrolases**	Hydrolysis reactions	Proteases, Lipases
4 **Lyases**	Non-hydrolytic and non-oxidative bond-breaking reactions	Aldolases
5 **Isomerases**	Isomerization reactions	Glucose isomerase
6 **Ligases**	Bond-formation reactions	Polydeoxyribonucleotide synthase

This huge variety of enzymes shares mainly the features laid out in Table 2[3–5].

Table 2. Common features of enzymes.

Advantages	Disadvantages
Very high enantioselectivity	Often low specific activity
Very high regioselectivity	Availability for selected reactions only
Active under mild conditions	Instability at extreme temperatures and pH and in aggressive solvents
Fewer by-products	Long development times for new enzymes
Can be degraded biologically	Some enzymes are still very expensive and require expensive cofactors

The properties of the enzymes previously laid out in Table 2 have enabled their industrial application beyond their natural metabolic functions, also accepting unnatural substrates [6]. Thus, their utilisation covers a wide-ranging production of human desired goods and needs – *e.g.* fine and bulk chemicals, human food and animal feed, pharmaceuticals and environmental technologies, among others – as well as analytical purposes. In fact, during the last few years the development of biotechnology – such as new molecular screening techniques and recombinant DNA technology – has allowed enzyme modification and its optimization for new synthetic applications and currently the vast majority of enzymes used as a biocatalyst, except for food processing, are recombinantly expressed by host organisms. Today some enzyme-manufactured products exceed quantities larger than 10000 t $year^{-1}$, such as ethanol – as a gasoline additive –, acrylamide and lactose-free milk, among others [3,7,8]. According to a

study by Freedonia Group Inc., the global enzyme market is expected to grow steadily and reach $7 bn in 2017 [9].

Nevertheless, the beginning of the use of enzymes did not start in the last few decades, but the utilisation of microorganisms such as enzymes as sources for alcoholic drinks production, cheese manufacture and bakery was already widespread among ancient people, long before their nature and properties were known. In the 1830s, *diastase* – a mixture of amylases – and *pepsin* were the first enzymes reported by Payen and Persoz and by Schwann, respectively. By the 1920s several different enzymes were known to exist. Between 1950 and 1970, new scientific and technical knowledge and market demands stimulated the further development of enzyme technology and in the 1960s and early 1970s commercial processes using enzymes began to appear widely [3,8,10].

1.1 Lipases

Lipases probably constitute one of the most frequently used enzymes in synthetic organic chemistry and in a broad range of biotechnological applications [11–15].

Lipases (EC 3.1.1.3) are triacylglycerol ester hydrolases whose natural function is to hydrolyse the ester bonds of acylglycerols – *i.e.* fats and oils – to fatty acids and glycerol [13] (Figure 1), thus widely found in most organisms from the microbial, plant and animal kingdoms [3,14]. Eukaryotes lipases are confined within lysosomes or in spaces outside cells performing lipids metabolism, adsorption and transport, while in lower eukaryotes and bacteria they can be either intracellular or secreted, hydrolysing lipids present in the environment and even act as virulence factors in some pathogenic organisms – *Candida albicans, Staphylococcus* and *Pseudomonas* species – [16].

A classification of lipases can be carried out based on their different kinds of selectivities towards their substrates: *chemo-*, *regio-* and *enantioselectivity* [17]; – in this thesis, the terms specificity and selectivity are used indistinctly as synonyms [18].

Lipases possess different regiospecificity towards acylglycerols. Non-selective lipases hydrolyse all three fatty acids of a triacylglycerol molecule, while 1,3-positional selective lipases hydrolyse preferably the ester bonds of positions sn-1 and/or sn-3 on the glycerol backbone, producing 2-monoacylglycerols instead of glycerol [17]. Different degrees of regiospecificity

are displayed by different lipases: *Rhizopus arrhizus*, for instance, has more pronounced 1,3-selectivity than *Rhizopus miehei* [19]. Some lipases also show different specificity towards different acylglycerols. For instance, *Malassezia globose* lipase was identified to be strictly specific for mono- and di- but not for triacylglycerols [20].

Moreover, lipases can be further classified based on distinct chemospecificitiy towards fatty acid chain length: usually lipases act on C4 to C18 chain fatty acids – rarely up to C22 –, but with different efficiencies. Isoforms of *Candida rugosa* lipase, for instance, have distinct chain length specificity: isoform 1 acts mainly on C8-C10, isoform 3 on short-chain and isoforms 2 and 4 on C16-C18 chains [16]. Furthermore, different preferences towards unsaturations are showed by lipases. This is the case for lipase B from *Geotrichum candidum*, which is highly selective for cisΔ9 unsaturated substrates [21].

Lipases can also distinguish between enantiomers in racemic mixtures, enantioselectivity being another characteristic displayed by these enzymes [17,22].

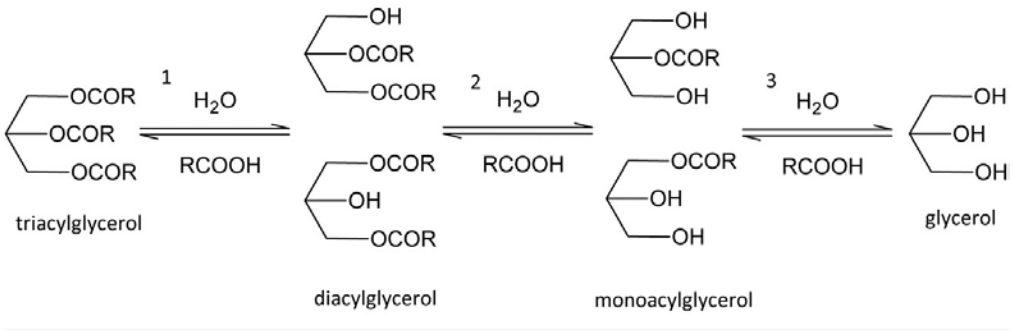

Figure 1. Triacylglycerol hydrolysis, yielding a free fatty acid and a diacylglycerol – reaction 1. Subsequently diacylglycerol is hydrolysed to monoacylglycerol releasing another free fatty acid molecule – reaction 2. Finally, monoacylglycerol is hydrolysed to glycerol releasing a further free fatty acid – reaction 3.

1.1.1 General Structure and Features of Lipases

An important and unique feature of lipases is their capacity to catalyse reactions at organic-water interfaces – a mixture of fats or oils and water, for instance – and in fact, they express generally higher catalytic activities in such systems than in aqueous solutions [11,23]. This phenomenon, which is not displayed by esterases, is known as *interfacial activation* and is

attributed to a mobile hydrophobic oligopeptide – called *lid* or *flap* – which covers the entrance to the active site (Figure 2). The contact between the lipase and an interface promotes the movement of this lid, allowing the substrate to enter the active site [3,11].

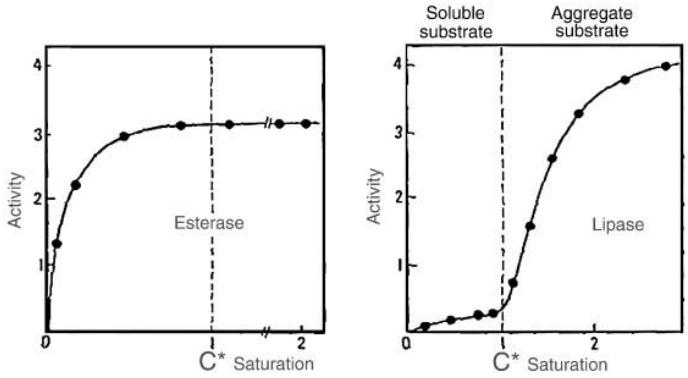

Figure 2. Representation of the main catalytic difference between esterases and lipases. Esterases perform their high catalytic activity on water-soluble substrates in aquous solutions, while lipases prefer organic-water interphases, due to their interfacial activation. Reproduced from [24], with permission of Elsevier.

However, this unique feature is not held in some lipases: lipases without a lid or with a mini-lid composed of only five amino acid residues, or with a lid but not interfacial activation phenomenon have been also described [14,16,23,25]. This suggests that interfacial activation may be linked to the lifetime of the open and closed forms of each lipase [23]. Thus, the only feature to distinguish perfectly lipases from esterases is that lipases readily accept water-insoluble substrates – *e.g.* long-chain fatty acids in acylglycerols –, while esterases prefer water-soluble compounds – short-chain fatty acids (Figure 2). However, it must be stated that most lipases are also active on water-soluble substrates [3,16,23].

Lipases display a wide variety in primary sequences with a range of molecular masses from less than 20 kDa, as in the case of lipase A from *Bacillus subtilis*, to about 60 kDa for larger fungal lipases, as in the case of *Geocthricum candidum* lipase. However, all lipases exhibit the same α/β-hydrolase fold structure [14,16] – common also in many other hydrolases [17] – and identical catalytic triad composed of Ser, His and Asp – sometimes Glu – [3] (Figure 3). Moreover, lipases, like most hydrolases, do not usually require coenzymes to carry out their catalytic activity – under certain conditions some mammalian lipases need coenzymes – [4,11].

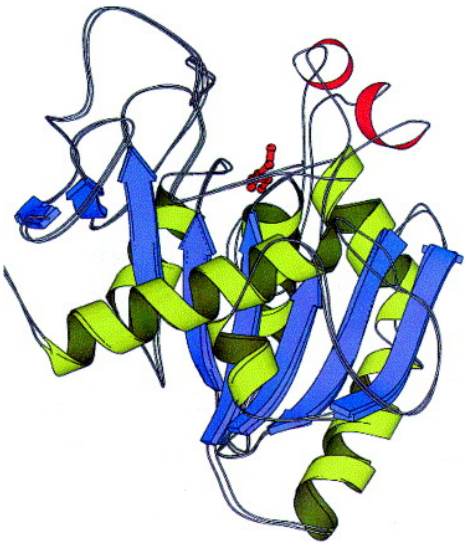

Figure 3. Crystal structure of the Thermomyces lanuginosus lipase. The β-sheet is shown in blue, surrounded by some helices in yellow, and the active serine site residue in red sticks, and the lid shown in red. Both the open and the closed conformation are shown superimposed. Reproduced from [26], with permission of Elsevier.

Glycosylations are usually found in eukaryotic lipases, with several functional roles – especially protein solubility, but also activity and stability in lower pH conditions – [16], and often glycosylation patterns are the only characteristic that distinguishes lipase isoforms produced by an organism [14].

The activity of the lipase is completely related to the amount of water surrounding it but with a high degree of variability among lipases. Some lipases, such as *Burkholderia cepacia* lipase, show maximal activity at high water activity, while others, such as *Rhizomucor miehei* lipase, behave oppositely [11]. In addition, taking into account that lipases are used in synthetic reactions, water plays an important role in reaction equilibria [14].

1.1.2 Catalytic Mechanism of Lipases

The mechanism of the lipase to catalyse ester hydrolysis is similar to carboxyl esterases and serine proteases and involves a first nucleophilic attack of the serine on the carbonyl carbon of the ester bond, yielding a covalent acyl-enzyme intermediate and releasing an alcohol – *i.e.* a

diacylglycerol would be released after forming a hydroxyl group in a triacylglycerol molecule – (Figure 4). This step is stabilised by the other two residues of the active site, histidine and aspartic acid. Then, a second nucleophilic attack occurs when the acyl-enzyme intermediate is hydrolysed by water, finally forming a carboxylic acid. Many different compounds can act as acyl donors and likewise, in addition to water, many nucleophiles compounds can perform the same role and break the acyl-enzyme intermediate [3,11,27,28]. Due to this broad substrate specificity, lipases can perform several reactions beyond their natural acylglycerol hydrolysis; some are listed below:

- *transesterification*: the acyl group of an ester is exchanged with an alkyl group of a nucleophile. The prefix *trans*- stands for the fact that an ester is exchanged from a donor to an acceptor molecule. The hydroxyl group is likewise exchanged. The nucleophile molecule is typically an alcohol, referred to as *alcoholysis*, but it can also be glycerol, for instance, called *glycerolysis*. Throughout this present dissertation transesterification refers to biodiesel synthesis.

- *esterification*: an ester is derived when the hydroxyl group of an acid is replaced by an alkyl group of a nucleophile. Throughout this present dissertation esterification refers to esters formation from free fatty acids with an alcohol – which is a very common example of esterification –.

- *interesterification*: two esters molecules exchange their organic groups R, acting both at the same time as acyl donor and acceptors.

- *acidolyisis*: an acyl group is exchanged between an ester and a carboxylic acid molecule. Thus, the ester becomes the carboxylic acid and *vice versa*.

However, the catalytic options of lipases spread to several other synthetic or non-conventional substrates and types of reactions, from using amines as nucleophiles compounds to performing aldol additions, as will be seen later [14,29,30].

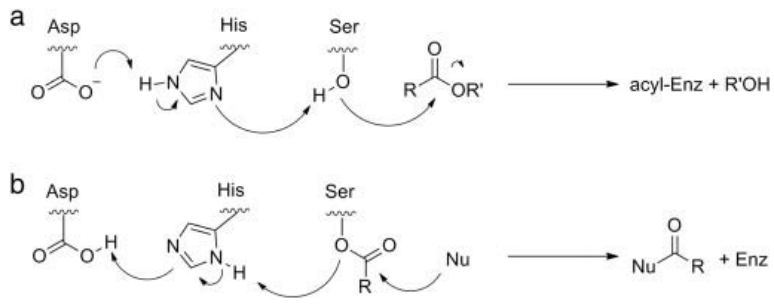

Figure 4. Steps of the lipase catalytic mechanism. An acyl-enzyme intermediate is formed by a serine nucleophilic attack on the carbonyl carbon, promoted by a histidine and an aspartate residue – these three amino acids form the catalytic triad – reaction a. This intermediate reacts with a nucleophile in a second nucleophilic attack, such as water, creating the product and returning the functional hydroxyl group to serine – reaction b. Reproduced from [31], with permission of Elsevier.

1.1.3 Sources of lipases

As we have seen throughout this introduction, lipases – like many other enzymes – are biocatalytical tools the use of which is expanding and drawing more attention over the last few years. Nowadays, several commercially available lipases from different organisms can be found; some examples are listed in Table 3 [15].

Table 3. Commercially available lipases

Type	Organism-Source	Commercial Form
Fungal	*Candida rugosa*	Powder
	Candida antarctia A/B	Immobilised
	Thermomyces lanuginosus	Immobilised
	Rhizomucor miehei	Immobilised/liquid
	Geotricum candidum	Liquid
Bacterial	*Burkholderia cepacia*	Powder
	Pseudomonas alcaligenes	Powder
	Pseudomonas mendocina	Powder
	Chromobacterium viscosum	Liquid
Animal	Pig pancreatic lipase	Granulated

Due to the current vast applications of lipases, their production is a crucial step. It begins with the expression of each lipase by its corresponding organism and a subsequent lipase extraction from it. However, this process may confront several drawbacks such as slow and not easy growing organisms, high demanding costs organisms and difficulties in recovering intracellular lipases, which may lead to low lipase production yields. These problems can be overcome expressing proteins in host systems, thus becoming heterologous proteins, which has been developed by the genetic engineering techniques of the last decades. This methodology allows the production of enzymes in microorganisms that can be grown easily and in cheap media, and the enzyme industry has taken advantage of it in order to improve the reproducibility and the yields of the production of enzymes [17]. For instance, the gene of the lipase from the fungus *T. lanuginosus*, used in the detergent industry, was cloned into *Aspergillus oryzae* and produced on a large scale (several 100 tons per year) by fermentations of this host system [14]. The expression system must be selected in terms of productivity, bioactivity, purpose, physicochemical characteristics of the interest protein and cost of the system itself [32]. The host system can be eukaryotic or prokaryotic, this last being the most utilised, as these organisms are generally easier to handle, have fast growths and reach high densities in inexpensive media. However, compared to eukaryotic organisms, they are not able to perform post-translational modifications, such as correct protein folding, glycosylations and correct formation of disulphide bridges [17]. *Escherichia coli* and *P. pastoris* are the most used host systems, accounting approximately for a half and a third of the total recombinant lipase expression, respectively [17].

1.1.4 Immobilisation of lipases

The major reasons for the application of immobilised enzymes are the reuse of these enzymes, especially if they are expensive, and easier product purification in order to lower the cost of biocatalytic processes [11]. The immobilisation of enzymes usually consists in binding them on the surfaces and/or within specific materials – also referred to as support –, via different kinds of interactions between the protein and the material itself. Several industrial applications employ immobilised enzymes; approximately 10 million tons of glucose-fructose syrup per year, for instance, are produced industrially by immobilised glucose isomerase and immobilised lipases produce more than 1000 tons per year of pharmaceutical and agrochemical intermediates [3]. Commercially available lipases are often already found as

immobilised preparations, as shown in Table 3. However, immobilisation may lead to some important drawbacks, such as the cost of materials for immobilisation, mass transfer limitations through particles – usually immobilisations are carried out with particle-shaped materials – and problems with cofactors and multi-enzyme reactions [3].

In addition to the advantages and disadvantages laid out above, enzyme activity, stability and selectivity may be altered by immobilisation, largely due to slight distortions in the enzyme structure – affecting the active site – caused by the interactions between the enzyme and the material where it is immobilised [33,34]. Usually enzyme activity decreases after immobilisation [11,33], although in some cases catalytic activity is not only kept but also improved. Lipases from *C. antarctica* – isoenzyme B –, *R. miehei* and *C. rugosa*, for instance, were reported to display hyperactivation after being immobilised by adsorption on a hydrophobic support, because they exhibit a stabilised open form afterwards [35]. Immobilisation also enhances enzyme stability against reaction conditions, such as temperature and inhibitors, avoiding activity lost throughout reaction time, compared to enzymes in solution. These advantages may be attributed to diverse reasons, such as the type of material, rigidification of the immobilised enzyme – *i.e.* short space between material surface and enzyme due to the amount of residues of the enzyme involved in anchorage to the support – and the type of interaction that generates the immobilisation [34]. Penicillin G acylase mutants become 300000 times more stable than enzyme in solution after immobilisation on disulphide/epoxide or glyoxyl supports following a patented methodology [36]. The immobilisation process may also affect enzyme selectivity, even altering enantioselectivity. For example, *C. rugosa* lipase preferred to catalyse the hydrolysis of (R)-2-phenyl-2-butyroylacetic acid when covalently immobilised on glutaraldehyde supports, while immobilised by adsorption on hydrophobic supports showed preference for S enantiomer [37].

Furthermore, the variations in enzyme behaviour described above are not only related to structural changes, but may also be greatly produced by the physicochemical properties of the enzyme surroundings – *i.e.* immobilisation material. Thus, hydrophobic and hydrophilic environments may promote gradients of compounds concentrations or partitions of compounds away from or towards the enzyme. This phenomenon can alter the inhibitors' effect on enzyme [33].

There are several principles and methods to immobilise enzyme and some of them are summarised in the next Table 4.

Table 4. Typical immobilisation methods employed in enzyme immobilisation

Immobilisation in carriers	Crosslinking	
	Binding to carrier	Adsorption
		Covalent binding
	Inclusion into carriers	Entrapment
		In microorganisms
Inclusion – enzyme in soluble form	Microencapsulation in membranes	
	Ultrafiltration systems	

Adsorption is based on physical interactions between the protein and the material and occurs when these interactions are strong enough, after bringing the enzyme solution and the material together. It is one of the simplest methods of immobilisation and cost-effective [3], and the kind of interaction force established depends on the chemical nature of the material. Several materials are able to perform this type of immobilisation. In the case of lipases, hydrophobic adsorption is perhaps the most common; actually, since lipases do adsorb on hydrophobic surfaces to a higher degree than other enzymes, this method is also used as a purification process [11].

Immobilisation by covalent binding consists of establishing chemical bonds between amino acids residues of an enzyme and reactive groups of the surface of the material. Thus, it is important not to involve residues of the active site of the enzyme in covalent bonds to avoid loss of activity.

1.1.5 Lipase applications in biotechnology

Lipases exhibit high chemo-, regio- and enantioselectivities and are highly active in non-aqueous solvents. These characteristics together with their ability to accept nucleophiles other than water, as previously mentioned, and also that they perform their activity in mild conditions, convert lipases into the third most commercialised enzymes, after proteases and carbohydrases [17], with a wide range of potential biotechnological applications, from industrial to organic synthesis and in research laboratories.

Moreover, with the purpose of addressing lipase activity, stability and specificity, protein engineering and mutagenesis methodologies – such as computer modelling and directed

evolution – have enlarged the use of these enzymes into new substrates and processes [26,38–40].

All this variety of natural and non-conventional substrates and reactions applications is widely referred to as *biocatalytic* or *enzyme promiscuity* [16,30,40].

1.1.5.1 Industrial applications

Lipases constitute one of the most important enzymes in practical applications, especially microbial lipases, mainly because of their versatility and ease of mass production [41,42]. The global demand for lipases is forecast to reach $ 345 million in 2017, according to Freedonia Group in 2014 [43]. Several examples of industrial uses of lipases are set out below. A more extensive list of lipase industrial applications can be found elsewhere [15].

A large sector of commercial application of lipases is the detergent industry, where they are added to some detergent formulations – other enzymes such as proteases, amylases and cellulases are also used in detergents. They increase the cleaning ability of detergents, hydrolysing fatty materials. This process can be carried out at low temperatures, which reduce washing energy consumption [41,44]. It is estimated that about 1000 tons of lipases are added to approximately 13 billion tons of detergent formulations every year [42]. The first commercial lipase was from *T. lanuginosus* developed by Novozymes and launched in 1988 [45]. Other lipases from *Pseudomonas mendocina*, expressed recombinantly by *Aspergillus oryzae* and *Bacillus* species, respectively, and *Pseudomonas pseudoalcaligenes* are or have been used in commercial detergents [41,42].

In the paper industry lipases can be used to remove the hydrolytic components of wood – triglycerides and waxes – in paper manufacture – a process known as *pitch control*. A method using *C. rugosa lipase* was developed and utilised by several paper manufacturers in Japan [44].

The regiospecificity exhibited by many lipases – towards sn-1 and sn-3 compared with sn-2 positions – is an excellent tool for the removal, incorporation or substitution of fatty acids on the glycerol backbone of oils and fats for other different fatty acids to produce specific-structured lipids (Figure 5). These tailored compounds show different physical and nutritional properties and are of great importance mainly in the food industry. For instance, stearic acid is

introduced at sn-1 and sn-3 positions of certain vegetable oils, obtaining a cheaper substituent which mimics cocoa butter's properties, which is a constituent of chocolate formulations. *R. miehei* has been used industrially to produce this substituent [11,46,47]. Human milk substitutes and incorporated omega-3 fatty acids in structured lipids are further examples of industrial applications using regiospecific lipases [11,48].

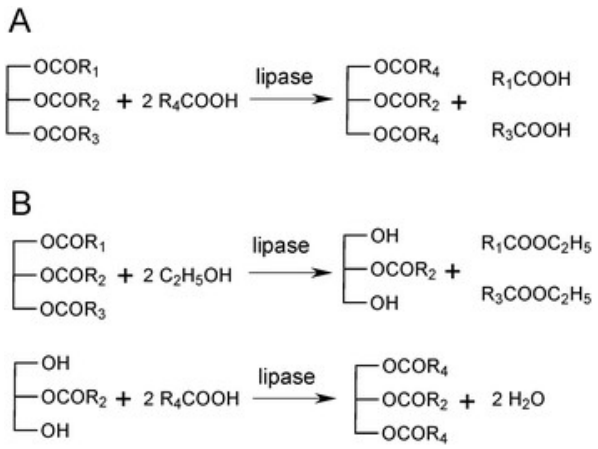

Figure 5. Preparation scheme of structured lipids catalysed by a 1,3-positional selective lipase. (A) One-step acidolyisis. (B) Two-step process; in the first step fatty acids in the sn-1 and sn-3 postions are removed by alcoholysis. Afterwards, 1-monoacylglycerol is acidolysed by a free fatty acid. Reproduced from [11], with permission of The Royal Society of Chemistry.

Lipases are also employed in the enrichment of polyunsaturated fatty acids – known as PUFAs – in mono- and diglycerides, which are used to produce several pharmaceuticals, such as anticholesterolemics and antiinflammatories [44]. Moreover, several other pharmaceutical processes are carried out by lipases [46]. For instance, the ability of lipases to perform resolutions of racemic mixtures is employed by BASF to produce chiral amines and chiral alcohols building blocks – which are used as intermediates of pharmaceuticals – by acylation of racemic mixtures (Figure 6) [11,49].

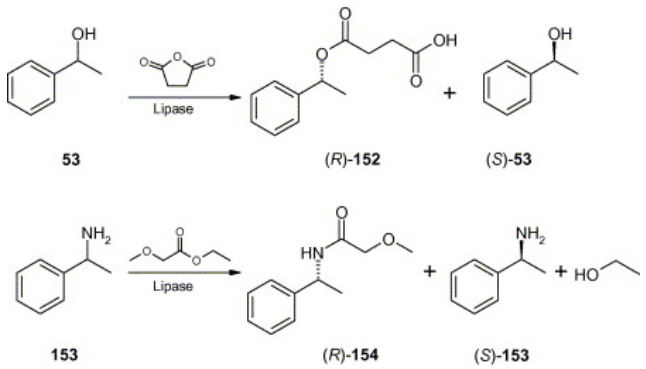

Figure 6. Examples of lipase resolution of racemic mixtures developed at BASF. Reproduced from [49], with permission of Elsevier.

1.1.5.2 Lipases in organic synthesis

The application of lipases extends beyond industrial uses to a wide variety of organic chemistry reactions as it is exemplified by the large number of publications in recent years. Chemo-, regio- and enantioselectivity together with their promiscuity to act on non-conventional substrates, as previously explained, are the main reasons for the versatility of lipases.

Furthermore, rational design and directed evolution have been recently utilised as tools of protein engineering to enhance and expand the activity of lipases to new catalytic uses [39].

Some examples of organic synthesis catalysed by lipases are set out below.

As previously discussed, other substances than water can act as nucleophiles in lipase catalysed reactions. *Aminolysis* refers to the use of amines as non-conventional nucleophile molecules for several lipases. Lipase B from *C. antarctica*, for instance, conducted the aminolysis reactions of linoleyl ethyl ester and methyl acrylate with various amine compounds [50,51]. Even aliphatic amines were utilised as acyl acceptors, as investigated using also *C. antarctica* lipase B [52]. Furthermore, the resolution of chiral amines via lipase enantioselectivity acylation has been greatly studied [27,53,54], and as previously mentioned, it is used in the pharmaceutical industry [11,27].

Enzyme resolutions are based on the fact that the enzyme catalyses just one enantiomer of the racemic mixture into a product that can be easily separated from the unreacted isomer – the reaction rate of the other enantiomer is slow enough to be neglected; sometimes it is referred to as kinetic resolution – [11]. Lipase catalysed resolutions are widely found in the literature

and are employed frequently to obtain enantiomerically pure compounds [49]. Their use in amines or in alcohols racemic resolutions are, in general, quite common, showing higher enantioselectivity for secondary alcohols compared to primaries; interestingly, lipases which perform high selectivity in carboxylic acids resolutions, are much less selective for alcohols, and *vice versa* [3].

An interesting example of lipase mediated resolution is in ibuprofen resolution (Figure 7). Ibuprofen – 2-(4-isobutylphenyl)-propionic acid – is a well-known nonsteroidal anti-inflammatory drug that presents two isomers, the (S)-enantiomer being about 160 times biologically more active than (R)-one. Lipase B from *C. antarctica* and *R. miehei* lipase, for instance, have been reported to conduct this resolution [55,56].

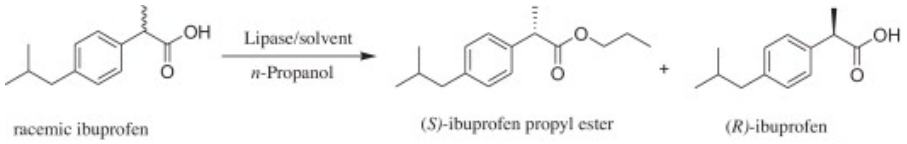

Figure 7. Lipase-catalysed enantioselective esterification of racemic ibuprofen. Reproduced from [57], with permission of Elsevier.

Nevertheless, kinetic resolution is not the only technique via lipase catalysis to obtain pure chiral compounds. In enzymatic desymmetrization, prochiral substrates are converted preferably into one enantiomeric product [49]. Dimethyl 3-phenylglutarate, for example, was asymmetrically hydrolysed to its (R)-monoester by ROL [58].

Aldol additions, conventionally displayed by lyases, have been shown in lipases, as a further example of their catalytic promiscuity. Lipase from porcine pancreas, and several other lipases, was reported to catalyse aldol reactions between acetones and aldehydes [59]. A mutant of lipase B from *C. antarctica*, whose serine in the active site was replaced with an alanine by site-directed mutation, was also reported to exhibit aldol addition activity [29].

1.2 Biodiesel

Biodiesel is defined as mono alkyl esters of long chain fatty acids produced by transesterification of triacylglycerols – as acyl donor – with a monohydric alcohol – as acyl acceptor – [60–62]. Vegetables oils and animal fats are typically used as triacylglycerols sources and various alcohols can be employed, such as methanol, ethanol and iso-propanol, although the first one is the most commonly utilised in industrial production because of its low cost and availability [60,61,63] – biodiesel derived from methanol is known specifically as fatty acid methyl esters, FAMEs – (Figure 8). Biodiesel can also be obtained by direct esterification of free fatty acids with alcohols.

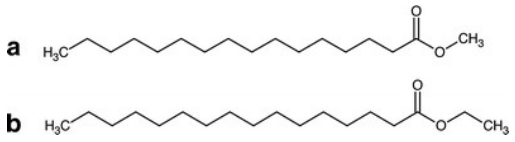

Figure 8. Chemical structures of methyl (a) and ethyl hexadecanoate (b). Reproduced from [64], with permission of Springer.

Transesterification involves three alcoholysis reactions of a molecule of triacylglycerol, producing three molecules of alkyl esters per triacylglycerol and consuming three molecules of alcohol. It is the same scheme reaction shown in Figure 1, but with alcohol and fatty acid alkyl esters instead of water and free fatty acids, respectively. Firstly, triacylglycerol is alcoholised to one molecule of biodiesel and a diacylglycerol. Subsequently diacylglycerol is alcoholysed to monoacylglycerol releasing another fatty acid alkyl ester molecule. Finally, the last alcoholysis takes places, converting monoacylglycerol to glycerol and releasing a further fatty acid alkyl ester. Thus, glycerol is the only final by-product of transesterification.

Nowadays biodiesel is most often used in blends with conventional diesel with which it is completely miscible [65].

The common international standards for biodiesel are EN 14214 and ASTM D 6751 [66].

1.2.1 Biodiesel properties

The direct use of vegetable oils – *i.e.* unmodified – in combustion engines may present several technical problems, principally high viscosity, low volatility – which leads to ash formation – and low stability against oxidation – which causes reactions of polymerization – [60,61,65,67]. Modification of oils and fats to alkyl esters via alcohol transesterification overcomes these problems, producing a fuel whose energy content and physical and chemical properties are similar to those of conventional diesel [61]. Some of these properties are listed below:

- *cetane number*: is the most common property of diesel and defines the ignition point; a higher cetane number indicates higher combustion efficiency and better ignition quality. It increases with increasing chain length and increasing saturations of fatty acids. Biodiesel has similar cetane numbers to conventional diesel – generally diesel has cetane numbers ranging between 40-50 – or even higher [60,61,68].
- the behaviour of biodiesel at low temperatures is another important parameter and it depends on the cloud point – *i.e.* the temperature at which fuel becomes cloudy due to solidification – and the pour point – *i.e.* the temperature at which fuel stops flowing –. Generally, biodiesel has higher cloud and pour point values compared to conventional diesel, which means that biodiesel has poorer qualities at lower temperatures. Better cloud and pour points are shown by alkyl esters from unsaturated oils, which make this biodiesel more suitable for cold climates [60,61,68].
- *flash point*: is the minimum temperature needed to ignite the fuel when exposed to a flame or a spark and varies inversely with the volatility of the fuel. Biodiesel shows a higher flash point, which makes it safer for transportation, handling and storage compared to conventional diesel [61,68].
- biodiesel has better lubrication properties than diesel [68].

Fuel properties depend not only on the unsaturations and chain length of the fatty acids – as set out above – but also on the type of alcohol used to transesterify the oil [61,68,69]. For instance, fatty acid methyl esters are more volatile than fatty acid ethyl esters – known as FAEEs. Actually, the biodiesel standards – DIN 51606, EN 14214 and ASTM D6751 – require or indirectly specify that biodiesel should be fatty acid methyl esters [65].

Currently, conventional fossil fuels present two main problems. On the one hand, there is a global consensus on the depletion of the sources of these fuels [60,61,68]. Since the beginning of the industrial revolution – between the late 18th and early 19th century – energy has

become a main factor for economic growth and since then its consumption has continued to increase. According to the International Energy Agency in its report “Energy and Climate Change” from 2015, energy demand is projected to grow by around 20% in 2030, and in particular the global petroleum demand is expected to be around 9% higher than today, reaching 99 million barrels per day in 2030 [70]. However, this growth will face the depletion of sources of fossil fuels, whose reserves are predicted by the World Energy Forum to run out in less than one century, although there is no exact date forecast [60]. On the other hand, the combustion of fossil fuels is significantly responsible for global warming due to its carbon dioxide emissions – a primary greenhouse gas.

Bearing in mind the properties of biodiesel pointed out in previous paragraphs, biodiesel has become a potential alternative fuel to deal with and solve the depletion and environmental problems caused by conventional fossil fuels. Mainly, fatty acid alkyl esters are a renewable source of energy and do not contribute to global warming when burned, because their use is a closed carbon cycle – carbon from carbon dioxide released after combustion is fixed and converted to biomass by photosynthesis, from which oil is produced again. Furthermore, the utilisation of biodiesel reduces the emission of atmospheric contaminants, such as carbon monoxide, particles in suspension and unburned hydrocarbons [61,62,65].

Atabani *et al*. [60] listed in detail the advantages and disadvantages of biodiesel and its use; they are summarised in Table 5.

Table 5. Advantages and disadvantages of biodiesel

Advantages of biodiesel
The oxygen content of biodiesel (10-11%) makes it a fuel with high combustion characteristics
Its use reduces net carbon dioxide emissions by 78% on a lifecycle basis when compared to conventional diesel
It is renewable, non-toxic, non-flammable, portable, readily available, biodegradable, sustainable eco-friendly and free from sulphur and aromatic content. This makes it an ideal fuel for heavily polluted cities
Its consumption helps rural development to restore unused land and a potential tool for rural employment
It has a similar and higher cetane number compared to conventional diesel
No need for drilling, transportation and/or refining like conventional diesel. Each country is able to produce biodiesel locally
It has better lubricity properties than diesel, improving fuel pumps and injectors performance, which increase engine efficiency
It is safer for transportation, handling and storing due to its higher flash point – 60-80°C for diesel and above 100-170°C for biodiesel
It can stimulate waste valorisation if it is made out of used cooking oils and lards
Its use may not require engine modifications when using blends with conventional diesel up to B20 – blends of biodiesel are numbered with a B and the percentage of biodiesel of the blend . Higher blends need some minor modifications
Disadvantages of biodiesel
Biodiesel has 12% lower energy content, which leads to an increase in fuel consumption
It has higher cloud and pour points, which makes it worse for cold weather conditions than diesel
Its nitrogen oxide emissions are higher
Its lower volatility causes the formation of deposits in engines
Due to its higher viscosity and lower volatility, higher injector pressure is needed
Biodiesel has low oxidation stability and in the presence of air it can be oxidised into fatty acids, which may cause corrosion
More than 95% of biodiesel is made from edible oil; this may cause economic and food supply problems, as food resources and crop fields are used for biodiesel production
Oils needs fatty acids removal before transesterification, which increases cost
Transesterification process has some environmental problems, mainly water disposal and water requirements

1.2.2 Biodiesel market, perspectives and feedstocks

Although the use of vegetable oil as fuel for engines dates back to the 1900s, its exploration started to draw attention in the 1980s [60,65]. Since then, its scientific development and research and its use has continued to grow, often induced by worldwide governmental policies, which have set targets for blending quotas and boosting biofuel technologies stimulated by financial and political support [71]. For instance, in 2008 the European Union proposed the requirement for 10% biofuels by 2020 [65].

The production of biodiesel has risen sharply in the last decade, from approximately 950 in 2000 to nearly 17000 million litres in 2010 with the European Union as the world's major producer, accounting for 53% of global biodiesel production [66]. In 2000 Biodiesel represented around 5% of the world's biofuel production and in 2011 biodiesel share accounted for around 20% of total biofuel production [66]. This increase seems to continue and biodiesel production is estimated to reach 41000 million litres in 2022, as reported by the United Nations [72].

The cost of the feedstock represents the main cost of biodiesel production, accounting for around 75% of the overall cost [60,63]. There are several potential sources of raw materials for biodiesel production and they can be classified as shown in Table 6 [60].

Table 6. Classification and examples of raw materials for biodiesel.

Main feedstocks	**Edible oils**	**Non-edible oils**	**Animal fats**	**Other**
Examples	Soybean	*Jatropha curcas*	Pork lard	Bacteria
	Rapeseed	Cotton seed	Beef tallow	Algae
	Sunflower	Tobacco seed	Fish oil	Microalgae
	Corn	Mahua	Chicken fat	Fungi

Despite the wide range of feedstocks, as shown above in Table 6, more than 95% of the world's biodiesel comes from edible oils, especially from rapeseed and sunflower oil [60]. These raw materials usually depend on regional production; rapeseed oil, for instance, is mainly used in European countries and Canada, soybean oil in the United States and Brazil and palm oil is utilised in Indonesia and Malaysia [65,73]. However, the use of edible oils has

several concerns, especially because it competes for food resources and available harvest lands, and it may also boost deforestation to create more farmlands [60,63].

Non-edible oils, animal fats and waste cooking oils are known as *second generation* biodiesel feedstock and they are a potential solution to reduce the utilisation of edible oils and environmental problems, which may also reduce biodiesel's production cost [60,63,73,74]. Moreover, microalgae have been recently appointed as the *third generation* biodiesel feedstock. Their higher growth rate and oil content compared to edible and non-edible vegetables, makes microalgae a promising feedstock [60].

1.2.3 Biodiesel production processes

Biodiesel can be synthesised by several transesterification methods. These methods can be classified into catalysed and non-catalysed processes. Catalysed process can be further classified, according to the nature of the catalyst, into chemical – *i.e.* alkaline and acid catalysts – and enzymatic biodiesel production. Supercritical methanol is used in non-catalysed transesterification.

1.2.3.1 Chemical catalysed transesterification

Chemical catalysis of fatty acid alkyl esters is based on the nucleophilic attack on the carbonyl group and can be carried out by several basic and acid compounds [75,76]. Currently the most widely-used industrial method is the alkaline – or basic – catalysis [60,61,77,78]. Acid catalysis is barely employed in biodiesel large scale production because it is more corrosive, exhibits slower reaction rates compared to an alkaline process – basic catalysis is around 4000 times faster – and requires high alcohol to oil molar ratios in order to achieve high yields [60,61,75].

Most commercial biodiesel production is obtained by using sodium and potassium hydroxide as catalysts; other basic catalysts, such as sodium and potassium methoxides achieve higher yields but they are costly [60]. In general, alkaline catalysts are widely regarded as a low cost catalyst able to perform transesterification in short times at medium temperatures – 60-80 °C – and to achieve high yields. However, energy consumption, difficult glycerol and catalyst recovery from reaction medium, generation of wastewater effluents and the subsequent

treatment – to reduce the environmental impact – are the disadvantages of the alkaline process. In addition, the presence of free fatty acids in the initial feedstock must be lower than 0.5% in order to prevent the formation of soaps, which leads to a severe reduction of the yield and hinders product recovery [61,76]. This is the case of waste cooking oils and animal fats – second generation feedstocks – which may not be suitable for alkaline catalysed biodiesel synthesis [76].

Acid catalysed transesterification includes catalysts such as sulfuric, hydrochloric, ferric sulphate, phosphoric and organic sulfonic acid [60]. This acid catalysed process is rarely applied in industrial biodiesel production, as mentioned above [61,76]. The main advantage of acid catalysis is that it does not produce saponification when raw materials with high free fatty acid content are used [61]. Therefore, a two-step catalysed transesterification can be employed to obtain biodiesel from high free fatty acids feedstocks, consisting of an acid catalysis – where free fatty acids are converted to esters – before the alkaline catalysed transesterification [79].

1.2.3.2 Enzymatic catalysed transesterification

The use of lipases as biocatalysts in biodiesel synthesis has drawn attention in recent years, as is shown by the increase in literature on this subject [80]. Compared to chemical processes, lipases catalysed transesterification has less energy consumption – it is performed in more gentle conditions –, does not generate waste water effluents and has easier catalyst recovery if lipases are immobilised. Moreover, lipases catalyse perfectly biodiesel production from feedstocks with high contents of free fatty acids – they esterify free fatty acids into esters. In contrast to these advantages, lipases are expensive, display slower rates compared to an alkaline process and are inactivated by alcohols [61,65,81]. Despite the benefits and advantages set out and the extensive scientific research, lipases have not yet been used on an industrial scale for biodiesel production, and only a pilot plant is reported to use lipases [78]. Recently, in 2014, Novozymes launched Eversa® Transform, the first commercial lipase – in liquid formulation – with proven performance to be implemented in scale enzymatic biodiesel process in the world [82].

The application of lipases in the synthesis of biodiesel is discussed more extensively later. The main advantages and disadvantages of chemical and enzymatic catalysed transesterification are summarised in Table 7 [60,61,78].

Table 7. Comparison of chemical – both alkaline and acid – versus enzymatic catalysed transesterification.

	Chemical catalysis		**Enzymatic catalysis**
	Alkaline	**Acid**	
Reaction rate	High	Slower than alkaline	Low
Reaction temperature	60-80°C	>100°C	20-50°C
Catalyst cost	Low	Low	High
Catalyst recovery	Difficult	Difficult	Easy
Glycerol recovery	Difficult (low grade)	Difficult (low grade)	Easy (high grade)
Free fatty acids	Soap formation	Not a difficulty	Not a difficulty
Environmental impact	Wastewater treatment	Wastewater treatment	No wastewater

1.2.3.3 Other transesterification methods

Several acid and basic solid materials can also be used as catalysts to perform biodiesel synthesis; their main advantage is that they can be easily separated and reused [76]. Alkali earth metals, single and mixed metal oxides and ion exchange resins are examples referred to in the literature [76,79,83].

A non-catalysed method to perform transesterification consists of mixing oil with alcohol at supercritical conditions. This process achieves the synthesis of biodiesel in very short times – 2-4 min – and due to the lack of a catalyst, purification of biodiesel and recovery of glycerol are much easier. However, high temperatures and pressures are needed – to set alcohol into supercritical conditions – and high alcohol to oil molar ratios are also needed [60,76].

1.3. Synthesis of biodiesel catalysed by lipases

In recent years the use of lipases to catalyse the formation of fatty acid alkyl esters has been widely explored, as a greener biodiesel synthesis compared to the conventional method, solving some of its drawbacks – as explained above. In this section the type of lipase, reaction system and biocatalyst formulation as well as the main factors affecting the course of the lipase transesterification are examined. In Table 8, several examples show the huge variety of possible combinations in lipase catalysed transesterification.

Table 8. Different examples of lipase catalysed transesterifications found in the literature.

Lipase	Biocatalyst Formulation	Reaction System	Substrates	Reference
Rhizopus oryzae	Whole cell	Solvent-free	Soybean oil/methanol	[84]
Thermomyces lanuginosus	Liquid	Solvent-free	Rapeseed oil/methanol	[85]
Candida antarctica B	Immobilised (Novozym 435)	t-butanol (solvent)	Cotton seed oil/methanol	[86]
Burkholderia cepacia	Covalent immobilisation	Solvent-free	Soybean oil/methanol	[87]
Candida antarctica B	Immobilised (Novozym 435)	Solvent-free	Waste cooking oil/ethanol	[88]
Candida rugosa	Liquid	-	*Jatropha curcas* seed oil/methanol	[89]
Burkholderia cepacia	Encapsulated	Solvent-free	Palm oil/methanol	[90]

1.3.1 Lipases used in biodiesel

Lipases used in biodiesel catalysis are commonly from fungi organisms, often produced by recombinant host microorganisms, the most widespread being lipase B from yeast *C. antarctica* [91]. Both extracellular and intracellular lipases have been studied, although the first are more employed. Intracellular lipases are used within microorganisms containing the enzyme – known as whole-cell biocatalysts – and filamentous fungi, such as *Aspergillus* and

Rhizopus, have been explored for this purpose. Although whole-cell biocatalysts offer a lower cost process compared to extracellular lipases, they have mass transfer limitations [81,91].

Regiospecificity is displayed by lipases towards the three positions of triacylglycerols, as explained above. Theoretically, non-specific lipases are more suitable than 1,3-positional selective lipases in biodiesel production synthesis because they act equally on the three fatty acids moieties to form alkyl esters, thus achieving ideally a 100% final biodiesel yield, while 1,3-positional selective ones would produce a 67% yield and 2-monoacylglycerols as a by-product instead of glycerol [11,78]. However, it should be stated that monoacylglycerols are a by-product of great interest – especially as emulsifiers –, with several applications in the food and pharmaceutical industries and in synthetic organic chemistry, where they are used as intermediates and as chiral building blocks [92,93]. Moreover, recently it has been reported that the presence of monoacylglycerols and/or free fatty acids in final biodiesel enhances its lubricity [94,95].

Furthermore, glycerol presents two problems regarding biodiesel production and its use. On the one hand, the market is already virtually flooded by glycerol production and it is forecast that its increasing generation may become an environmental problem [96]. On the other hand, glycerol presence in biodiesel causes various technical problems when used in engines, including coking, increase in viscosity and polymerisation, reducing the efficiency of the engine; actually, EN 14214 states that traces of glycerol may be removed up to 0.02% [94]. In addition, the removal of glycerol of final biodiesel involves energy consumption and waste water generation [94]. Moreover, glycerol causes activity loss in immobilised lipases, as it is further explained later.

Therefore, although the use of 1,3-positional selective lipases leads to a reduction in alkyl esters yield, their application should be considered as it produces monoacylglycerols instead of glycerol, avoiding its drawbacks.

Despite their regiospecificity, there are several examples of pronounced 1,3-positional specific lipases that synthesise alkyl esters with yields over 67% – *R. oryzae* [97] and *T. lanuginosus* [98], among others. This higher yield is related to the acyl migration phenomenon, which promotes the conversion of 1,2- to 1,3-diacylglycerols and 2- to 1(3)-monoacylglycerols, allowing regiospecific lipases to act on these substrates. This phenomenon is resulted from the spontaneous movement of acyl groups in an acylglycerol molecule – in this case fatty acids in a glycerol skeleton – from hydroxyl group in position sn-2 to the adjacent one position sn-

1(3), and is affected by temperature and acyl chain length [99,100]. Acyl migration is also dependent on solvent properties, decreasing its rate with the increasing of polarity [101].

As previously explained, regiospecificity regarding the type of acylglycerol – i.e mono- di- or triacylglycerol – is also exhibited by lipases. For example, the slowest alcoholysis step catalysed by Novozym 435 – *i.e. C. antarctica* lipase – is the conversion of diacylglycerol to an alkyl ester and a monoacylglycerol, while the fastest step is the subsequent alcoholysis, from monoacylglycerol to another fatty acid alkyl ester and glycerol [91].

Thus, it can be concluded that regiospecificity of lipases is a crucial parameter in the progress of biodiesel synthesis; lipase choice can be fitted to the different variables in biodiesel production, such as the characteristics of the feedstock or the desired by-product. For instance, combinations of different regiospecific lipases, such as 1,3-selective Lipozyme TL IM – *i.e. T. lanuginosus* – with less regiospecific Novozym 435 lipase have been successfully employed [102].

1.3.2 Reaction system

The reaction system or environment within which a reaction is carried out has a crucial role in the reaction progress and it should fit the properties and nature of substrates and products. In particular, biodiesel synthesis involves different compounds that differ greatly in their properties, especially with regard to their polarity. Oils and fats, diacylglycerols, fatty acid alkyl esters, as well as free fatty acids are non-polar species, while the alcohol used as acyl acceptor – especially methanol –, glycerol and water – if it is present in the system – are polar compounds; the polarity of monoacylglycerols is between non-polar and polar species mentioned. Furthermore, the reaction environment must be suitable to keep lipase activity.

The two main systems utilised in enzymatic transesterifications involve organic media. Different compounds are diluted in an organic solvent or alternatively, in solvent-free reactions, where the substrates act as organic solvent themselves – mainly fats or oils, because their molar volume are much bigger than alcohol ones, so in a solvent-free system oil is the major compound in volume –. The application of organic solvents protect lipases from denaturation by alcohols and increase the reaction rates because the viscosity of reaction mixtures is reduced; by contrast, solvent-free systems offer environmental and economic advantages – no solvent recovery is necessary –, but have mass transfer limitations due to oil

viscosity and low solubilisation of alcohols in oil – which may lead to enzyme inactivation, as it is further extensively discussed – [61,78,91,103].

A common feature of these two reaction systems is that their content of water is low [11]. Although water can also act as substrate, a minimum amount of water is necessary to keep the catalytic conformation of enzymes – it is also referred to as structural water or water layer coating the enzyme – [103–105]. However, there are large differences among enzymes concerning how their catalytic activities are influenced by water activity; maximal activity of *B. cepacia* lipase, for instance, is shown at high water activity, while *C. antarctica* lipase B presents an optimum activity at low water activity [11].

This essential water layer coating the lipase is strongly influenced by the hydrophobicity of the solvent used, since more hydrophilic organic solvents interact and remove this water. The hydrophobicity of a solvent is a parameter defined by the logarithm of the partition coefficient of the solvent between 1-octanol and water – usually referred to as logP. In general, the enzyme activity and stability is low in hydrophilic solvents – logP < 2 –, moderate in solvents with logP 2-4 and higher in more hydrophobic environments – logP > 4. Hydrophobic organic solvents such as isooctane, n-heptane, n-hexane, petroleum ether and cyclohexane are widely used in lipase transesterifications. However, tert-butanol is also utilised, since its moderate polarity or hydrophilicity – logP = 0.79 – improves the solubility of short chain alcohols and glycerol – which may also affect the lipase negatively, as later discussed – [81,91].

Moreover, both organic solvent and free-solvent systems may become biphasic systems if water is added to the systems, creating a non-polar or non-aqueous phase and an aqueous phase. Thus, the most hydrophilic species, such as alcohols, glycerol and to a lesser extent monoacylglycerols, split between the two phases.

1.3.3 Biocatalyst formulation

Lipases, as well as other enzymes, can be used as free or immobilised formulations or preparations. Free lipase formulations, known also as liquid-lipase, consist of soluble enzymes in buffers – sometimes contain stabilizers to avoid enzyme denaturation [91]. Thus, its application converts both free-solvent and organic solvent systems into biphasic systems, with one non-polar and an aqueous phase. The appearance of this second phase leads to distribution of some compounds between the two phases, especially alcohols and glycerol.

Free lipase formulations are low cost biocatalyst preparations, but they are barely employed in biodiesel synthesis, because immobilised lipases show several better advantages – as previously set out –, such as easier biocatalyst recovery and reutilisation, and increase of enzyme stability due to higher structural rigidity [91]. However, the use of soluble formulations is significantly cheaper because it avoids the additional cost of immobilisation. Studies of biodiesel synthesis using soluble *C. rugosa* and *T. lanuginosus* lipases, for instance, have been reported [89,106,107].

Lipases immobilised by adsorption are one of the most commonly used formulations and a wide variety of support materials have been utilised, such as silica gels, macro- and microporous resins, acrylic resins and diatomaceous earths, among others. However, the major drawback of adsorption is that lipases may be stripped off the support, due to weak adhesion forces between the enzyme and support [108]. *C. antarctica* lipase immobilised on acrylic resin – known by its commercial name Novozym 435 – is widely used in biodiesel synthesis.

Covalently attached lipases are also employed in the synthesis of fatty acid alkyl esters, with several examples with a wide range of lipases in literature. For instance, *B. cepacia* lipase was covalently immobilised on polyacrylonitrile nanofibers and ROL on a glutaraldehyde-treated polymethacrylate amoni-epoxide carrier, respectively [87,109].

Other immobilisation techniques have been also explored in biodiesel synthesis. For example, *B. cepacia* lipase is reported to have been used cross-linked and encapsulated in transesterification studies, respectively [90,110].

1.3.4 Alcohol inactivation, glycerol inhibition and water content

A major issue explored in the literature is the detrimental effect on lipases activity caused by alcohols, which reduce the lifetime of lipases in transesterifications [61,108]. Although ethanol and methanol are widely recognised to have stronger negative effects on lipases comparatively to other alcohols, they are the most common alcohols used as acyl acceptors in biodiesel synthesis, especially methanol, because of its economic feasibility and accessibility [61,103,108,111,112] and also because alkyl esters from methanol are more volatile than from ethanol [61]. However, methanol has a more negative impact on lipases than ethanol [112].

Despite the literature available, the origin of the negative effect of methanol is not yet properly understood, as one can guess from the non-specific terms used to describe it, such as inactivation, deactivation, inhibition or denaturation [103]. Some works have pointed out that proteins are inactivated by insoluble short chain alcohols, such as methanol and ethanol – poorly soluble in oil –, which interact with the essential water molecules – *i.e.* structural water or water layer – necessary for the maintenance of the active structure of the enzyme [91]. It was also suggested that high concentrations of alcohols may induce alterations of the intra-protein hydrophobic interactions, leading to unfolding of the enzyme followed by irreversible deactivation [103]. Lipase deactivation was concluded to be inversely proportional to the number of carbon atoms on linear lower alcohols, and lower in the case of branched alcohols than in linear alcohols [113]. Nevertheless, some lipases are more tolerant to methanol than others, such as those from *Pseudomonas* genus, while *C. rugosa* lipase, for instance, appears to be more methanol sensitive [103].

Several methodologies have been proposed and employed to overcome methanol and ethanol negative effects. Stepwise addition or feeding strategy consists of splitting the total stoichiometric amount of alcohol into little amounts that are added to the reaction at specific times. This strategy prevents the formation of insoluble alcohol within the reaction medium and it has been widely utilised in transesterification studies [61,91,103,114]. The replacement of methanol or ethanol by less harmful acyl acceptors or the use of solvents has also been explored [61,115].

Loss of lipase activity is also reported, caused by glycerol, after it is produced as a by-product in transesterification [61,91,108]. The low solubility of glycerol in hydrophobic organic solvent and solvent-free systems induces its adsorption on lipase immobilisation carriers and forms a hydrophilic environment, which reduces hydrophobic substrates accessibility to lipases [116]. Thus, the glycerol negative effect is attributed to mass transfer limitations in immobilised enzymes, not to enzyme inhibition in the strict sense [78]. Lipases that exhibit 1,3-positional selectivity may represent an advantage, because no glycerol is formed.

Apart from its essential role in the catalytic conformation of lipases – as explained above –, water can play other roles in enzymatic transesterifications. Water presence converts the system into a biphasic system, whose interface has generally an important impact on lipase activity due to its interfacial activation – as previously mentioned. In contrast, an excess of water may lead to an increase in triacylglycerols hydrolysis and as a consequence, free fatty acids content in the final product [91,108].

1.3.5 Kinetics of transesterification

The mechanism of lipase catalysed transesterifications is generally represented by Ping-Pong Bi Bi models (Figure 9) [103]. In particular, there are two viewpoints of the mechanism but not with a general consensus between them. Some authors explain transesterification as a direct alcoholysis of the fatty acids moieties in the glycerol backbone [88,117,118], while transesterification is understood by others as a two-step reaction, which consists of a first acylglycerols hydrolysis and the subsequent esterification of the liberated free fatty acids [97,119,120].

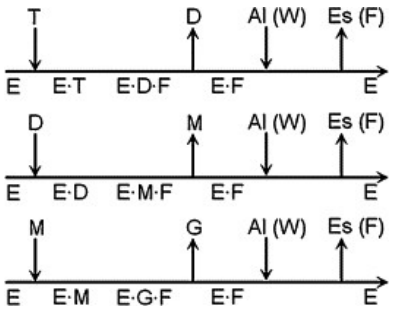

Figure 9. Schematic diagram of Ping Pong Bi Bi mechanism for transesterification and hydrolysis of acylglycerols. E: enzyme, T: triacylglycerol, D: diacylglycerol, Al: alcohol, W: water, Es: alkyl fatty acid ester, F: free fatty acid, M: monoacylglycerol and G: glycerol. E·T, E·D·F, E·F, E·D, E·M·F, E·M and E·G·F stand for the different enzyme complexes, respectively. Reproduced from [121], with permission of Elsevier.

2. *Rhizopus oryzae* lipase. State of art

As previously explained, the work of the present thesis has been focused on the use of rROL, expressed recombinantly in *P. pastoris* by the same research group.

2.1 Rhizopus oryzae lipase

R.oryzae is a filamentous and lipolytic fungus [122] and one of the three groups in which *Rhizopus* species are mainly divided, including *R. microsporus* and *R. stolonifer* [123]. Several *Rhizopus* species – *R. arrhizus*, *R. delemar*, *R. javanicus*, *R. niveuas* and *R. oryzae* – were latterly recognised to be the same organism and renamed as *R. oryzae*, in spite of minor variations; for instance, ROL differs by only two substitutions in the amino acid sequence from *R. delemar*, *javanicus* and *niveaus* lipases – His134 is asparagine and Ile234 is leucine in ROL – [123,124].

R. oryzae only produces extracellularly one form of lipase which, as well as other *Rhizopus* lipases and the closely related *R. mihei* lipase, has a high 1,3-regiospecificity towards acylglycerols [122,124]. Its structure is represented in Figure 10.

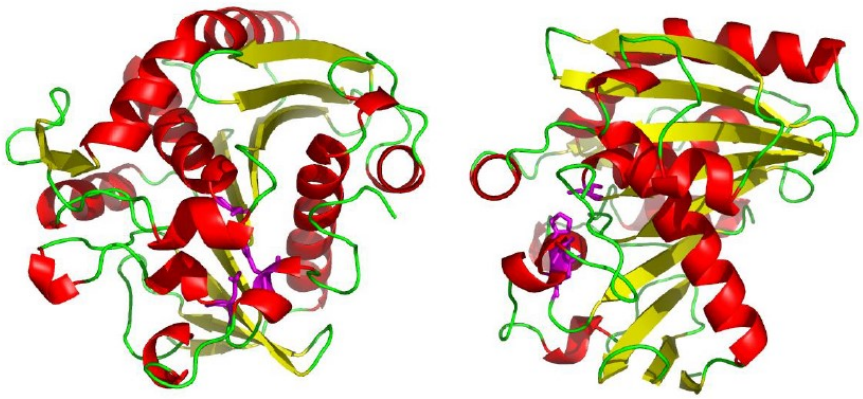

Figure 10. Structure of the *Rhizopus oryzae* lipase. The amino acids of the catalytic triad are coloured in purple [125].

The molecular weight of the lipase was determined to be 32 kDa and the isoelectric point is 6.85. It possesses four potential sites of N-linked glycosylation and three disulphide bonds, between amino acids 152 and 391, 163 and 166, and 358 and 367 [122].

The precursor amino acid sequence of the native ROL consists of 392 amino acids residues. The first 26 residues correspond to a signal sequence – pre-region –, which promotes the direct membrane translocation of the protein; the next 97 belong to a pro-region, whose function is to decrease the toxicity of the lipase itself when it is still inside the cell and to support the folding of the lipase; the last 269 correspond to the mature sequence of the lipase [122,126]. However, the secreted form of the lipase was reported to be made up of 297 residues, 269 of which correspond to the mature lipase and the remaining 28 to the last part of the pro-region [122]. This remaining part was found to have an important role in the specific activity, regio- and stereoselectivity [127].

ROL is widely employed as a biocatalyst in several different reactions. For instance, it has been explored in the synthesis of lysophosphatidic acid and phosphatidic acid by acylation of glycerophosphate [128], in the synthesis of diacylglycerols by hydrolysis of soybean oil [129] and in the enantioselective hydrolysis of α-acetoxy aryl alkyl ketones [130]. It is also broadly used in enzymatic synthesis of biodiesel, as several published works in the literature can be found.

2.2 Recombinant expression of *Rhizopus oryzae* lipase

The lipase of *R. oryzae* has been cloned and expressed in heterologous host systems, such as *E. coli*, *Saccharomyces cerevisiae* and *P. pastoris* [124,126,131]. The use of *E. coli* as expression system for ROL generally leads to the formation of inclusion bodies and inactive protein, which involves later purification and refolding processes [124,126,132] – however, it was reported that soluble ROL was expressed, using *E. coli* Origami as host system [132]. These problems related to the employment of *E. coli* as expression system are overcome when *S. cerevisiae* is used instead, expressing successfully extracellular active ROL [131].

Regarding the expression of ROL in *P. pastoris*, the mature sequence of the lipase was cloned under the promoter of alcohol oxidase – P_{AOX} – and in the frame of the α-factor prepro signal sequence from *S. cerevisiae* to target the protein to the secretory pathway – *i.e.* to express the lipase extracellularly – [124,133].

This expression system has been widely studied within the research group where this present thesis has been developed and used to produce the lipase which this thesis is based on [134–137]. ROL is obtained by *P. pastoris* cultivations and concentrated and purified as described elsewhere [122].

2.3 Characterization and application of recombinant *Rhizopus oryzae* lipase

rROL produced in the research group was characterised and compared to a native one. Firstly, two bands of around 34 kDa were detected for the recombinant lipase. The differences of the two bands were attributed to post-translational modifications, after an N-terminal analysis revealed that both bands have the same amino acid sequence [122].

Furthermore, the molecular weight of the recombinant lipase was determined to be slightly lower than the commercial one. This was attributed to the presence of a pre-pro-sequence in the mature commercial lipase – as previously described –, while the recombinant one was determined to have only 4 amino acids attached to the mature enzyme, by n-terminal analysis: the first two amino acids belong to the final sequence of the α-factor from *S. cerevisiae* and the next two to the restriction site where the ROL gene was cloned in pPICZα [122,124].

Moreover, the specific activity of the recombinant lipase was concluded to be more than 40 times higher and less affected by ionic strength than the native one [122]. The specificity towards triacylglycerols was similar for both lipases but completely different towards *p*-nitrophenol esters; native lipase hydrolysed preferably short carbon esters, while the recombinant one exhibited a preference towards longer chains [122]. This difference between both lipases may be attributed to the difference in amino acid sequences between both lipases described in the previous paragraph, and also to post-translational modifications of *P. pastoris* and the presence of an esterase in the commercial ROL powder, as reported by Guillen *et al.* [122].

Various synthesis applications have been carried out using rROL produced in the research group as a biocatalyst – apart from biodiesel synthesis, which is the aim of the present thesis. The lipase was employed in the synthesis of ethyl butyrate and acetylation of cortexolone in two different studies, respectively [138,139]. The 1,3-regiospecificity exhibited by this lipase has been also explored in the synthesis of structured lipids; acyl positions sn-1 and sn-3 of olive oil were substituted with caprylic and capric acids – C8 and C10 carbon chains – in order to

obtain MLM triacylglycerols – *i.e.* triacylglycerols containing medium-chain fatty acids (M) at sn-1 and sn-3 positions and a long-chain fatty acid (L) at position sn-2 – [140,141]. Also, human milk fat substitutes were also synthesised via acydolysis of tripalmitin with oleic acid catalysed by rROL [142].

3. Results and Discussion

This section provides a brief overview of the most illustrative results obtained during the thesis and their discussion. A more detailed description and discussion of the results can be found in the published and submitted journal articles, at the end of this booklet.

3.1 Effect of water and free fatty acids on lipase inactivation by methanol

Part of the experimental work of the thesis was aimed at the study of methanol's detrimental effect on rROL and its reduction. This work was performed by transesterifications of olive oil in free-solvent media, with the lipase immobilised by adsorption. It must be stated that, although olive oil is edible and also a too expensive substrate to be converted into biodiesel, it was chosen as substrate; olive oil is nearly all made up of oleic acid, which makes analytical quantifications easier because there is a smaller variety of fatty acids and their respective esters.

For this purpose, an experimental design was done in order to build a response surface model based on three factors or variables: methanol to oil molar ratio – within range 3:1-6:1 –, water amount – within range 0-10% based on oil weight – and the number of fractions in which methanol is added – methanol stepwise addition within range 1-7 additions. This methodology allowed the effect of these three variables and the relationships between them on the final biodiesel yield to be explored.

The model obtained has an adjustment of r^2 0.9593 and the molar ratio variable was statistically determined to have little influence on biodiesel yield over the range studied. The model suggests the presence of water reduces the inactivation effect of methanol on the lipase, allowing the addition of methanol in less fractioned stepwise addition strategy whereas if no or little water is in the system, methanol must be added to the reaction in more additions. Therefore, water content is strongly related to enzyme inactivation by methanol.

Methanol is not completely soluble in oil and forms drops within the reaction; due to its polarity, this non-soluble methanol is able to interact and remove structural water – also referred to as essential water layer –, necessary to keep the active form of the lipase, causing lipase inactivation. When water is added to the reaction medium, an aqueous phase is formed,

which solubilises the non-soluble methanol of the oil phase and thus reduces its negative effect. However, water promotes hydrolysis of triacylglycerols.

Another parameter that was shown to reduce methanol inactivation was the presence of free fatty acids in the initial substrate. A series of mixtures of olive oil and free oleic acid – within the range of 0-20% of free oleic acid based on olive oil weight – were transesterified, and the biocatalyst was recovered at the end of the reactions and reused in repeated reactions. The results showed that both reaction rate and biocatalyst stability improved when the content of oleic acid increased from 0 to 20%. The reaction rate, for instance, was nearly doubled when 10% of free oleic acid was employed, compared to the experiments using no free oleic acid. On the other and, the biocatalyst kept around 90% of its activity after 10 reaction cycles when 10% of free oleic acid was used, while in absence of acid there was an 80% of activity lost after 10 cycles.

The improvement of the initial reaction rate was attributed to the reduction of the viscosity of the reaction medium due to the presence of oleic acid – free fatty acids are less viscous than oils –. Thus, the reaction rate increased as a consequence of lower mass transfer limitations. However, the improvement of the activity stability of the biocatalyst was not attributed to a reduction of viscosity, but to polarity increase in the reaction medium. Adding free fatty acids to the medium increases its polarity and therefore methanol becomes more soluble in it, reducing the presence of drops of non-soluble methanol capable of producing lipase inactivation, as explained previously.

3.2 *Rhizopus oryzae* lipase specificities

Different experiments and their results have been used to explore rROL specificity. In particular, the specificity towards acyl acceptors – methanol versus ethanol –, towards the different fatty acids present in oils and towards different acylglycerols – 1-, 2-monoolein and triolein – was evaluated.

Specificity towards ethanol and methanol as acyl acceptors in transesterification were examined comparing methanolysis of ethyl oleate versus ethanolysis of methyl oleate. Higher initial reaction rates were obtained in ethanolysis, so rROL is more specific towards ethanol.

Lipases display different chemospecificity towards fatty acids regarding their carbon chain length and the presence of unsaturations, as explained in the introduction. Palmitic, stearic, oleic, linoleic and linolenic acid are the most frequent fatty acids in triacylglycerols of olive oil – as well as the majority of vegetable oils –; from various experiments, the esters of these 5 fatty acids were quantified and plotted individually versus reaction-time. Since the relative proportions of all fatty acid methyl esters were constant throughout reaction-time, it can be stated that rROL has the same specificity towards these 5 different fatty acids.

The 1,3-positional selectivity of rROL was also studied, carrying out the alcoholysis of 1- and 2-monoolein, respectively. In the case of 2-monoolein, all biodiesel synthesised was demonstrated to not come directly from 2-monoolein, but from 1-monoolein produced from the acyl migration of 2-monoolein. These results show that rROL exhibits a high 1,3-positional selectivity.

1-monoolein was also compared to triolein in order to study lipase acylglycerol specificity, concluding that rROL was more specific towards 1-monoolein than triolein. 1-monoolein was consumed around 4.5 times faster than triolein by rROL in transesterification. Furthermore, transesterifications of triolein were carried out with stoichiometric limitation amounts of alcohol. Once the alcohol was almost finished, the newly formed 1-monoolein from 2-monoolein by the acyl migration phenomenon were rapidly alcoholised to biodiesel and glycerol. Since no more alcohol was present in the reaction medium, backwards transesterification occurred, combining dioleins and alkyl esters, forming triolein and alcohol, which was used in the alcoholysis reaction of 1-monoolein. This regeneration of triolein as a consequence of alcoholysis of 1-monoolein also shows the specificity of rROL towards 1-monoolein compared to triolein.

3.3 Comparison between recombinant and commercial *Rhizopus oryzae* lipase

The specificity of the recombinant lipase was compared to a commercial one, evaluating the consumption rates of 1-monoolein and triolein of both lipases. The commercial lipase was 2.8 times faster for 1-monoolein compared to triolein, slightly lower than recombinant lipase – 4.5 times, as explained previously –. This difference may be attributed to differences in post-translational modifications of the producing host *P. pastoris* strain, the presence of an esterase in the commercial ROL powder and also that rROL lacks the pre-pro-sequence of the mature

commercial one, as set out in the previous section *2.3 Characterization and application of recombinant Rhizopus oryzae lipase*.

3.4 Interfacial activation of *Rhizopus oryzae* lipase

Unlike other enzymes, lipases perform their catalytic activity in organic-water interphases, a phenomenon known as interfacial activation, as extensively explained in the introduction. This feature was explored in the case of rROL. Three transesterifications experiments combining different biocatalyst preparations and reactions systems were carried out: soluble lipase in an organic-water system, immobilised lipase in an organic system – the immobilised form is necessary to maintain lipase activity in no-water system – and immobilised lipase in an organic-water system. Around 20-fold higher reaction rates were achieved when catalysis was performed by immobilised lipase in an organic monophasic system compared to the other two biphasic systems. This suggested that no interfacial activation is necessary for rROL to exhibit high catalytic activity.

3.5 Transesterification reaction pathway

Although free fatty acids were shown to prevent lipase inactivation by methanol, their concentration remained constant in the experiments described above in section *3.1 Effect of water and free fatty acids on lipase inactivation by methanol*, and apparently it seemed that lipase did not catalyse their esterification with methanol to biodiesel. For this reason, experimental work was carried out to elucidate their role in biodiesel synthesis reaction. As explained in the introduction, in the literature two viewpoints of the reaction pathway of transesterification exist: one-step reaction assumes that the synthesis of biodiesel takes places by direct alcoholysis of triacylglycerols, while the two-step reaction assumption states that triacylglycerols are first hydrolysed and the subsequent liberated fatty acids esterified to biodiesel.

Since it is not possible to trace the origin of a methyl oleate – from a free oleic acid molecule or from an oleate bound in a triacylglycerol –, an experiment transesterifying a mixture of triolein and 20 % free linoleic acid – base on triolein weight – was carried out. Total free fatty acid concentration remained constant, although when free fatty acids were specifically

analysed, it was discovered that free linoleic acid decreased at approximately the same rate as free oleic acid increased. This phenomenon demonstrated that the so-called two-step reaction exists, since water released when free linoleic acid was esterified by methanol was used by the lipase to hydrolyse triolein and produce free oleic acid. If two-step is the reaction pathway, it should be expected that if less free linoleic acid is used, a lower methyl oleate rate should be achieved. A second experiment was carried out using 10% of free linoleic acid and it was unexpectedly detected that methyl oleate was formed at a faster rate. Therefore, the transesterification reaction also involves the direct one-step alcoholysis of triacylglycerols.

4. Conclusions

This section provides a brief overview of the most illustrative conclusions of the thesis. More detailed conclusions can be found in the published and submitted journal articles, found at the end of this booklet.

Lipase inactivation by methanol is caused when non-soluble methanol within organic phase interacts and removes structural water molecules of the lipase.

Methanol's detrimental effect can be avoided if methanol is added in stepwise addition. The more water added to the reaction medium, the less methanol fractioned stepwise addition is required to prevent lipase inactivation. However, the presence of water may lead to non-desired hydrolysis of triacylglycerols.

Contrary to conventional alkaline biodiesel catalysis, lipases perform transesterification of substrates containing initially free fatty acids. Moreover, polarity of free fatty acids improves methanol solubilisation in the reaction medium, reducing lipase inactivation.

rROL is more specific towards ethanol than methanol as acyl-acceptor.

rROL displays the same specificity towards palmitic, stearic, oleic, linoleic and linolenic acids, which are the most frequent fatty acids found in vegetable oils.

About 4.5 times higher specificity towards 1-monoolein than triolein is exhibited by rROL. Moreover, rROL expressed negligible activity towards 2-monoolein.

Commercial ROL also exhibits higher specificity towards 1-monoolein than triolein, but less pronounced than the recombinant one. This difference might be attributed to differences in post-translational modifications of the producing rROL host *P. pastoris* strain, the presence of an esterase in the commercial ROL powder and also to the presence of a pre-pro-amino acid sequence in the mature commercial ROL, which is lacking in the recombinant one.

Biodiesel synthesis or transesterification reaction was demonstrated to be a combination of two different pathways: one-step reaction, consisting of a direct alcoholysis of triacylglycerols, and a two-step reaction, which includes a first hydrolysis of triacylglycerols and the subsequent esterification of the released fatty acids.

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Contributions

Participation in the following published works was also done

Enzymatic biodiesel synthesis from yeast oil using immobilized recombinant *Rhizopus oryzae* lipase. Susan Hartwig Duarte, Gonzalo Lázaro del Peso Hernández, Albert Canet, Maria Dolors Benaiges, Francisco Maugeri, Francisco Valero. Bioresource Technology, 2015, 183:175-180.

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Appendix

Publication I. Albert Canet, M. Dolors Benaiges, Francisco Valero. Biodiesel Synthesis in a Solvent-Free System by Recombinant *Rhizopus oryzae* Lipase. Study of the Catalytic Reaction Progress. Journal of the American Oil Chemists's Society, 2014, 91:1499-1506.

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ORIGINAL PAPER

Biodiesel Synthesis in a Solvent-Free System by Recombinant *Rhizopus oryzae* Lipase. Study of the Catalytic Reaction Progress

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Abstract The recombinant 1,3-positional selective *Rhizopus oryzae* lipase (rROL) was used to synthesize biodiesel and monoacylglycerols simultaneously. The reaction was carried out in a solvent-free system with the enzyme immobilized on octadecyl-Sepabeads. Using response surface methodology, the methyl ester yield was optimized by means of the study of the effect of water, substrate molar ratio (methanol:olive oil) and methanol stepwise addition. It was concluded that in order to prevent enzyme inactivation by methanol, alcohol should be added slowly; otherwise a large amount of water would be present. Taking the best conditions, a 50.3 % yield was achieved in 3 h, which corresponds to 75.4 % of the acyl groups at the 1,3-position undergoing transesterification. It was also concluded that methyl esters result from the esterification of the free fatty acid hydrolyzed by the enzyme and also from a direct transesterification of oil. In addition, the fatty acid selectivity of rROL was found not to favor one fatty acid in olive oil over another.

Keywords Immobilization · Biodiesel · Monoacylglycerols · Recombinant *Rhizopus oryzae* lipase · Response surface model

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Introduction

Recently biodiesel (monoalkyl esters of long-chain fatty acids) has shown itself to be an alternative diesel fuel because of its favorable properties, environmental benefits and the fact that it is derived from renewable biological resources [1]. Currently, enzymatic production of biodiesel represents an environmentally more attractive option to the conventional process. The employment of lipases as biocatalysts allows mild reaction conditions and the use of waste oils [2–4]. In addition, the immobilization of lipase on a support combines these advantages with those of the heterogeneous catalysis, like catalyst recovery and reuse [5]. New immobilized biocatalysts are needed to achieve higher enzyme activity and enhance enzyme stability and thus, reduce the cost of biodiesel production [6]. Moreover, the use of solvent-free systems has important benefits compared to organic solvent ones in terms of cost reduction and environmental benefits [1].

On the other hand, another important problem is that the glycerol produced tends to adsorb onto enzyme immobilization carriers, causing a partial loss of the activity of the lipase [4, 7]. In addition, high levels of glycerol in the fuel cause various problems in diesel motors [8]. Therefore, glycerol traces must be removed before the final biodiesel use.

Recent investigations have shown that minor biodiesel compounds, including free fatty acids and monoacylglycerols, are responsible for the lubricity of low-level blends of biodiesel with conventional diesel, since pure methyl esters exhibit a reduced lubricity power when compared to that of biodiesel containing these compounds. Thus, the simultaneous synthesis of biodiesel and monoacylglycerols could be a solution [8]. No glycerol is produced as a by-product and the monoacylglycerols obtained can serve as

emulsifiers in the food, pharmaceutical and cosmetic industries [9].

In this study, it was proposed to use the 1,3-positional selective recombinant *Rhizopus oryzae* lipase (rROL) to synthesize biodiesel and monoacylglycerols simultaneously from olive oil, to avoid the generation of glycerol as co-product. The recombinant ROL used is a promising new biocatalyst for biodiesel production that showed a 44-fold higher specific activity compared to a commercially available lipase obtained directly from *R. oryzae*, and a higher specificity towards the *p*-nitrophenol ester of long chain length [10].

rROL was immobilized by adsorption, because among immobilization techniques, it is simple and allows retention of high catalytic activity for lipases [11]. Among different supports, octadecyl-Sepabeads were chosen because this support revealed a high stability of rROL in a previous study [12]. Immobilization on this support is by hydrophobic interaction and it has been reported to permit hyper-activation and stabilization of lipases [13]. Methyl ester yield was optimized by means of a response surface methodology (RSM) studying the effect of water, substrate molar ratio (methanol:oil) and programming used for methanol stepwise addition.

Materials and Methods

Materials

Methanol and heptane were purchased from Panreac (Barcelona, Spain) (both of high grade) and the olive oil virgin type was a domestic use one. The lipase colorimetric kit used for the activity assay was obtained from Roche (Roche kit 11821792, Mannheim, Germany). Octadecyl-Sepabeads (Resindion rsl, Binasco, Italy) were a gift from Professor J.M. Palomo from the Instituto de Catálisis y Petroleoquímica (CSIC, Madrid). Standards of methyl palmitate, methyl stearate, methyl oleate, methyl linoleate, methyl linolenate and oleic acid were purchased from Sigma-Aldrich (St Louis, USA).

Lipases

Recombinant *R. oryzae* lipase was produced by the Bioprocess Engineering and Applied Biocatalysis group of the Universitat Autònoma de Barcelona (UAB). This lipase was obtained by a mixed substrate fed-batch cultivation of a recombinant *Pichia pastoris* Muts strain using methanol as the inductor at a set-point of 2 g/l and sorbitol as cosubstrate at a constant specific growth rate of 0.01 h^{-1} [14]. The culture broth was centrifuged and micro-filtered to remove the biomass. The supernatant was concentrated by ultrafiltration with a Centrasette® Pall Filtron system (New York, USA) equipped with an Omega membrane with a 10-kDa cut-off, and subsequently dialyzed against 10 mM Tris-HCl buffer pH 7.5 and thereafter lyophilized [10].

Immobilization on Sepabeads

The support Sepabeads were pre-treated by incubation in 80 ml of an acetone–water solution (v/v 50 %) for 30 min, followed by solution removal by vacuum filtration. After that, 30 ml of a solution of 5 mM phosphate buffer at pH 7 containing an approximate rROL activity of 10,000 UA/ml was prepared, dissolving lyophilized lipase under magnetic stirring for 1 h at 4 °C. The solution was then centrifuged for 20 min at 12,000 rpm (98 mm radius) and then the supernatant was mixed with 1,500 mg of pretreated Sepabeads (containing about 60 % of moisture) in a roller MoviROL (P-selecta, Abrera, Spain) for 6–7 h at 4 °C. After a contacting time of 6–7 h, the solution was removed by vacuum filtration. Immobilized biocatalyst was then dried with silica gel, at room temperature, in a desorber until its weight reached a constant value (between 1 and 3 days). Finally, the support with the immobilized lipase was stored at −20 °C.

The activity and protein amount in the final biocatalyst were about 170 UA/mg biocatalyst and 13.5 mg protein/g biocatalyst, respectively, where one unit of lipase activity (UA) was defined as the amount of lipase necessary to hydrolyze 1 μmol of ester bond per minute under assay conditions. These values were calculated as the difference between the activity and the protein concentration in the initial and final supernatant, divided by the weight of support.

Lipase Activity and Protein Concentration

The lipase activity assay was carried out using a Cary Varian 300 spectrophotometer (Varian, Palo Alto, USA) at 30 °C in 200 mM Tris-HCl buffer at pH 7.25 using the Roche lipase colorimetric kit, as previously mentioned [15]. Protein concentration was determined by the method of Bradford using bovine albumin as the standard [16]. Samples were analyzed in duplicate.

Fatty Acid Methyl Esters and Oleic Acid Analysis

Methyl esters and oleic acid sample concentration were analyzed with a 7890A gas chromatography (Agilent Technologies, Santa Clara, USA) equipped with a capillary column 1909BD-113 (30 m × 0.32 mm × 0.25 μm, Agilent Technologies) and an auto-sampler. The column temperature was held at 190 °C for 9 min, raised to 250 °C

at 20 °C/min and kept at this temperature for 13 min. The temperature of the injector and the flame ionization detector were 250 and 300 °C, respectively. Helium was used as the carrier gas at a constant flow of 0.9 ml/min.

Enzymatic Reaction

All reactions were done in 1.5-ml HPLC vials inside an incubator (IKA KS 400 ic, Satufen, Germany) at 30 °C and under continuous stirring at 200 rpm (20 mm orbital diameter). The reaction mixture contained 1 g of olive oil and 40 mg of immobilized biocatalyst, and the reactions were carried out for 3 h. The amount of water added to the reaction media, the substrate molar ratio (methanol:olive oil) used and the number of the stepwise methanol addition was performed according to the experimental design presented in Table 1. All reactions were done in duplicate.

Sampling

To determine the amounts of methyl esters and free oleic acid by gas chromatography, samples (approximate 150 µl) of the enzymatic reactions were withdrawn at predetermined time intervals of 13 min. One 1.5-ml HPLC vial was used for each experimental point. First, the samples were filtered with a 0.45-µm PVDF filter (Millipore, Billerica, USA) to remove immobilized lipase in order to stop the reaction, then they were centrifuged at 10,000 rpm for 3 min. After that, 10 µl of the organic layer (if water is used in the reaction, a small polar layer does appear after centrifugation) were taken with a micropipette, weighed in an analytical balance and diluted with heptane.

Experimental and Statistical Design

A five-level, three-factor central composite design study was used to determine the best conditions to carry out the enzymatic reaction. This experimental design required eight factorial points, six axial points and nine central points. The substrate molar ratio (3:1–6:1 methanol:olive oil) and the water amount (0–10 % based on oil weight) were two of the studied variables, which are the most commonly used to study transesterification reactions [1, 17]. Due to ROL being 1,3-positional selective, all the experiments were done with excess methanol. It is widely reported that an excess of methanol is needed to achieve higher yield, but a too high level of the methanol:olive oil molar ratio causes a decrease in the yield probably because methanol causes lipases inactivation [5]. A third studied variable was the number of steps of the stepwise methanol addition (1–7 steps), because a stepwise addition is reported to reduce lipase methanol inactivation [17, 18]. The response variable was the methyl ester yield, measured at 3 h. The experimental design is presented in Table 1. The experimental data obtained were fitted to a quadratic polynomial equation as follows:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i \cdot X_i + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{(i+j+1)} \cdot X_i \cdot X_j + \sum_{i}^{3} \beta_{(i+6)} \cdot X_i^2 \quad (1)$$

where Y is the response variable (methyl ester yield, %), X are the independent variables and β the different regression coefficients for the independent, linear, interaction and quadratic terms. Coefficient estimations as well

Table 1 Experimental setup for a five-level, three-factor surface response design and experimental data

Exp.	X_1	X_2	X_3	Methyl esters yield (%)	Exp.	X_1	X_2	X_3	Methyl esters yield (%)
1	3.6 (−1)	2 (−1)	2 (−1)	5.62	13	4.5 (0)	5 (0)	1 (−1.68)	2.14
2	3.6 (−1)	2 (−1)	6 (1)	53.71	14	4.5 (0)	5 (0)	7 (1.68)	52.11
3	3.6 (−1)	8 (1)	2 (−1)	48.47	15	4.5 (0)	5 (0)	4 (0)	49.04
4	3.6 (−1)	8 (1)	6 (1)	51.68	16	4.5 (0)	5 (0)	4 (0)	50.69
5	5.4 (1)	2 (−1)	2 (−1)	1.24	17	4.5 (0)	5 (0)	4 (0)	50.19
6	5.4 (1)	2 (−1)	6 (1)	59.82	18	4.5 (0)	5 (0)	4 (0)	51.38
7	5.4 (1)	8 (1)	2 (−1)	45.02	19	4.5 (0)	5 (0)	4 (0)	51.93
8	5.4 (1)	8 (1)	6 (1)	56.16	20	4.5 (0)	5 (0)	4 (0)	51.90
9	3 (−1.68)	5 (0)	4 (0)	48.89	21	4.5 (0)	5 (0)	4 (0)	51.12
10	6 (1.68)	5 (0)	4 (0)	49.93	22	4.5 (0)	5 (0)	4 (0)	51.20
11	4.5 (0)	0 (−1.68)	4 (0)	4.03	23	4.5 (0)	5 (0)	4 (0)	51.01
12	4.5 (0)	10 (1.68)	4 (0)	51.84					

X_1 = methanol:oil molar ratio [mol methanol/mol oil], X_2 = water amount [% based on oil weight] and X_3 = stepwise methanol addition [number of methanol additions]. Codified values for variables given in parentheses

as their statistical significance were evaluated by Matlab (R2008b, Natick, USA) toolbox Regstats.

Methyl Ester Yield Determination

Due to the high viscosity of oil and methyl esters, samples of the reaction media were weighed because it is not possible to take or measure properly a specific volume with a micropipette. To be able to know the volume taken, it is necessary to know the density value of the sample and thus finally calculate the methyl ester concentration and its yield. However, density varies slightly throughout the reaction, mainly because of not using any solvent. Thus, the density value used is 0.887 mg/ml, which is the mean value between the densities of the oil used and methyl oleate.

Methyl ester yield is defined as the ratio (as a percentage) between methyl ester moles and the total initial fatty acids contained in the oil used. The methyl ester proportion is defined as the ratio between each methyl ester and the sum of all the methyl esters.

Results and Discussion

Response Surface Model

In this study, response surface methodology (RSM) was used to understand the effect and the relationship between three important parameters in lipase-catalyzed biodiesel synthesis such as methanol: oil molar ratio, water content in the reaction media and the number of the methanol stepwise additions. The experiments were carried out in a solvent-free medium, in order to achieve a more environmentally-friendly bioprocess, at 30 °C, the optimal temperature determined in previous studies [10].

The experimental results shown in Table 1 were used to estimate the coefficients of the quadratic polynomial equation (Eq. 1) described in the Materials and Methods section. The values of the estimated coefficients are presented in Eq. 2 where X_1, X_2 and X_3 correspond to the coded variables methanol: olive oil molar ratio, water amount added based on oil weight and the number of stepwise methanol additions, respectively:

$$\begin{aligned} Y = {} & 50.80 + 0.33 \times X_1 + 11.81 \times X_2 + 15.02 \times X_3 \\ & - 0.09 \times X_1 \times X_2 + 2.30 \times X_1 \times X_3 \\ & - 11.54 \times X_2 \times X_3 + 0.78 \times X_1^2 - 6.81 \times X_2^2 \\ & - 7.10 \times X_3^2. \end{aligned} \quad (2)$$

The value of r^2 adjusted of 0.9583 was obtained indicating a good fit of the model. Another important statistical data is the p value for the estimated parameters, which indicates the significance of these coefficients on the response. All coefficients without significance (p value >0.05) are those related to the factor X_1 which codifies the methanol:olive oil molar ratio (Table 2), suggesting that this factor has little influence on the response model over the range studied (3:1–6:1), and did not cause loss of enzymatic activity although methanol was present in a stoichiometric excess. In order to minimize methanol employment, the molar ratio of 3:1 was selected as the most appropriate. The coefficients related to methanol:olive oil molar ratio were then deleted from Eq. 1. The resultant model provided the same value for the coefficients involving X_1 and X_3 in Eq. 1 and a value for r^2 adjusted of 0.9593.

The contour plot representing the methyl ester yield predicted as a response variable of the model is shown in Fig. 1 as a function of the water content (X_2) and the number of methanol additions (X_3). A maximum methyl ester yield occurs for low water content and highly fractioned methanol, and for high water content and minimally fractioned methanol. This means that the increase in water amount allows the use of a fewer fractioned methanol stepwise additions; therefore the water content is strongly related to enzyme inactivation by methanol.

Probably, when a low water content was used, the methanol, which is not solubilized in oil, inactivates the enzyme, removing its structural water. When external water is added to the system, it forms a separate aqueous phase that contains solubilized methanol, thereby reducing inactivation of the enzyme. Nevertheless, an excess of water may promote a rise in oil hydrolysis and consequently transesterification is slowed [4] [19]. So, in order to obtain a maximum methyl ester yield and to avoid any possible enzyme denaturation, the values of selected parameters were 4 % of water and seven methanol additions, in order to reduce the methanol amount in each addition.

The effect of water content, methanol concentration and stepwise addition was also studied separately with a recombinant ROL, immobilized on Amberlite IRA-93. The best performance was obtained with a methanol to oil ratio of 4.8 and a methanol addition in four steps. These values are quite similar to those obtained in this paper. However, maximum biodiesel yield was obtained with a water content of 60 % by oil weight [20], higher compared to the value obtained in this work (4 %). This difference between these two recombinant proteins is discussed below.

Time Evolution of the Transesterification Reaction

A transesterification reaction was carried out under the optimal conditions, described in the previous subsection. The time evolution of methyl ester and oleic acid

Table 2 *p* values of each coefficient in Eq. 2

Parameter	β_0	β_1	β_2	β_3	β_4	β_5	β_6	β_7	β_8	β_9
p value	6.1×10^{-15}	0.8	4.1×10^{-8}	2.3×10^{-9}	0.9	0.1	1.2×10^{-6}	0.4	8.6×10^{-6}	5.6×10^{-6}

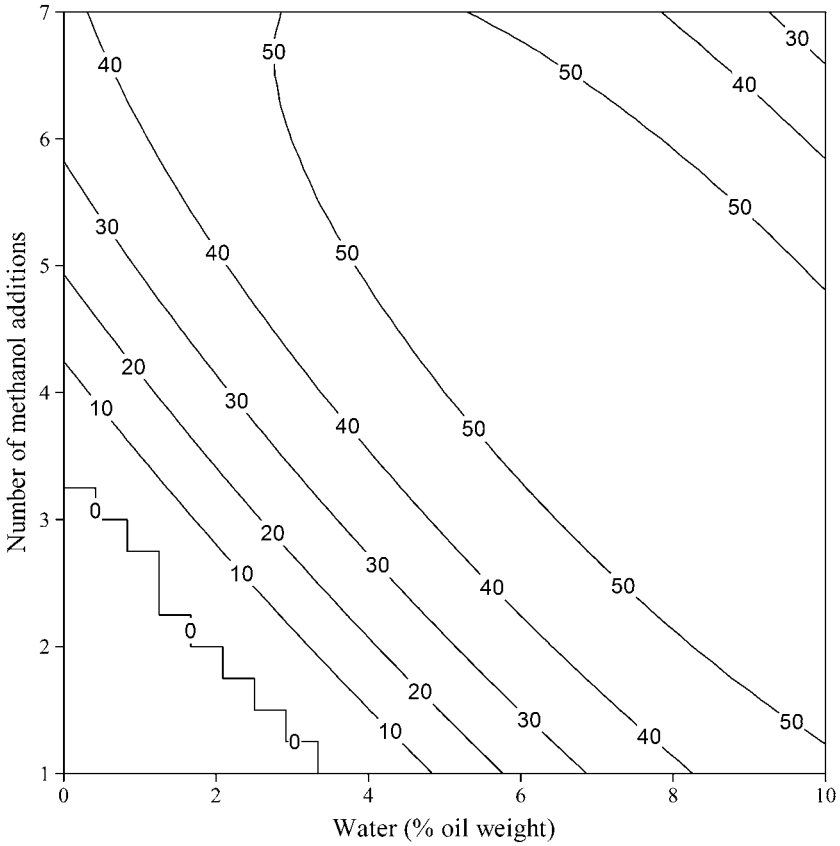

Fig. 1 Contour plot representing the response surface of the methyl ester yield as a function of the number of additions (stepwise methanol addition) and water amount. The methanol:olive oil molar ratio is set at 3:1. The *contour line* represents the methyl esters yield (%). The experiments were carried out at 30 °C and using 40 mg of biocatalyst

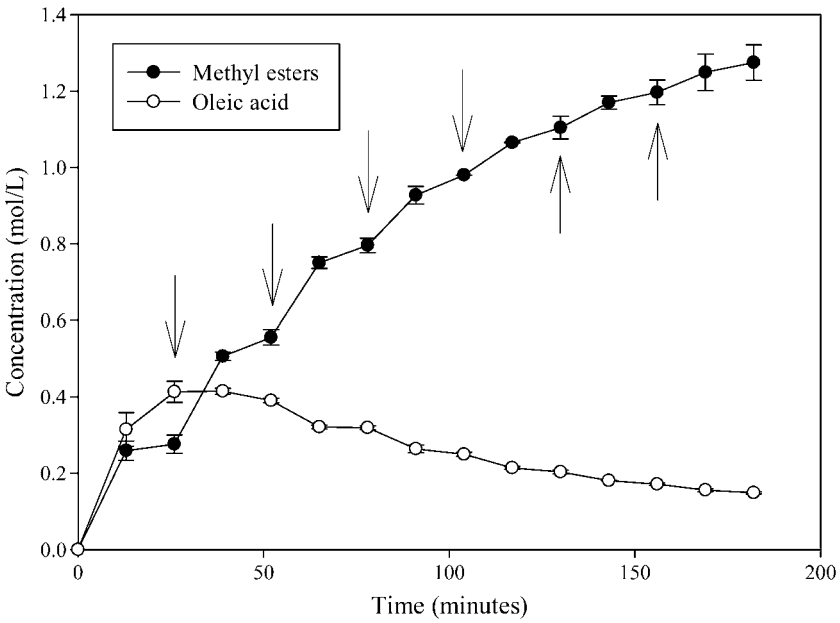

Fig. 2 Methyl ester and oleic acid concentrations *versus* time. The reaction conditions were the optimal conditions (3:1 methanol:olive oil ratio, 4 % of water amount based on oil weight and seven methanol additions) found by the response surface model (Fig. 1), using 40 mg of biocatalyst as in the surface response model study at 30 °C. The *arrows* indicate methanol additions

concentration is shown in Fig. 2. After the first addition of methanol (at time zero), a free fatty acid maximum was reached, its concentration being higher than that of methyl ester. Moreover, after the first methanol addition, lipase tends to hydrolyze instead of transesterifying the oil, probably due to the water amount being higher than that of methanol. When a second methanol addition is carried out, the concentration of free fatty acid begins to decrease.

Another important fact is that the methyl ester synthesis rate is higher than free oleic acid consumption rate. It appears that methyl esters are derived from both the esterification of the free fatty acid hydrolyzed by the enzyme and from direct transesterification of oil (Fig. 2). These results disagree with some published reports [18, 21], which stated that transesterification consisted of a succession reaction mechanism, where the oil was first hydrolyzed to free fatty acid and the latter is subsequently esterified. However, another study indicates that methyl esters are obtained by direct transesterification of oil [22]. A further explanation could be that for the succession reaction mechanism, the second reaction (i.e. esterification) is fast enough to not allow free fatty acids to accumulate in the reaction media.

Furthermore, in the first three intervals between methanol additions the methyl ester synthesis rate decreased with time, likely because methanol was almost completely consumed. After the third methanol addition, the reaction progressed more slowly than before; therefore, methanol started to accumulate in the system. This behavior has also been reported previously, using a recombinant ROL and a five stepwise addition strategy [20], although the slowdown in the methyl ester synthesis was observed at a higher yield. The difference between the two studies may be explained by different optimal water amounts (60 % based on oil weight for the cited study *versus* 4 % in this study), but also because the support and immobilization, covalent binding for the cited study and adsorption in this study, and finally because previous biochemical characterization of rROL used in this work showed differences compared to the native ROL, due to different post-translational modifications [10].

Another important fact is that methanol stepwise addition strategy has important effects on the time course of the reaction. Fewer fractioned methanol addition strategies tend to produce periods of time where there is a slow methyl ester synthesis rate, because methanol is exhausted [17, 23–25], while higher fractioned methanol additions strategies tend to generate more continuous evolutions and therefore avoid these periods of time, which means a more efficient process.

After 3 h of reaction, the methyl ester concentration reached 1.3 ± 0.1 mol/L, which corresponds to 50.3 ± 2.0 % yield, while the yield of a sample taken at 21 h was 58.9 ± 3.9 %. Taking into account the 1,3-positional selective of rROL, the value at 21 h corresponds to 88.4 % of the maximum yield.

Table 3 Methyl esters yield (%), re-using enzyme after its recuperation at 2 and 21 h

Recuperation time (h)	First use (methyl esters yield, %)	Second use (methyl esters yield, %)
2	40.96 ± 0.92	38.84 ± 2.08
21	58.31 ± 0.64	54.67 ± 1.25

The reaction was carried out with 1 g olive oil, a 3:1 methanol:olive oil molar ratio, methanol fractioned in seven additions, 40 mg of biocatalyst and 40 µl of water

Error bars reflect standard deviation with two replicates

Compared with a previously published that used another rROL, the reaction time to achieve similar conversion or yield has been drastically reduced from 24 [20] to 3 h (Fig. 2). Although it is difficult to compare the lipase activity of the biocatalyst between different laboratories, it seems that the recombinant ROL produced by our group has a great activity reducing biodiesel synthesis time, even working at a lower concentration. However, in the cited research the biocatalyst seems to show higher stability [20].

Biocatalyst Stability

In order to know a possible enzyme inactivation due to methanol presence, the biocatalyst was removed from the reaction media by vacuum filtration and used again. The results are shown in Table 3. No significant differences were observed when biocatalyst was reused (about 6.2 %). Thus, methanol had no or little inactivation effect on the immobilized lipase under the studied conditions, although the biocatalyst was in contact for several hours with a large amount of non-reacted methanol. Taking into account that methanol is more soluble in methyl esters than in oil [26], as the reaction advances inactivation should decrease.

Biocatalyst Amount

In order to know the effect of the biocatalyst amount on the reaction rate, transesterification was carried out with different amounts of immobilized lipase. The reaction performance is presented in Fig. 3. In the range from 10 to 40 mg, the reaction rate increased as the support particles amount increased, which is what would be expected when increasing catalyst in a bioconversion. However, when 80 mg of immobilized rROL was used, the reaction rate was similar to that observed with only about 40 mg of biocatalyst. It may be explained by the existence of methanol mass transfer limitations between the oil and the

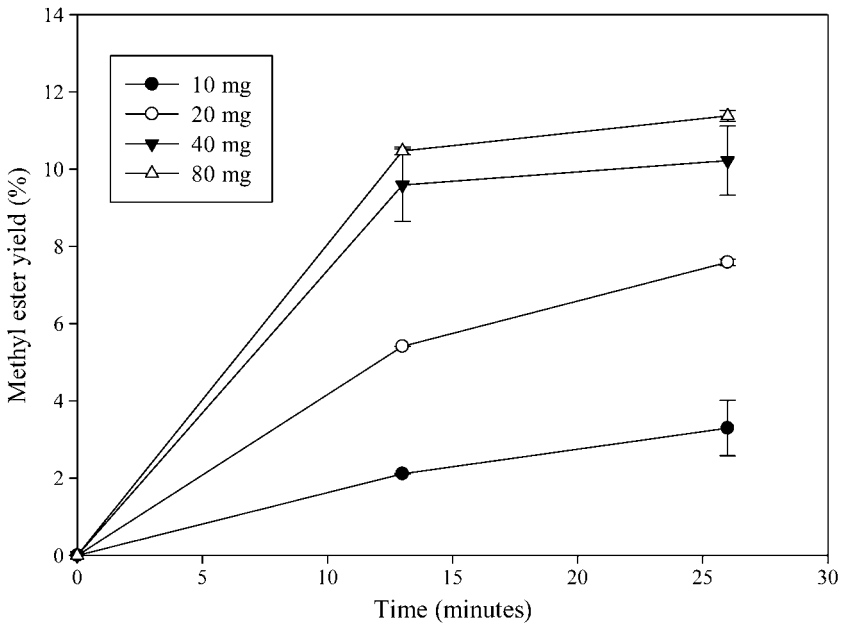

Fig. 3 Methyl ester yield *versus* time as a function of the biocatalyst amount used based on oil weight. The reactions were carried out under the optimal conditions given in Fig. 2 but with one methanol addition employed

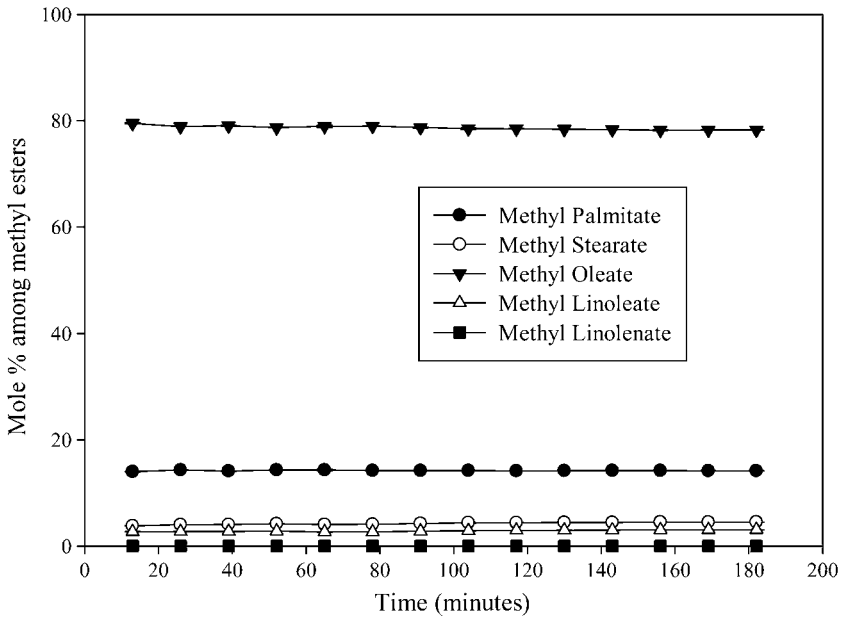

Fig. 4 Time-course of methyl ester concentration in the reaction medium, showing the composition of each methyl ester. The data was obtained from the experiments used to generate Fig. 2

aqueous phases, bearing in mind that the support particles' external and internal mass transfer limitations are not dependent on the amount of these particles.

Fatty Acids Specificity

As mentioned before, one of the main advantages using biodiesel as a fuel is that waste cooking oil and non-edible oils can be both used as feed stocks. So, these feedstocks can have a very diverse origin, and therefore different fatty acid composition [27]. For this reason, it is interesting to know whether the enzyme is specific towards the different fatty acids present in oils. In order to study this aspect, the proportion of each methyl ester was evaluated during the time course of the reaction. The results are presented in Fig. 4. It can be seen that the percentage of the composition of each methyl ester remained constant during the time course of the reaction, which means that the lipase is not specific towards any particular fatty acid (C16:0, C18:0, C18:1, C18:2 and C18:3), at least under the conditions studied. Thus, *R. oryzae* lipase is very useful to transesterify any oil that is mostly composed of the same fatty acids contained in olive oil regardless of the fatty acid proportions.

Conclusions

This work has aimed at studying and coupling biodiesel and monoacylglycerol synthesis, avoiding the formation of glycerol as a by-product. Under optimal conditions, a yield of 50.3 % was achieved in 3 h (taking into account the 1,3-positional selective of rROL, this value corresponds to a 75.4 % of maximum yield). Also, it has been found that water strongly determines the strategy of methanol stepwise addition to reduce enzyme inactivation. Further results suggested that the enzyme has the same specificity for the different fatty acids present in the oil. Thus, rROL seems to be a potential biocatalyst for biodiesel synthesis.

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Research paper

Lipase-catalysed transesterification: Viewpoint of the mechanism and influence of free fatty acids

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ABSTRACT

A series of lipase-catalysed transesterification experiments were carried out to study the effect of the presence of free fatty acids on synthesis reaction rate and the stability of the biocatalyst, and also to elucidate the underlying process, which remains a subject of debate. Based on the results, the reaction rate and biocatalyst stability increased with increasing content in free fatty acids of the reaction mixture. Also, tests carried out with a mixture of triolein and linoleic acid revealed that the transesterification is a combination of direct alcoholysis of triacylglycerols and a two-step reaction involving hydrolysis of acylglycerols and further esterification of previously released free fatty acids. The time course of triacylglycerols and diacylglycerols revealed that the enzyme is similarly selective for both types of substrate.

1. Introduction

Biodiesel is a mixture of monoalkyl esters of long chain fatty acids (FAME) with chemical and physical properties similar to those of conventional diesel, which make it a promising alternative to diesel fuel [1]. Biodiesel is obtained by transesterification of triacylglycerols – mainly vegetal fats – with short-chain alcohols such as methanol, most often in alkaly catalysis [1]. However, catalysed transesterification in a basic medium requires large amounts of energy and water for purification [1,2]. Also, the formation of soaps from substrates containing more than 0.5 wt% free fatty acids has an adverse impact on yield [2]. This makes the alkaline process unsuitable for substrates such as waste cooking oil [1]. On the other hand, acid catalysis allows oil to be treated with large amounts of free fatty acids but occurs at a much lower rate than alkaline catalysis [3].

In recent years, lipase-catalysed transesterification has become an effective alternative to basic and acid catalysis for biodiesel production. Enzyme-based transesterification uses less energy than chemically catalysed processes; also, unlike basic catalysis, it can be used with substrates containing free fatty acids [4].

Although lipases are widely known to carry out transesterification in the presence of free fatty acids [1,2,4], their effect on transesterification has scarcely been studied to date [5–7]. The mechanism for the reactions catalysed by lipases has been represetend by Ping-Pong models [8,9] but in the case of enzyme-based transesterification it has been explained in the light of two different viewpoints [10]. In one, lipase synthetises esters by direct alcoholysis of triacylglycerols in a single step [8,11,12]. The other involves hydrolysis of triacylglycerols and subsequent esterification of the resulting fatty acids [10,13–15].

In this work, we examined the effects of free fatty acids on the rate of the transesterification reaction and the stability of the biocatalyst. To this end, we performed series of experiments intended to elucidate whether the transesterification process involves a single step or two.

The enzyme used was recombinant lipase from *Rhizopus oryzae* obtained by culturing *Pichia pastoris* [16]. This lipase, which is a1,3-positional selective, was previously used in immobilized form on a support that was immersed in a solvent-free medium to develop an environmentally friendly process. The enzyme has also been used for transesterification in a solvent-free system [17].

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2. Materials and methods

2.1. Materials

High-grade methanol and heptane were purchased from Panreac (Barcelona, Spain). The oil used was virgin-grade olive oil for household use. The substrate triolein was purchased from TCI Europe N.V. (Zwijndrecht, Belgium). The other substrates (methyl oleate and methyl linoleate) were obtained from Sigma–Aldrich (St Louis, MO, USA), and so was the derivatizing reagent (MSTFA). High-grade standards of linoleic acid, stearic acid, oleic acid, linoleic acid, methyl palmitate, methyl stearate, methyl oleate, methyl linoleate, methyl linolenate, monoolein (DL-α-monoolein) and diolein (1,3-diolein) used for calibration were also supplied by Sigma–Aldrich. High-grade triolein was purchased from Acros Organics (Geel, Belgium).

The support for lipase immobilization, Relizyme OD403/S, was obtained from Resindion S.r.l. (Binasco, Italy), and the lipase colorimetric kit used to assess enzyme activity (ref. 11821792) from Roche (Mannheim, Germany).

2.2. Lipase

Recombinant *R. oryzae* lipase was produced by the Bioprocess Engineering and Applied Biocatalysis group of the Universitat Autònoma de Barcelona (UAB). The enzyme was obtained by fed-batch cultivation of a recombinant *P. pastoris* strain using methanol as inductor [16]. The culture broth was centrifuged and microfiltered to remove biomass, after which the supernatant was concentrated by ultrafiltration on a Centrasette® system from Pall Filtron (New York, USA) furnished with an Omega membrane of 10-kDa cut-off, and subsequently dialysed against 10 mM Tris–HCl buffer at pH 7.5 and thereafter lyophilised [18].

2.3. Determination of lipase activity and total protein

Lipase activity was determined in 200 mM Tris–HCl buffer at pH 7.25 at 30 °C, using the Roche lipase colorimetric kit on a Varian 300 spectrophotometer from Cary (Palo Alto, CA, USA) [19]. Total protein was determined by using the Bradford method with bovine albumin as standard [20]. Both enzyme activity and total protein were determined in triplicate.

2.4. Lipase immobilization

A volume of 100 ml of 5 mM phosphate buffer at pH 7 containing about 5000–6000 UA lipase/ml was used to dissolve lyophilised rROL under magnetic stirring at 4 °C for 1 h. The solution was then centrifuged and the supernatant mixed with 1000 mg of pre-treated support at 4 °C for immobilization under slow stirring with a roller for 7 h. Then, the solution was vacuum-filtered and the biocatalyst washed with 300 ml of the same initial phosphate buffer. Finally, the biocatalyst was dried to constant weight in a desiccator containing silica gel and stored at −20 °C until use.

The support was pre-treated by mixing 1000 mg of Relizyme OD403/S with 100 ml of water–acetone solution for 30 min. Then, the solution was vacuum-filtered and washed several times with water to completely remove the acetone.

2.5. Transesterification experiments

All transesterification reactions were carried out in 10 ml vials at 30 °C under continuous stirring in an incubator (20 mm orbital diameter, IKA KS 400 ic, Staufen, Germany). All reaction media contained 32 000 UA of biocatalyst.

The reactions used to study free fatty acids were carried out by mixing specific amounts of olive oil and oleic acid with a final weight of 8 g and adding 160 μl of methanol. For catalyst stability tests, the biocatalyst was allowed to settle at the end of the reaction and the medium removed. The vials containing the biocatalyst were stored at 4 °C until reuse.

Transesterification process was studied by using 8 g of two different mixtures of triolein and linoleic acid with 160 μl of methanol.

The time course of acylglycerols was studied by using 8 g of two different mixtures of triolein and oleic acid to which methanol was added stepwise at 30 or 60 min intervals. Seven methanol additions of 160 μl each were carried out in each experiment.

The densities of triolein, olive oil and oleic acid are very close so the final volume reached is the same for all the experiments described above.

2.6. Sample preparation and determination of methyl esters, free fatty acids and acylglycerols

Samples of the reaction mixture were withdrawn at preset intervals and passed through a PVDF filter of 0.45 μm pore size from Millipore (Billerica, MA, USA) to remove all biocatalyst. This was followed by storage at −20 °C until analysis for methyl esters, free fatty acids and acylglycerols. Analyses involved centrifuging the samples at 10 000 rpm for 3 min, withdrawing an aliquot of 10 μl from each with a micropipette, weighing on analytical balance and dilution with heptane. Weighing was required because the high viscosity of acylglycerols and also, to lesser extent, methyl esters, precluded accurate withdrawal of a given volume with a micropipette [17]. The average standard errors for the compound concentrations in each sample was 2.57%.

Methyl esters (viz., methyl palmitate, stearate, oleate, linoleate and linolenate) and free fatty acids (viz., palmitic, stearic, oleic and linoleic) were analysed on a 7890A gas chromatograph from Agilent Technologies (Santa Clara, CA, USA) equipped with a G4513A auto-sampler and a 19095N-123 INNOWAX (30 m × 0.53 mm × 1 μm), both from Agilent Technologies. The software used was Agilent ChemSation from Agilent Technologies. The initial oven temperature, 130 °C, was raised to 240 °C at 16 °C/min and held at that level this for 24 min. The temperatures of the injector and flame ionization detector were 250 and 280 °C, respectively. Helium at a constant flow rate of 3.699 ml/min was used as carrier gas. All samples were centrifuged at 10 000 rpm for 3 min prior to analysis. No further sample preparation was needed.

Mono-, di- and triolein were analysed with the same gas chromatograph and auto-sampler as the methyl esters and free fatty acids. A capillary column BD-EN14105 (10 m × 0.32 mm × 0.1 μm, Part number 123-BD01 from Agilent Technologies) was used tied to an on-column inlet with a high-temperature retention gap. The initial oven temperature, 50 °C, was held for 1 min, raised to 180 °C at 15 °C/min, then to 230 °C at 7 °C/min and 370 °C at 10 °C/min, and held for 5 min. The flame ionization detector was kept at 380 °C. Helium at a constant flow rate of 3 ml/min was used as carrier gas. Samples were prepared somewhat in accordance with European standard EN14105. 100 ml of centrifuged and diluted sample in heptane (as previously described) were derivatized with 10 μl of MSTFA at room temperature mixing for 3 min. This was followed by addition of 0.8 ml of heptane after 30 min. It should be noted that chromatographic peaks for acylglycerols are somewhat difficult to integrate because the different fatty acids combine into similar acylglycerol structures that are difficult to separate [21]. This led us to estimate a molar balance for each sample – the combined amount of triolein, diolein and monoolein should be constant in all experiments – the average error in which was

estimated to be 7.27%.

3. Results and discussion

Fig. 1.1 depicts the alcoholysis of triacylglycerols in a single step. As can be seen, an acyl donor – usually methanol – breaks the ester bond in the triacylglycerol skeleton and causes the formation of an ester bond between the acyl donor and a hydroxyl group in the glyceride structure.

The two-step reaction (Fig. 1.2) involves hydrolysis of the triacylglycerol molecule to release a free fatty acid, followed by esterification of the fatty acid by an acyl donor. This is a cyclical process because water released in the esterification reaction hydrolyses a fatty acid moiety in a glyceride. The fact that, as stated above, lipase-catalysed transesterification can occur in the presence of free fatty acids, led us to examine their influence on methyl esters and the two-step reaction proposed for the process.

3.1. Influence of free fatty acids on transesterification

The effect of free fatty acids on transesterification was examined by using different mixtures of olive oil with variable concentrations of free oleic acid over the range 0–20%. Fig. 2 shows the time course of FAME. Increasing the free fatty acid concentration increased the initial FAME production rate, albeit not proportionally; thus, the rate increased from $1.8 \cdot 10^{-4}$ mol/min in the absence of added acid to $2.6 \cdot 10^{-4}$ and $3.6 \cdot 10^{-4}$ mol/min in the presence of 5 and 20% added acid, respectively. Similar results were obtained by Li S. et al. and Du W. et al. [5,7]. The fact that the FAME production rate increased with increasing amount of free fatty acids suggests that the transesterification reaction is a two-step process. However, the concentration of free fatty acids remained virtually constant except when no acid was added (Fig. 2). The increase in transesterification rate can be ascribed to a decrease in viscosity as more free oleic acid was added to the reaction mixture. With no acid added, the oleic acid concentration increased slightly as a result of the water initially contained in the biocatalyst facilitating hydrolysis of triacylglycerols by lipase.

Fig. 3 illustrates the effect of the initial presence of free fatty acids on biocatalyst stability. As can be seen, stability increased with increasing initial concentration of free fatty acids. Above 10%, the biocatalyst was reusable over up to 10 cycles with a loss of activity of only about 10%; in absence the FFA, however, the catalyst lost 50% of its activity after 7 cycles and was almost inactivated after 10. The same behaviour was observed by Du W. et al. [5]. Loss of activity is widely attributed to methanol inactivation, due to the contact of insoluble methanol with the enzyme [6,17]. Adding FFA

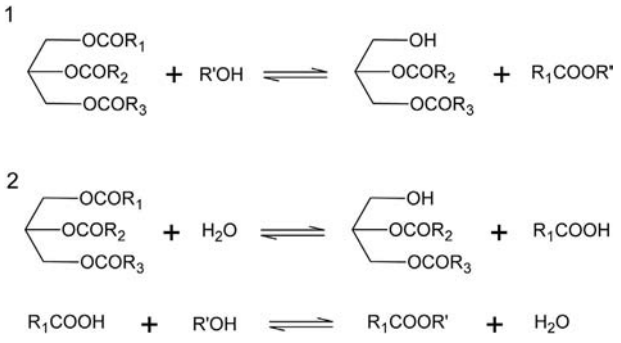

Fig. 1. Proposed transesterification processes found in literature. Subfigure 1 corresponds to one-step reaction direct triacylglycerol alcoholysis. Subfigure 2 corresponds to two-steps reaction involving a first hydrolysis and a second esterification.

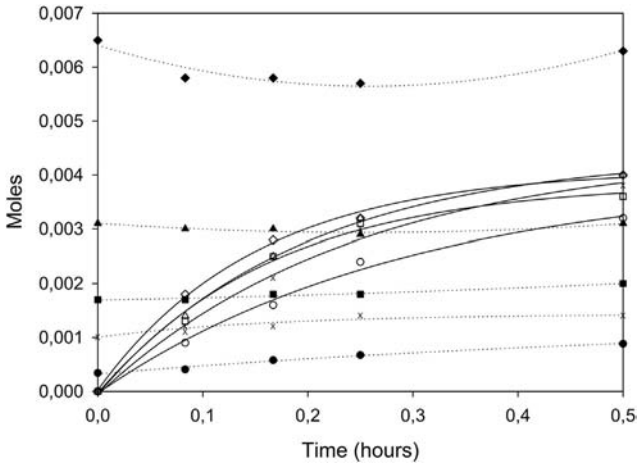

Fig. 2. Methyl esters formation and free fatty acids evolution for substrates with different amounts of free oleic acid. Dot lines and black symbols correspond to free fatty acids and solid lines to methyl esters. ◊: 20% free oleic acid, Δ: 10% free oleic acid, □: 5% free oleic acid, x: 2.5% free oleic acid and ○: 0% free oleic acid.

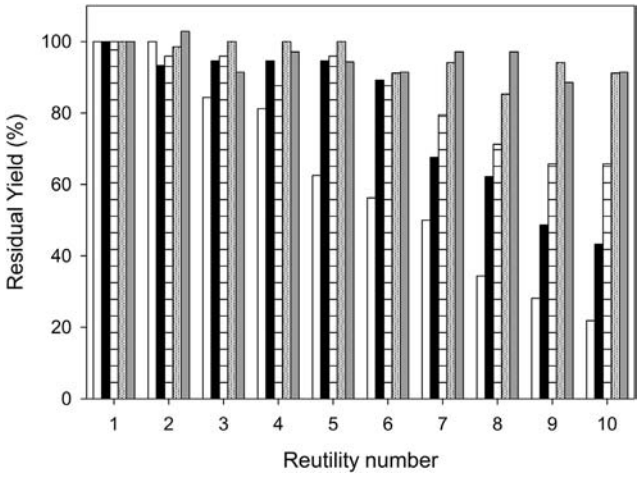

Fig. 3. Residual yield for substrates with different amounts of free oleic acid. The yield is normalized to the first one. Empty bar: 0% free oleic acid, Solid bar: 2.5% free oleic acid, Striped bar: 5% free oleic acid, Dot bar: 10% free fatty acid and Grey bar: 20% free oleic acid.

to the reaction increases the polarity of the medium and therefore methanol becomes more soluble in the medium [6], reducing enzyme inactivation. A similar explanation is found in the literature [5], stating that the lower the value of logP, the higher lipase tolerance to methanol.

3.2. Elucidation of the transesterification process

As stated before, the concentration of free oleic acid remained constant throughout the transesterification reaction (Fig. 2). Therefore, the reaction must have occurred by direct alcoholysis of triacylglycerols (Fig. 1.1) or new oleic acid molecules coming from triacylglycerols hydrolysis, meaning that the real transesterification process is the two-step reaction (Fig. 1.2).

The fate of free fatty acids (FFA) was traced in an experiment using triolein and free linoleic acid in order to check whether FFA came from the hydrolysis of triacylglycerols since the hydrolysis of triolein would have given oleic acid as the sole fatty acid. Linoleic acid was chosen because it has the same carbon chain length as

oleic acid plus an additional double bond, and also because both the ester and acid forms of the two acids can be quantified separately by gas chromatography. In addition, we assessed lipase selectivity towards oleic and linoleic acid by using an equimolar mixture of the two acids. As can be seen from Fig. 4, rROL was identically selective for both acids. This result is consistent with previous reports [17].

Two different experiments using two different initial linoleic acid concentrations were performed. Figs. 5 and 6 show the variation of the concentrations of oleic and linoleic acids, and their corresponding FAME, at an initial linoleic acid concentration of 10% and 20%. As can be seen, the total acid concentration remained virtually constant in both experiments. This suggests that the water molecule released when a linoleic acid molecule was esterified was rapidly used by lipase to hydrolyse triolein to a free oleic acid molecule. Also, the hydrolysis reaction was faster than the esterification reaction – otherwise, the amount of free oleic acid formed moles appeared would not have been the same as that of linoleic acid consumed and the total concentration of acid would not have remained unchanged as a result. Therefore, it can be clearly concluded that the transesterification reaction occurs at least by the so-called two-step process (Fig. 1.2) by which water released by esterification of a free fatty acid is rapidly used to hydrolyse a triacylglycerol and produce another molecule of free fatty acid, after which the process is restarted. If this assumption is correct, the initial reaction mixture should only contain free linoleic acid and the initially formed esters should be linoleate esters mainly since virtually no free oleic acid would have yet been released. In the presence of 10% linoleic acid, however, the amount of methyl oleate at the beginning of the bioprocess exceeded that of methyl linoleate. Also, the initial reaction rate should have increased with increasing free fatty acid concentration, but the initial rate of methyl oleate formation rate was virtually the same in both experiments. Therefore, the transesterification reaction also involves direct alcoholysis of triacylglycerols.

In conclusion, lipase-catalysed transesterification is a combination of two processes, namely: direct alcoholysis of triacylglycerols in a one-step reaction and a two-step hydrolysis of triacylglycerols followed by an esterification.

One other major inference from the results with 10 and 20% linoleic acid (Figs. 5 and 6, respectively) is that the mole balance for this acid differed between the two experiments. The total initial amount of free linoleic acid should have been the combination of the final amount of free linoleic acid and that of linoleate ester. This

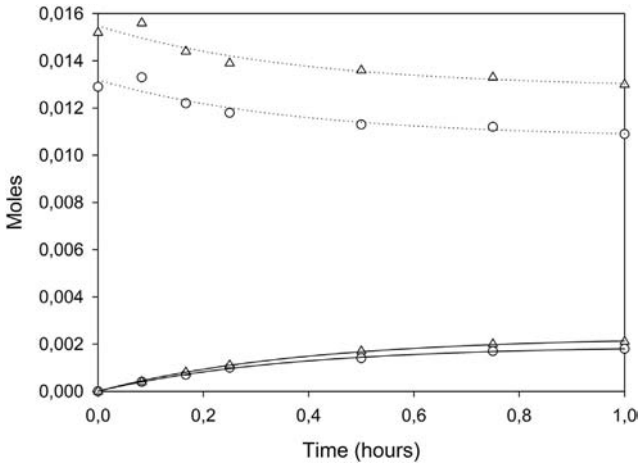

Fig. 4. Methyl esters formation and free fatty acids evolution. The reaction was carried out using an equimolar mixture of linoleic and oleic acid. Dot line corresponds to free fatty acids and solid line to methyl esters. Δ: linoleic and ○: oleic.

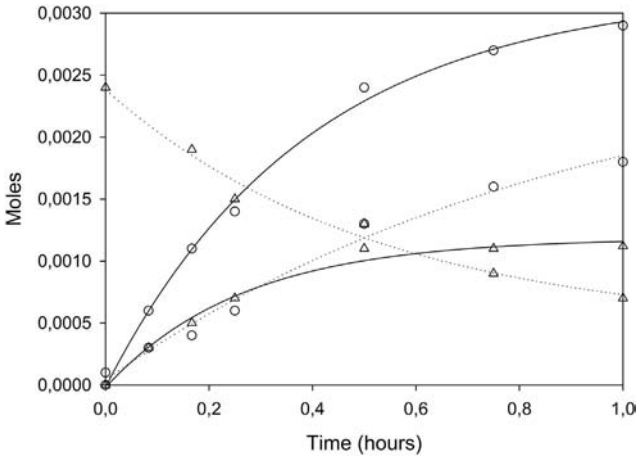

Fig. 5. Methyl esters formation and free fatty acids evolution. The reaction was carried out using a mixture of 90% triolein and 10% free linoleic acid (in weight). Dot line corresponds to free fatty acids and solid line to methyl esters. Δ: linoleic and ○: oleic.

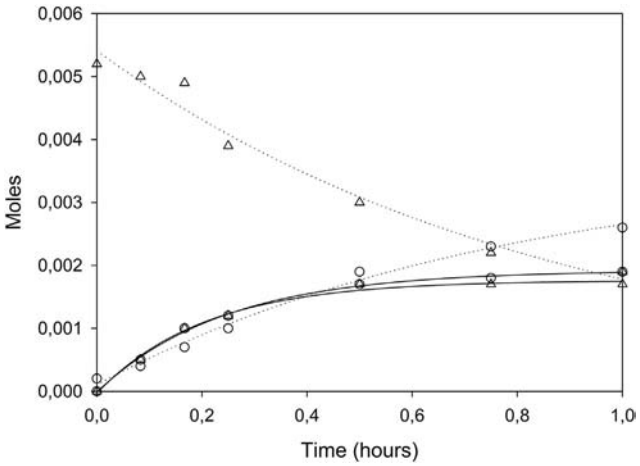

Fig. 6. Methyl esters formation and free fatty acids evolution. The reaction was carried out using a mixture of 80% triolein and 20% free linoleic acid (in weight). Dot line corresponds to free fatty acids and solid line to methyl esters. Δ: linoleic and ○: oleic.

was not the case, however, because some free linoleic acid was incorporated by the enzyme into the diacylglycerols or monoacylglycerols formed by transesterification (Fig. 7). As can be seen from Fig. 6, although transesterification stopped within 30 min owing to the depletion of methanol, linoleic acid continued to be consumed and oleic acid formed.

3.3. Evolution of acylglycerols

Lipase-catalysed transesterification involves a number of compounds including triacylglycerols that are converted into diacylglycerols and then into monoacylglycerols with formation of esters. Also, the activity of lipase is known to be closely linked to the polarity of the substrate and the reaction rate to depend on its structure [22]. As a result, lipase selectivity towards acylglycerols

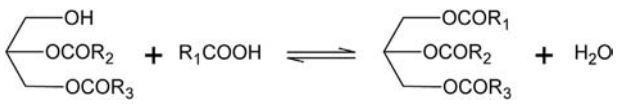

Fig. 7. Incorporation of free fatty acids to acylglycerols.

during transesterification may change not only because acylglycerol structure changes by effect of the conversion of triacylglycerols into mono- and dioacylglycerols, but also because these substrates differ in polarity.

This led us to examine the evolution of all species involved in the transesterification reaction including acylglycerols. The analysis of acylglycerols is complicated by the large number of chromatographic peaks given by the large variety of long-chain fatty acids present in oil. An experiment was thus performed with triolein as the sole substrate that yielded, diolein and monoolein alone. The experiment was carried out in the presence and absence of free oleic acid in order to assess its potential effects on lipase selectivity towards acylglycerols. Seven different methanol additions were done corresponding to the stoichiometric molar relationship to triolein. This stepwise addition procedure was repeated at 30 and 60 min intervals to assess lipase inactivation by methanol throughout the transesterification reaction.

Figs. 8 and 9 show the results obtained with methanol additions every 30 min, with 20 and 0% of initial free oleic acid, respectively. Adding methanol at 30 min intervals to a medium containing triolein but no free fatty acids (Fig. 9) led to gradual accumulation of excess alcohol not used in the reaction and to inactivate the enzyme after the third addition. This was not the case in the presence of 20% of free oleic acid (Fig. 8) because the reaction was faster, so methanol accumulated to a lesser extent between additions; also, as noted earlier, the presence of oleic acid reduced the inactivation effect of methanol.

Figs. 10 and 11 show the results obtained with methanol additions at 60 min intervals, with 20 and 0% of initial free oleic acid, respectively. With 20% of free oleic acid (Fig. 10), the addition interval had virtually no effect on the reaction and the FAME formation profile was independent of the rate of methanol addition, comparing it to the case of adding methanol every 30 min (Fig. 8). In the absence of free fatty acids (Fig. 11), however, adding methanol every hour prevented inactivation of the enzyme – which occurred with methanol additions at half-hour intervals (Fig. 9) – and the reaction yield was higher as a result.

As previously found, the concentration of free oleic acid remained virtually constant throughout the reaction (Fig. 2). This was also the case with these experiments (Figs. 8–11), but only between the first and second methanol addition. After that, the concentration of free oleic acid decreased as the reaction developed. As stated above, free fatty acids reduce lipase inactivation by

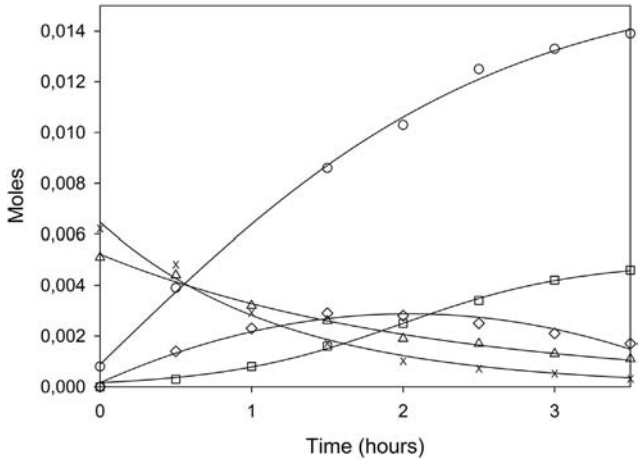

Fig. 8. Evolution for all the species involved in transesterification reaction. Methanol was added every half hour and the reaction medium contained initially 20% of free oleic acid. x: triolein, ◊: diolein, □: monolein, Δ: free oleic acid and ○: methyl oleate.

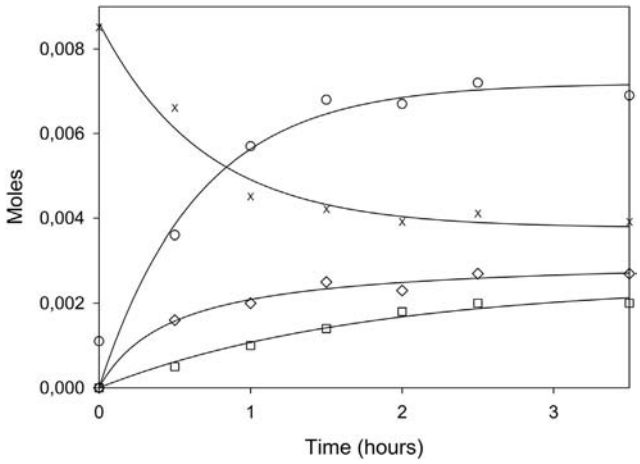

Fig. 9. Evolution for all the species involved in transesterification reaction. Methanol was added every half hour and the reaction medium contained initially 0% of free oleic acid. x: triolein, ◊: diolein, □: monolein, Δ: free oleic acid and ○: methyl oleate.

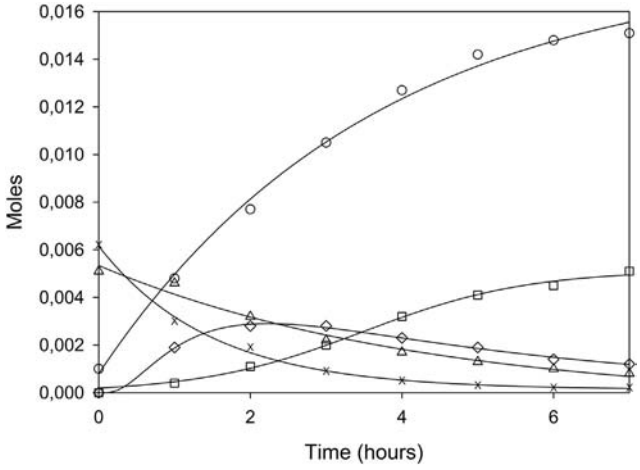

Fig. 10. Evolution for all the species involved in transesterification reaction. Methanol was added every hour and the reaction medium contained initially 20% of free oleic acid. x: triolein, ◊: diolein, □: monolein, Δ: free oleic acid and ○: methyl oleate.

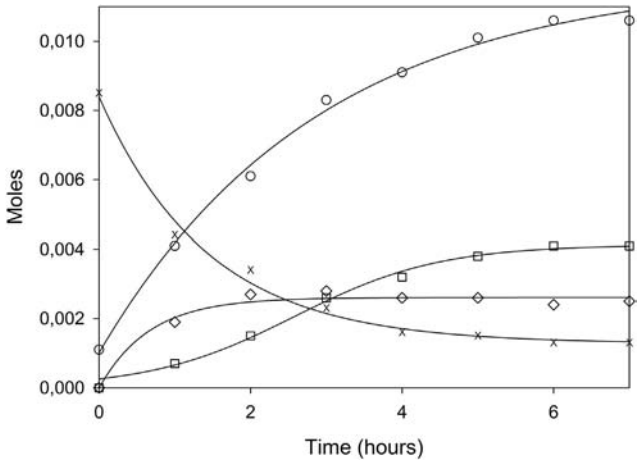

Fig. 11. Evolution for all the species involved in transesterification reaction. Methanol was added every hour and the reaction medium contained initially 0% of free oleic acid. x: triolein, ◊: diolein, □: monolein, Δ: free oleic acid and ○: methyl oleate.

effect of the high polarity of methanol; therefore, a decrease in free fatty acid concentration can lead to inactivation of the enzyme. This was not the case, however, probably because diacylglycerols, monoacylglycerols and esters present in the reaction medium were more polar than triacylglycerols and hence similar to free fatty acids in their effect. It can thus be concluded that lipase stability against methanol was not specifically improved by free fatty acids, but rather by polar enough substances to buffer the high polarity of the alcohol.

No significant differences in lipase selectivity towards triacylglycerols and diacylglycerols were observed (Figs. 8–11). If rROL had been more selective towards triacylglycerols than diacylglycerols, diolein and triolein would have accumulated to some extent rather than being thoroughly consumed from the reaction medium. In fact, the amount of diolein decreased even in the presence of substantial amounts of triolein.

4. Conclusions

One of the main advantages of lipase-catalysed over basic-catalysed transesterification is that the former process can be carried out in the presence of free fatty acids. Also, as shown here, free fatty acids increase the reaction rate and the stability of the biocatalyst. In fact, free fatty acids protect the enzyme by effect of their polarity buffering the high polarity of methanol. This also seems to be the case with other transesterification substrates such as mono- and diacylglycerols.

This study also demonstrates that transesterification is a combination of two processes, namely: direct alcoholysis of triacylglycerols and a two-step reaction involving hydrolysis of triacylglycerols followed by esterification of previously released free fatty acids.

Also, rROL is similarly selective towards tri- and diacylglycerols, so no diacylglycerol accumulation occurs during the reaction – if it does at any time, the amount of methanol to be added should be altered to avoid it and prevent inactivation of the enzyme.

Acknowledgements

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Publication III. Albert Canet, M. Dolors Benaiges, Francisco Valero, Patrick Adlercreutz. Alcoholysis reactions catalysed by free or immobilised *Rhizopus oryzae* lipase for biodiesel production.

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Alcoholysis reactions catalysed by free or immobilised *Rhizopus oryzae* lipase for biodiesel production

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ABSTRACT

Transesterification of triolein with methanol or ethanol to biodiesel was studied using recombinant *R. oryzae* (rROL) lipase as catalyst. Both rROL and a commercially available *R. oryzae* lipase (ROL) had higher specificity towards ethanol than methanol and towards 1-monoolein than triolein, this difference being more pronounced in the case of the recombinant one. In addition, both enzymes were highly 1,3-positional selective and did not accept 2-monoolein as substrate. Methanol caused more lipase inactivation than ethanol.

A comparison was made of free and immobilised rROL in water/organic biphasic and organic reaction systems working with heptane as a solvent. The immobilised lipase in a system without added water had more alcoholysis activity, with initial rates around 20 times higher than for the free lipase in a biphasic system. The highest yield, > 90 %, was also obtained in this low water system with a molar ratio of ethanol to triolein of 3. It was shown that efficient acyl migration occurred at moderate alcohol concentrations, but with alcohol to triolein molar ratios higher than 3 acyl migration was slow, which caused low biodiesel yields.

1. Introduction

Biodiesel is a mixture of fatty acids monoalkyl esters (FAMEs) and a biodegradable and renewable alternative fuel source with several advantages versus current diesel fuel [1,2]. It is produced industrially from short chain alcohols and triacylglycerols, typically vegetable oils using alkali catalysis [3].

Compared to the alkali process, lipase-catalysed transesterification has several advantages; mainly that lipases perform their conversions in milder conditions [4] and are able

to catalyse both transesterification and esterification, which allows the use of cheaper raw materials with high amounts of free fatty acids for biodiesel production [5]. For these reasons, this process has drawn huge scientific attention during the last 10 years [6].

However, the use of lipases in biodiesel synthesis has many limitations and drawbacks, therefore in recent years there have been an increasing number of scientific publications studying different lipase transesterification aspects. One of the main bottlenecks for the industrial application of the enzymatic process is the high cost of the catalyst – i.e. lipase – compared to the alkali catalyst, such as NaOH [6]. On the one hand, the use of recombinant lipases is a well-accepted way to reduce the cost of the biocatalyst [7] and currently many published studies on transesterification used recombinant lipases [8–11]. On the other hand, immobilisation of lipases is generally used to obtain reusable enzymes preparations and enhances its stability [12], which also contributes to cost reduction. However, currently there is great interest in using free lipase liquid formulations for biodiesel production [9,13,14].

Methanol is the most widely used acyl-acceptor in transesterification because of its economic feasibility and accessibility [15], although it is reported to hamper lipase activity. Its negative effect is still not completely understood, as one can guess from the non-specificity of the terms used to describe it, such as inactivation, inhibition or denaturation [16]. One possible explanation is that lipases are easily inactivated in the presence of short chain alcohols, which may interact with water molecules necessary to keep the native structure of the lipase [5], although ethanol is reported to inactivate the enzyme to a lesser extent [1,15]. Thus, this is another major drawback in lipase-catalysed transesterification and several strategies have been studied to avoid specially methanol inactivation. The stepwise addition of methanol is often used to prevent lipase inactivation in published work, achieving high biodiesel yields [17–20].

In a previous publication [19], it was observed that water in the reaction medium minimized the negative effects of methanol. Water content in the reaction medium plays an important role in lipase-catalysed reactions. A minimum amount of water is needed to keep lipase conformation, but an excess of water may lead to hydrolysis [5].

A wide range of lipases are 1,3-positional selective, which means that these enzymes are specific to hydrolyse ester bonds positions 1 or 3 in triacylglycerols [4]. Several examples of transesterifications described in the literature used this type of lipase from *R. miehei* [13,21,22], *R. oryzae* [13,19,23,24], *P. fluorescens* [13] or pig pancreatic lipase [25]. Although the utilization of these lipases leads theoretically to a maximum theoretical yield of 66.7%,

higher yields were often observed [13,23,24,26,27], due to acyl migration, which promotes the conversion of 1,2- to 1,3-diacylglycerols and 2- to 1(3)-monoacylglycerols, achieving an overall conversion to glycerol and biodiesel.

In this present study, we have carried out comparative experiments studying the differences between using immobilised and free lipase preparations. Quantification analysis of free oleic acid, methyl and ethyl oleate, 1(3)- and 2-monoolein, 1,3- and 1,2-diolein and triolein have been made in order to understand the whole transesterification progress and also to study acyl migration in the different reactions done. In addition, experiments comparing the lipase specificity towards different alcohols (i.e. ethanol and methanol) and acylglycerols (1-monoolein and triolein) were carried out. This work has been done with a recombinant 1,3-positional selective *Rhizopus oryzae* lipase (rROL), which was previously used in several biodiesel synthesis studies [3,8,19,28] and also in other lipase studies [29–32]. Experiments using a commercial *R. oryzae* lipase (ROL) were also carried out in order to compare both lipases.

2 Materials and methods

2.1. Materials

High-grade n-hexane, n-tetradecane, methanol and methyl *t*-butyl ether were purchased from Merck (Darmstadt, Germany) and n-heptane from VWR Chemicals (Stockholm, Sweden). Analytical grade ethanol (99.5%) was obtained from Solveco (Rosersberg, Sweden). Ethyl oleate, methyl oleate and oleic acid were from Aldrich. Silica gel and *p*-nitrophenyl butyrate were purchased from Sigma-Aldrich (St Louis, Missouri, USA). Accurel MP1000 was from Membrana GmbH (Obernburg, Germany) and Relizyme OD403/S from Resindion S.r.l. (Binasco, Italy). *N*-methyl-*N*-trimethylsilyheptafluorobutyramide (MSHFBA) was obtained from Macherey-Nagel (Düren, Germany) and triolein from Larodan Fine Chemicals (Malmö, Sweden), which contain traces of diolein and free oleic acid. Other chemicals were of analytical grade.

2.2. Lipases

Recombinant *R. oryzae* lipase (rROL) was produced in the Bioprocess Engineering and Applied Biocatalysis research group in the Universitat Autònoma de Barcelona (UAB) as stated in previous work [3]. The enzyme was produced by fed-batch cultivation of a recombinant *P.*

pastoris strain using methanol as inductor. The culture broth was centrifuged and microfiltered to remove biomass, after which the supernatant was concentrated by ultrafiltration in a Centrasette® system from Pall Filtron (New York, USA) equipped with an Omega membrane of 10-kDa cut-off and subsequently dialysed against 10 mM Tris-HCl buffer at pH 7.5 and thereafter lyophilised.

Commercial *R. oryzae* lipase (ROL; product number 80612-25G) was purchased from Sigma-Aldrich.

2.3. Lipase activity

The hydrolytic activity of the lipase was analysed via spectrophotometric assay based on *p*-nitrophenyl butyrate hydrolysis, as described in a previous study [33]. The absorbance of the p-nitrophenol formed was measured continuously at a wavelength of 400 nm in a thermostat spectrophotometer at 25°C using pH 7 sodium phosphate buffer. *p*-nitrophenyl butyrate initial concentration was 0.4 mM in the cuvette and the enzyme concentration was chosen so that it allowed linear absorbance measurements for 2 min.

2.4. Commercial R. oryzae lipase immobilisation

1.5 g of lyophilised powder ROL were dissolved in 30 ml 50mM phosphate buffer at pH 7 for 30 min at room temperature under magnetic stirring. The enzyme solution was then mixed with 1.5 g of Accurel MP1000, which were previously mixed with 4.5 ml ethanol, and the immobilisation was set at slow agitation at room temperature for 5 hours. After that the solution was vacuum-filtered and the immobilised lipase or biocatalyst rinsed with 10 ml of the same initial buffer. The biocatalyst was finally dried to constant weight in a silica gel desiccator and stored at -20°C.

2.5. Recombinant R. oryzae lipase immobilisation

rROL was immobilised by adsorption on Relizyme OD403/S. 0.7 g of lyophylised powder rROL were dissolved in a volume of 50 ml 5mM phosphate buffer at pH 7 under magnetic stirring at 4°C for 1 hour; then the solution was centrifuged and the supernatant kept for immobilisation. Meanwhile, 2.8 g of Relizyme OD403/S were mixed with 40 ml of water-acetone (50/50 v/v) solution for 30 min; after that the solution was removed by vacuum-filtration and washed several times with water to remove acetone. The pre-treated support – i.e. Relizyme OD403/S – was then mixed with the supernatant lipase solution for 7 hours at 4°C under mild agitation. Then the immobilisation was stopped by vacuum-filtration, rinsing the

immobilised lipase with 200 ml of the initial buffer. Finally, the biocatalyst was dried to constant weight in a silica gel desiccator and stored at -20°C until use.

Lipase activity measurements were taken of the supernatant before and after immobilisation. Lipase activity per g of immobilised lipase was calculated as the ratio between the lipase activity disappearance of the supernatant before and after the immobilisation, and the weight of the amount of support used.

2.6. 1- and 2-monoolein preparation

75 mg immobilised ROL were added to 1 g triolein dissolved in 6 ml methyl *t*-butyl ether. 0.25 ml ethanol were initially added and 0.3 ml ethanol more after 45 min. The reaction was set at 37°C under 1000 rpm agitation in a thermoshaker for 6 hours. After that, the immobilised lipase was removed from the reaction medium by vacuum filtration and the biocatalyst was washed with 5 ml methyl *t*-butyl ether in order to recover all the 2-monoolein. The solvent was then evaporated from the reaction medium using a rotary evaporator with a water bath at 30°C. The substance left (mainly 2-monoolein, dioleins, triolein and ethyl oleate) was mixed and dissolved in 10 ml heptane and the mixture was set to -20°C overnight. After that 2-monoolein precipitated and it was separated from the other compounds and heptane by vacuum filtration, washing it with 10 ml pre-cooled heptane at -20°C. The remaining solvent in 2-monoolein was removed by rotary evaporator and stored at -20°C.

1-monoolein was synthesised by 2-monoolein acyl migration by adding 0.1 g silica gel and 3 ml heptane to 0.3 g 2-monoolein and setting the reaction at 70°C under 1000 rpm agitation in a thermoshaker for 24 hours. The silica was removed from the reaction medium by vacuum filtration and the solvent by rotary evaporator. 1-monoolein was stored at -20°C.

2.7. Transesterification experiments

All reactions were carried out in 4.5 ml glass vials in a thermoshaker at 37°C under 1000 rpm agitation. Duplicates of each experiment were conducted. Three types of transesterification experiments using triolein as substrate were done, varying lipase preparation and reactions systems: free rROL – i.e. liquid lipase solution in water/organic biphasic system –, immobilised rROL with added water – water/organic biphasic system – and immobilised rROL without water – organic system –. Reactions were prepared with 0.265 g triolein dissolved in 1.6 ml heptane (160 mM triolein). Different triolein to acyl acceptors molar ratios were studied, adding different amounts of ethanol and methanol at the beginning of the reaction. 0.05 g lyophilised powder rROL were dissolved in 1 ml water, centrifuged and 0.4 ml

of the supernatant were added to the reactions for free rROL catalysed reaction experiments. For the experiments with immobilised rROL, the same total lipase activity as in free rROL catalysis experiments was used, adding approximately 0.08 g of biocatalyst in each reaction; 0.4 ml water were added to some of them.

2.8. Alcoholysis experiments

All reactions were carried out in 4.5 ml glass vials in a thermoshaker at 37°C under 1000 rpm agitation. Duplicates of the experiments were performed. Two alcoholysis experiments were carried out in order to study lipase specificity towards ethanol and methanol. On the one hand, 0.266 g methyl oleate were dissolved in 1.6 ml heptane (480 mM methyl oleate) with 0.052 ml ethanol (480 mM ethanol) catalysed by 0.4 ml free rROL, which were prepared as in transesterification experiments. On the other hand, the same reaction was done, but using ethyl oleate as acyl donor and methanol as acyl acceptor.

2.9. Comparison recombinant/commercial lipase

All reactions were carried out in 4.5 ml glass vials in a thermoshaker at 37°C under 1000 rpm agitation. Duplicates of the experiments were done. Transesterification comparison reactions using triolein, 1-monoolein or 2-monolein as substrates were performed dissolving it in 1.6 ml heptane to an initial concentration of 100 mM. These experiments were catalysed with 0.1 ml of free rROL and free ROL solution preparations, prepared as previously described. Compared to the previous transesterification experiments described above, the initial substrate concentration and the amount of lipase liquid preparation used were reduced (100 instead of 160 mM and 0.1 instead of 0.4 ml, respectively) in order to avoid the formation of emulsions. 0.026 ml ethanol were added as acyl acceptor. In the case using 2-monoolein, a control reaction was set with the same conditions but without lipase, in order to quantify and discount the acyl migration from the results.

2.10. Sample preparation and GC analysis

Sample preparation and GC analysis were almost the same as previously described [33]. Oleic acid, methyl and ethyl oleate, 1- and 2-monoolein, 1,2- and 1,3-diolein and triolein concentrations in the samples were analysed in a 430-GC gas chromatograph with an auto-sampler CP-8400, both from Varian (Agilent Technologies Inc., Santa Clara, USA), equipped with a flame ionisation detector and a capillary column (CP8907 VF-1 ms 15 m x 0.25 mm x 0.25 µm) from Agilent Technologies. Helium was used as a carrier gas and the temperature of both detector and injector was 350°C. The starting oven temperature was 180°C; it was

maintained for 2.5 min; then the temperature was increased at 10°C/min to a temperature of 340°C and held for 26 min.

Samples were withdrawn at selected times from the organic phase of the reaction media and derivatised by silylation by adding an equal volume of MSHFBA and incubating them at room temperature for 30 min. An equal volume of ethanol was added afterwards to stop the silylation reaction. Samples were then diluted with a n-tetradecane solution (10 mM in hexane), used as internal standard.

3. Results and discussion

Fig. 1 shows the complete transesterification process from triolein to glycerol as a succession of single acylglycerol alcoholysis reactions (Fig. 1, reactions 1,2,3,6 and 7), catalysed by a non-regioselective lipase. However, the alcoholysis from 2-monoolein to glycerol (Fig. 1, reaction 3) is negligible when using 1,3-selective *R. oryzae* lipase, and thus acyl migration reactions (Fig. 1, reactions 4 and 5) are needed to reach all the way to glycerol. A corresponding reaction scheme could be drawn for hydrolysis with water and oleic acid replacing alcohol and alkyl oleate, respectively.

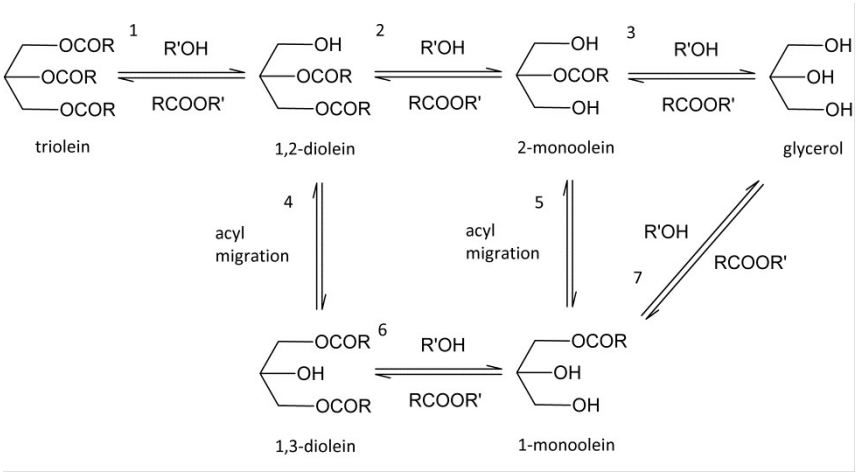

Figure 1

Complete alcoholysis of triolein to glycerol and free fatty acids (reactions 1, 2, 3, 6 and 7). Acyl migration phenomenon converts 1,2-diolein and 2-monoolein to 1,3-diolein and 1(3)-monoolein (reactions 4 and 5), respectively. If a 1,3-positional selective lipase is used, reaction 3 is negligible. Hydrolysis has the same reactions scheme, with water instead of alcohol and free fatty acids instead of alkyl esters.

3.1. Lipase specificity towards acyl acceptor

Although ethanol causes less lipase inactivation than methanol [1,15,34], the latter is still used the most in biodiesel synthesis, because of its low price and good accessibility [15]. Since both alcohols can be used, it is interesting to compare the lipase specificity towards ethanol and methanol. Two different alcoholysis experiments were carried out, between methyl oleate and ethanol and between ethyl oleate and methanol, both catalysed by free rROL in a water/heptane biphasic system. Higher initial reaction rates were achieved using ethanol – - 0.39 and 0.40 mM/min for methyl oleate consumption and ethyl oleate formation, respectively – compared to the ones when using methanol – 0.24 and -0.33 mM/min (Table 1). Altogether the results indicate that ethanol is a more efficient acyl acceptor in the alcoholysis reactions catalysed by rROL.

Table 1

Initial formation/consumption rates (mM/min)

Initial substrates	Methyl Oleate	Ethyl Oleate
Methyl oleate + ethanol	- 0.39±0.01	0.40±0.01
Ethyl oleate + methanol	0.24±0.03	- 0.33±0.05

3.2. Comparison between recombinant and commercial R. oryzae lipase

The expression of recombinant enzymes in suitable host microorganisms is a widely used method to reduce difficulties and costs in their production. However, the properties of a recombinant enzyme are not always identical to those of the wild type enzyme. In the present study, substrate specificities of the recombinant and commercial lipases were examined and compared in order to elucidate possible differences. Both lipases had higher specificity towards ethanol than methanol but the difference was considerably larger for the recombinant enzyme (Table 2).

Table 2

Initial rate ratios

Rhizopus oryzae lipase	Ethanol/methanol	1-monoolein/triolein
Recombinant	2.23±0.43	4.43±0.41
Commercial	1.55±0.47	2.82±0.95

Lipases are known to display significant differences in terms of acylglycerols specificities, regioselectivities and stereoselectivities [35,36]. In the present study, the consumption rates of 1-monoolein were 4.43 and 2.82 times faster than those of triolein for rROL and ROL (Table 2), respectively, which means that both enzymes are more specific for 1-monoolein than triolein, in agreement with the specificity of lipases from *C. antarctica B* and *R. miehei* but different from lipases from *R. arrhizus* and *T. lanuginosus* immobilised on polypropylene and used in methyl t-butyl ether without water addition [36]. Comparing recombinant and commercial *R. oryzae* lipase, it is clear that rROL is even more specific for 1-monoolein, which might be caused by differences in post-translational modifications of the producing host *P. pastoris* strain, as described in a previous report [29]. Importantly, both lipases expressed negligible activity in alcoholysis of 2-monoolein, since 2-monoolein

disappearance rates for both lipases were the same as in the control reaction without lipase and attributed to acyl migration. This shows that both lipases are highly 1,3-specific under these conditions.

3.3. Lipase preparation and reaction system comparison

Lipases in nature usually catalyse reactions at organic-aqueous interfaces and actually these enzymes display higher catalytic activity under such conditions than in homogeneous solution; this phenomenon is known as interfacial activation [37]. In biodiesel production, quite promising results have been obtained with the lipase dissolved in the aqueous phase of a biphasic system with the oil as organic phase [38,39]. Nevertheless, immobilisation is an attractive methodology to enhance lipase activity and facilitate lipase reuse [37]. Here, three different systems with different rROL preparations were studied: free rROL (biphasic system), immobilised rROL without added water (organic phase) and for comparison also immobilised rROL with 0.4 ml water added (biphasic system).

Initial reaction rates for biphasic systems increased slightly by increasing alcohol to triolein molar ratio, except for the case of free lipase with ethanol, which remained constant (Table 3). Comparing both biphasic systems, the initial rates were higher for free lipase regardless of alcohol type, most probably because the immobilised system added more mass transfer limitations – the particles – to the liquid/liquid ones – i.e. the interface between organic and aqueous phases –. However, the highest rates were achieved for reaction systems catalysed by immobilised rROL without added water, being around 20 times higher than those of biphasic systems for both alcohols. This suggests that for the immobilised lipase no liquid/liquid interface is necessary to achieve high lipase alcoholysis activity.

Table 3

Initial rate of alkyl ester formation in triolein transesterification (mM /min)

System	Ethanol to triolein molar ratio				
	1	2	3	4.5	6
Free enzyme – biphasic			1.1±0.1	1.0±0.1	1.0±0.0
Immobilised enzyme – biphasic			0.2±0.1	0.4±0.1	0.5±0.1
Immobilised enzyme – organic	21.3±0.5	24.1±0.7	22.1±1.0	15.7±0.0	10.0±0.4
System	Methanol to triolein molar ratio				
	1	2	3	4.5	6
Free enzyme – biphasic			0.5±0.1	0.6±0.0	0.8±0.1
Immobilised enzyme – biphasic			0.3±0.1	0.3±0.0	0.4±0.1
Immobilised enzyme – organic	22.7±1.0	21.0±1.8	9.5±0.7	1.2±0.2	0.3±0.1

Another important result from the organic phase reactions is that reaction rates decreased dramatically when the alcohol to triolein molar ratio was increased from 3 to 6, especially in the case of methanol, where the initial reaction rate decreased from 9.5 to 0.3 mM/min. This might be explained by the fact that adding more alcohol to the system generated a separate alcohol phase which caused enzyme inactivation. In the biphasic systems, the alcohol was diluted in the aqueous phase, and thereby its inactivating effect was decreased. In all three reaction systems the reaction rates were higher when ethanol was used as acyl acceptor, in agreement with previous results (Table 2).

Product compositions in terms of oleic acid equivalents for the three systems at the end of the reaction are shown in Figs. 2, 3 and 4, respectively. Even though in both biphasic systems there was water, little oleic acid was formed compared to alkyl esters, and some hydrolysis occurred even in the reactions in organic systems (Fig. 4), probably because there was some remaining water inside the immobilised lipase.

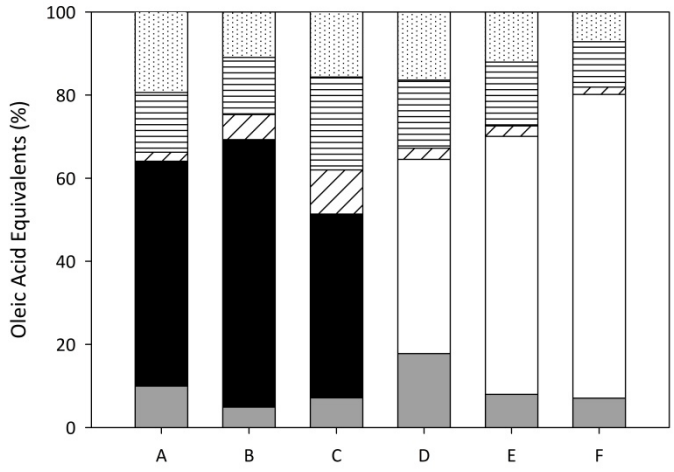

Figure 2

Product composition after 24 hours. Alcoholysis of triolein catalysed by 0.4 ml free rROL formulation, using 0.265 g triolein in 1.6 ml heptane and different ethanol and methanol to triolein molar ratios at 307C and 1000 rpm agitation. Legend: A=3 ethanol to triolein molar ratio, B=4.5 ethanol to triolein molar ratio, C=6 ethanol to triolein molar ratio, D=3 methanol to triolein molar ratio, E=4.5 methanol to triolein molar ratio and F=6 methanol to triolein molar ratio. Legend bars: Grey bar=oleic acid, Black bar=ethyl oleate, White bar=methyl oleate, Diagonal-stripped bar=monooleins, Stripped bar=dioleins and Dot bar=triolein.

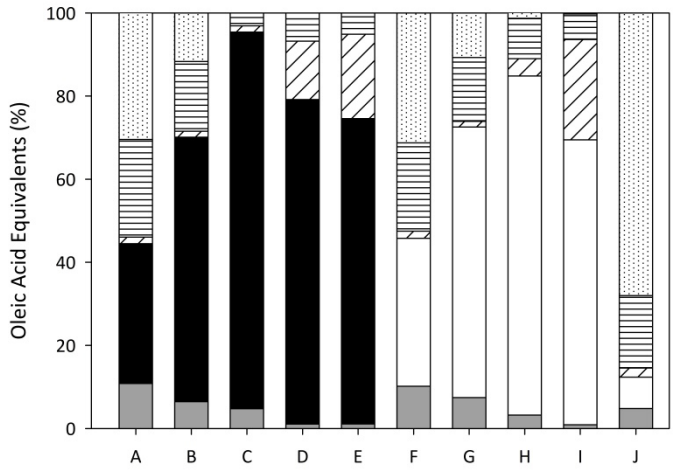

Figure 3

Product composition after 24 hours. Alcoholysis of triolein catalysed by immobilised rROL, using 0.265 g triolein in 1.6 ml heptane, 0.4 ml water and different ethanol and methanol to triolein molar ratios at

37°C and 1000 rpm agitation. Legend: A=3 ethanol to triolein molar ratio, B=4.5 ethanol to triolein molar ratio, C=6 ethanol to triolein molar ratio, D=3 methanol to triolein molar ratio, E=4.5 methanol to triolein molar ratio and F=6 methanol to triolein molar ratio. Legend bars: Grey bar=oleic acid, Black bar=ethyl oleate, White bar=methyl oleate, Diagonal-stripped bar=monooleins, Stripped bar=dioleins and Dot bar=triolein.

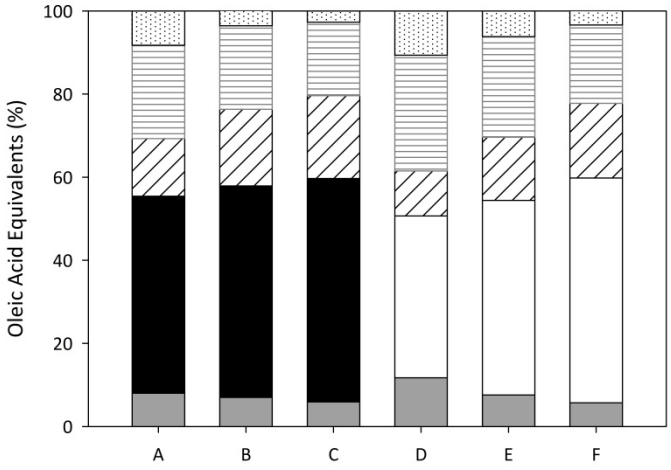

Figure 4

Product composition after 24 hours. Alcoholysis of triolein catalysed by immobilised rROL, using 0.265 g triolein in 1.6 ml heptane and different ethanol and methanol to triolein molar ratios at 37°C and 1000 rpm agitation. Legend: A=1 ethanol to triolein molar ratio, B=2 ethanol to triolein molar ratio, C=3 ethanol to triolein molar ratio, D=4.5 ethanol to triolein molar ratio, E=6 ethanol to triolein molar ratio, F=1 methanol to triolein molar ratio, G=2 methanol to triolein molar ratio, H=3 methanol to triolein molar ratio, I=4.5 methanol to triolein molar ratio and J=6 methanol to triolein molar ratio. Legend bars: Grey bar=oleic acid, Black bar=ethyl oleate, White bar=methyl oleate, Diagonal-stripped bar=monooleins, Stripped bar=dioleins and Dot bar=triolein.

The reaction system with water catalysed by immobilised rROL ended with higher contents of monooleins (Fig. 3), compared to those obtained for free lipase systems (Fig. 2), most probably due to mass transfer limitations, as previously discussed, which also explain the low initial reaction rates achieved by this system (Table 3). A molar ratio methanol:triolein of 6 inactivated the immobilised rROL in the organic system, as the high amount of unreacted triolein shows (Fig. 4).

In agreement with the observed initial reactions rates, yields for biphasic systems increased by increasing alcohol to triolein ratio, except for the case of free lipase with ethanol, in which ethanol induced inactivation seemed to occur (Table 4). The yields achieved in the systems without added water were higher compared to the ones of the two biphasic systems, reaching 100 and 82.6% yields for ethanol and methanol, respectively (Table 4).

Table 4

Alkyl oleate yield (%)

System	Ethanol to triolein molar ratio				
	1	2	3	4.5	6
Free enzyme – biphasic			57.2±2.0	64.2±2.3	51.3±1.9
Immobilised enzyme – biphasic			50.0±0.8	52.3±5.0	54.0±2.3
Immobilised enzyme – organic	38.4±1.3	67.9±1.1	100.7±7.0	81.2±0.8	75.6±1.1
System	Methanol to triolein molar ratio				
	1	2	3	4.5	6
Free enzyme – biphasic			45.9±1.7	61.0±2.9	71.2±2.5
Immobilised enzyme – biphasic			38.2±8.0	46.3±0.5	51.1±2.3
Immobilised enzyme – organic	40.5±1.3	63.3±1.7	82.6±1.7	68.6±1.5	7.8±4.8

Bearing in mind the 1,3-positional selectivity of the *R. oryzae* lipase, yields over 66.7% should not be expected. However, higher yield values were achieved, especially for reactions with immobilised enzyme without added water, because acyl migration allows the conversion of 2-monoolein to 1-monoolein, which can be easily used by rROL to produce glycerol. Analysis of glycerol in the reaction systems is difficult, so to further monitor the occurrence of these reactions, an acylglycerol balance was set up for each experiment, consisting of the sum of triolein, 1,2- and 1,3-diolein and 2- and 1-monoolein. This total amount of acylglycerols should be constant if further conversion to glycerol does not occur (reactions 3 and 7 in Fig. 1). On the other hand, if acyl migration occurs, rROL can alcoholyse 1-monoolein, thereby decreasing the total acylglycerol concentration. It can be concluded that acyl migration occurred in nearly all reactions, reaching 10.0 and 28.5% remaining acylglycerols for ethanol and methanol, respectively, in systems without added water at a molar ratio of 3 (Table 5). One important aspect is that, despite achieving 81.2, 75.6 and 68.6% yields (Table 4) in the reactions with immobilised enzyme without added water for alcohol to triolein ratios of 4.5 and 6 for ethanol and 4.5 for methanol, respectively, the acylglycerols balance values were still high – 54.0, 69.9

and 81.2%, respectively – (Table 5). This observation is also in agreement with the product compositions of these experiments (Fig. 4), where the monoolein concentrations were higher than in other cases, meaning that only limited acyl migration had occurred, in contrast to the fast acyl migration when a molar ratio of 3 was used. These results agree with the ones found by Li et al. [27], who stated that increasing the polarity in the solvent phase decreased the acyl migration rate. So, the greater the amount of alcohol used, the higher the polarity in the reaction system and the slower the acyl migration. This phenomenon is observed in Figs. 5 and 6, which correspond to the first 3 hours' evolution of the reactions catalysed by immobilised rROL in organic system when 3 and 6 alcohol to triolein molar ratios were used, respectively. In the case of the lowest alcohol to triolein molar ratio used (Fig. 5), 2-monoolein reached a maximum concentration at 30 minutes and started to decrease as a consequence of acyl migration, while in the highest alcohol molar ratio case (Fig. 6), 2-monoolein reached a maximum concentration at 60 minutes and remained almost constant.

Table 5

Ratio: Final Acylgycerols / Initial Acylglycerols (%)

System	Ethanol to triolein molar ratio				
	1	2	3	4.5	6
Free enzyme – biphasic			50.3±1.7	49.7±8.9	94.2±5.9
Immobilised enzyme – biphasic			87.4±2.6	90.5±7.0	88.6±4.2
Immobilised enzyme – organic	79.6±2.7	43.5±1.8	10.0±2.3	54.0±1.9	69.9±2.2
System	Methanol to triolein molar ratio				
	1	2	3	4.5	6
Free enzyme – biphasic			48.4±4.0	42.1±3.2	28.2±2.6
Immobilised enzyme – biphasic			82.4±9.2	86.3±4.4	83.3±9.2
Immobilised enzyme – organic	77.0±1.7	36.5±0.9	28.5±1.1	81.2±2.5	103.2±9.7

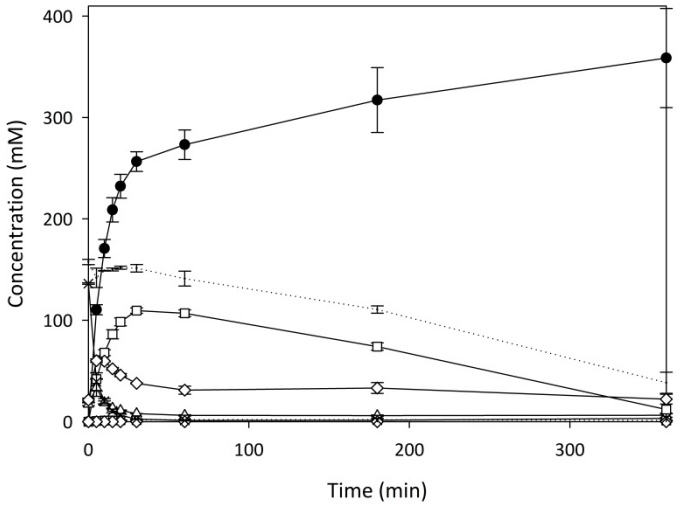

Figure 5

Compounds evolution in triolein alcoholysis catalysed by immobilised rROL with 3 ethanol to triolein molar ratio at 37°C and 1000 rpm. Legend: ●=ethyl oleate, Δ=free oleic acid, □=monoolein (continuous line=1-monoolein, dot line=2-monoolein), ◊=diolein (continuous line=1,2-diolein, dot line = 1,3-diolein), x = triolein, dot line=all acylglycerols (the sum of 1- and 2-monolein, 1,2- and 1,3-diolein and triolein)

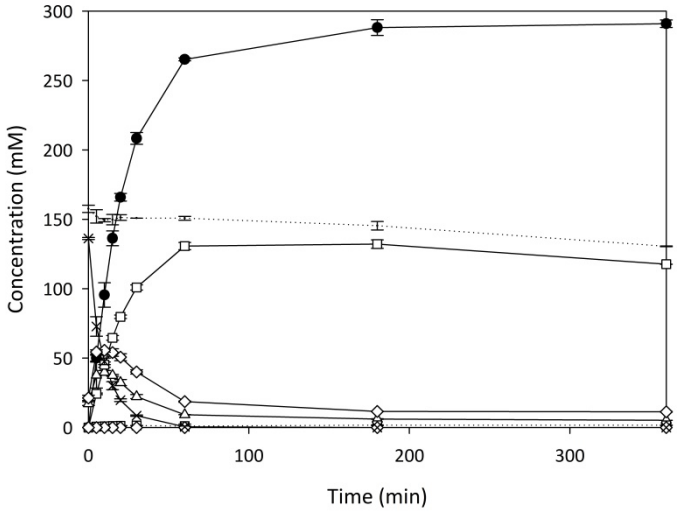

Figure 6

Compounds evolution in triolein alcoholysis catalysed by immobilised rROL with 6 ethanol to triolein molar ratio at 37°C and 1000 rpm. Legend: ●=ethyl oleate, Δ=free oleic acid, □=monoolein (continuous line=1-monoolein, dot line=2-monoolein), ◊=diolein (continuous line=1,2-diolein, dot line = 1,3-diolein), x = triolein, dot line=all acylglycerols (the sum of 1- and 2-monolein, 1,2- and 1,3-diolein and triolein)

Monoolein and diolein represented in Figs. 2, 3 and 4 are the sum of the isomers 1- and 2-monoolein, and 1,2- and 1,3- diolein, respectively. In all cases (except when using an alcohol to triolein molar ratio of 1 and 2 described in the next section), 2-monoolein was over 90% of the total monoolein and 1,2-diolein almost 100% of the total diolein (data not shown). This shows that the lipase-catalysed alcoholysis of the acylglycerols formed by acyl migration is fast enough to prevent building up high concentrations of them.

3.4. Transesterification at small alcohol to triolein molar ratios

The highest initial reaction rates were observed at alcohol to triolein molar ratio of 3 and were 22.1 and 9.5 mM/min for ethanol and methanol, respectively (Table 3). Therefore, even lower molar ratios were tried to test whether even faster rates could be achieved. It was discovered that in the case of ethanol the decrease in the molar ratio did not enhance the initial reaction rate (Table 3). In contrast, when the methanol to triolein molar ratio was reduced from 3 to 2, the initial rate was more than double, increasing from 9.5 to 21.0 mM/min, but it remained nearly constant when the molar ratio was further reduced to 1. Thus, the maximal initial reactions rates are nearly the same for both alcohols.

Figs. 7 and 8 show the time course of the reactions with a molar ratio of alcohol to triolein of 1 for both alcohols. In the reaction with ethanol, triolein was initially consumed to a concentration of 38 mM after 15 minutes, when its concentration started to increase to reach around 60 mM (Fig. 7). During the first 15 minutes rapid conversion of triolein and alcohol to alkyl ester, 1,2-diolein and 2-monoolein occurred. In this period a major part of the ethanol was consumed. Between 15 and 180 minutes acyl migration was prominent, directly seen as the formation of 1,3-diolein and to some extent 1-monoolein (Fig. 1, reactions 4 and 5). Most of the 1-monoolein formed was, however, converted rapidly to glycerol and ethyl oleate (Fig. 1, reaction 7) as indicated by the reduction in total acylglycerols. No accumulation of 1-monoolein was observed, because of the high lipase specificity towards this substrate as previously concluded, so lipase alcoholises it rapidly. Since the ethanol concentration was low during this period, ethanol necessary for the 1-monoolein alcoholysis must be obtained somehow from another reaction: 1,2-diolein reacted with ethyl oleate with the formation of triolein and ethanol (Fig. 1, reaction 1 backwards). This phenomenon is shown by the increase in triolein and decrease in 1,2-diolein occurring in parallel with the decrease in total acylglycerols (due to the formation of glycerol). After 180 minutes, acyl migration continued while otherwise only minor net changes were observed. Qualitatively the same behaviour was observed when using a methanol to triolein molar ratio of 1 (Fig. 8). Furthermore, these

phenomena also occurred when an alcohol to triolein molar ratio of 2 was used (data not shown). In contrast, when molar ratios of alcohol to triolein of 3, 4.5 and 6 were used this phenomenon did not take place (Figs. 5 and 6 for 3 and 6 ethanol to triolein molar ratios; other data not shown), because there was enough acyl acceptor (alcohol) for all the fatty acids in the triolein substrate.

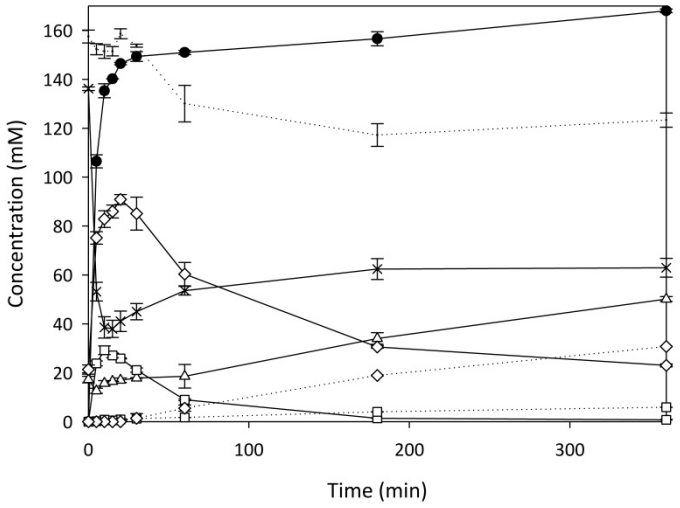

Figure 7

Compounds evolution in triolein alcoholysis catalysed by immobilised rROL with 1 ethanol to triolein molar ratio at 37°C and 1000 rpm. Legend: ●=ethyl oleate, Δ=free oleic acid, □=monoolein (continuous line=1-monoolein, dot line=2-monoolein), ◊=diolein (continuous line=1,2-diolein, dot line = 1,3-diolein), x = triolein, dot line=all acylglycerols (the sum of 1- and 2-monolein, 1,2- and 1,3-diolein and triolein)

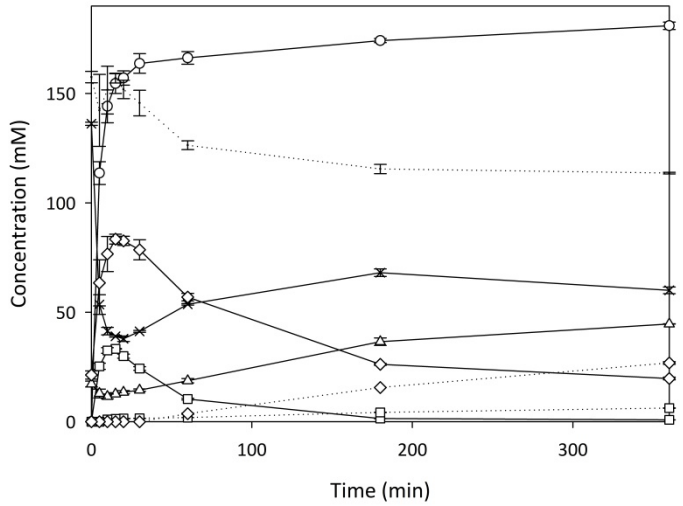

Figure 8

Compounds evolution in triolein alcoholysis catalysed by immobilised rROL with 1 methanol to triolein molar ratio at 37°C and 1000 rpm. Legend: ○=methyl oleate, Δ=free oleic acid, □=monoolein (continuous line=1-monoolein, dot line=2-monoolein), ◊=diolein (continuous line=1,2-diolein, dot line = 1,3-diolein), x = triolein, dot line=all acylglycerols (the sum of 1- and 2-monolein, 1,2- and 1,3-diolein and triolein)

4. Conclusions

This present work showed that recombinant and commercial *R. oryzae* 1,3-positional selective lipases display higher specificities towards 1-monoolein than triolein, being 4.43 and 2.82 times bigger, respectively. This difference between the two lipases might be caused by differences in post-translational modifications of the producing host *P. pastoris* strain. Both lipases showed to be slightly more specific for ethanol than methanol, and methanol had a bigger lipase inactivation effect.

A comparison between the use of immobilised recombinant lipase and free lipase liquid preparations was carried out, showing important differences. Immobilised rROL in a system without water added achieved more than 20-fold higher initial alcoholysis reactions rates, which indicated that no interfacial activation is necessary to achieve high lipase alcoholysis activities. Furthermore, the highest yields were also obtained in the case of immobilised lipase without added water. Acyl migration was shown to occur especially at lower alcohol to triolein molar ratios, making it possible to reach yields higher than the

expected 66.7% for a 1,3-specific lipase. Higher alcohol concentrations reduced the acyl migration rates and thereby the biodiesel yield.

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