# UAB

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# Enzymatic synthesis of biodiesel from high free fatty acid feedstock using a recombinant *Rhizopus oryzae* lipase

Memòria per optar al Grau de Doctor per la Universitat Autònoma de Barcelona

dins el Programa de Doctorat en Biotecnologia, sota la direcció dels doctors

## Maria Dolors Benaiges Massa i Francisco Valero Barranco

**Kírian Bonet Ragel**

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Maria Dolors Benaiges Massa, Professora titular, i Francisco Valero Barranco, Catedràtic, membres del Departament d'Enginyeria Química, Biològica i Ambiental de la Universitat Autònoma de Barcelona,

CERTIFIQUEN:

que el biotecnòleg Kírian Bonet Ragel 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 "Enzymatic synthesis of biodiesel from high free fatty acid feedstock using a 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 18 d'abril de 2018.

Dra. Maria Dolors Benaiges Massa               Dr. Francisco Valero Barranco

# AGRAÏMENTS

En aquest sentit sóc de poques paraules, però segur que tothom que ho llegeixi se sentirà d'alguna manera partícip d'aquesta tesi.

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The present thesis is focused, in general terms, on the enzymatic synthesis of biodiesel using a recombinant *Rhizopus oryzae* lipase (rROL), expressed in a methylotrophic yeast (*Pichia pastoris*) as a cell factory and immobilised onto a polymethacrylate support. The main feature of this enzyme is its regioespecificity, which allows to catalyse the alcoholysis of *sn*-1 and *sn*-3 ester bonds of the triglyceride into two fatty acid alkyl esters (biodiesel). The use of rROL becomes a key factor since glycerol is not formed as a by-product in favour of 2-monoglyceride.

The first part of the thesis is focused on the evaluation of a novel feedstock as a substrate for biodiesel production. Alperujo oil is a vegetable oil, which can be representative of other high-FFA feedstocks, considered waste-oils. Preliminary studies are performed to find out the role of this FFA in terms of initial reaction rate and stability of the rROL. Moreover, the enzyme has been immobilised by covalent binding to ensure its stability and recovery.

During the following parts, emphasis is put on the improvement of the enzymatic reaction itself using 10-mL vials. Temperature and initial water activity are set up in order to increase initial rate and enzyme stability. In addition, the two most used acyl-acceptors are compared. Methanol and ethanol are added using three stepwise strategies: one, five and ten pulses. Stability and productivity are also compared in order to find out the best one.

Then, scale up to a 50-mL stirred-reactor is carried out by reproducing the previous experiments. Initial rates and stability are compared. Further analysis allowed to calculate enzyme's half-life times and productivities in the different reactions. Semi-continuous addition of the acyl-acceptor which best results were obtained with, is attempted by using an automatised micro-burette.

Last chapter is focused on the simulation of an industrial process of enzymatic production of biodiesel using all the previous obtained results. Specific software (SuperPro Designer®) is used to raise and develop a process to produce and purify the biodiesel as well as its by-product (2-monoglyceride). Then, viability studies are performed and some modifications are suggested in order to find out a profitable and feasible process.

La tesi que es presenta a continuació està encarada, en termes generals, en la utilització d'una lipasa recombinant de *Rhizopus oryzae* (rROL) expressada en un llevat metilotròfic (*Picchia pastoris*) i immobilitzada en un suport de polimetacrilat, per a la síntesi enzimàtica de biodièsel. La característica més important d'aquest enzim és la seva regió-especificitat, que permet catalitzar l'alcohòlisi dels enllaços èsters *sn*-1 i *sn*-3 del triglicèrid en dos alquil-èsters d'àcid gras (biodièsel). La utilització de la rROL esdevé un punt clau per al procés ja que no es forma glicerol, sinó 2-monoglicèrid, un producte de valor afegit.

La primera part de la tesi està enfocada en l'avaluació d'una nova matèria primera com a substrat per a la producció de biodièsel. L'oli d'*orujo* (o de pinyolada) és un oli vegetal que pot ser representatiu d'olis amb alt contingut en àcids grassos lliures, com la majoria d'olis de rebuig. Així doncs, es realitzen estudis preliminars per conèixer el rol d'aquests àcids grassos en termes de velocitat inicial de reacció i estabilitat de la rROL. D'altra banda, es procedeix a la immobilització de l'enzim per enllaç covalent per tal d'assegurar-ne l'estabilitat i reutilització.

Després, l'èmfasi es posa sobretot en la millora de la reacció en vials de 10 mL. Per tal de millorar la velocitat inicial de reacció i l'estabilitat de l'enzim, es realitzen proves a diferents temperatures i activitat inicial d'aigua. També es compara la forma d'addicionar els dos acceptors d'acil més utilitzats actualment, el metanol i l'etanol, en reaccions d'un, cinc i deu polsos. Després, es compara l'estabilitat i es calculen les productivitats per tal de trobar el més adequat.

Seguidament es duu a terme el canvi d'escala del procés a través d'un reactor de 50 mL reproduint els experiments anteriors. Estudis posteriors permeten calcular la vida mitja de l'enzim en les diferents reaccions, com també les productivitats. Finalment, s'addiciona de forma semi-continua, per mitjà d'una micro-bureta automatitzada, l'acceptor d'acil amb el que s'obtenen els millors resultats.

L'últim capítol es basa en la simulació d'un procés industrial per a la síntesi enzimàtica de biodièsel amb els resultats prèviament obtinguts. Es planteja un procés per a la producció i la purificació tant del biodièsel com del subproducte, 2-monoglicèrid i s'utilitza un software específic (SuperPro Designer®) per al desenvolupament del projecte. Se n'estudia la viabilitat i els canvis suggerits per aconseguir la seva rendibilitat.

# INTRODUCTION

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# 1. INTRODUCTION content

## 1.1. Enzymes and catalysis

In chemical terms, catalysis *is the acceleration of a chemical reaction by a catalyst* [1]. Specifically, it means the reduction of the necessary *activation energy* to convert a substrate to a product. This action is carried out by a component – catalyst – that does not change the extent nor the equilibrium of the reaction. Ideally, catalyst must be present in the same initial conditions at the end of the reaction. However, they are not consumed, they can be altered and reduced along their use so they cannot be used indefinitely.

Enzymes are a well-known kind among lots of catalyst types that are responsible for carrying out biocatalytic reactions, thus they are also referred as biocatalyst. They are crucial for life and evolution, as they perform a huge number of reactions for cell metabolism.
Not only enzymes have an important role on life but also most of the processes and materials: fermentation of foods and beverages, improvement of detergents, textile bleaching, etc [2]–[4].

First appearance of term *enzyme* was during the second half of the 19th century, when Wilhelm Kühne wrote in German about "the unformed or not organized ferments, whose action can occur without the presence of organisms and outside of the same must be called enzymes." However, the first enzyme discovery reported but without using its scientific term was in 1833 by French chemist Payen, who described the reactions and industrial applications of the diastase.

Enzymes are polypeptides, formed essentially by a primary structure or sequence of amino acid residues. Each primary structure is organised in three-dimensional forms as strands or coils called the secondary structure. Again, these forms are rearranged in another three-dimensional structure – tertiary –, which finally give the shape of the protein. Some complex enzymes are assembled by subunits of tertiary structures called monomers forming a large enzyme that could be functional as well.

There is a wide range of enzymes presents, considering that they are formed by the 20 amino acids: the possible primary structure could be described as $20^n$. Although they can be very huge molecules, the most important part of an enzyme is the active site, which is in fact, where the reaction takes place. Enzymes hold their active site in a very little space, compared with the entire protein, formed by three unique amino acids called the catalytic triad. Studies have shown that the major part of hydrolases and transferases such as lipases [5], proteases [6] or peptidases

[7] have this triad. Nevertheless, classification of this huge variety of enzymes is not especially done considering the active site but the reaction that carries out on it. There are six big families differing between each other in the reaction that they catalyse. Table 1.1. shows the given name for each type by the Enzyme Commission (EC) [8].

**Table 1.1.** Enzyme families classified by Enzyme Commission

| Class | Name | Reaction catalysed | Example |
|-------|------|--------------------|---------|
| EC 1 | Oxidoreductases | To catalyse oxidation/reduction. | Oxidase |
| EC 2 | Transferases | To transfer a functional group from one substance to another. | Transaminase |
| EC 3 | Hydrolases | Formation of two products from one substrate. | Lipase |
| EC 4 | Lyases | Non-hydrolytic addition or removal of groups from substrates. | Decarboxylase |
| EC 5 | Isomerases | Intramolecular rearrangement. | Isomerase |
| EC 6 | Ligases | To join two molecules by synthesis of new C-O, C-S, C-N or C-C bonds. | Synthetase |

Moreover, in the last decades, since new biotechnological tools have been developed – DNA sequencing, protein modification, gene expression, etc –, the use of enzymes has also experimented an increase. These modifications and improvements have implied the acceptance of unnatural substrates for most of the enzymes, thus intensifying the range of possible reactions.

As mentioned above, this vast variety of biocatalysts and reactions has been used since first enzyme was discovered. Their excellent properties - listed in Table 1.2. - make these molecules perfect to handle a wide variety of processes that implies the production of goods such as food industry, bulk chemicals, drugs and pharmaceutical components, environmental technologies, clothing manufacturing and combustibles among others [9]–[11] reaching a global demand of $8 billion in 2017 [12].

**Table 1.2.** Enzyme features classified in advantages and disadvantages.

| Advantages | Disadvantages |
|---|---|
| Very high enantioselectivity | Low specific activity compared with chemical catalysis |
| Very high regioselectivity | Availability for selected reactions only |
| Active under mild conditions | Inactivation by high temperatures, pH or aggressive solvents |
| Fewer by-products | Long development times for new enzymes |
| Can be degraded biologically | Expensive and co-factor requirements |

## 1.2. Lipases

### 1.2.1. Lipase definition and activity

Lipases – triacylglycerol ester hydrolases – are hydrolytic enzymes (EC 3.1.1.3). Their natural function is to catalyse the hydrolysis of ester bonds of a triacylglycerol backbone into fatty acids (Fig. 1.1.), but they can also perform a great number of reactions depending on the reaction medium and conditions. These great features and protein engineering progresses have made lipases to become one of the most used enzymes by industry – dairy, baking, paper, oil, pharmaceutical, etc. [13] –. Although they represented no more than 4% of the global enzyme market on 1989 [14], nowadays they represent besides carbohydrases and proteases, the 70% of all enzyme sales [15].
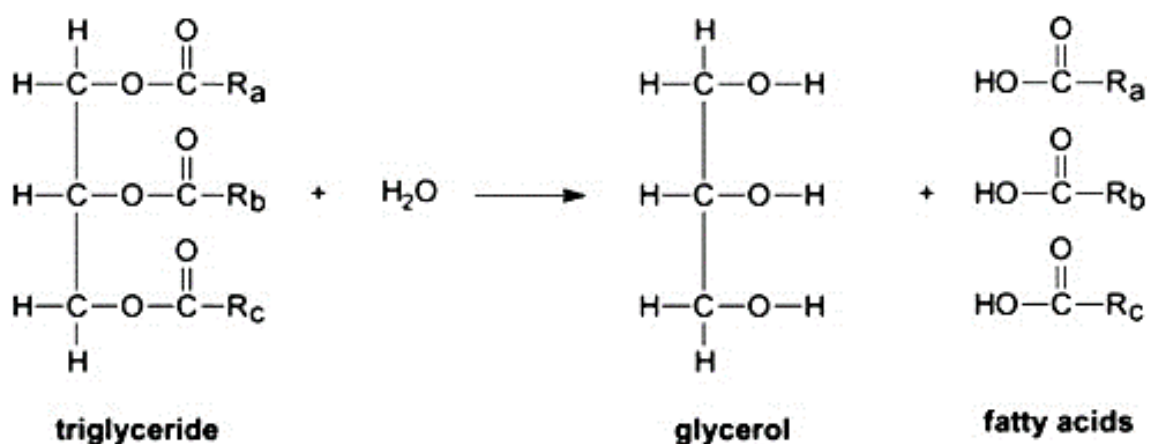


**Figure 1.1.** Natural reaction of a lipase: hydrolysis of triglyceride producing glycerol and fatty acids [16].

There are more than 400 types of lipases [17] classified according to the organism they belong to [18]–[22].

Another way to classify lipases is depending on their localisation, so they can be found in some common source tissues such as blood plasma, brain, kidney, lung among 280 others [17]. This huge number of localisations leads lipases to have many natural substrates, up to one hundred [17]. Even thought, as it was mentioned above, enzymes are characterised by their high substrate promiscuity [23], [24] which is also present in most of lipases. Thus, more than one thousand other reactions can be catalysed by these enzymes [17], [25].

Despite this promiscuity, it should be stated that exist different kinds of selectivities or specificities towards their substrates, which can be *regio-*, *chemio–*, and *enantioselectivity* [26].

Lipases exhibit different regiospecificity towards acylglycerols. They can be divided whether they catalyse the complete hydrolysis of the triacylglycerol molecule into glycerol and fatty acids or whether they prefer to hydrolyse only *sn*-1 and/or *sn*-3 positions on the glycerol backbone. In this case, it should be referred as positional-selective lipases or 1,3-lipases which are relevant especially in the manufacture of structured lipids [27]. However, there are different degrees of regioselectivity shown by each lipase. For instance, *Rhizopus niveus* lipase only shows 1,3-regiospecificity and does not show activity over *sn*-2 position, *Pseudomonas sp.* lipases display more activity in *sn*-1,3 than *sn*-2. Moreover, *Arthrobacter sp.* lipases show equal preference for both reactions, while *Candida antarctica A* lipase has demonstrated more predilection for position *sn*-2 than *sn*-1,3 [28]. Additionally, some lipases such as *Rhizopus miehei* and *Thermomyces lanuginosus* show less regioselectivity for monoacylglycerols than for triacylglycerols [29].

In terms of chemiospecificity, lipases can be further classified based on the differences in the subtrate that they act on – length and unsaturations –. Usually, lipases prefer fatty acids formed by chains of 4 to 18 carbons. For instance, *Candida rugosa* lipases are non-chemiospecific towards the length of the fatty acid, but it has been reported that has low activity towards long and polyunsaturated ones [30]. However, isoform 1 prefers chains from 8 to 10 carbons, isoform 2 and 4 acts on C16-18 fatty acids while isoform 3 does it on short fatty acids [2].

Finally, lipases show enantioselectivity, which make them enzymes capable to distinguish enantiomers in racemic mixtures [31], [32]. This feature is especially important in pharmaceutical field, performing racemic resolutions where enantiomers of a same product can show different activity [33].

### 1.2.2. Structure and features of lipases

Lipases show a wide variety of forms especially according to its organism. One of the smallest lipase can be found in a concrete *Rhizopus* strain (17.5 kDa) [34] while the largest one comes from *Candida rugosa* (about 60 kDa) [35]. From structural point of view, most of lipases are formed by α/β hydrolase fold [36]–[38].

Their catalytic triad is generally the same, consisting in a trypsin-like triad composed by three amino acids: Serine (Ser), Histidine (His) and Aspartic acid (Asp). It has been the focus of several studies to modify or improve lipase activity or other characteristics [6], [39], [40].

The most important feature of lipases is their capability to carry out reactions at organic-water interfaces. It is called *interfacial activation* and it has been reported that lipases even can show higher activities in these kind of medium than in aqueous solutions [41]. This property is not found among enzymes such as esterases, which only acts on molecules that are soluble in water [42] [Fig. 1.2.].
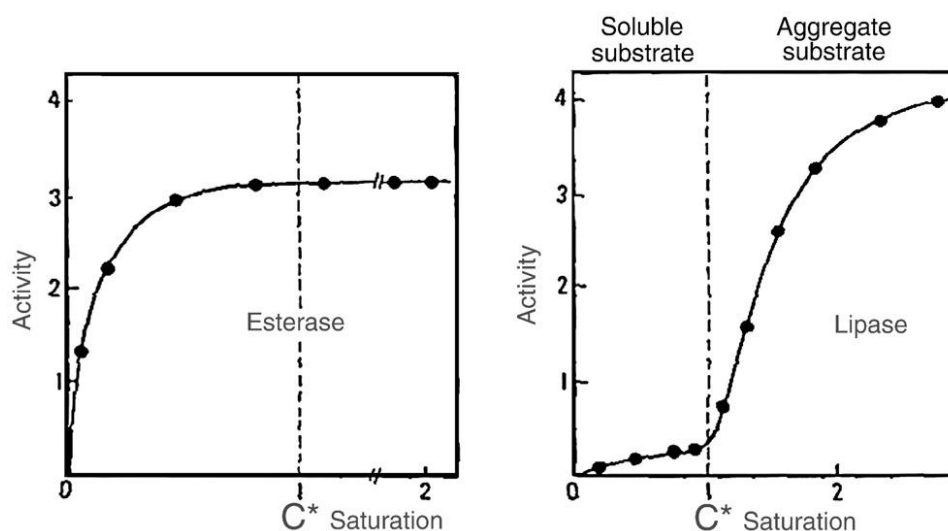


**Figure 1.2.** Graphical representation of the main catalytic difference between esterases and lipases. Esterases perform their high catalytic activity on water-soluble substrates in aqueous solutions, while lipases prefer organic-water interphases, due to their interfacial activation [43].

There is a well-known domain called *lid* or *flap* in the protein structure of the lipase that is the responsible for this unique characteristic. It is a very interesting part, evidence of that can be found in lots of studies that have focused on it. Technically, the lid is a stabilised and mobile amphipatic region that covers the active site of the lipase whose length, complexity and mobility depends on the type of the enzyme [44]–[46]. In contact with interface, the lid become displaced, exposing hydrophobic residues and revealing the catalytic site (Fig. 1.3.). Recent

studies, using different techniques such as enzyme immobilisation, mutagenesis in addition with computer simulations, have brought more information about the lid behaviour of some lipases [45], [47] and their role on substrate specificity and thermostability [48]. In some cases, the flap plasticity has been found to be enhanced depending on the substrate [49]. However, when this part is removed from some lipases like *Candida antarctica* A, interfacial activation was lost while stability, activity and stereoselectivity were retained in similar values in wild-type ones [46].
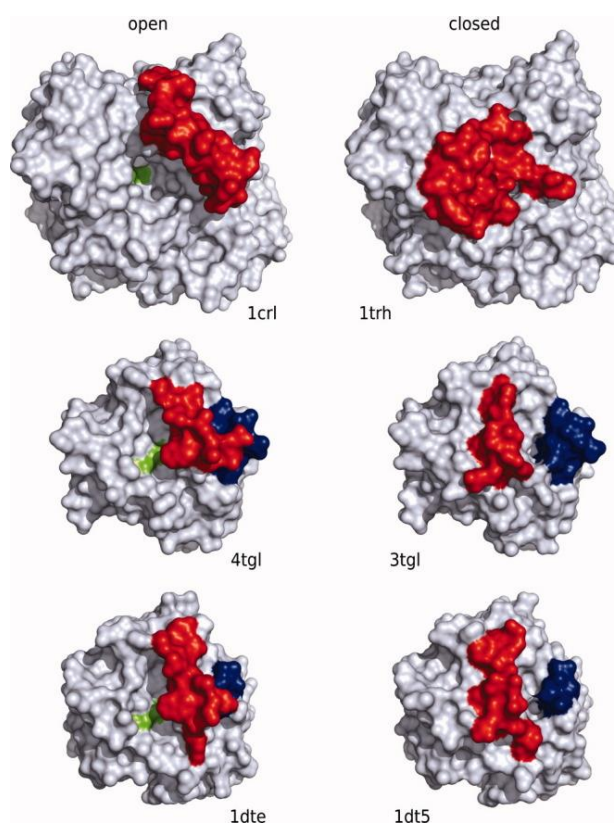


**Figure 1.3.** Overview of the open (left) and closed (right) structures of the lipases CRL (top), RML (middle), and TLL (bottom). The lid is marked in red, the catalytic triad in green, and the flexible loop in TLL and RML in blue [45].

### 1.2.3.　Lipase reactions

Lipases have evolved to catalyse cleavage of ester bonds through hydrolysis, but as any biochemical reaction, the reverse synthesis reaction also takes place on the molecular level.

Hydrolysis. As it is scientifically well known and as many studies stated, taking a closer view of the catalytic mechanism, the reaction occurs via bi-bi ping-pong mechanism [50]–[53]. First, nucleophilic attack on the carbonyl carbon of the ester bond takes place involving the hydroxyl residue of the triad serine, stabilised as well by aspartic acid and histidine. This first step yields

a covalent acyl-enzyme intermediate and an alcohol. Next step implicates a second nucleophilic attack on this intermediate carried out by a water molecule, forming the resulting carboxylic acid [54].

In addition, since lipases are stable in non-aqueous media, they can also catalyse other reactions using extra nucleophiles agents rather than water, like alcohols [55]. This variety of co-substrates extends the range of reactions beyond their natural one – e.g. transesterification, acidolysis, esterification, etc. – and makes lipases one of the most promiscuous enzymes present in nature [Fig 1.4.].
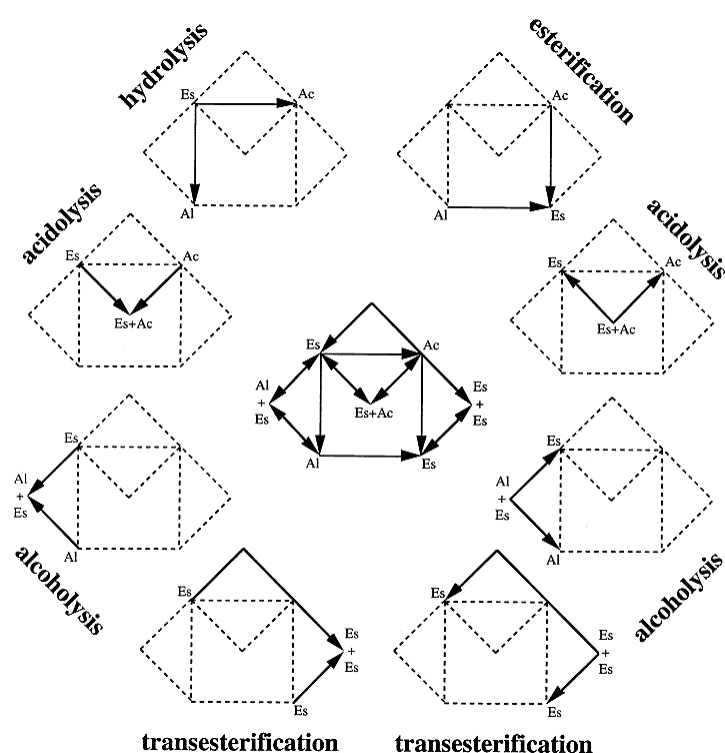


**Figure 1.4.** Wireframe of the lipase-catalysed reaction domain. *Es*, *Al* and Ac stand for esters, alcohols and acids, respectively. Reactants are depicted before the beginning of arrows, products are depicted after the end of arrows [56].

It should be noted that all such reactions are expressible as combinations of reversible hydrolyses of different reactants/products [56], [57]:

Transesterification. Here, the acyl group of an ester – or triacylglrlycerol – switches with the alkyl groups of a nucleophilic agent, usually an alcohol – therefore, called alcoholysis –. It is one of the most important and industrially used reaction, mainly in biodiesel production. As it is, this term will be used equally for biodiesel synthesis in this thesis from now on.

Interesterification. In this case, two ester molecules exchange their alkyl group from each other. Both moieties act as nucleophile and acyl acceptor.

<u>Esterification.</u> An alkyl group of a nucleophilic agent attacks the hydroxyl radical of an organic acid. The resulting product is an ester. Here in this thesis, the synthesis of biodiesel through free fatty acids and alcohols will be referred as direct esterification.

<u>Acidolyisis.</u> Like in interesterification reaction, an exchange of groups takes place. Here, an ester group is switched with the alkyl moiety of an acid.

<u>Aminolysis.</u> This reaction is not a common one among lipases. In this case, carboxylic esters are converted to the corresponding carboxylic amides [58], [59].

### 1.2.4. Lipase sources, recombinant expression and protein engineering

The raising of industrial use of lipases, has extended the range of its commercial availability either obtaining them from their natural source or also from other kind of hosts. As said before, one can found lipases in each of the five living being superkingdoms, but the most commonly used lipases come from animals, fungi and bacteria [2], [60]–[62].

There are many companies selling preparations of lipases for their use at lab or industrial scale. Table 1.3. shows a compilation of some of the most important commercial lipases classified according to the organism.

The expression and subsequent purification and formulation of the lipase from its original organism may generate some drawbacks for enzyme companies, especially in terms of industrial production due to some problems. Major complications come from organism growing, which can be complex and slow as well as from purification or extraction, leading to lowering the yield of production. In these cases, the most common purpose to overcome these constraints is the recombinant expression of the enzyme using more useful or easier to handle organisms. Taking in advantage all the biotechnological progresses up to date, including DNA sequencing, protein engineering and high technology implemented in bioprocess engineering, enzyme companies have adapted their production developments into easier and cheaper ones [63], [64].

Instead of purifying the final enzyme from the organism, another existing tool is the whole-cell biocatalyst that means using the entire organism with the target enzyme inside as a catalyst. Comparing with purified enzyme, this strategy can overcome problems of loss of stability and damage from external environment [26], [65]. Furthermore, because of last developments on proteomics, another promising methodology is the expression of these enzymes on the surface of the cell thus avoiding mass transfer bottlenecks in the membrane or toxic substrates inside

the cell [66]. However, in the case of lipases this kind of strategy has one major drawback. In order to reach high levels of protein using the native organism, lipids as a carbon source are needed but most of them are aerobic ones, so the medium required for their grow becomes more complex [67].

**Table 1.3.** Most used commercial lipases, their trade name and activity [13], [68], [69].

| Source | Organism | Trade Name | Manufacter | Activity |
|---|---|---|---|---|
| Bacterial | *Pseudomonas aerugionas* | Lipase LIP | Toyobo™ | >500 U/g |
| | *Pseudomonas fluorescens* | Lipase AK | Amano™ | >18,500 U/g |
| | *Burkholderia cepacia* | Lipase PS | | >30,000 U/g |
| | *Burkholderia cepacia* | Lipase SL | Meito™ | >60,000 U/g |
| | *Alcaligenes sp.* | Lipase PLC | | >30,000 U/g |
| | *Pseudomonas stutzeri* | Lipase TL | | >50,000 U/g |
| Fungal | *Candida antarctica* | Novozym 435 | Novozymes™ | >10000 PLU/g |
| | *Thermomyces lanuginosus* | Novozym TL IM | | >20,000 U/g |
| | *Candida cylindracea* | Lipase MY | Meito™ | >30,000 U/g |
| | *Candida rugosa* | Lipase Type VII | | >700 U/mg |
| | *Mucor javanicus* | Lipase M | Amano™ | >10,000 U/g |
| | *Rhizopus oryzae* | Lipase F-AP15 | | >150,000 U/g |
| | *Apergillus niger* | Lipase A | | >50,000 U/g |
| Animal | *Sus scofa (pig)* | Lipomod 224P (Type II) | Biocatalyst™ | 100-400 U/g |

Thus, heterologous expression of lipases, combining protein engineering and modernisation of biochemical processes, have raised as a promising strategy in the last decades. If we look in the literature, one will see that a huge variety of lipases are produced using this technique nowadays. For instance, *Thermomyces lanuginosus* lipase is produced on a large scale – about 100 tons per year – by non-native organism, as it is the *Aspergillus oryzae* fungus [57]. There are two general categories for lipase heterologous expression: prokaryotic and eukaryotic. In the

case of prokaryotic, bacteria *Escherichia* coli stood out for the most used organism [70]–[73] due to some advantages such as high-cell densities, short grow, it is a well-known organism with lots of engineering tools as well as metabolic information available. In contrast, eukaryotic hosts have become preferable ones because of their ability to perform post-translational modifications among other advantages. For instance, *Saccharomyces cerevisiae* has been the first yeast used for heterologous protein expression taking advantage on the large amount of information about its genetics and physiology and its classification as GRAS organism [67], [74].

Another single-celled eukaryotic fungal organism is *Pichia pastoris*, which presents all the previous mentioned advantages of yeast producing recombinant processed proteins and its genetic manipulation is currently easy to handle due the information and tools available [75], [76].

Other systems for heterologous protein production are based on animal cells, including mammalian or insects as the most used [67], [77]. However, these hosts are still one-step behind yeast because of their high complexity, cost and investment requirements. In addition, these systems still have a slow grow and they are not able to achieve cell densities.

Once the host is chosen, many modifications at different levels can be made to improve some facts of the lipase. As it is explained in [26], there is a huge range of reported genetic modifications to boost lipase properties. For instance, using different kinds of rational design mutagenesis like site-directed mutagenesis (SDM), which allows substitution of amino acid residues with another one, or performing site saturation mutagenesis (SSM) substituting one amino acid by another of the remaining 19 to create a more variated library of mutants [78], [79]. In the case of directed evolution, error-prone Polymerase Chain Reaction (eqPCR) can be used as well as chemical mutagenesis or UV irradiation [80]. If these modifications are done near or in the active site of the lipase, a change in their conformational state may occur thus improving their activity. Another kind of modifications are those performed at the substrate-binding site or even in the lid. That means, changing the lipase ability to interact with these substrates, thus modifying the chemo-specificity. Some studies have even inverted the enantio-selectivity of some lipase from S- to R- [81], [82].

### 1.2.5.    Immobilisation of lipases

Use and application of enzymes in industry has always been linked to the economical sustainability of the process. Immobilisation of enzymes has an important role on this field

because in most of cases their price becomes a very significant part of the total process cost [4], [83]–[85]. Advantages of immobilisation includes lowering the cost due to enzyme reuse, easier biocatalyst and product recovery and sometimes improving of enzyme stability and activity. Some cases showed hyperactivation of the lipase after immobilisation due to the final open form of it [86]. In contrast, after an immobilisation some drawbacks may appear like mass transfer limitations, loss of enzyme activity and additional steps and associate costs. Equally to previous advantages, if the interaction between the carrier and the enzyme excessively affects the conformation of it or its active site, it can suffer undesirable variations in the activity, selectivity or stability [87], [88].

As well as other enzymes, lipases can be immobilised using most of the existing methods developed for enzyme immobilisation (Table 1.4. and Figure 1.5.).

**Table 1.4.** Principal enzyme immobilisation methods [41], [89].

| Immobilisation in physic supports | Adsorption (inorganic supports, organic polymers, mesoporous silica, organic solvent, protein-coated microcrystals |
|---|---|
| | Entrapment (sol-gel materials, organic polymers) |
| | Covalent (epoxy, agarose, nanoparticles) |
| | Cross-linking (enzyme crystals, enzyme aggregates) |
| | Whole-cell (inner-cell, membrane displaying) |
| Inclusion | Surfactant (ion-paired, coated, micro-emulsions, organo-gels) |

Adsorption is the simplest method to immobilise any kind of enzymes. Normally, hydrophobic and ion reversible interactions between lipase and the carrier occur [90], [91]. Sometimes, adsorption can cause a conformational change in the lipase, enhancing its activity [92]. Different kind of supports can be used, like porous inorganic carriers – weak interaction – or mesoporous silica, an interesting methodology because of the possibility to modify the materials in terms of size, pore diameter, etc [93], [94]. Another type of adsorption material are organic polymers, which are the most common commercial immobilised lipase preparations available such as Novozym 435™ (*Candida Antarctica B* lipase immobilised in polymethayacryldivinylbenzene), but also methacrylate (Sepabeads™) or polypropene (Accurel MP-1000™) are other organic supports used.

In the case of entrapment, the most important technique is sol-gel immobilisation in aqueous silica-based materials like hydrolysable and alkyl trimethoxysilane. Sol-gel immobilisation was found to be more effective than adsorption probably due to less enzyme leakage from the adsorbed carrier [95]. Finally, organic polymers are also used to entrap lipases. Polymerisation and crosslinking, for instance using poly-vinyl alcohols (PVA) networks, stands out as a classical way [96].

The case of covalent binding is one of the mostly common used way to immobilise lipases because the huge variety of available methods described. Although leakage of the lipase is nearly negligible, one should consider that covalent modifications of enzyme's groups might cause permanent inactivation but also try to immobilise the lipase in the opened form. As said, covalent immobilisation includes a high number of methods, but epoxy-carriers and agarose-based supports are one of the most used [97], [98].
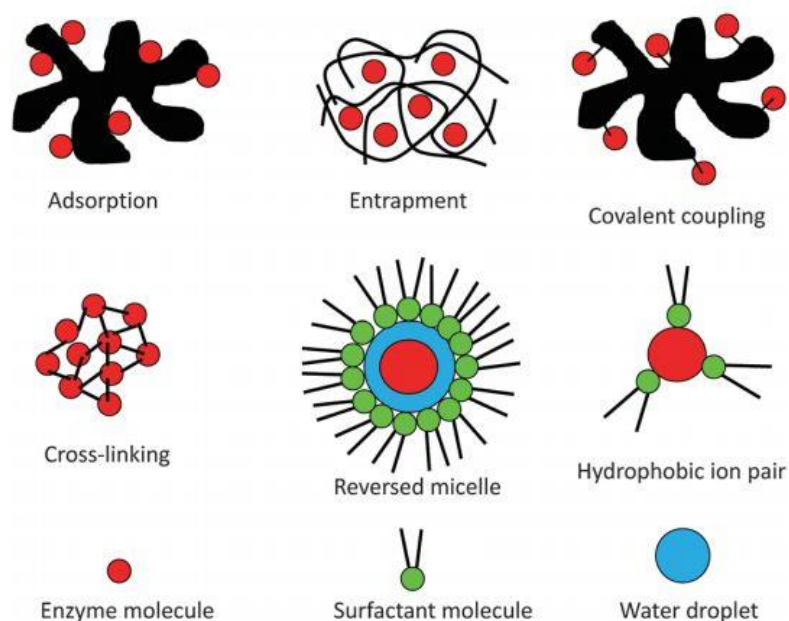


**Figure 1.5.** Schematic presentation of the most common lipase immobilisation methods [99].

Crosslinking immobilisation uses the enzyme itself as aggregates through covalent binding of its amino groups. Glutaraldehyde in solution is the most common cross-linking agent [100]. There are two important methods: CLECs and CLEAs. Cross-linked Enzyme Crystals are based in the crystallisation of the enzyme, which is the critical step, followed by glutaraldehyde action [101], [102]. In contrast, Cross-linked Enzyme Aggregates are formed by adding a precipitant solution – e.g. ammonium sulphate – before the cross-linking agent [103], [104]. However, crosslinking immobilisation is also used in combination of adsorption to avoid its typical enzyme leakage.

### 1.2.6. Uses of lipases

As mentioned before, lipases can perform a large variety of reactions due to their enantioselectivity, regiospecificity and chemoselectivity even in organic media. In addition, the wide range of co-substrates available apart from water make these enzymes one of the most commonly used in almost every industrial field [13]. A key factor that also has enhanced this fact is all the current biotechnological methodologies implemented in all levels such as protein engineering or heterologous protein expression [60], [105].

Food industry is one of the most important industrial field where lipases play a significant role on it [106]. Another sector where lipases participate is in the synthesis of structured lipids or human milk infant substitutes. In the case of structured lipids, fats and oils are modified to get high nutritional values or to be more suitable for food applications, for instance diets and pharmaceutical uses. Lipases can help in modifying the original fat content chemical properties [107]. The major triglyceride present in human milk is unsaturated at the sn-1,3 positions and saturated at the sn-2 position. Palmitic acid (C16:0) represents 20–33% of the total fatty acids with one-third located at the sn-2 position [13]. Usually, 1,3-specific lipase such as *Rhizopus sp.* are responsible for carrying out reactions using tripalmitin with unsaturated fatty acids that resulted in 1,3-diunsaturated-2-saturated triglycerides [108], [109] (Fig. 1.6.).
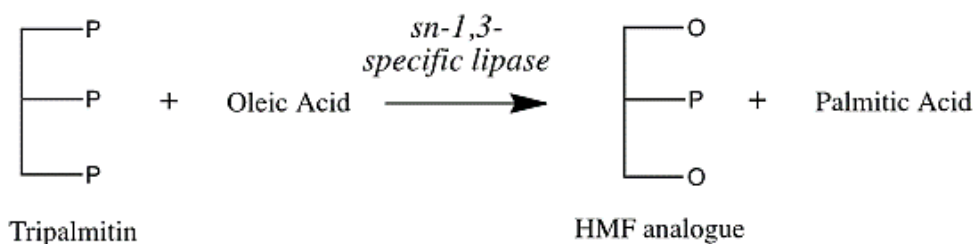


**Figure 1.6.** Reaction schematic for production of an *sn*-OPO human milk fat analogue; P and O represent palmitic and oleic acids, respectively [110].

Another important field of lipase implementations is detergent industry. These enzymes increase the cleaning capability of the product due to their ability to hydrolyse fatty matter – dirt in general – at low temperatures. It is reported that, approximately, 1000 tons of lipases are added to 13 bilion tons of detergent formulation every day. Here, *Thermomyces lanuginosus*,

*Bacillus sp.* and *Pseudomonas sp.* lipases have been frequently used in commercial detergents [111]–[113].

Lipases are enzymes with a high versatility due to the advantages laid down before such as its substrate promiscuity and large number of reactions possible.

For example, the use of lipases is very interesting in the pharmaceutical field because of its enantio- and regioselectivity, as well as for its ability to resolve racemic mixtures which is an important feature for drug production [33], [114]. A case that has been strongly studied is ibuprofen enzymatic synthesis, since lipases – especially *Candida rugosa* – have been discovered to efficiently yield this product. Ibuprofen is a global-widely used nonsteroidal anti-inflammatory drug that presents two isomers: R-enantiomer has 160-fold lower biological activity than S- one [115], [116]. Using modern currently available tools, such as computer aided modelling or testing, some immobilisations have even enhanced the enantioselectivity property as reported in some studies [117], [118].

Lipases can also resolve chiral amines via enantioselectivity acylation [41] and some others can perform aldol additions [119], [120].

A large explanation of lipase industrial applications variety can be found in more detailed reviews [13], [26].

## 1.3. Biodiesel

### 1.3.1. Definition

As an alternative petroleum-based fuel, biodiesel is defined as mono alkyl esters from long chain-fatty acids (FAAE). Although pure vegetable oils can be used directly in engines, first, their high price compared to fossil fuel and second, their high viscosity and free fatty acids (FFA) presence, which can lead to engine damage by polymerisation and oxidation [84] make the alkyl esters of these fatty acids more suitable as fuel source.

The use of biodiesel is not a new technology, but it has been taken seriously since petroleum production will increase from 98.3 million barrels per day in 2015 to 113 in 2040 [121] and their reserves have been forecasted to be depleted in 2050 [122]. Furthermore, a fact that has helped biodiesel to be an emerging source is the global warming problem [123].

Briefly, biodiesel is produced through transesterification of triacyclglycerols present in vegetable oils and animal fats, and methanol as acyl-acceptor [124], [125]. There are such many ways to produce it, which are explained in more details along the next lines.

### 1.3.2.    Biodiesel in Europe and World

European Union also has been concerned about fossil fuel exhaustion issue applying eco-friendly policies to be implemented in every EU country to reduce greenhouse gas (GHG) emissions. Thus, by 2020, EU aims to have 10% of the transport fuel come from renewable source, currently being at 5.5% [126]. In addition, European Commission has also published another policy framework for climate and energy in the period from 2020 to 2030 in complementation with last one detailed. These new policies propose to reduce a 40% the total GHG emissions in 2030 compared to 1990 [127].

Although the application of all these reforms, one big problem is still present in Europe. As shown in Fig. 1.7., production capacity of European biorefineries decreased from 24900 ML in 2011 to 21140 in 2016, representing a negative 15.10%. In addition, another worrying fact is that the production is always between the 40-60% over total capacity, demonstrating something is happening in biodiesel factories [128]. European biodiesel production remained between 13100 and 13500 ML which represents about 40-43% of global production. However, despite of having biorefineries stopped or closed, Europe still need to import some extra quantities of biodiesel – about 9600 ML in the last 6 years –. This seems a contradictory circumstance at first, but when one realises that importing biodiesel from Indonesia and Argentina – between 2011 and 2013 – and from Malaysia – last four years – is cheaper than produce it in some country in EU, this fact is more understandable [129].

In terms of global production, biodiesel synthesis increased 700% between 2005 and 2015, reaching a total production of 31600 ML and is expected to rise by another 35% by 2025 [131].
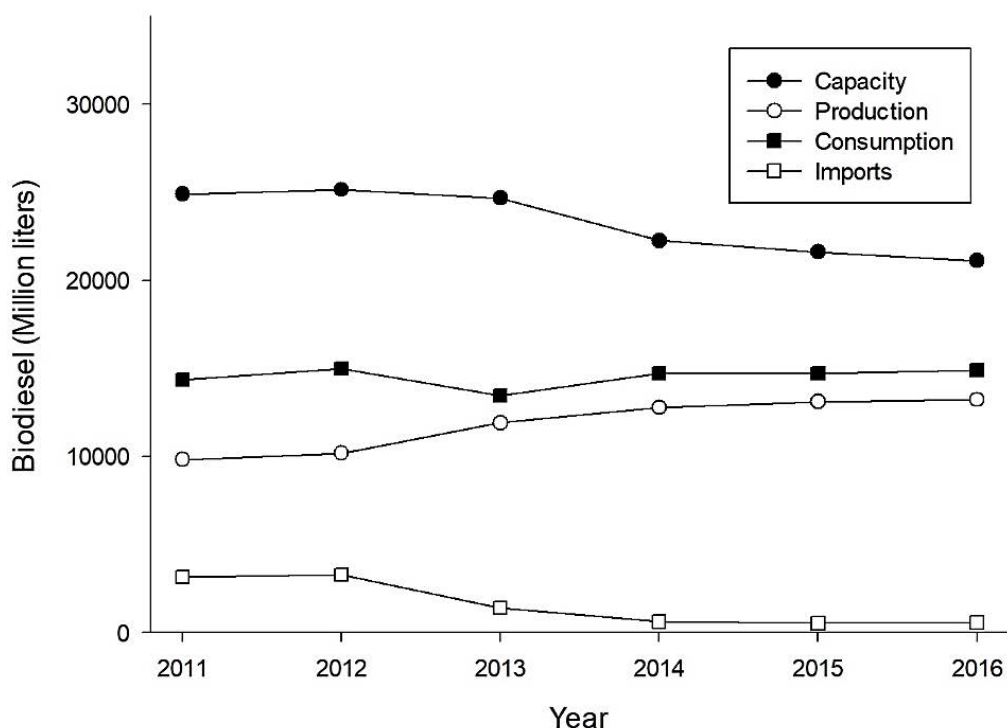
**Figure 1.7.** Evolution of biodiesel status in EU: production capacity, production, actual consumption and imports. Data from [130].

### 1.3.3. Biodiesel properties

As said before, although vegetable oils can be directly used as a fuel source, their high viscosity, FFA content and some other properties can lead to gum or ash formation due to their oxidation, polymerisation and carbon deposition [84]. These properties became more suitable for the use in diesel engines by converting vegetable oils to alkyl esters. Biodiesel produced from lipid components are mainly comprised of five different FAAES, which are palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (18:3), varying this composition according to sources and growth conditions. Some properties are inherent to fatty acids – e.g. cetane number, viscosity, oxidative sensibility – and some others are related to production or storage – e.g. acid value, free total glycerol, carbon residue, water presence –. The most important are laid down here [124], [132], [133].

- Cetane number (dimensionless): referred to the ignition delay time of a fuel. Higher cetane numbers indicate reduced ignition delay time, which means better combustion. This property is based in hexadecane combustion behaviour (CN=100). Biodiesel shows CN values about 46-70 which is slightly higher than the diesel CN value, about 47-55.

- Viscosity (cS): a property that increases with chain length and saturation. Higher viscosities may tend to form droplets upon injection, leading to poorer atomisation during the combustion resulting in operation problems and carbon deposits. It is one of the major problems associated with biodiesel (3.7-5.8 cS) compared to diesel (1.9-3.8 cS).

- *Cold flow*: these properties are influenced by the source of the crude oil they are made from, how they are refined and if they are blended to improve their performance. There are two important cold flow parameters: cloud point (K) and pour point (K). The first one is referred to the temperature when wax crystals start to appear due to solidification. While in the major cases fuel can be used without problems below this point, it must be used above the cold filter plugging point, which crystals are aggregated in sufficient amounts to plug the filter. Biodiesel cloud point varies from 262-289 K compared with 256-265 K in diesel. The other parameter is the pour point (K), which is the lowest temperature where fuel is observed to flow. In this case, biodiesel value is about 258-286 K while diesel one is between 237-243 K. That means that biodiesel still has poorer values than petrodiesel in terms of cold flow parameters. Even though, it depends on the oil source. It has been reported that while increasing the amount of saturated fats – e.g. coconut and palm oil – and thus increasing stability and CN number, conversely, the cold flow properties decrease [134]. That is why monounsaturated and polyunsaturated fats – e.g. canola, safflower and sunflower oil – are used in cold-weather countries.

- Oxidative stability: it is a property related with the content of unsaturated fatty acid chains that affects especially allylic and *bis*-allylic $CH_2$ positions. It is affected also by large storage times and conditions and by the material container too [135]. The chemical composition of biodiesel fuels makes it more susceptible to oxidative degradation than fossil diesel fuel. Oxidation stability ranges between 3 and 6 hours minimum but almost anti-oxidants additives are required.

- Flash point (K): refers to the minimum temperature to ignite a volatile material. It varies inversely with the volatility of the fuel, thus in the case of biodiesel this temperature is higher (408-423 K) than diesel (325-350 K), which makes it safer for transportation and handling. This value depends not only on the unsaturations and chain length but also on the alkyl moiety of the ester. For example, fatty acid methyl esters – coming from methanol – are more volatile than fatty acid ethyl esters – coming from ethanol –.

Some countries stablished quality standards in in order to control that every used biodiesel fits within certain value ranges. The most important standards are EN 14214 from EU and ASTM D6751 from USA and Canada [125].

Considering all these properties that have been pointed out above, biodiesel has raised as alternative to fossil fuels due to two important facts: as a solution to the imminent petroleum depletion and to the environmental issue derived from its extraction, treatment and use. As it is mainly known, biodiesel is a renewable source of energy and it does not contribute to global warming due to its closed carbon cycle. This means that the carbon from carbon dioxide produced after combustion can be fixed again to obtain new biomass that will be used as oil source without increasing the atmospheric carbon releasing [136].

Furthermore, a fact that is drawn from these properties is such great advantages [9], [124], [133], [137]–[143] that biodiesel has as a fuel source (Table 1.5.).

**Table 1.5.** Advantages and disadvantages of biodiesel compared to common fuel [124], [137]–[139], [141], [142], [144]–[146].

| | |
|---|---|
| **Advantages** | Biodiesel has 10–11% of oxygen; this makes biodiesel a fuel with high combustion characteristics. |
| | It reduces net carbon dioxide emissions by at least 78% on a lifecycle basis. |
| | Biodiesel has higher cetane number – depending on the vegetable oil –, reducing the ignition delay. |
| | It is safe for transportation, handling, distribution, utilisation and storage due to its higher flash point. |
| | Biodiesel has better lubricity properties which decrease engine wear, tear and increases engine efficiency. |
| | Each country has the ability to produce biodiesel as a locally produced fuel, thus no need for drilling, transportation, or refining like petroleum diesel. |
| | Biodiesel reduces the environmental effect of a waste product and can be made from used cooking oils and lards. |
| | Biodiesel may not require engine modification up to B20 – 20% biodiesel in diesel –. However, higher blends may need some minor modification. |
| **Disadvantages** | Biodiesel has 12% lower energy content than diesel, this leads to an increase in fuel consumption of about 2–10%. |
| | It has higher cloud and pout point – depending on vegetable oil –, which can make it unfeasible for cold climates. |
| | It has relatively higher viscosity – 11-18 times diesel – and lower volatility than diesel thus needs higher injector pressure. |
| | Oxidation stability of biodiesel is lower than that of diesel. It can be oxidised into fatty acids in the presence of air and causes corrosion of fuel tank, pipe and injector. |
| | Due to the high oxygen content in biodiesel, advance in fuel injection and timing and earlier start of combustion, biodiesel produces relatively higher NOx levels than diesel in the range of 10–14% during combustion. |
| | As more than 95% of biodiesel is made from edible oil, there have been many claims that this may give rise to further economic problems. |
| | Chemical transesterification process is complex, as most of vegetable oils require expensive fatty acid separation or use of less effective or expensive acid catalysts. In addition, chemical transesterification has some environmental effects such as waste disposal and water requirement for washing, soap formation, etc. |

### 1.3.4. Production of biodiesel: substrates and catalysts

The emerging concern about global warming has resulted in an increasing of biodiesel production year by year as commented above. Biodiesel can be produced through several methods divided into two main groups: catalysed and non-catalysed processes and using a huge range of substrates. The first group includes transesterification using chemical – alkaline and acid – or biocatalysis – using enzymes – and the second one comprises a most novel technique, supercritical transesterification.

### 1.3.4.1. Biodiesel feedstocks: first-, second- and third-generation

Globally, there are more than 350 oil-bearing crops identified as potential sources for biodiesel production. A summary of some of the most used is shown in Table 1.6. [147]:

**Table 1.6.** Classification of the most used feedstocks depending on the source and biodiesel type produced from them.

| Edible oils | Non-edible oils | | |
| | Vegetable oils | Waste oil / Animal fats | High-lipid organisms |
|---|---|---|---|
| Soybean | *Jatropha curcass* | Waste cooking oil | Bacteria |
| Rapeseed | Tobacco seed | Beef tallow | Microalgae |
| Sunflower | Cotton seed | Pork lard | Algae |
| Corn | Mahua | Fish oil | Fungi |
| **1st generation BD** | **2nd generation BD** | | **3rd generation BD** |

As said above, the wide range of available feedstocks represents one of the most important factors for producing biodiesel. It is estimated that about 84% of the biodiesel production is obtained globally by rapeseed oil, which happens to be an edible oil. Similarly, other edible oils such as sunflower oil, palm oil and soybean oil also contribute substantially [124]. That means that edible oils are the most used, nowadays. Some studies have reported that feedstocks represent about 70-80% of the final biodiesel cost in chemical synthesis [141], [148], [149]. This high percent has led to evaluate which are the most suitable vegetable oils in terms of productivity per hectare in order to reduce costs. For instance, a worldwide used oil like soybean

oil has a yield of 446 L/ha/year while palm oil has one of 5950L/ha/year [147], [150]. These differences have marked the evolution on their sales in global market.

However, despite producers have been considering all this data, the use of edible oils has some concerns, especially as they can compete for food resources and available lands for harvesting in addition with the problem associated with deforestation [151], [152]. Very large portions of land were needed to cultivate the first generation of biodiesel crops for them to contribute significantly to the world's fuel demand, which created serious ecological imbalances as countries around the world began cutting down forests for plantation purposes [148].

This is where alternative feedstocks have raised as promised substitutes of edible oils. The use of non-edible oils, animal fats or waste oils for second-generation biodiesel production have experienced an increase during the last years [126], [153] to reduce the dependency on first-generation biodiesel – from edible oils –. The most common used oils are *Jatropha carcass* oil [141], [142], [154], karanja oil and tobacco seed oil [155], [156] conforming the non-edible vegetable oils. In addition, waste-cooking oil [157]–[159] and animal tallows and lards [9], [143] are also used.

As first-, second-generation biodiesel can be used directly or performing minor changes in a diesel engine and it has comparable power, brake-specific fuel consumption and brake thermal efficiency. However, same as first-generation, the formation of oxides of nitrogen and possible engine corrosion is a matter of concern [160]. In addition, biodiesel produced from waste vegetable oils and animal fats can enhance the greenhouse gases (GHG) emissions savings associated with the cultivation of the biomass, processing, transport and distribution (Table 1.7.).

**Table 1.7.** Default GHG savings for some first-generation biodiesel compared with second-generation one [126].

| Raw material | Default GHG savings |
|---|---|
| Rapeseed | 38% |
| Soybean | 31% |
| Sunflower | 51% |
| Palm oil | 56% |
| Waste vegetable oil / animal fats | 83% |

Although second generation feedstocks do not typically affect the human food supply chain and can be grown in wastelands, they may not be abundant enough to replace much of our total transportation fuels [148]. Furthermore, their yields and productivities are still low in comparison with some edible-oils because of the lack of efficient technologies for the commercial exploitation of wastes for biofuels production. In addition, arable lands are still needed for most of them, although the subsequent quality of the obtained oil [161], [162].

In contrast, third-generation biodiesel has been developed recently to be obtainable from single-cell organisms such as microalgae, yeast, molds, cyanobacteria and bacteria [125], [163], [164]. Most especially, microalgae are being considered as the most promising choice for biodiesel production because they offer many advantages including a photosynthetic efficiency higher than terrestrial plants and the possibility of using non-arable land for cultivation [165], [166] and for their high lipid accumulation [147], [167].

The major disadvantage of this technology is the elevate cost associate to the investment, culture and harvesting of microalgae itself. It has been estimated that the total cost performance for one litre of microalgal oil is $2.4 while it is about 3 or 4 times less for vegetable oil. These high prices are associated with the lack of high-efficient methods although the constant developing of the process [147].

Table 1.7. summarises the major advantages and disadvantages between the three biodiesel categories [162].

**Table 1.7.** Principal advantages and disadvantages of 1st, 2nd and 3rd generation biodiesel.

| Feedstock | Advantages | Disadvantages |
|---|---|---|
| 1st Gen | Environmentally friendly | Directly related to edible biomass |
| | Economic and social security | Limited feed stocks |
| 2nd Gen | Environmentally friendly | The seeds are weak competitors with weeds |
| | They grow on marginal land | They require moist soil and do not do well in arid climates if planted |
| | Not competing with food | High FFA content |
| 3rd Gen | Total carbon emissions would be reduced substantially | The oil found in algae tends to be highly unsaturated |
| | Low water use | The cost of algae-base biofuel is much higher |
| | High flexibility to strain selection | High FFA content |

### 1.3.4.2. Chemical transesterification

It is basically referred to an exchange of alkoxy group of an ester compound – lipid sources like oil or fats – with an aliphatic alcohol in the presence of an alkaline or acid catalyst.

The most common used method is homogeneous alkaline-catalysed transesterification using NaOH or KOH as studies are published year after year [124], [125], [168]. In the case of acid catalysis, the most employed catalyst is $H_2SO_4$ [142], [169], [170]. Although transesterification using acid catalysts is much slower – about 4000 times – than that obtained from alkali catalysis, it can be performed if high content of water and FFAs are present in the feedstock [168]. Other problems associated are its possible corrosive behaviour and high alcohol requirements [124], [147].

Heterogeneous catalysis is also a great technique as solid catalyst can be recycled and re-used several times with better separation of the final product. This type of catalysis can be in basic or acid form. Most used materials are mesoporous and silicate, sulphonated and tungstated zirconia [166], [171].

Commercial biodiesel is mainly produced using homogeneous basic catalysis – sodium and potassium hydroxide – and methanol as primary alcohol. This reaction results in fatty acid methyl esters (FAME) and glycerol (Fig. 1.8.). In general, these kinds of catalysts are widely used due to its low cost, their capability to perform short reactions at low-medium temperatures – a range between 60-80°C – achieving high yields. Even these advantages, alkaline catalysis are hindered by the high energy requirements, difficult glycerol and catalyst recovery and disposal of waste water, creating a big impact on the environment [124].

$$CH_2-OCOR^1$$
$$|$$
$$CH-OCOR^2 \quad + \quad 3CH_3OH \quad \underset{NaOH,\ \Delta T}{\overset{Catalyst}{\rightleftharpoons}} \quad \begin{matrix} CH_2OH \\ | \\ CHOH \\ | \\ CH_2OH \end{matrix} \quad + \quad \begin{matrix} R^1COOCH_3 \\ R^2COOCH_3 \\ R^3COOCH_3 \end{matrix}$$
$$|$$
$$CH_2-OCOR^3$$

Triglyceride  Methanol  Glycerol  Methyl esters
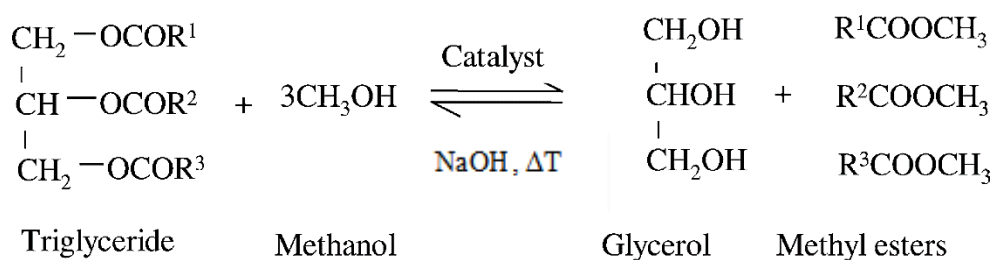
**Figure 1.8.** General overview of transesterification of triglycerides to methyl esters with methanol and alkali catalyst [172].

However, the right method is normally chosen depending on the feedstock that will be used as a substrate for biodiesel production in order to achieve higher yields, less by-products formation and in order to lower final cost of the process.

### 1.3.4.3. Enzymatic transesterification

As laid down above, one of the major disadvantages that both second- and third-generation biodiesel feedstock share is their high FFA content. This circumstance becomes more concerning in the case of low-quality substrates such as waste cooking oil or animal fats, because the FFA content is even higher [83]. FFA values can range depending on the substrate – waste fryer grease, 5.6%; waste cooking oil, 7.25%; fat from meat, 11%; brown grease, 40%; acid oil, 59.3% [153] –. But not only waste oils have high FFA contents, microalgae also do [173], [174].  The problem appears when performing the widely used alkali transesterification with basic catalyst, which can interact with these FFA to produce soaps. Once saponification takes place, it hinders yield and difficulties glycerol separation. Thus, in order to use these high-FFA feedstocks, a neutralisation step must be done before transesterification to reduce the content to lower than 3-5% [146], [172], increasing the final cost of the process.

Is in that way where enzymatic catalysis and the use of lipases stand trying to solve the FFA content issue. 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 [124], [151]. Advantages and disadvantages of enzymatic catalysis in contrast to homogeneous are laid down in Table 1.8.

**Table 1.8.** Most important advantages and disadvantages of homogeneous – alkaline and acid – and enzymatic catalysis [124], [147].

| | Homogeneous chemical catalysis | | Enzymatic catalysis |
|---|---|---|---|
| | **Alkaline** | **Acid** | |
| **Reaction rate** | High | Low | Low |
| **Reaction temperature** | Low-medium | High | Low |
| **Catalyst cost** | Low | Low | High |
| **Catalyst recovery** | Difficult | Difficult | Easy |
| **Glycerol recovery** | Difficult – Low grade | Difficult – Low grade | Easy – High grade |
| **FFA content** | Saponification | Compatible | Compatible |
| **Environmental impact** | Wastewater treatment | Wastewater treatment | Low |

## 1.4.    Biodiesel synthesis through enzymatic catalysis using lipases

As seen before, enzymatic biodiesel production can offer some advantages compared with traditional synthesis, as it is an energy-consuming and non-environmentally friendly process. Biocatalysis suggests important advantages such as easy purification steps and synthesis of higher-quality biodiesel with minimal amounts of down processing and potential energy savings. In other words, and considering all the enzymes properties laid down before, biocatalytic biodiesel production processes could be more highly selective and efficient with less by-products formation [175].

Despite of all these facts, industrialisation of this process is still the main bottleneck due to the high price and low worldwide availability or non-optimal operational features of the current available enzymes [176]. Although this drawback, industrial-scale plants operating using lipases for biodiesel synthesis have been already stablished, for instance, in China: Lvming Co. Ltd (Shangai) has a production of 30,000 ton/year and it uses immobilised lipase from *Candida sp.*

and waste cooking oil as feedstock. In addition, Hai Na Bai Co. Ltd. (Hunan) has a production capacity of 20,000 ton/year using Novozyme 435 lipase, but the feedstock it is unknown [177].

As extensively explained before, lipases are enzymes that have a growing potential in industrial processes due to their high reaction versatility promoted by substrate promiscuity. In general, commercial lipases can be found in two forms: immobilised or lyophilised powder formulations produced by fermentative processes from *Aspergillus niger sp.*, *Aspergillus oryzae sp.*, *Candida rugosa sp.* and *Rhizomucor miehei* [178].

### 1.4.1. Source of lipases

Lipases used for biodiesel production are mainly microbial – bacterial or fungal – origin because they are produced extracellularly and they are almost homogeneous in lipolytic activity terms. In contrast, mammalian and plant lipases can contain interfering enzymes and some may require a co-factor. Although that, it has been reported that fungal lipases have better transesterification activity than bacterial ones [159].

The most used lipases are non-specific for triglycerides because of higher yields achieved, such as *Candida Antarctica*, *Candida rugosa*, *Pseudomonas cepacia* and *Rhizomucor miehei* [179]. However, regiospecific lipases – those than hydrolyse ester bonds of triglycerides depended on their position on the glycerol backbone – have been raised as a promised alternative to the non-specific. Despite this, these non-specific lipases can obtain a final total yield – ideally 100% – acting on the three fatty acids of triglyceride [41]. In contrast, when most common regiospecific lipases – 1,3-positional selective – are used, only a 67% of final yield – acyl migration apart, which will be explained later – can be achieved but at the same time monoglycerides are produced instead of having glycerol as a by-product. If separation and purification is carried out properly, high-grade monoglycerides can be obtained, which are currently products of industrial interest. These by-products have a wide range of industrial applications, concretely on food and pharmaceutical area as emulsifiers or surfactants in contrast to glycerol, currently considered as a bulk chemical [26], [124], [178]. In addition, some studies have reported some benefits in biodiesel properties, like lubricity, when monoglycerides are present on it [138], [180], [181].

It is worth to mention that higher yields using these lipases can be obtained due to a non-enzymatic phenomenon called *acyl migration* which promotes spontaneous movement of the *sn*-1,2 to the adjacent ones. Once the acyl group is in position *sn*-1 or *sn*-3, it can be hydrolysed again by the lipase, increasing the final yield [26], [124]. Acyl migration can be enhanced by

several factors such as the polarity of solvents, water activity, temperature, pH, substrate specificity and stereospecificty [25].

### 1.4.2. Biodiesel synthesis reaction overview

There are a huge number of factors that can have an important role in the reaction of transesterification, starting with the feedstock pre-treatment in order to have a high-quality substrate that will affect the following steps. Immobilisation of the lipase is always a significant part in the process, as it will certainly determine the total cost of it. In terms of the transesterification reaction itself, solvent presence, inactivation of alcohol, formation of glycerol and water content must be considered as well. Finally, the operation has to be performed in the proper reactor in order to achieve higher yields and less biocatalyst inactivation.

#### 1.4.2.1. Feedstock pre-treatment

Quality of feedstock is a key factor for production of biodiesel because can determine the best option to produce and purify it. Common methods are chemical refining like neutralisation of FFA forming soaps or precipitation of phospholipids [151], [182], physical refining such as acid or water-degumming [153], [182] but also enzymatic treatment is used trying to reduce initial FFA content [183].

#### 1.4.2.2. Lipase formulation: soluble or immobilised?

The use of lipases for biodiesel synthesis has been constantly developing. However, utilisation of these enzymes may suppose a heavy burden on total process cost [83]–[85]. Thus, selecting a properly formulation for the use of lipases is currently one of the most important factors on biodiesel production processes. Two main methods can be chosen depending on the environment of the reaction.

In one hand, simplest and cheapest form is the use of free soluble lipases [151] in the presence of organic solvents and low water-containing systems. However, the current existing little literature shows that it is not such an appropriate method due to its several disadvantages compared with immobilisation. Most important drawback is the high difficulty, or even the impossibility, to recover and reuse the lipase [184].

On the other hand, as said before, there is a wide range of lipase immobilisation possibilities specialised for biodiesel synthesis. Most used and simple preparation is adsorption on physical supports like inorganic carriers, mesoporous silica, organic polymers, etc. For example, *Rhizopus oryzae* lipase (ROL) was attached to hydrophobic carrier [185], and *Pseudomonas fluorescens* lipase was immobilised to polysterene [186]. Novozyme 435, a worldwide-known commercial lipase is also immobilised on acrylic resin by adsorption. Despite of being one of the most used technique, it has some drawbacks, as binding forces are usually weak. Thus, depending on the system – agitation, polarity, etc. – lipases may be leaked from the support [41], [177].

In contrast, several studies have focused on covalent immobilisation due to its high binding force – avoiding enzyme stripping – and the possibility to use a wide range of carriers. Even though, it can induce a limited degree of inactivation because of three-dimensional modifications. Covalent attachments of lipases are already well studied as one can find several works in the literature. For instance, CALB was covalently immobilised in nanoparticles [187] and CRL was attached to polymer-coated microspheres for biodiesel production [188].

In general, one of the best advantages of any kind of immobilisation is the recovery possibility at the end of the reaction. Thus, the biocatalyst can be re-used again, whether its activity has not been lost, improving the final cost of the process.

### 1.4.2.3. The use of solvent

Setting up a proper reaction system is also a key factor that has an important role on the transesterification reaction. In the case of biodiesel synthesis environment, usually compounds which differ on their polarity met up. The primary substrate – oil and fats – as well as diglycerides, fatty acids and the product itself – alkyl esters, biodiesel – are non-polar compounds, while polar species comprises alcohols, water and the common by-product, glycerol. Monoglycerides' polarity is placed between polar and non-polar compounds.

Transesterification reaction can be carried out using two main systems: monophasic, thus using an organic solvent as reaction matrix or allowing substrates acting as the solvent themselves – solvent-free – [189]; or biphasic, by using immiscible solvents and aqueous buffers [25], therefore and forming an interface. In the first case, adding an organic solvent to the medium reduces the viscosity of the reaction, protecting lipases from alcohol gradients that can damage them – this issue will be discussed later – and it also increases the reaction rate [185] but also increasing the solubility between glycerol and alcohol [190]. Mass transfer limitations may occur when no solvent is used due to high viscosity of oils and low solubility of alcohols on them [191].

Advantages of solvent-free systems are the easier recovery of the final product because of the absence of solvent and eco-friendlier environment.

Biphasic reactions, as interface is created, interfacial lipase activation is promoted using free lipase [192]. In addition, it simplifies the separation of glycerol since it resides in the aqueous fraction, as well as it facilitates the reutilisation of lipase [193].

### 1.4.2.4. Effect of alcohols

Currently, the most used alcohols for biodiesel enzymatic alcoholysis are methanol and ethanol, and in less proportion propanol, butanol, *tert*-butanol, etc [124], [194], [195]. They are used in different alcohol:oil ratios. However, as will be later explained, both alcohols have an important negative effect on lipases hindering their activity and affecting the possible re-use [191], [196], so the overall yield of enzyme-catalysed reaction depends on the interplay between reaction velocity and the rate of enzyme denaturation. The reason why methanol and ethanol, even these drawbacks, are still the most used is because their economic feasibility and availability [52], [124]. Separately, methanol reactivity is higher than ethanol. However, FAME are more volatile than FAEE [124], and methanol has more negative effects on lipases [197].

This adverse impact has been observed in several studies but researches do not agree on what exactly triggers this inactivation – although it has been described using other terms such as *denaturation*, *deactivation* or *inhibition* [52] –. Some works have pointed out that short-chain alcohols, like methanol, may interact with essential water molecules that surround the active site of the enzyme, also called *structural water* [198], [199]. Some others also suggested that high concentrations of short-chain alcohols might induce variations on the intra-protein hydrophobic interactions, resulting in an unfolding of the enzyme followed by irreversible deactivation. Nevertheless, differences on methanol tolerance have been found depending on the lipase species. For instance, lipases from *Pseudomonas sp.* seems to be more methanol-acceptant than *Candida rugosa* lipase [200].

Alternatives to reduce deactivating effect of methanol will be discussed later, but the most used strategy is stepwise addition [124], [151], [152], [198], [201], [202]. This approach avoids formation of alcohol droplets or concentration gradients by adding those in pulses or in a semi-continuous addition.

### 1.4.2.5. Water content

The presence of this compound is a key factor in organic and biodiesel synthesis reactions. Water concentration can affect the equilibrium of the reaction and it can promote undesired ones like hydrolysis and FFA formation. However, a minimal monolayer of water on the surface of enzyme is required to maintain the three-dimensional structure of enzyme [198], [203]. When the amount of water is high enough to convert the system into a biphasic system, an interface it is created affecting – either positively or negatively – the lipase activity. As explained later, lipases – depending on species and genus – have different sensitivity to water activity [189], [204], [205].

### 1.4.2.6. Effect of glycerol

Despite of having higher yields, glycerol as by-product is obtained when using non-specific lipases. Glycerol has been considered as an important problem during biodiesel synthesis processes due to several reasons. First, nowadays this polyol compound is tagged as a *bulk chemical* because it is worldwide and easy availability and its constantly increasing generation may cause environmental problems. Second, glycerol poses a potential problem as it is known to inhibit immobilized lipases, most likely by clogging of the catalyst particles [124]. Also, if final biodiesel contains some glycerol impurities, it can damage engines causing technical problems due to polymerisation and viscosity [206]. Thus, some strategies have been developed in order to minimize glycerol negative effect, such as direct dialysis [202] or using *tert*-butanol, which dissolves better the glycerol than the most used alcohols [207].

### 1.4.2.7. Transesterification reaction kinetics

Lipase-catalysed biodiesel synthesis reaction is one of the most discussed issues about biodiesel in general due to the possible viewpoints that are available [185].

In general terms, it is widely accepted that Ping-Pong bi-bi with competitive alcohol inhibition is the current mechanism that defines biodiesel synthesis [196], [200], [208] (Fig. 1.9.). That means a first interaction between the enzyme and triglyceride – diglyceride or monoglyceride – and the consequent formation of the triad enzyme-fatty acid-diglyceride – monoglyceride or glycerol backbone – before the hydrolysis of the ester bond. Subsequently, a diglyceride is released and an alcohol enters the site active and forms the consequent alkyl ester.
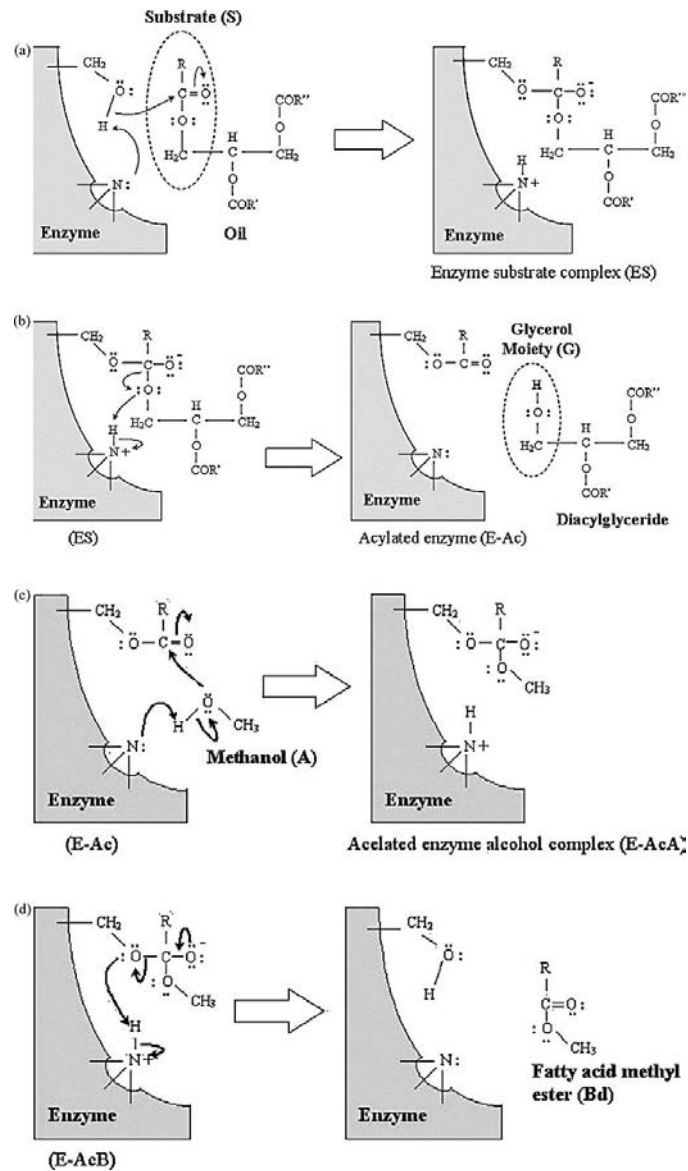
**Figure 1.9.** Molecular overview of Ping-Pong Bi-Bi mechanism of transesterification reaction [209].

The other case defines transesterification as the direct alcoholysis of fatty acid moieties from triglycerides in a comprised Ping-Pong bi-bi reaction [157], [159].

However, some studies stated that biodiesel synthesis – formation of fatty acid alkyl esters – may occur by the combination of the two viewpoints mentioned above (Fig. 1.10): direct alcoholysis of triglycerides (Reaction A) and a two-step reaction involving hydrolysis of triglycerides followed by esterification of previously released free fatty acids (Reactions B and C) [185]. Thus, it is assumed that both pathways – Reaction A and Reactions B and C – may occur simultaneously during the biodiesel synthesis.
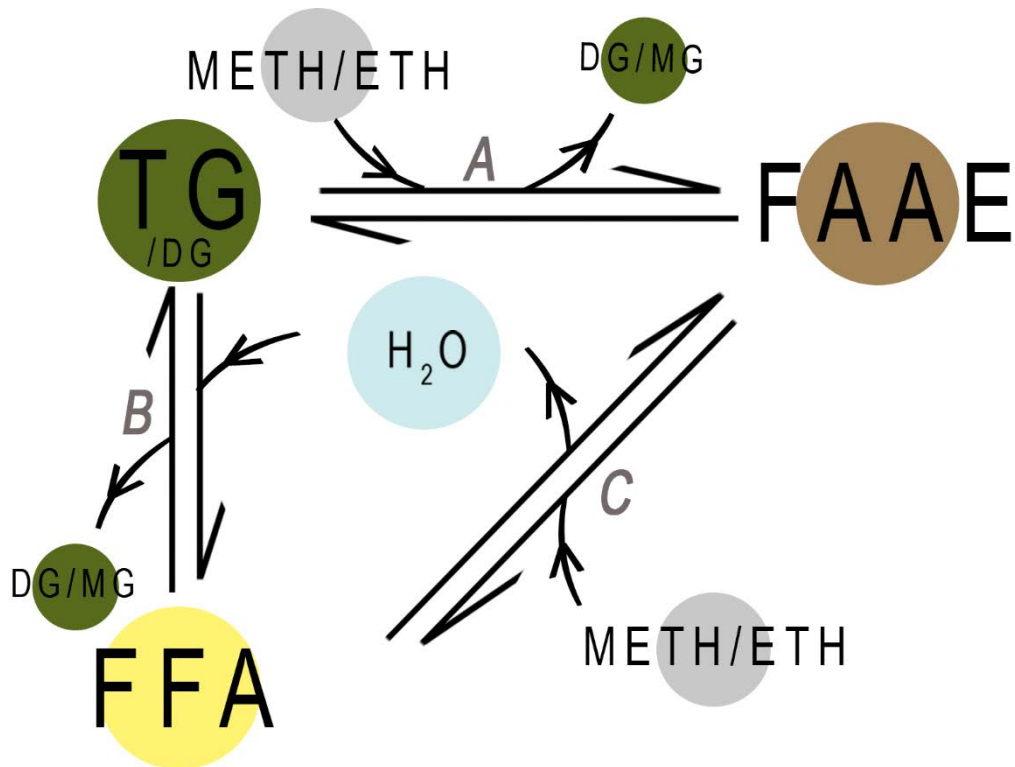
**Figure 1.10.** Schematic visualisation of the three main reactions involved in biodiesel synthesis through recombinant ROL. Reaction A: transesterification of triglycerides (TG) or diglycerides (DG) to biodiesel (FAAE) using acyl-acceptor (ETH/METH) releasing diglycerides (DG) or monoglycerides (MG). Reaction B: hydrolysis of TG or DG into free fatty acids (FFA) releasing DG or MG. Reaction C: direct esterification of FFA into FAAE. Water moiety ($H_2O$) is consumed in reaction B and produced in reaction C.

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# STATE OF THE ART

recombinant *Rhizopus oryzae* lipase
and its applications

**2**

## 2. STATE OF THE ART CONTENT

The present thesis has been carried out focusing on the utilisation of a *Rhizopus oryzae* lipase (ROL) as catalyst for biodiesel production. Next lines define and provide a context for this work, placing it at its starting point.

### 2.1. Recombinant expression of *Rhizopus oryzae* lipase

Heterologous expression of proteins has raised as a promising approach in the last decades. In the case of *R. oryzae* lipase (Fig. 2.1), bacterial and fungal organisms have been used as host to produce it. Coliform bacterium *Escherichia coli* was used in several works, but it was rapidly discarded due to the formation of inclusion bodies which difficulted the extraction and purification of the lipase [1], [2]. Therefore, the yeast *Saccharomyces cerevisiae* was the selected organisms as host, overcoming the problems associated with inclusion bodies [2].
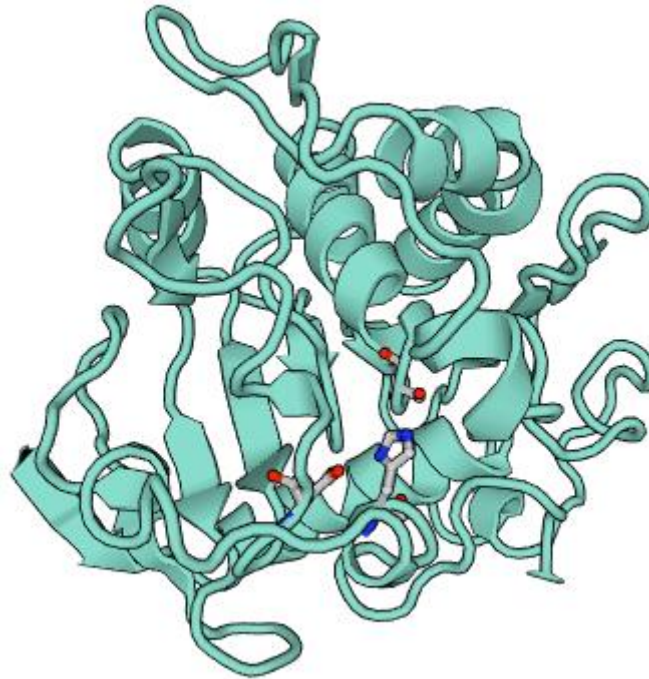


**Figure 2.11.** Three-dimensional representation of *Rhizopus oryzae* lipase. Active site is shown coloured in red and blue [3].

In the case of the research group in which this thesis has been carried out, ROL has been expressed through a wide range of alternatives working with the well-known methylotrophic yeast *Pichia pastoris*. Heterologous expression using the promoter of the alcohol oxidase (P*AOX*) and others like formaldehyde dehydrogenase (P*FLD*) in *Pichia pastoris* has been studied [4]–[7]. In the case of P*AOX*, which is a methanol-inducible promoter, it has some described advantages

such as high productivity, the capacity to grow in a minimal medium at high cell densities, low levels of endogenous protein secretion and the ability to efficiently secrete heterologous protein [8]. In addition, optimisation of a feeding strategy using mixed substrates based on the monitoring and control of methanol concentration in a fed-batch cultivation was achieved, increasing 5-fold the enzyme productivity [9], [10].

Most recently, group works have been focused on optimising protein expression – whether of ROL or another kind of protein – based on system biology, macrokinetic and physiologic parameters such as oxygen or carbon sources [11]–[13].

## 2.2. *Rhizopus oryzae* lipase

*Rhizopus oryzae* was previously called *R. arrhizus.* However, taxonomic diversity of the filamentous fungi *Rhizopus* genus was reinvestigated and several species were combined into three new groups [14]: *Rhizopus oryzae* group, *Rhizopus microspores* group and *Rhizopus stolonifera* group. In addition, recent studies combined *R. delemar* and *R. javanicus* into the first group, and classified *R. niveus* as the old *R. delemar* [15]. Although there have been reports of reclassification based on DNA–DNA hybridization and isozyme analysis, this arrangement is accepted as the standard classification of this genus [16].

The expression of lipolytic enzymes from *R. oryzae* strain has been already described [1], [17], [18]. *R. oryzae* only produces one form of extracellular lipase, which it has been extensively studied. The most characteristic of its features is that it has a high 1,3-regiospecificity towards triglycerides [19].

Characterisation of a recombinant ROL (rROL) done by the research group determined that the molecular weight of lipase was 32 kDa and the isoelectric point was 6.85, and 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 [19]. The same work determined that rROL is 40 times more active than native lipase and it is less affected by ionic strength.

In addition, molecular weight was determined to be about 34 kDa for the native ROL (nROL). To explain this, it should be noted that the precursor amino acid of the nROL comprise 392 residues (Fig 2.2). These 392 amino acids are divided in three parts: the first 26 correspond to a *pre*-region with a signal sequence function, promoting direct membrane translocation. The second part, involving the next 97 residues, corresponds to a *pro*-region whose function is to decrease the toxicity of itself for the cell. Last part, including 269 residues, corresponds to the mature sequence of the lipase [19]–[21]. However, that is not exactly as commented since the native

secreted form of the fungal lipase includes 28 residues from the *pro*-region, conforming a protein made up of 297 amino acids [19], [21]. In the case of 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 [1].
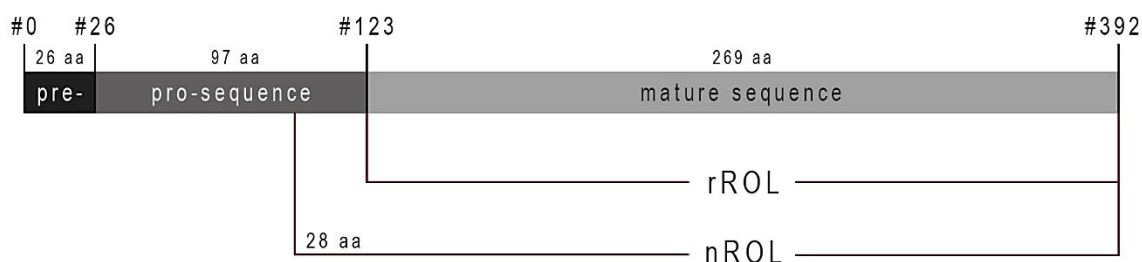


**Figure 2.2.** Schematic representation of recombinant ROL and native ROL.

## 2.3. *Rhizopus oryzae* lipase applications

The recombinant lipase from *R. oryzae* expressed in *Pichia pastoris* by the research group has been used as biocatalyst in several reactions taking advantage of its 1,3-regiospecificity; for instance, for the preparation of chenodeoxycholic esters [22], acetylation of cortexolone [23] as well as synthesis of hydroxy monodeprotected glycals [24]. It also has been used to produce human milk fat substitutes [25], [26] structured lipids [27]–[29], to improve the synthesis of ethyl butyrate [30] or even as a test for immobilisation in a re-valorised support such as discard bovine bone [31].

However, the most explored application using rROL is, by far, the synthesis of biodiesel. One of the first works done was to carry out solvent-free reactions and to optimise through a response surface methodology (RSM) the methanol addition strategy in order to reduce its damage to lipase as well as the amount of water present in the system [32]. High lipid content oils, like yeast oils, have been used as feedstocks for biodiesel production using rROL immobilised in adsorption carrier and using *n*-hexane, *iso*-octane and *tert*-butanol as solvents [33] as well as non-edible oils like *Jatropha carcass* oil [34]. Finally, it should be stated that works done by Canet et al. [35]–[37] have been taken as basis for current biodiesel researchers in the group and also for the present thesis. For instance, it was found out that rROL performs two simultaneous reactions: direct alcoholysis, and hydrolysis of triglycerides and further esterification of the released fatty acids during the biodiesel synthesis reactions. In addition, it was also found that
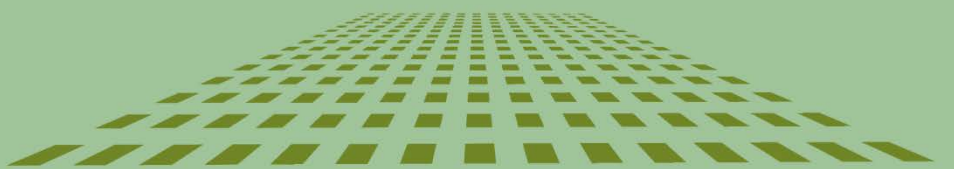
rROL increases its activity as more oleic acid is present in the substrate [37]. Moreover, it was studied that no interfacial activation is needed for rROL to achieve high reaction rates and it also was observed that acyl migration in monophasic systems is restricted as more polar solvent – methanol, for instance – is added [35]. Finally, flow regime of real reactor and the mass transfer problems involved in a solvent-free system working with lipase immobilized by adsorption were studied through a comparison between a packed-bed and a stirred tank reactor using olive oil [36].

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# OBJECTIVES

**3**

The following lines expose the general aim that have been pursued for the attainment of the present thesis as well as other partial objectives that will be focused in the subsequent chapters.

## 3.1. Main objective

The main objective of the present work is to bring light to the utilisation of any non-edible or waste oil – with high content of free fatty acids – for the synthesis of biodiesel through enzymatic catalysis using a covalently immobilised recombinant *Rhizopus oryzae* lipase expressed in *Pichia pastoris*.

## 3.2. Partial objectives

The succeeding chapters will be showing the results obtained of the agreed objectives listed below:

- Exploring the utilisation of a feedstock with a high content of free fatty acids (*alperujo* oil) very common in the territory.

- Comparison of two kinds of covalent immobilisation in terms of enzyme load and reaction yield.

- Application of a pseudo-optimised transesterification reaction by studying two key parameters, such as temperature and water activity, as well as the best of three stepwise addition strategies of two acyl-acceptors – methanol and ethanol – in a lab-scale vials. Stability of the biocatalyst, an important factor in biodiesel synthesis, was also studied.

- Stirred mini-reactor approach. Fluid performance studies and alcohol addition strategies implementation.

- Semi-continuous addition of alcohol implementation in stirred mini-reactor by automatized micro-burette in order to achieve higher yields and less biocatalyst damage.

- Process flow design and economic study for a simulated industrial enzymatic plant to synthesise 35000 tons per year of biodiesel.

METHODS

4

# 4.  METHODS content

### 4.1. Heterologous expression of the lipase

Recombinant 1,3-regiospecific *Rhizopus oryzae* lipase was produced by the research group based on optimisations done by studies and works previously cited.

### 4.1.1. Strain

Wild type *Pichia pastoris* X-33 strain containing the vector pPICZαROL was used for the heterologous expression of recombinant ROL under the control of the promoter $P_{AOX1}$ (Mut$^+$ phenotype) [1]. Strategy used for production was: initial glycerol batch phase (GBP), transition phase (TP) and finally a methanol-induction phase (MIP) by applying a methanol non-limited fed-batch strategy (MNLFB).

### 4.1.2. Pre-inoculum preparation

*P. pastoris* was cultured in 500 mL beakers at 30°C, 150 rpm (HT Multitron incubator, Infors AG, Bottmingen, Switzerland), for 24 hours – approximately, according to previously kinetics studies – in YPD medium (10 g of yeast extract, 20 g of peptone, 20 g od D-glucose and 500 µL of antibiotic zeocin per litre of distilled water, pH 7.4). The culture was then centrifuged and the resulted biomass re-suspended in bioreactor culture medium. Cells were used as inoculum for a 5 L-Applikon Biobundle bioreactor (Applikon Biotechnology B.V., Delft, Netherlands). Initial fermentation volumes were 2L and $OD_{600}$ about 2.5 [2].

### 4.1.3. Batch phase

A defined medium with glycerol, macro-elements, trace salts, biotin and antifoam was initially used. During this stage pH at 5.5 was controlled by addition of $NH_4OH$ 30% (v/v) and dissolved oxygen (DO) was kept higher than 25% air saturation. Stirring rate was set between 600 and 1000 rpm and 1 vvm of air was introduced in order to maintain this oxygen concentration value. [2].

### 4.1.4. Transition phase

Glycerol (50% w/w) and pure methanol (both solutions with 5 mL of trace salts solution and 2 mL of biotin solution per litre) were used as feeding using pre-programmed additions for 5 hours: in the case of glycerol, decreasing feeding rates under carbon limited conditions was applied. For methanol, a constant feeding rate was added to assure a methanol concentration lower than inhibitory concentration to favour the depression of $P_{AOX1}$ [3]. In this phase and next one, pH was controlled using KOH 5 M because $NH_4OH$ could interfere with methanol probe signal (Methanol Sensor System, Raven Biotech Inc., Vancouver, Canada).

### 4.1.5. Methanol induction

Cells were grown under methanol non-limited conditions; which concentration was controlled at 3 g $L^{-1}$ performing a predictive-PI control strategy [4]. Nitrogen supplementation using $NH_4CL$ solution (200 g of $NH_4CL$, 5 mL of trace salts and 2 mL of biotin per litre of distilled water) was added. Its flow rate was directly linked to methanol one and it was calculated using an estimated ammonium chloride/methanol yield of 0.12 g $g^{-1}$.

DO at 25% respect to air saturation was controlled by a cascade-based control scheme, in which both stirring – between 800 and 1100 rpm – and total inlet gas flow rate and additional pure oxygen – between 0-1 vvm – were used [2].

It should be noted that complementary analysis, such as dry cell weight, off-gases ($O_2$ and $CO_2$), mass and macroscopic balances were not performed since the only aim of previously described method was the production of the enzyme of interest.

### 4.2. Lipase recovery

After centrifugation of the medium at 9000 rpm for 15 min, as *P. pastoris* expresses heterologous lipases extracellularly [5], [6], the supernatant was kept for next recuperation steps.

First, the supernatant containing the lipase was micro-filtered using 0.70 µm glass fibre prefilters (Merck Millipore Ltd. Tullagreen, Carrigtwohill Co., Cork, Ireland) and then using 0.45 µm aqueous nylon membranes (Merck Millipore Ltd. Tullagreen, Carrigtwohill Co., Cork, Ireland) to remove remaining cells and to eliminate some other impurities.

Next step was to concentrate 10x the lipase extract by ultrafiltration using a 10 kDa cut-off Centrasette membrane (Pall Filtron, New York, USA) to reduce the undesired protein load and then a diafiltration in a Tris-HCl 10mM ph=7 buffer was done.

The final extract was lyophilised and stored at 4°C.

Samples from every step were taken and lipolytic activity (see 4.3.) and total protein analysis (see 4.4.) were performed.

## 4.3. Lipolytic activity analysis

Lipase activity – whether from lipase concentration process or from immobilisation – was determined using the Roche colorimetric assay kit (Roche AG, Basel, Switzerland) as follows: 0.3 ml of substrate (1,2-O-dilauryl-rac-glycero-3-glutaric-(methylresoru-fin)-ester) was mixed with 0.5 ml Tris-HCl buffer (200 mM, pH 7.25) and 0.5 mL of diluted sample in a thermostatically controlled cuvette, at 30°C. The increase in absorbance at 580 nm was followed for 7 min with an UV-Vis Cary Varian 300 spectrophotometer (Varian Inc., Palo Alto, California, USA; now Agilent Technologies, Santa Clara, California, USA). The absorbance increase per second was calculated from the slope of the curve and correlated to the lipolytic assay using pH-stat analysis [5], [7]. One unit of lipolytic activity was defined as the amount of lipase necessary to hydrolyse 1 μmol of ester bond per minute under assay conditions. Assays were performed in triplicate with an estimated RSD of 5%.

## 4.4. Total protein analysis

Extracellular protein concentration was determined with the Pierce Coomassie (Bradford) Protein Assay Kit (Thermo Fisher Ltd, Waltham, Massachusetts, USA) according to the manufacturer's instructions. Bovine serum albumin (BSA) was used as standard for the calibration curve. Assays were performed in triplicate with an estimated RSD of 5%.

## 4.5. Lipase covalent immobilisation of HFA and HFAGlut

Two types of biocatalysts resulting from the same support have been used in the present thesis: in one hand, rROL immobilised on non-treated commercial ReliZyme HFA403/S (Resindion S.r.l., Binasco, Milano, Italy) called HFA, and in the other hand, rROL immobilised on the same HFA but previously treated with ethylenediamine and glutaraldehyde (HFAGlut).

HFA403/S is a polymethacrylate carrier with a particle size about 100-300 μm and with an average diameter pore of 40-60 nm. It is described as an amino-epoxide carrier, with a minimum oxirane content of 30 μmol/g wet.

### 4.5.1.  Pre-treatment of HFA (HFAGlut)

As said above, HFA was pre-treated with ethylenediamine and glutaraldehyde. It has been reported that performing this modification with a spacer arm, added between the support and the enzyme, minor steric hindrances are present [8].

Thus, the carrier was incubated in 100 mL of 1M ethylenediamine solution pH 10 per 1 g of dry support under orbital incubator during 4 h at 60 °C. Then, the solution was rinsed under vacuum filtration. After that, the carrier was incubated in 100mL of 2.5 w/v glutaraldehyde solution (in phosphate buffer 0,1 M pH 7.25) at pH 8 on a roller during 2 h at room temperature. Finally, support was rinsed again under vacuum filtration [9].

### 4.5.2.  Lipase immobilisation

A total volume of 100 mL of 0.1M phosphate buffer pH 7.5 per 1 g of dry support containing the desired rROL activity – depending on the case – was prepared, dissolving lyophilised lipase under mild magnetic stirrer for 30 min at 4°C. After that, the solution was centrifuged at 12000 rpm for 10 min. Then, the supernatant was incubated with 1 g of treated carrier on a roller shaker for at least 42 h at 4°C. A lipase solution without support was also left as a blank control. Samples from both solutions were taken and analysed (see 4.3 and 4.4). Biocatalyst final activity were calculated as the difference between the activity and the protein concentration in the initial and final supernatant, divided by the weight of dry support [10]. Once immobilisation was done, immobilised biocatalyst was rinsed with phosphate buffer previously referred under vacuum filtration. Finally, it was dried on silica gel at room temperature until its weight reached a constant value and then stored at 4°C.

## 4.6.  Biodiesel feedstock

Raw alperujo oil was kindly donated by Dr. Eulogio Castro (Dept. of Chemical, Environmental and Materials Engineering; University of Jaén, Spain). It was collected from Sierra Mágina olive oil extraction mill (Mancha Real, Jaén, Spain). Three types of substrates were used:

- Centrifuged alperujo – hereinafter referred as *initial* – obtained by centrifugation (5 min at 4500 rpm) in order to mechanically dewax it.

- Neutralised centrifuged alperujo was obtained by adding the necessary volume of a sodium hydroxide solution to neutralise the total FFA amount present in the initial substrate. This volume (V, in L) was calculated from (Eq. 1):

$$V = \frac{m_o \cdot A}{M \cdot C} \quad (1)$$

where $m_o$ is the total substrate amount (in g) to be neutralised; A, the acidity of the substrate (values from 0-1, see 4.7); M, is the oleic acid molar mass (in g mol$^{-1}$); C, sodium hydroxide solution concentration (in mol L$^{-1}$).

Here was assumed the fact that all substrate acidity came from the oleic acid presence. In order to assure the total neutralisation of the substrate, a 10% more of solution volume was added. Then, the final volume was mixed with the substrate under magnetic stirrer for 20 min at room temperature. Next step was to heat up the solution at 60°C for 20 min. Due to the soap formation during the process it is desirable to clean up the substrate with distilled water at 80 °C under magnetic stirrer, and then separate it by decantation.

- Supplemented centrifuged alperujo was obtained by adding the necessary amount of oleic acid to match the original substrate acidity. This substrate is used in order to ensure the actual role of FFA. The total amount of oleic acid needed is obtained here (Eq. 2):

$$A = \frac{m_{oleic}}{m_{oleic} + m_o(1 - AN)} \quad (2)$$

where A is the substrate acidity (values from 0-1, see 4.7); $m_{oleic}$, the total amount of oleic acid (in g); $m_0$, the amount of substrate to be treated (in g); AN, the neutralised substrate acidity (values from 0-1, see 4.7).

## 4.7. Acidity determination

In order to determine the total acidity of the substrates, acid-base titration was used. The method was carried out following European protocols: 702/2007 of 1991R2568.

## 4.8. Water activity pre-equilibrium

Saturated salts were employed in order to achieve desired initial water activities [11]. The salts used were: LiBr ($a_w$=0.066), KOH ($a_w$=0.093), NaI ($a_w$=0.397), NaBr ($a_w$=0.560), NaCl ($a_w$=0.755), $K_2SO_4$ ($a_w$=0.976).

All reaction components were pre-equilibrated separately overnight with each salt-hydrates in a jar with tight fitting lid [12].

## 4.9. Transesterification reactions

All biodiesel synthesis reactions are referred as *transesterification reactions* in the present thesis in order to simplify the lecture.

### 4.9.1. Transesterification reactions in 10 mL-vials

All reactions were carried out in duplicate in 10-mL closed vials, using an incubator KS 400 (IKA GmbH & Co. KG, Staufen, Deutschland) under orbital stirring at 350 rpm, at different temperatures depending on the experiment (30°C, 40°C, 50°C). Free-solvent reactions with 8 g of alperujo oil and the total amount of dry biocatalyst corresponding to 32000 UA were employed – approximately a 2-3 %wt respect substrate –. The total stoichiometric amounts (2:1 alcohol to oil ratio) of methanol and ethanol – depending on the experiment – were added.

Volumes of methanol or ethanol corresponding to the 12% of final yield was added in order to calculate initial rates of some reactions to prevent enzyme inactivation.

In complete reactions, stepwise strategy was used in three different ways to add the total stoichiometric volume: one single pulse at the beginning of the reaction, five pulses with the same volume and ten pulses with decreasing volumes along the time.

Samples were withdrawn using 1-mL syringes with 0.8 mm-needles and then filtered with 4 mm-aqueous Millex membranes (Merck-Millipore, Billerica, Massachusetts, USA)

Stability-testing reactions were carried out by leaving the biocatalyst deposited on the bottom of the vial and removing the medium above. Then, vials containing the biocatalyst were stored at 4°C until the next reaction.

### 4.9.2. Transesterification in 50mL-minireactor

Reactions were carried out following the same method as previously described (see 4.8.1) using a 50 mL-minireactor HME-R50 (Scharlab, SL., Barcelona, Catalunya). This equipment is provided with a mechanical stirring system with an agitation range between 100-700 rpm. It is also equipped with a heating plate – up to 250°C –. The mini-reactor has four inputs: one for stirring axis, one for temperature probe, one for a condenser and last one for alcohol adding and sample withdrawing.

A change of scale (5-fold) is applied respect to vial volumes. Thus, 40 g of alperujo oil and the total amount of dry biocatalyst corresponding to 160000 UA were employed.

As said above, stability-testing reactions were carried out by leaving the biocatalyst deposited on the bottom of the mini-reactor and removing the medium above. Then, tank containing the biocatalyst were stored at 4°C until the next reaction.
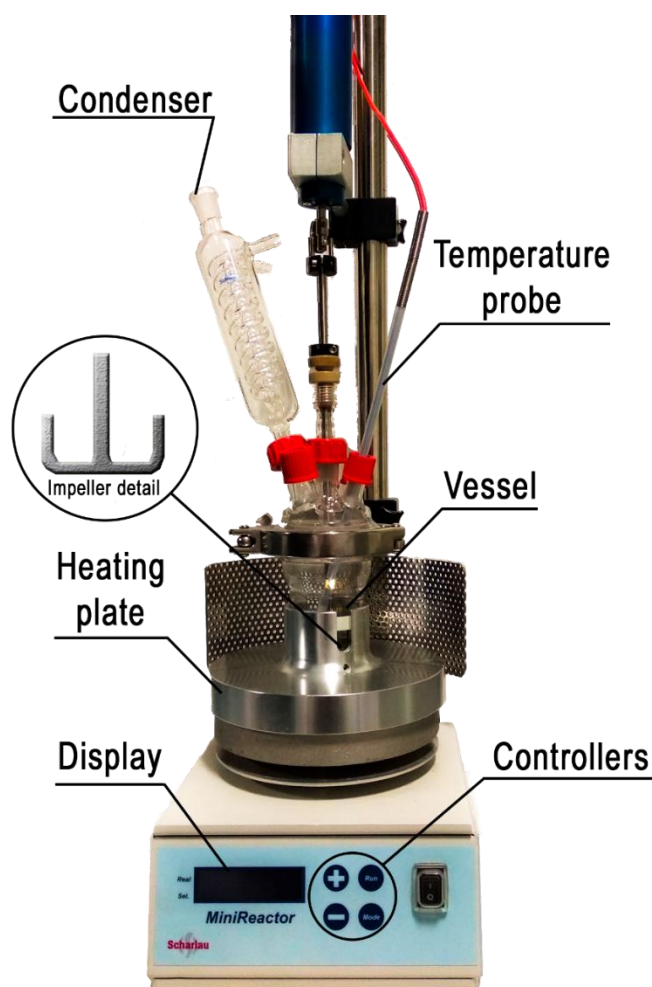


**Figure 4.12.** Main parts of the 50 mL-reactor used in the present thesis and schematic representation of the impeller.

### 4.9.3. Semi-continuous addition using micro-burette

Different exponential addition strategies were applied using a Hamilton 500 µL-micro-burette (Reno, Nevada, USA) and a mechanical dispenser connected to a CPU running a substrate addition software developed for fermentation processes adapted to the performance of enzymatic bioreactor.

### 4.10. Enzyme Particle Concentration

The percentage of enzyme particle suspension in front different impeller speeds: 100, 160, 260 and 360 rpm. Simulated reactions were prepared using 40 g of alperujo oil and the corresponding amount of biocatalyst. Samples of 0.5 mL were taken just from under the vortex effect, in the middle of the reactor. Then, samples were filtered, washed using heptane to remove oil impurities, dried overnight using silica-gel sieves and weighed [13]. Enzyme Particle Concentration (EPC) was calculated as follows (Eq. 3):

$$EPC\ (\%vol) = \frac{Enzyme\ weight\ (g)}{Actual\ enzyme\ concentration\ (g\ mL^{-1}) \cdot 0.5\ mL} \cdot 100 \qquad (3)$$

### 4.11. Sample treatment

Since the low volumes of withdrawn samples – mainly fatty acids alkyl esters and some unreacted oil – are highly viscous, volume measurements could not be accurately achieved. Thus, mass measurements – about 10 mg per sample – were performed considering approximately the reaction medium density each time. Then, samples were diluted with HPLC grade *n*-heptane (Sigma-Aldrich Co., Sant Louis, Missouri, USA). After that, 50 µL of internal standard – methyl heptadecanoate in *n*-heptane, 1.928 mg mL$^{-1}$ – and 50 µL of diluted sample were mixed in HPLC vials ready for analysis.

### 4.12. Fatty acid alkyl esters and oleic acid analysis

Fatty acid alkyl esters, oleic acid standards were purchased in Sigma-Aldrich Co. (Sant Louis, Missouri, USA) and treated as method followed in 4.11.
FAME, FAEE and oleic acid sample concentrations were quantified using a modified method previously used by the group [10]. A 7890A Agilent Gas Chromatography equipment (Agilent

Technologies, Santa Clara, USA) with a capillary high-polarity-column HP-INNOWAX 19095N-123 (30 m x 0.53 mm id, x 1.0 µm) were used.

Inlet temperature was set to 300°C and 6.5 psi. Initial oven temperature was set to 150°C with no hold time. Then, a ramp of 24°C min$^{-1}$ until 240°C was performed with a hold time of 17 min. Total method time was 20.75 min, reducing a nearly a 50% of the total time respect the previous method. Detector temperature was set to 320°C with a H$_2$ and airflow of 40 mL min$^{-1}$ and 400 mL min$^{-1}$, respectively.

Values of RSD for FAME/FAEE and oleic acid was determined as 3% and 7%, respectively.
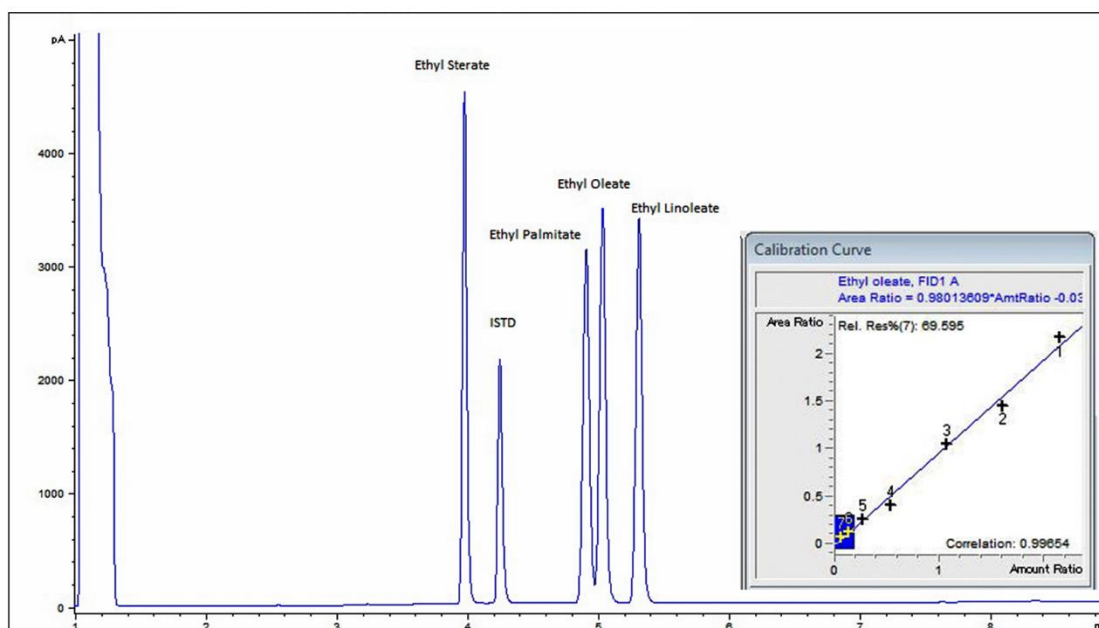


**Figure 4.13.** Example of chromatogram for FAEE and calibration curve obtained via gas chromatography.

## 4.13.   Economic evaluation software

SuperPro Designer®, version 7.0, was used since this software facilitates modelling, evaluation and optimization of integrated processes in a wide range of industries, such as biodiesel. Some works where SuperPro Designer® is used for the modelling of biodiesel synthesis processes can be found in the literature [14], [15].

Simulation was divided into two parts: the batch phase comprising the substrate treatment and transesterification reaction followed by the continuous phase including the downstream and purification of biodiesel and by-products.

## 4.14. References

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# RESULTS I

## First evaluation of the use of alperujo as a substrate for enzymatic biodiesel synthesis by covalent-binding immobilisation

# 5

# 5. RESULTS I. CONTENT

## 5.1. Introduction

Production of biodiesel (mono-alkyl esters of long chain fatty acids) is widely implanted and described nowadays, due to the fossil fuel reserves depletion.

The most common way to produce it is through chemical transesterification using a basic catalyst, mainly. There is a wide range of substrates able to produce biodiesel through this method. Most of them are vegetable oils such as corn, palm, cottonseed, sunflower or soybean [1], [2]. However, the disposition of agricultural lands to biodiesel producing companies, and the subsequently utilisation of these edible oils, generates a constant ethic conflict.

Thus, the feedstocks for biodiesel production has experienced a change recently in order to keep away this problem. Nowadays, edible oils should be avoided for this application in preference to the non-edible vegetable oils as well as waste oils from industry or restaurant sector [3]. Another source is the derived lipid from microalgae. Species with high lipid content and relatively small cultivation areas are *Chlorella* and *Dunaliella*, whose biodiesel productivity can be up to 800 times more than the productivity when using oils from crops [4]. In addition, oleaginous yeasts have been studied as lipid source. Advantages when using oleaginous yeasts, such as *Candida* sp., are the high amount of accumulated lipids in its biomass (>20% w/w), its short life cycle and its production non-dependent to climate factors [5].

The major problem that appears when using these substrates is the high content of free fatty acids. To carry out the alkali catalysed transesterification correctly, FFA values lower than 0.5-3% are needed [3], [6]. However, it should be noted that the range of FFA values in non-edible oils or fats can significantly vary, even reaching high values [7]. In these cases, the reaction stops because of the soap formation due to the basic catalyst [3], [8]. So, basically, the substrates are previously pre-treated in order to reduce this FFA content and to remove some impurities and other components [9]. It is known that this process may take some time and also it may add some costs to the final process. In this way, alternatives have been developed in order to avoid the problem of saponification, and also to enhance productivity and environmental benefits. It has been reported some alternative methods in order to carry out direct esterification of FFA with solid acid catalyst, which reduce the FFA levels during the biodiesel synthesis reaction [10]. Biodiesel synthesis through enzymatic biocatalysis has been applied by far as the most attractive solution to this problem. Lipases – triacylglycerol acyl hydrolase EC 3.1.1.3 –  are the enzyme which catalyses biodiesel synthesis and they are also used for huge other applications [11], [12].

In the present thesis, alperujo oil (Fig 5.1.) was used as a substrate for biodiesel production. Alperujo, in its initial form, it is a non-edible oil that comes from the olive extraction processes.

Concretely, the residual by-product of the olive mechanical press still contains a small amount of oil that can be extracted with solvents [13], [14]. After a drying the by-product, an extraction with hexane is performed (crude alperujo). Then, a distillation and refinement of the alperujo to become edible is needed [15].
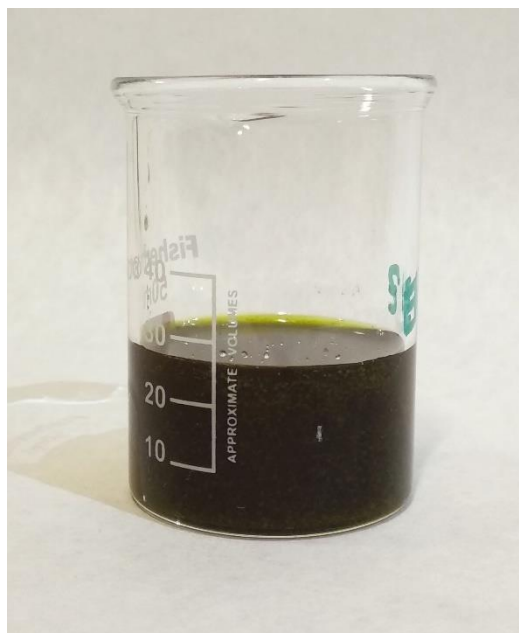


Figure 5.14. Alperujo oil, used as biodiesel feedstock.

It is a by-product easily available in Mediterranean regions – only Spain generates approximately 132000 tons of refined orujo per year, depending on the season [13], [16] – so it can be a low-cost feedstock.

Alperujo oil composition consists of pieces of skin, pulp, stone and seed – of the total olive weight, the pulp forms about 70-90%, the stone 9-27% and the seed about 2-3% [17] –. However, depending on the olive type, climate and extraction methods all these proportions can significantly change.

During the extraction, if high temperatures are used, toxic compounds such as phenols and benzopyrenes are produced [13], [17]. High-concentrated phenolic compounds can lead to serious ecological problems once it is scattered on the soil, so it is non-friendly to the environment [18]. Benzopyrenes are carcinogenic fatty elements that can enter the cell membranes and cause cellular oxidation leading to cell aging and even death [13], [19], [20]. Thus, biodiesel synthesis using raw alperujo oil could be sustainable way to revaluate it. In this study a first approach to produce biodiesel from a high-FFA content non-edible feedstock – alperujo oil – through enzymatic transesterification using recombinant ROL is proposed. Some

recent works have demonstrated the efficiency of regioespecific fungi lipases, such as ROL or *Rhizomucor miehei* lipase (RML), by keeping glycerol in monoglycerides form [21], [22]. Monoglycerides can be left in the final biodiesel in certain concentrations to increase lubricity or purified in order to obtain a value-added by-product.

Since benefits of immobilisation have been widely described before [23], [24], covalent attachment of the enzyme was used and evaluated in this study. Recent works of the group successfully achieved the adsorption immobilisation of this enzyme and its use for biodiesel synthesis [25]–[27] but covalent immobilisation was never performed for this purpose. Principals advantages of covalent respect adsorption immobilisation is the decrease of enzyme leaking due to the attachment itself. However, it can produce physical modifications on the enzyme that may lead to alterations on the activity.

Thus, here it is presented a brief study of the robustness of the biocatalyst comparing a covalent immobilisation on commercial polymethacrylate epoxy-amino support (HFA) with an glutharaldehyde-treated HFA carrier (HFAGlut) immobilisation. Several studies have used this carrier modification since it promotes the addition of a spacer arm between the enzyme and the carrier itself [28]–[30]. This alteration can decrease the present steric hindrance [31]. In addition, it is widely described the utilisation of glutaraldehyde to improve enzyme stability by multipoint or multi-subunit immobilisation [32].

Therefore, rROL immobilised into both carriers – forming the subsequent biocatalyst rROL-HFA and rROL-HFAGlut – were tested using a alperujo oil, briefly characterised. The role of the free fatty acids present in the substrate were evaluate in front the biocatalyst.

## 5.2. Results and discussion

### 5.2.1. Acidity and fatty acid content

Table 5.1. shows the substrates acidity characterisation – see 4.7. in Materials and Methods –. Supplemented alperujo acidity fitted to the acidity value of initial alperujo, demonstrating the correct application of the oleic acid supplementation method – see 4.6. in Materials and Methods –. This type of substrate was used at the end of the chapter in order to elucidate the actual role of FFA. In addition, it should be noted that it was considered that the low percentage

of neutralised alperujo acidity were negligible to better understand the subsequent reaction results.

**Table 5.1.** Acidity values of substrates.

| Substrate | Acidity (%) |
|---|---|
| Initial alperujo | 18.93 ± 0.93 |
| Neutralised alperujo | 1.705 ± 0.03 |
| Supplemented alperujo | 17.99 ± 0.33 |

Table 5.2. shows the main fatty acid content of alperujo. Alperujo oil is a direct sub-product from the olive oil crop; therefore, oleic acid is the main fatty acid present in it, representing an 89% of the total fatty acid content, followed by palmitic and linoleic acid, representing a 5.53% and 3.32%, respectively. Moreover, these values indicate that alperujo oil is composed mostly by a monosaturated fatty acid (89.73%), followed by saturated (6.78%) and polyunsaturated (3.49%). Biodiesel made from saturated fats has higher melting point, a disadvantage compared with unsaturated, which have better cold weather properties. However, highly unsaturated biodiesel may require oxidative stabilisers to be used safely as fuel [33]–[35].

**Table 5.2.** Fatty acid composition (%wt) of *alperujo* obtained by saponification.

| Fatty Acid | Composition (%) |
|---|---|
| Palmitic Acid (16:0) | 5.53 ± 0.20 |
| Steraric Acid (18:0) | 1.25 ± 0.15 |
| Oleic Acid (18:1) | 89.73 ± 0.64 |
| Linoleic Acid (18:2) | 3.32 ± 0.24 |
| Linolenic Acid (18:3) | 0.17 ± 0.09 |

### 5.2.2. Biocatalyst comparison: transesterification reactions in 10-mL vials

Transesterification reactions were carried out using 8 g of substrate in 10-mL vials at 30°C – see 4.9.1. in Materials and Methods– as well as in solvent-free medium for several reasons, but firstly to avoid later stages for biodiesel recovery [23]. In this study, four methanolysis reactions

were proposed according to each type of substrate – initial and neutralised, see 4.6. in Material and Methods – and biocatalyst – rROL-HFA and rROL-HFAGlut, see 4.5. in Material and Methods –. The time evolution of oleic acid and the reaction yield (FAME) – see 4.11. in Materials and Methods – are shown in Fig. 5.2. A-D. It is important to note here that according to the low amount of methanol added to prevent methanol inactivation [25], only 12% was the maximum yield to be achieved in the reactions, since initial rates were the relevant parameter to be studied here.
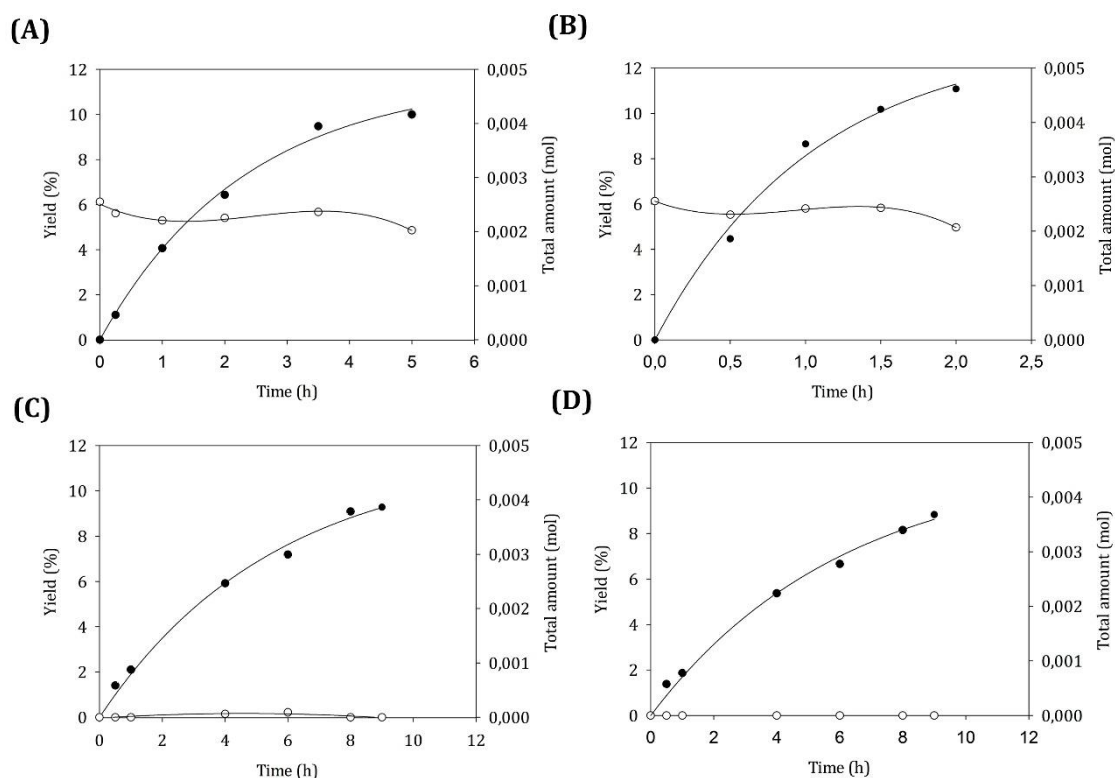


**Figure 5.2.** Time evolution of FAMEs yield (●) and oleic acid total amount (○) in the transesterification reaction using initial alperujo oil as a substrate and rROL-HFA (**A**) or rROL-HFAGlut (**B**) as a biocatalyst, or using neutralised alperujo oil as substrate and rROL-HFA (**C**) or rROL-HFAGlut (**D**) as a biocatalyst.

Firstly, reaction times were quite different in some cases. In the case of using initial alperujo as a substrate (Fig. 5.2. A-B), the maximum yield was achieved at 3.5 h when rROL-HFA was used as a biocatalyst and 2 h in the case of rROL-HFAGlut, resulting in a 2.6-fold higher initial rate in the second reaction (Table 5.3.), just for the only reason that rROL was immobilised in the glutharaldehyde-treated HFA support, instead of the commercial one. As said, it is known that this method allows improving the immobilisation rates by orienting the enzyme in a different way [36], extending its spacer arm between the support and enzyme, giving more versatility [32].

However, when neutralised substrate was used (Fig. 5.2. C-D), it took such higher times to reach the maximum yield (9%) than when oil with FFAs was used. In the case of the reaction catalysed by rROL-HFA biocatalyst, it took 8 h and the reaction catalysed by rROL-HFAGlut biocatalyst took up to similar time, 9.5 h. Observing figures 5.2. A and 5.2. B, it would seem that this enhancement on the initial reaction rate would came from the immobilisation method, but yield behaviours when neutralised alperujo was used led to think that there would be some kind of different reaction mechanisms. Indeed, it is clear that free fatty acids – mostly, oleic acid – played an important role in reaction kinetics because the absence of this component slowed the reaction up to 10 times.

Moreover, as shown in Fig. 5.2. A-B, the total amount of oleic acid remained stable during the both reactions, suggesting that it did not react with acyl donor. However, it is known that the directly FAMEs synthesis reaction by oleic acid and methanol in free-solvent media is fully described [37]. Therefore, it seems reasonable to expect that the minimal water resulting from the direct esterification reaction would lead in a continued oleic acid obtaining by hydrolysis of triglycerides. Nevertheless, reactions were not completed as it were performed using low volumes of methanol. Whole reactions were performed afterwards comparing two acyl-acceptors but also trying to elucidate the free fatty acids behaviour in some way. In fact, other studies demonstrated that transesterification is actually a combination of two processes, namely: direct alcoholysis of triglycerides and a two-step reaction involving hydrolysis of triglycerides followed by esterification of previously released free fatty acids [27].

**Table 5.3.** Initial rate values for each reaction.

| Substrate | Biocatalyst | Initial rate ($\mu$mol FAME mL$^{-1}$ min$^{-1}$) |
|---|---|---|
| **Initial *alperujo*** | rROL-HFA | 2.493 |
| | rROL-HFAGlut | 6.482 |
| **Neutralised *alperujo*** | rROL-HFA | 0.748 |
| | rROL-HFAGlut | 0.640 |

### 5.2.3. Stability-testing reaction cycles

It has been widely reported that lipases have an important decrease of their activity due to the methanol presence in the reaction media and specifically, when it is prolonged over long periods of time [3], [21], [38]–[40].

In order to know how the two tested biocatalysts worked in the presence of methanol, a serial of biodiesel synthesis reaction cycles was carried out. The final yield of each reaction was compared to first one to determine the decreasing of the biocatalyst activity. The results are shown in Fig. 5.3. A-D.

Lipase activity was quite preserved when initial alperujo oil was used as a substrate, regardless of the utilisation of both biocatalysts as shown in Figure 5.3. A-B. When rROL-HFA biocatalyst was used, a 36.5% of activity was lost during 10 batch cycles – summing up a total of 35 hours of reaction –. It is also true that just the 21% of activity was lost during 9 batch cycles – a total of 18 hours of reaction – regarding the case when rROL-HFAGlut was used.
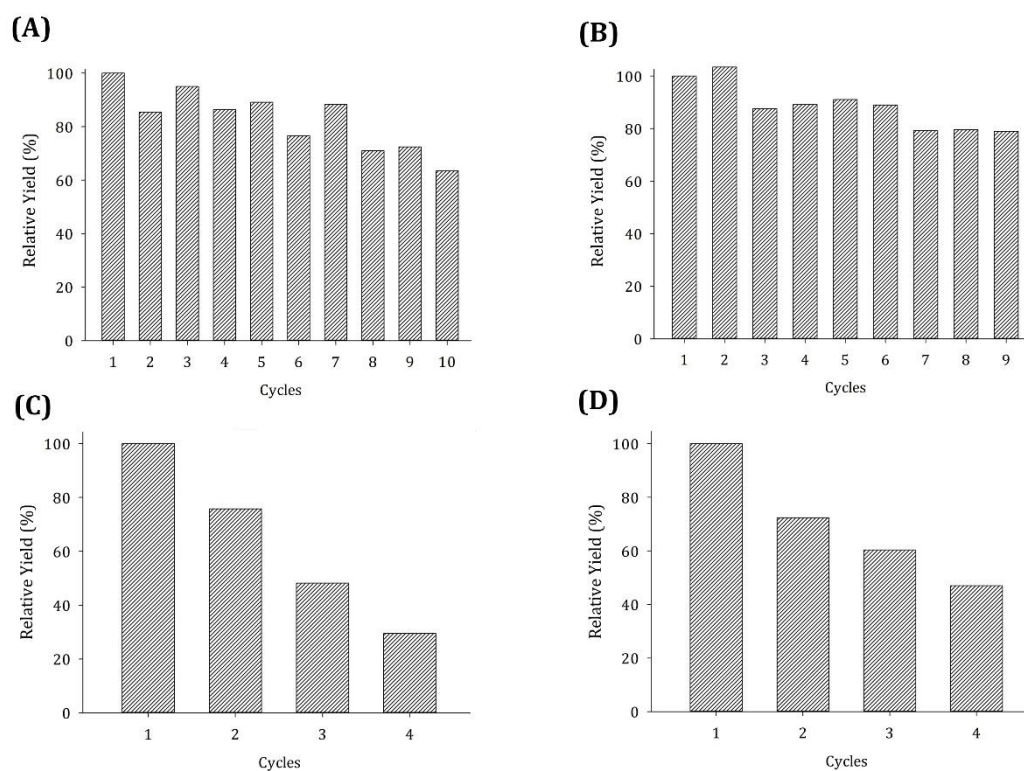


**Figure 5.3.** Relative yield (considering first reaction achieved yield as the 100 %) using the rROL-HFA (**A**) or rROL-HFAGlut (**B**) as a biocatalyst and the initial alperujo as a substrate, or using the neutralised alperujo and the rROL-HFA (**C**) or rROL-HFAGlut (**D**) as a biocatalyst, after cycle reactions.

On the other hand, lipase activity lost more than 50% of the initial activity, concretely more than 70% and 53% when neutralised alperujo and rROL-HFA and rROL-HFAGlut were used, respectively (Fig. 5.3. C-D). In both cases it occurred after 4 batch cycles, however considering that the biocatalyst spent up to 32 and 36 hours – rROL-HFA and rROL-HFAGlut, respectively – in contact with the methanol, these results were only comparable to the results when initial

alperujo and rROL-HFA were used, when the activity loss were 37% respect the first reaction after a similar time (35 h). Therefore, these results led to think that the absence of FFAs in the substrate not even have adverse consequences in terms of initial rate but also for the biocatalyst stability.

Comparing the loss of lipase activity after 18 hours working with initial alperujo – 5 and 9 cycles in the case of rROL-HFA and rROL-HFAGlut, respectively – it was the same in both cases, about the 20%. So, it is clear that, the support used and the immobilisation method did not affect the enzyme stability; also regarding no significant differences when neutralised alperujo was used.

To conclude, it is demonstrated that this major loss in the lipase activity came from the presence of methanol in the reaction media, and the consequent lipase exposure to it during long terms of time. On the other hand, FFAs presence seems to reduce negative effect of methanol in a same exposure time and in addition it allows faster reaction rates.

### 5.2.4.  Supplemented oil reactions

It is known that alperujo oil contains a lot of components that make this substrate different from other vegetal feedstocks. Most of these components are lignin, hemicellulose, cellulose and fats [14]. In order to assure that the great differences in the initial reaction rate between neutralised and initial oil, came from the presence of FFAs, a verification test was carried out. Thus, a new substrate was prepared – see 4.6. in Materials and Methods –. Basically, a specific amount of oleic acid was added to the neutralised oil to simulate the initial one acidity, about 19%.

rROL-HFAGlut was selected as a biocatalyst, because resulted in the best option since it has been demonstrated that the initial reaction rate and stability was much better than when rROL-HFA was used.

As shown in Fig. 5.4. A, the yield behaviour of the rROL-HFAGlut working with supplemented substrate was almost identical when compared to the rROL-HFAGlut using initial alperujo. About a yield of 11% was achieved in both cases in 2 hours of reaction. Furthermore, initial reaction rate was maintained, as shown in Table 5.4. The slightly higher value of oleic acid amount in the supplemented oil reaction would come from the consideration that neutralised oil was supplemented entirely with oleic acid, whereas the initial oil acidity may come from different components.

In order to determine and compare the stability of the biocatalyst when it is used in supplemented substrate reactions, a serial of reaction cycles was carried out following previous described steps (Fig. 5.4.B). The biodiesel production capacity loss was only an 8.2% during 5 batch cycles. Comparing this results with the biocatalyst stability when using initial oil (Fig. 5.3.B)

it was slightly similar, since only a 10% activity was lost during 10 hours in both cases. Thus, it is clear that the presence of FFAs in the substrate is the reason of a favourable increasing of the initial reaction rate and also in the enzyme stability. Free fatty acids are more polar than triglycerides, thus adding these compounds in such a high concentration increases the polarity of the medium. Then, methanol – polar specie – becomes more soluble reducing high concentration gradients and avoiding to a lesser extent lipase damage [41], [42].

**(A)**     **(B)**
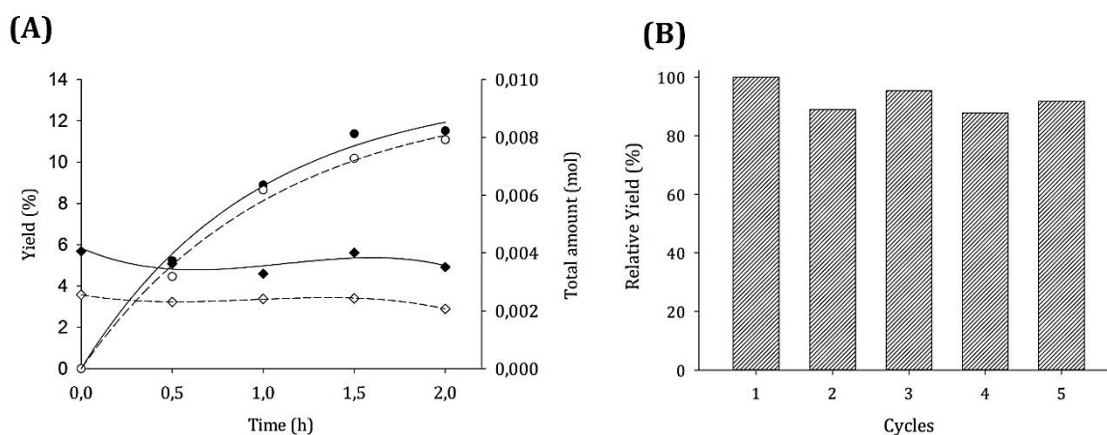


**Figure 5.4. A**: Time evolution of FAMEs yield when using initial (○) or supplemented (●) oil, and oleic acid total amount present in media when using initial (◊) or supplemented oil (♦), both reactions using rROL-HFAGlut as a biocatalyst. **B**: Relative yield – considering the first reaction yield as 100% – when using supplemented oil as a substrate after cycle reactions.

**Table 5.4.** Initial reaction rate values when different substrates were used.

| Biocatlayst | Substrate | Initial rate (µmol FAME mL$^{-1}$ min$^{-1}$) |
|---|---|---|
| rROL-HFAGlut | Initial *alperujo* | 6.482 |
| | Supplemented *alperujo* | 7.280 |

## 5.3.    Conclusions

Activation using ethylenediamine and applying the treatment with glutaraldehyde on the carrier resulted in an enhancement of the initial transesterification rate not observed when no modification was done.

In terms of substrate suitability, performing these briefs studies, it can be said that alperujo oil stands as a promising biodiesel feedstock due to its properties. Besides that, it is pretty available

and it can be used as a model of high FFA feedstocks. In addition, synthesis of biodiesel from this oil could allow its revaluation as a main part of this sustainable process.

Finally, in terms of reaction kinetics, FFAs presence on this feedstock seems to in to increase polarity in the medium, which provided higher initial reaction rates and enhanced the enzyme stability along the biocatalyst reuses. Reactions spent up to 4 times less when alperujo oil was used instead of neutralised.

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# R E S U L T S II

## Comparative stepwise addition study
## of methanol and ethanol as acyl-acceptor

# 6

# 6. RESULTS II. CONTENT

## 6.1. Introduction

In the last decades, the use of lipases for biodiesel production in front alkali-catalysed transesterification has arisen due to its advantages, such as less energy consumption, more environmental-friendly process because it does not generate as much as waste than the chemical one, and the immobilisation of catalyst turns its recovery much easier [1], [2]. In addition, another advantage of using lipases is its perfect compatibility with FFAs, which are present in the major of non-edible oils. It has been widely reported, not only the possibility of synthesise biodiesel by the direct esterification of FFAs [3]–[5], but also the reaction benefits when using substrates with high FFA content [6], [7].

The major problems present in enzymatic-catalysed transesterification, aside the high cost of the enzyme [8], [9], is the inhibition of the lipase by the acyl acceptor. Short-chain alcohols like methanol, ethanol and others like *tert*-butanol or propanol are the most widely used due to their high availability, low price and reactivity against triglycerides [10].

Methanol has been reported to be the principal cause of enzymatic deactivation during the transesterification reaction [2], [11]–[13]. Nevertheless, the origin of the detrimental effect of methanol is still poorly understood, as one can guess also from the non-specificity of the terms used to describe it, such as inactivation, deactivation, inhibition, or denaturation [14]. This hindering effect of methanol is even more acute when free-solvent systems are used. The most convincing hypothesis is to assume that high concentrations of this alcohol takes off the structural water surrounding the active site [6], [15]. Although this major drawback, methanol is still the most used alcohol due to its availability and economic feasibility [9].

Likewise, ethanol – not as harmful for the lipase as methanol – has been also used widely for biodiesel production [16]–[19], because of some advantages such as major solubility in triglycerides [20] and its low toxicity and easier to handle in front methanol. In addition, is a preferred alcohol in transesterification reaction compared to methanol because it is derived from agricultural products and is renewable and biologically less objectionable in the environment [21].

Nevertheless, the problem of alcohol inactivation can be partially overcome by several strategies. For instance, one of the most used is the addition of organic solvent to the medium in order to improve solubility of these alcohols and reducing the viscosity of the medium [22], [23]. But the use of organic solvents is undesirable due to increased costs, environmental concerns, and the need for further downstream processing [23], [24].

Another strategy which is also widely implemented, evenmore in free-solvent medium, is stepwise addition of the alcohol [9], [25]–[27]. If correctly applied, this method avoids lipase inactivation due to low concentrations of alcohol present in the system, since it is added as system requires it [28], [29]. It should be noted that, although this strategy is the most common used one, not all lipases are equally inhibited by alcohols. For instance, non-regiospecific lipases like *Candida rugosa*, *P. cepacia* and *P. flourescens* are tolerant to the inhibition of methanol [8] in contrast to *Candida Antarctica* lipase which is found to be very sensitive to this alcohol [14]. Since tolerance to methanol seems to be inherent of lipases, several studies have been focusing on developing novel and improved enzymes through protein engineering methods [30], [31].

Other key parameter in enzymatic reactions is the water activity ($a_w$). Some studies have stated that this parameter is important in order to achieve higher yields because it is directly linked to the reaction's thermodynamics [32], [33] as water activity defines the total amount of water present in the system which is available to react. In addition it is also linked to the hydrolytic activity of some lipases [34], [35].

In this work, lipase dependence on initial water activity and temperature has been studied, as well as the utilisation of methanol and ethanol as an acyl acceptor via different stepwise-addition strategies. Recombinant 1,3-regioespecific *Rhizopus oryzae* lipase (rROL) covalently-immobilised in HFAGlut support in a free solvent media was used. As said, this kind of lipases synthesises biodiesel without forming glycerol as a by-product. As several studies have stated that glycerol may cause inactivation of lipases by adsorbing on the carrier forming a hydrophilic environment [2], [36] thus increasing mass transfer limitations and reducing final biodiesel yield. Instead of glycerol, 2-monoacylglycerol is produced, which is a product with an added-value mainly used as emulsifier, lubricant and food surfactant [37], [38]. While non-specific lipases are the most used for biodiesel production, the use of positional-specific such as *Rhizomucor miehei* lipase (RML) have shown a great results and efficiency [39], [40].

As said before, alperujo is an easy available by-product form the olive oil extraction processes [41]. Its high content in FFA – a value between 19-24%wt –, besides organic matter, makes this oil a perfect model for waste oils [7].

In this study it is assumed that the total amount of biodiesel produced came from both reactions (Fig. 1.10.): transesterification of triglycerides and direct esterification of FFAs whether free in medium or previously hydrolysed from triglycerides, as it is raised by other works [6], [42], [43] and no acyl-migration occurred since it is favoured by polar systems and long-term reactions [44].

## 6.2. Results and discussion

### 6.2.1. Effect of the water activity

Some studies have stated that one of the most important reaction parameter, especially related with the kinetics, is the water activity ($a_w$) [45]. Even though the reaction media contain mainly organic solvent and/or substrates, some water is needed to keep the enzyme active, called structural water [46]. Lipolytic activity of lipases is affected by a wide range of water activity values, depending on the specie and genus [33], [47]. Even more, the optimal water activity value differs significantly depending on the enzyme surround and the reaction system [2], [47]. In this work, a recombinant *Rhizopus oryzae* lipase was used in free-solvent medium and it is worth noting that activity water effect in these kind of reaction media is yet understudied.

Thereby, a set of methanolysis reactions with 8 g of alperujo oil at 30°C were carried out pre-equilibrating the system (see Materials and Methods 4.8) with six different initial water activity values trying to cover the entire range – from 0.033 to 0.976 –. Initial reaction rate – in µmol FAME $mL^{-1}$ $min^{-1}$ – was calculated for each reaction adding one pulse of methanol representing a 12% of total stoichiometric volume, in order to avoid inactivation effect on biocatalyst, since only the calculation of initial rates was relevant here.

Figure 6.1 shows that when lowest $a_w$ were tested, i.e. total amount of water present in the medium was insignificant, enzyme activity was hindered due to this water absence. In addition, when high $a_w$ were applied, for instance using $K_2SO_4$ with $a_w$ = 0.976, synthesis of biodiesel was no favoured since water amounts in the system led to promote hydrolysis of triglycerides. Then, a curve with a maximum peak was expected as it was observed when KOH salt was used in the pre-equilibrium at $a_w$ = 0.093 with an initial rate of approximately in 24 µL FAME $mL^{-1}$ $min^{-1}$. Some studies have reported matching cases for the same *Rhizopus oryzae* lipase [20]. At this point, sorption isotherms of similar substrates showed that initial moisture content was approximately 1%, while pre-equilibrating with $K_2SO_4$ moisture content represented a 15-20% [48], [49]. In addition, low initial rates exposed in previous chapter (see Table 5.3.), showed that reactions occurred in a system with high $a_w$, with a value about 0.8-1.0.

In the following experiments, pre-equilibrium of all reaction components separately at $a_w$=0.093 were carried.
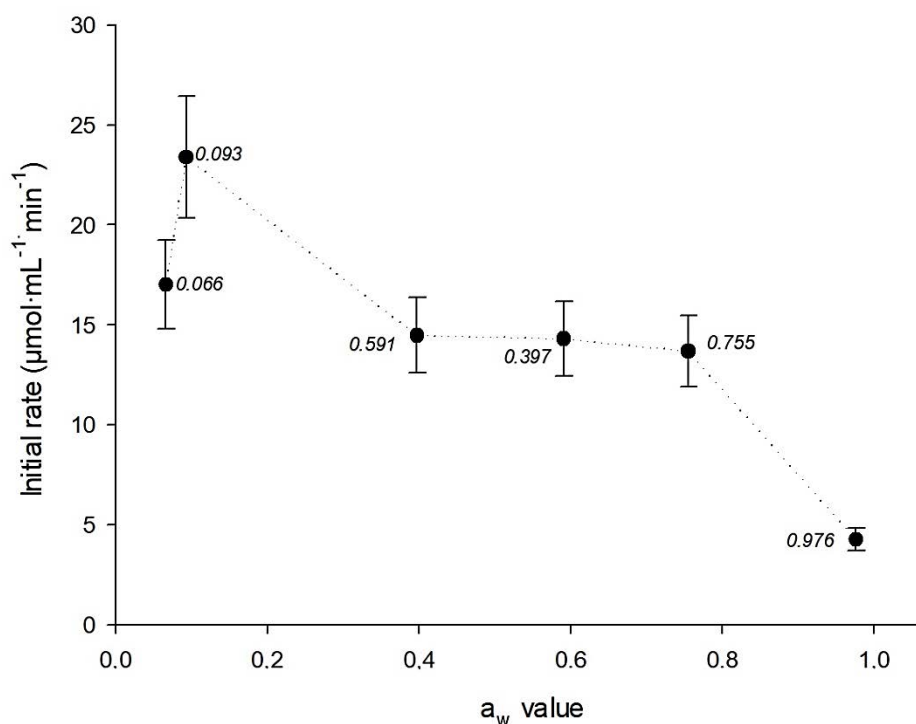
**Figure 6.1.** Initial reaction rate profile of recombinant *R. oryzae* immobilised in HFAGlut support as a function of water activity at 30°C using alperujo as a substrate.

### 6.2.2. Effect of the temperature

As it is known, temperature is also a key parameter in enzyme-catalysed reactions. Higher temperatures induce higher reaction rates and may reduce mass transfer limitations because of the reduction of substrate viscosity. However, higher temperature values can inactivate enzymes. Even though recent studies had well characterised the optimal temperature for free lipase activity [50], three mild temperatures were tested to observe which promoted better transesterification rate of immobilised lipase. In order to study enzyme stability, reactions were carried out in cycles – reusing the biocatalyst – to see the activity loss at 30°C, 40°C and 50°C. Methanol addition was performed in five pulses of methanol – 2:1 oil to alcohol molar ratio – to reduce alcohol inactivation [26].

As shown in Figure 6.2., higher biocatalyst's stability was obtained when reactions were carried out at 30°C, with an activity loss of 62% in the fourth cycle – total time in contact with methanol of 20h –. Although higher yields and faster reactions were achieved at 40°C and 50°C, these high temperatures were detrimental for biocatalyst life since its activity loss was 90% in 17h (4 cycles) for 40°C experiment, and 95% in 10h (3 cycles) for the 50°C. In that way, it was preferable to

maintain the enzyme activity to reuse it in further reactions than achieving higher yields but hindering enzyme life.
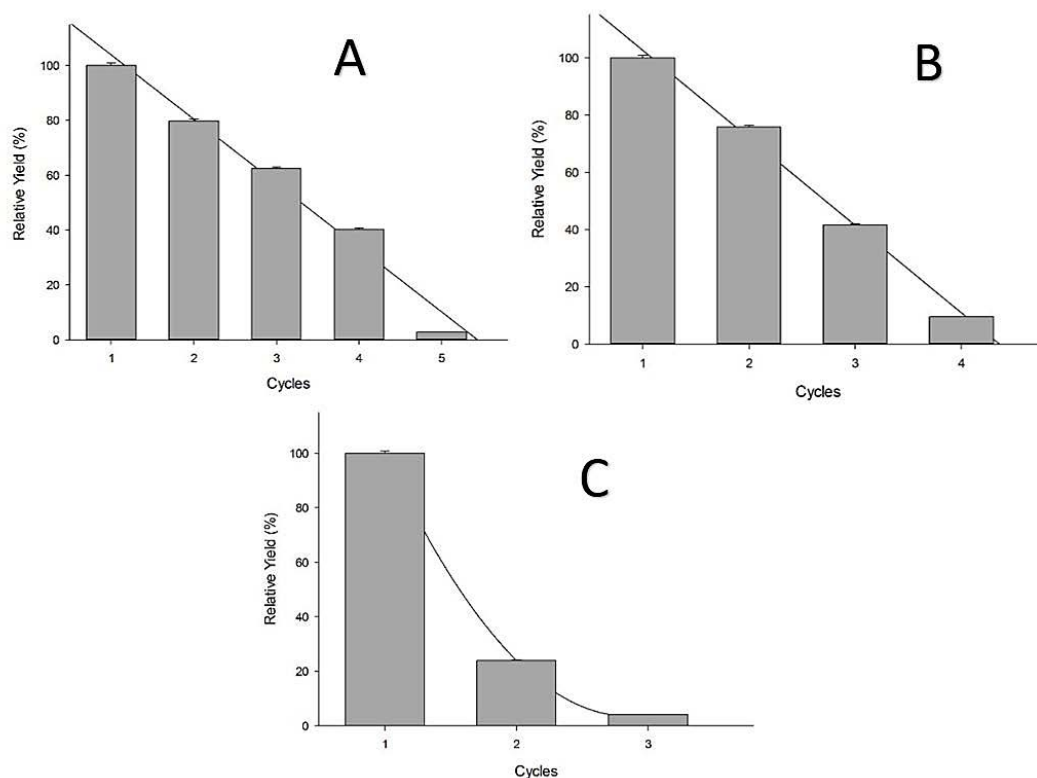


**Figure 6.2.** Relative yield – considering first reaction yield as 100% – of 5-pulse methanolysis reactions at three different temperatures (A, 30°C; B, 40°C; C, 50°C).

Productivity values were calculated for the first 10h. Table 6.1 shows how, in the case of 30°C and 40°C, productivities were nearly the same – about 41 µmol of FAME per minute – while at 50°C it was very low since enzyme stability was completely hindered due to high temperature. However, taking into account that 10 hours represented different number of cycles for each reaction – 2 cycles in the case of 30°C and 3 cycles in the case of 40°C and 50°C – the volumetric productivity was very different. Thus, a 1.5-fold higher value was achieved when reactions were carried out at 30°C, evidencing that milder temperatures resulted in better results in terms of global productivity.

**Table 6.1.** Productivity values of biodiesel synthesis reactions by stepwise addition along the stability tests (10 hours).

|  | 30°C | 40°C | 50°C |
|---|---|---|---|
| **FAME produced (mols)** | 0.0247 | 0.0248 | 0.0129 |
| **Productivity (µmols min$^{-1}$)** | 41.21 | 41.33 | 2.15 |
| **Volumetric productivity (µmols min$^{-1}$ mL$^{-1}$)** | 2.32 | 1.55 | 0.081 |

### 6.2.3. Enzymatic load in HFAGlut support

It has been previously demonstrated that treatment of commercial support HFA with glutaraldehyde has better results in terms of initial reaction rate and also in terms of biocatalyst stability when a high-FFA feedstock is used. In addition, it was tested several water activities pre-equilibration and three temperatures. Water activity of 0.093 and 30°C reactions showed the best results. Next experiments were carried out to determine the possible load of rROL without hindering the initial rate in a biodiesel synthesis reaction. Since enzymes are expensive, the key here is to use the minimum amount of rROL to get the maximum initial rate.

Several reactions with 8 g of substrate at 30°C using set of biocatalysts with increasing load of enzyme were carried out. A low-volume pulse of methanol corresponding a 12% of total yield was added in order to avoid lipase inactivation. Pre-equilibration of all components separately were performed in a water activity of 0.093.

As shown in Fig 6.3., a nearly linear relationship between initial rate and enzymatic load is present, up to 300 UA mg$^{-1}$ support. Over this value no increase in initial rate was observed due to mass transfer limitations.
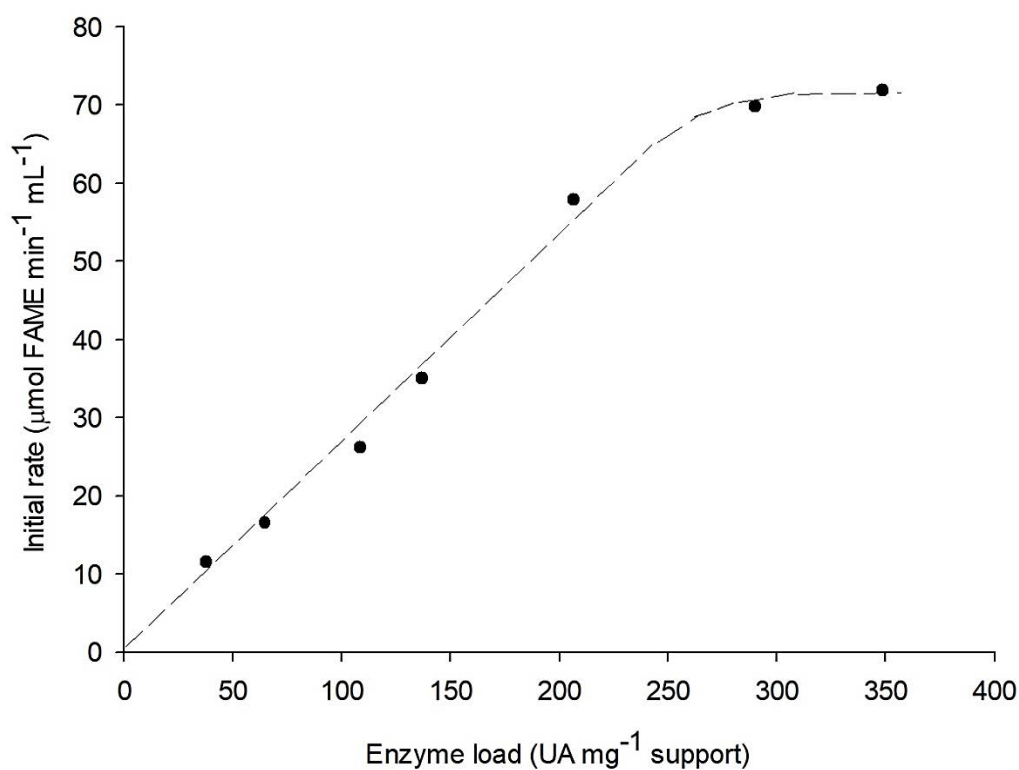


**Figure 6.3.** Correlation between initial rate of transesterification reaction and enzyme load in support.

Thus, it was decided that the next experiments would be performed using biocatalysts with a rROL load value within a 250-300 UA mg$^{-1}$ of support range in order to get the maximum reaction rate but spending the less enzyme.

### 6.2.4. Effect of stepwise addition, comparing methanol and ethanol as acyl-acceptor

Methanol and ethanol have been the most commonly used acyl acceptors in biodiesel synthesis since these compounds are easily available and not as expensive as could be alcohols with longer carbon chains such as *iso*-propyl alcohol [51] or butanol [52], [53]. However this advantage, it has been widely reported that methanol is one of the most harmful alcohol and may cause lipase deactivation [9], [14], therefore some strategies have been proposed in order to avoid this enzymatic damage that impact on the activity of the subsequent reuses. Adding water to the system reduces high concentrations of methanol, but it may promote the undesired hydrolysis reaction [26], [54], as seen before. Here is presented a comparison of one the most frequently used methods, the stepwise addition of the acyl acceptors, as well as a comparison between methanol and ethanol [55]–[57].

As shown in Figure 6.4., adding the total stoichiometric volume of methanol for 8 g of substrate – approximately 0.75 mL – at once were detrimental for the lipase's activity and only a yield of 2.84% was achieved – considering the maximum yield is 66.67% due to the *sn*-1,3-regioespecificity of the lipase –. Another data confirming this low initial rate was the oleic acid behaviour, which seemed to be maintained constant along the reaction.

Moreover, adding the same stoichiometric amount of ethanol – approximately 1.05 mL – resulted in a reaction with a 49.61% yield in 360 minutes with a decreasing of the oleic acid. This evidenced the both widely known reactions: transesterification and esterification. As said in the introduction, hydrolysis of triglycerides into free fatty acids and their following esterification to fatty alkyl esters are already described [6]. Thus, the decreasing of accumulation of oleic acid observed in the case of ethanolysis means that esterification rate was higher than formation rate from hydrolysis.

**Figure 6.4.** Time evolution of FAMEs/FAEEs yield and oleic acid of 1-pulse transesterification reaction using methanol and ethanol. Reaction conditions: 8g of alperujo oil, 2:1 alcohol to oil molar, 30°C and 350 rpm.

In order to evaluate the lipase stability at these conditions, a cycle-reactions were carried out, reusing the final biocatalyst with fresh substrate. Five ethanolysis reactions – a total of 30 hours in contact with ethanol – were performed. As it can be seen in Figure 6.5., a 52.4% of the initial activity was retained in the case of ethanol. Methanolysis stability reactions were not carried out since the low value obtained in the first cycle.

**Figure 6.5.** Relative yield – considering first reaction yield as 100% – of 1-pulse ethanolysis reactions. Methanolysis reaction is not shown due to the low yield achieved.

Figure 6.6. shows both methanolysis and ethanolysis reaction with the same previous conditions, same acyl acceptor's stoichiometric amount but added by 5 pulses of equal volumes. In the case of methanol, due to its lower initial rate calculated before the experiment, pulses were added every 60 minutes. The final yield achieved was 48.06% in 300 minutes, a 17-fold improvement of the previous result just doing it stepwisely.

In the case of ethanol, pulses were added every 40 minutes because of higher reaction rate. Here, an improvement of 17% was achieved in terms of final yield, obtaining a 58.16% in just 200 minutes, nearly a time reduction of 45%.

These results match with some previous studies reporting that stepwise addition of ethanol may increase both the final yield and immobilised lipase's performance in free-solvent [28], [59] or in solvent system compared with the same strategy using methanol [60].
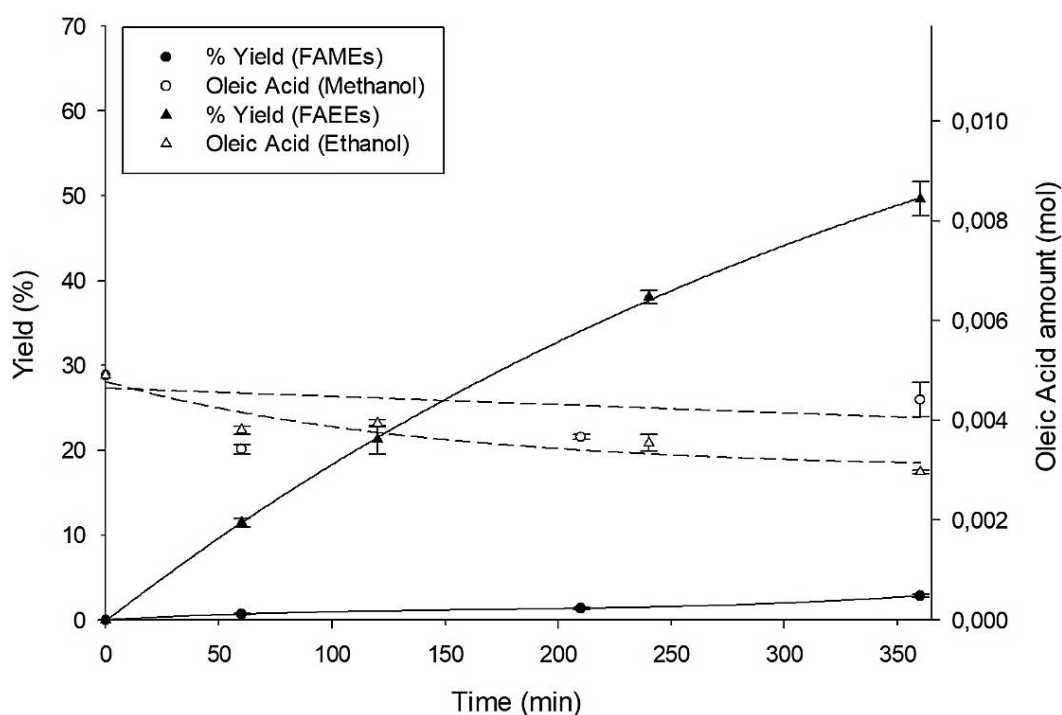
**Figure 6.6.** Time evolution of FAMEs/FAEEs yield and oleic acid of 5-pulse transesterification reaction using methanol and ethanol. Each point corresponds to an alcohol pulse, except the final point. Reaction conditions: 8g of alperujo oil, 2:1 alcohol to oil molar, 30°C and 350 rpm.

The slow feeding of methanol into the medium allowed enough enzyme stability to catalyse the esterification and transesterification reactions. In contrast to 1-pulse methanolysis reaction, when methanol was added using five pulses, decreasing of free oleic acid was also observed. The same behaviour was observed in the ethanolysis reactions but with a higher reaction rate for FAEE synthesis.

In terms of stability (Figure 6.7.), during 5 cycles of methanolysis reaction, rROL-HAFAGlut lost nearly the whole capacity of synthesising biodiesel, reducing the initial activity up to 97.3%. It is clear that, performing a fifth cycle was detrimental for the lipase's activity, since a 40.26% of initial activity remained after the fourth cycle (20 hours). In the case of ethanol, along the same 20 hours (6 cycles), more than 90% of lipase activity was retained. Comparing these results with one-step addition (Fig. 6.5.), dividing the total amount of ethanol into 5 pulses lead to increase stability 1.5-fold, from nearly 60% during 18-24 hours to 90% of retained activity in the same time. In the case of methanol, the improvement was still higher, since using one-step addition no reaction was occurred.
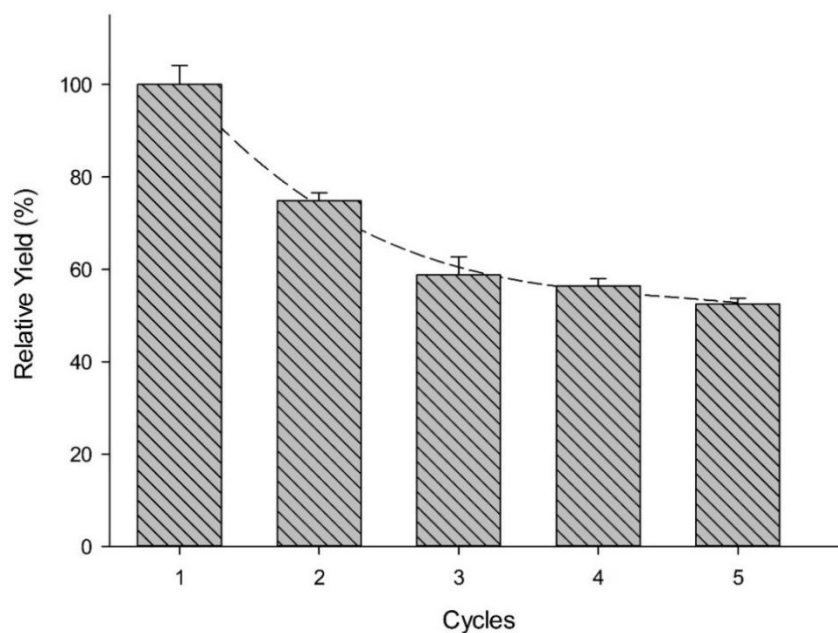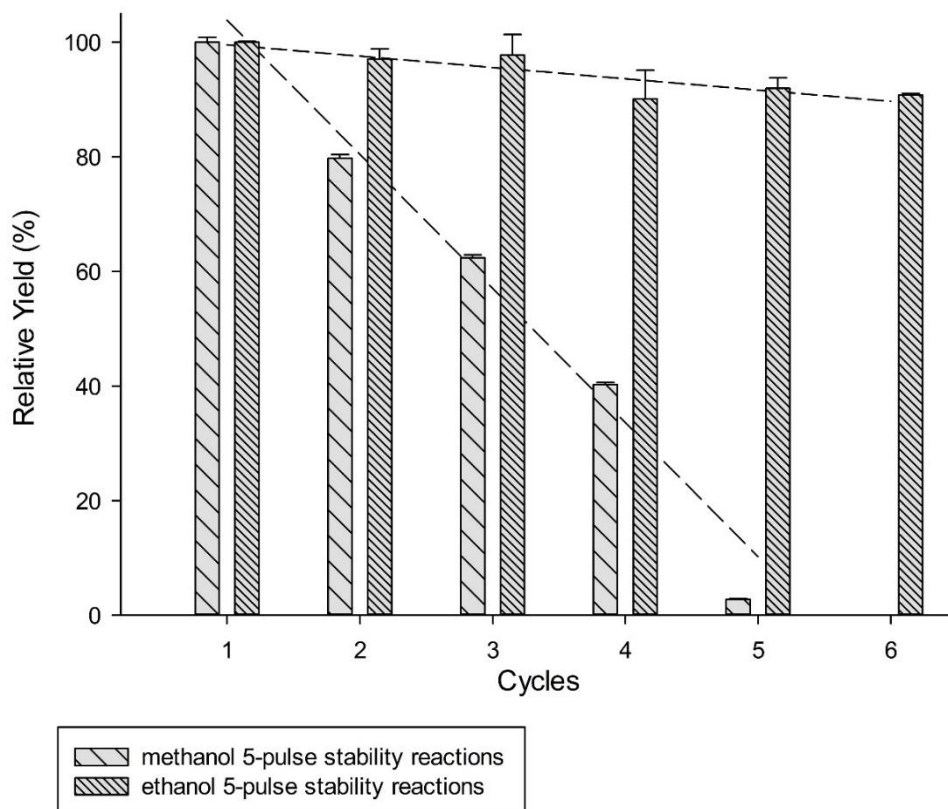
**Figure 6.7.** Relative yield – considering first reaction yield as 100% – of 5-pulse methanolysis and ethanolysis reactions.

Thus, adding the acyl acceptor stepwisely, reduced the damage on the lipase obtaining higher yield and also retaining more activity at the end of the cycles. Even so, harmful effects of methanol were still present.

Next experiments were carried out adding the same amount of alcohol but dividing it into ten pulses with decreasing volumes and increasing the addition frequency along the time (Table 6.2.). This strategy was chosen in order to emulate the yield evolution in the 5-pulse reactions, trying to add alcohol as the reaction needed it. Consequently, methanol or ethanol accumulation in the system was reduced and yield and stability should be enhanced indeed.

In the case of methanolysis reaction, shown in Figure 6.8, a final yield of 57.16% was achieved in 360 minutes, which means a relevant increasing of up to 19% respect 5-pulse addition. In the case of ethanolysis, a 60.25% of yield was achieved – which represents the 91.28% of the theoretical maximum yield – in 260 minutes, an increase of 3.59% compared with 5-pulses ethanolysis.

**Table 6.2.** Volumes of methanol and ethanol added for each pulse and frequency applied. For both cases alcohol to oil molar ratio of 2:1 was chosen.

| Pulse | Methanol | | | | Ethanol | | | |
|---|---|---|---|---|---|---|---|---|
| | Volume (mL) | Time (min) | Total time (min) | Methanol added (mL) | Volume (mL) | Time (min) | Total time (min) | Ethanol added (mL) |
| 1 | 0.103 | 60 | 60 | 0.103 | 0.174 | 40 | 40 | 0.174 |
| 2 | 0.103 | 50 | 100 | 0.206 | 0.158 | 30 | 70 | 0.332 |
| 3 | 0.091 | 50 | 150 | 0.297 | 0.126 | 30 | 100 | 0.458 |
| 4 | 0.091 | 40 | 190 | 0.389 | 0.111 | 30 | 130 | 0.569 |
| 5 | 0.080 | 40 | 230 | 0.469 | 0.111 | 25 | 155 | 0.680 |
| 6 | 0.080 | 35 | 265 | 0.549 | 0.095 | 25 | 180 | 0.775 |
| 7 | 0.069 | 30 | 295 | 0.617 | 0.095 | 25 | 205 | 0.870 |
| 8 | 0.057 | 25 | 320 | 0.674 | 0.063 | 20 | 225 | 0.933 |
| 9 | 0.037 | 25 | 340 | 0.711 | 0.063 | 20 | 245 | 0.996 |
| 10 | 0.020 | 20 | 375 | **0.731** | 0.054 | 15 | 260 | **1.050** |



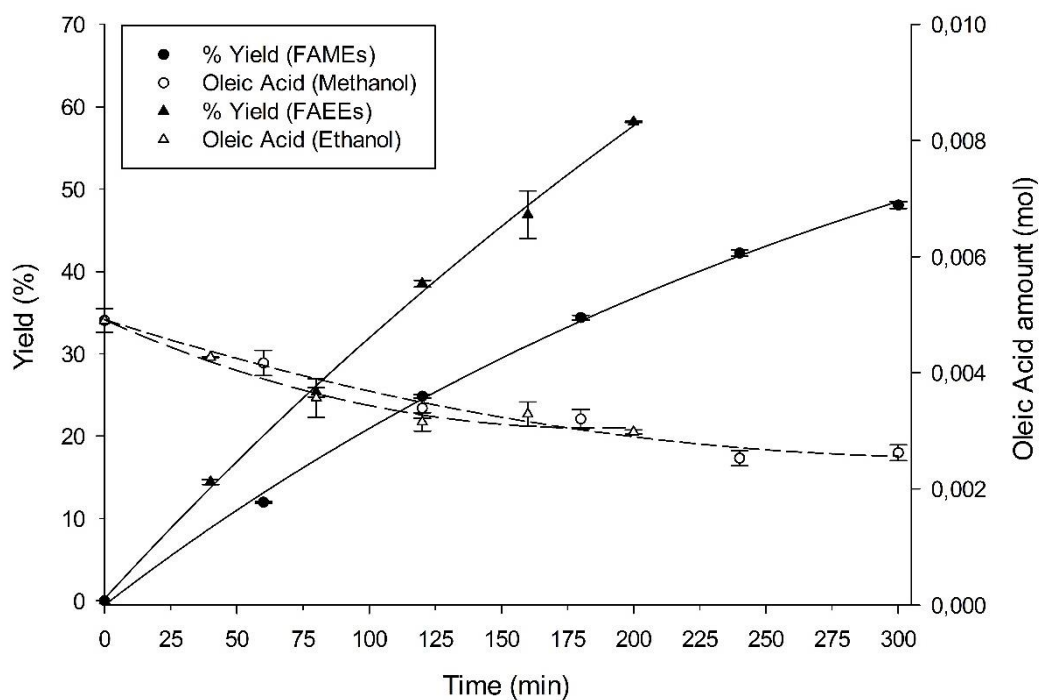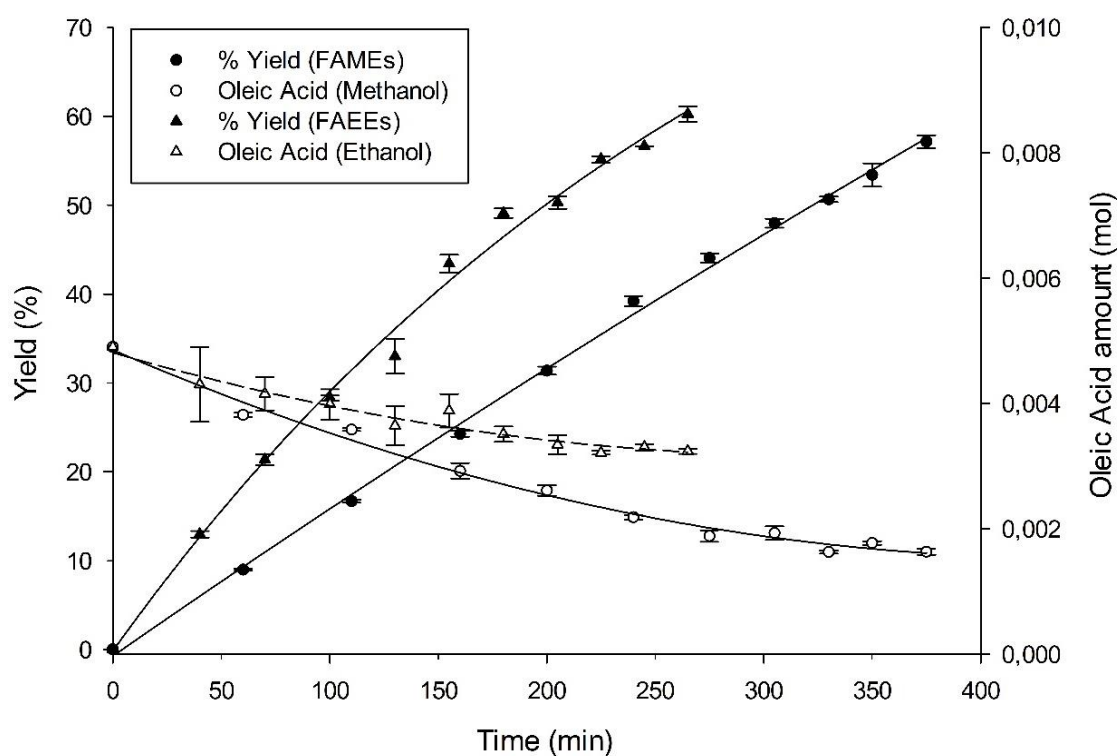**Figure 6.8.** Time evolution of FAMEs, FAEEs yield and oleic acid of 10-pulse transesterification reaction using methanol and ethanol. The first 10 points correspond to the 10 pulses. Reaction conditions: 8g of alperujo oil, 2:1 alcohol to oil molar, 30°C and 350 rpm.

These results showed that dividing methanol, avoiding the inactivation lipase to a lesser extent, allowed to obtain higher yields.

In addition, in contrast to 5-pulse reactions where the decrease of total amount of free oleic acid was similar for both alcohols, when ten pulses were added the disappearing of this compound was faster in methanolysis than in ethanolysis, which remained similar as in the previous case.

In order to elucidate the reason for this behaviour, an experiment was performed in the same conditions but using oleic acid as the main substrate instead of alperujo oil. Fig 6.9. shows that direct esterification using methanol and ethanol resulted in the same methyl or ethyl oleate formation rate. As expected, initial rate of direct esterification using both alcohols resulted to be slower that initial rate of alkyl esters formation in the reaction with alperujo (Fig 6.8.) where transesterification of triglycerides and esterification of free fatty acids were co-existing. Therefore, if one considered that esterification rates using both alcohols were similar, it was clear that direct transesterification via ethanol should be higher than using methanol. In contrast, as said before, free oleic acid was observed to be lower in 10-pulse than in 5-pulse methanolysis. This could happen due to the slow feeding of this alcohol when ten pulses were added which led to promote more esterification of free oleic acid.

Thus, considering the higher reaction rate of FAEE synthesis, it could be stated that ethanol resulted in a better acyl-acceptor than methanol in this system.
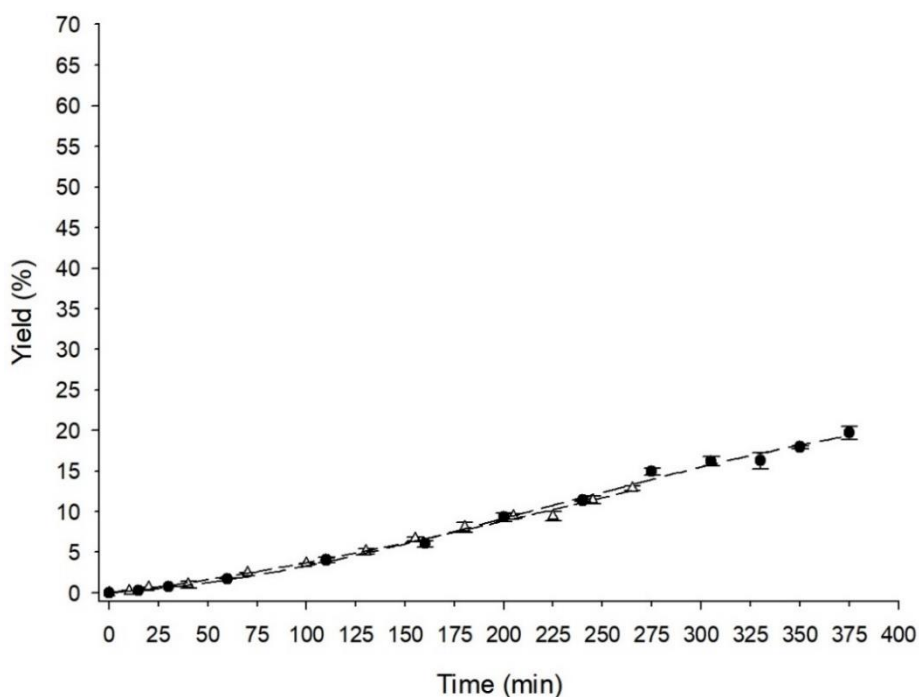


**Figure 6.9.** Time evolution of yield of direct esterification of 8g of oleic acid using methanol (●) and ethanol (Δ) as acyl-acceptors, at 30°C and 350 rpm.

In terms of stability, shown in Figure 6.10., the differences seen in previous experiments get narrower. After 30 hours of methanolysis (5 cycles), the activity of the rROL-HFAGlut was decreased only in a 12.31% respect the first reaction. It was a notable improvement compared with the 5-pulse methanolysis, which lost a 60% of the initial activity just in 20 hours (Figure 6.7). On the other side, an 88.11% of activity was retained in 7 cycles when ethanol was used, which corresponds to 30.3 hours of reaction.

It is clear that, as long as the total amount of alcohol needed was divided, the higher enzymatic stability was achieved, particularly in the case of methanol. As said before, methanol can cause inactivation of the lipase, even higher if no solvent is used, but this problem could be solved by using a properly chosen strategy of stepwise addition [12], [61], [62].
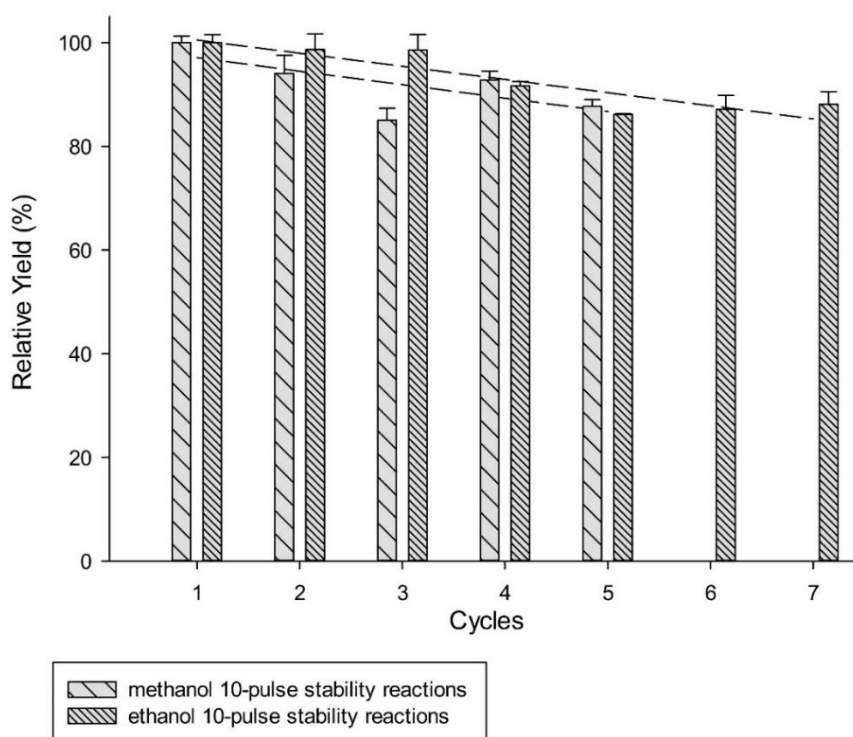


**Figure 6.10.** Relative yield – considering first reaction yield as 100% – of 10-pulse methanolysis and ethanolysis reactions.

Table 6.3. shows the calculated productivity for each reaction cycles up to approximately 20 hours to give an overview of all reactions. Methanolysis reactions' productivity were 1.68-fold lower than ethanolysis when 5-pulse reactions were employed, and 1.08-fold lower than ethanolysis when 10-pulse were carried out. Comparing both methanolysis reactions, the final

productivity increased a 52.17% when the methanol was added in 10 pulses compared with 5-pulse methanolysis, demonstrating the stepwise effect in such a harmful alcohol. On the other hand, a decreasing of a 2.58% of the final productivity were obtained when ethanol was added using the 10-pulse stepwise addition, since times between pulses in this case were overestimated, reducing productivity.

**Table 6.3.** Productivity values of biodiesel synthesis reactions by stepwise addition along the stability tests

| Reaction | Productivity ($\mu$mol biodiesel $min^{-1} mL^{-1}$) |
|---|---|
| 5-pulse methanolysis | 0.69 |
| 5-pulse ethanolysis | 1.16 |
| 10-pulse methanolysis | 1.03 |
| 10-pulse ethanolysis | 1.13 |

A fact that can be drawn from this was that, as the total amount of acyl acceptors was divided, the differences of the harmful effect between them were minor, due to the capability of the lipase to handle the added volume. For the case of ethanol, this statement was not as clear as in the case of methanol, due to the higher times employed in 10-pulse reactions which reduced productivity achieved since no substantial yield enhancement was observed.

## 6.3. Conclusions

Recombinant *Rhizopus oryzae* lipase covalently immobilised in HFAGlut support was used as a biocatalyst in the biodiesel synthesis reaction using alperujo oil. Previous pre-equilibration of the system was carried out at several water activity values in order to study the influence on initial rate, giving better results when it was pre-equilibrated at $a_w$ = 0.093 using KOH. Moreover, three temperatures were tested to elucidate which promoted better transesterification. While 40°C and 50°C gave promising results in terms of initial rate, but they were finally discarded in favour of 30°C since enzyme stability was hindered at these temperatures.

In addition, enzymatic load was calculated by studying its correlation against initial rate, resulting in a maximum load of 250-300 UA $mg^{-1}$ support without mass transfer limitations.

Finally, methanol and ethanol as acyl acceptors were compared. In general terms, ethanolysis initial reaction rate was higher than when methanol was used as acyl-acceptor. Adding all alcohol at once, ethanol gave better results regarding the final yield and enzymatic stability than

methanol. However, if the stepwise additions were incremented, the difference between the two acyl acceptors became closer. When ten pulses were added, the ethanolysis reaction rate was higher than methanolysis one, but in contrast, the lipase activity along the reuses remained nearly the same in both reactions.

Still in lab-scale vials (10 mL), and pending the further scaling up to larger volumes, these results automatically ensured in applying a semi-continuous or fed-batch system in order to add the chosen acyl acceptor.

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# RESULTS III

## Scaling up to lab-scale stirred mini-reactor and first approach to semi-continuous addition

**7**

# 7. RESULTS III. Content

## 7.1.  Introduction

Large-scale production of biodiesel using low-value feedstocks as substrates have been in constant development [1]–[3] since edible seed/vegetal oil biodiesel has been criticised due to its low sustainability and potential conflict with food, fibre production and for the use of arable land, besides high water and fertiliser requirements [4], [5]. Most of these low-value feedstocks are waste oils like animal fats from industry or waste cooking oil from restauration [6]–[8] or basically non-edible vegetable oils, such as *Jatropha carcass* or *Mahua indica* [9]–[11]. The principal advantage of using low-cost feedstocks, apart from solving the ethical problem of "*fuel versus food*" previously commented, is that biodiesel produced from them is also perfectly suitable as a biofuel since no difference in engine performance and exhaust emissions – except $NO_x$ – have been found compared with other biodiesels [12]–[14].

Enzymatic catalysis has arisen as one of the most promising methods to synthesise biodiesel by using these kind of substrates [2]. The most common method is using an immobilised lipase – it allows reutilisation of the enzyme [15], [16] – and stepwise addition of short-chain alcohols like methanol and ethanol [2].

One of the parameters that also deserves attention, and sometimes is forgotten by researchers, is the *vessel* or *container* where the reaction takes place, i.e. the reactor [17].

Screw-capped vials are the most used as reaction vessels at lab-scale level since low volumes of substrate are needed, about 5-20 mL. In addition, low amounts of biocatalyst are used, thus reducing the cost of the preliminary studies [18], [19]. The major disadvantage of these kind of reactors is that usually temperature is given by thermal bath and orbital shaker or magnetic stirrer are used as agitation system [20]–[23], in contrast to larger reactors which can dispose space for probes and stirrer axis.

On the other hand, large vessels with volumes about 50 mL to 1-2 L are used also at lab-scale level since are reproductions of industrial-scale ones. All considerations must be taken into account when selecting the most suitable bioreactor for biodiesel production, whether the size and the type. Working with packed-bed reactors (PBRs), in contrast to stirred-tanks (STRs), damaging of the support can be avoided and volumes are reduced increasing volumetric productivity [24]. These reasons make PBRs one of the most used at lab-scale level, obtaining high biocatalyst stability [25]–[28]. However, despite the use of co-solvent, the immiscibility of substrate, glycerol accumulating and channelling flow make packed-bed reactors still a challenging system. Fluidised-bed reactors can overcome these problems but they require low amounts of enzyme per volume in the reactor, decreasing overall reactor efficiency in favour of

packed-bed reactors. In addition, complex changes in the flow pattern within these reactors causes unexpected effects upon the conversion rate [17].

Stirred-tank reactors, whether in batch or continuous operation, remains one of the best systems to synthesise biodiesel due the high yields achieved [29]–[31]. Optimal mechanical agitation – i.e. without shear stress on the biocatalyst – ensure good mixing of the parts and reduce mass transfer limitations avoiding the use of co-solvent in the most of cases. In addition, working with STRs in continuous mode can be implemented using multiple tanks operating in series in order to reduce the total reaction volume. The main advantage of this design is the possibility to add separation tanks for disposal products or by-products between reactors in order to avoid inhibitions [32].

Finally, development of integrated or coupled reactors have been studied recently, e.g. inclusion of a CSTR to PBRs as an inventive step to avoid enzyme inhibition due to insoluble methanol droplets [33].

Next scaling-up steps include implementing pilot plant reactors with volumes from 10 to 50L and up to 100 L [34]. The main problem when scaling-up enzymatic process such as biodiesel synthesis is the total amount of enzyme/biocatalyst needed. It is known that its cost may represent up to 60-70% of the total one [19]. Thus, researchers must ensure that the chosen biocatalyst should be enough stable in order to be cost-effective [35].

Some companies already use lipases as biocatalyst for biodiesel production at industrial scale. In fact, nowadays, Chinese companies have successfully established this process. Hunan Rivers Bioengineering Co. Ltd. (Hunan, China), uses Novozym 435 lipase in STRs, operates with a designed capacity of 20000 tonnes per year. Other company, Luming and Environmental Protection Technology Co. Ltd. (Shanghai, China), which uses spent frying oil as substrate in STRs, has a production line capacity of 10000 tonnes per year of biodiesel [36]. Moreover, the American company Piedmont Biofuels (North Carolina, USA) established in 2012 a new technology (FAeSTER) for a continuous biodiesel production using immobilized or liquid enzyme [37]. As well, Aemetis Inc. has recently developed an enzymatic biodiesel plant in India with a total capacity of 50 million gallons per year, supplying in addition refined glycerine to pharmaceutical and industrial customers [38].

In the present chapter, the stablished parameters for biodiesel synthesis in 10 mL-vials determined in Chapter 6, were implemented in a lab-scale stirred reactor of 50 mL. An attempt to scale-up the process to a large volume – up to five times – and to achieve the same results

was performed. Moreover, semi-continuous addition of ethanol was carried out using different addition profiles via automatised micro-burette.

## 7.2. Results and discussion

### 7.2.1. Enzyme Particle Concentration

Since one of the most important modification using vials or reactors was the stirring system, it was significant to know how it would affect over the particle dynamics. Homogenous suspension mechanism was governed by the bulk liquid recirculation. The particles were lifted from the solid particles pile or solids layer formed on the bottom by the circulation flows. In order to know which was the minimum stirred speed to obtain a higher homogeneous suspension compared with the theoretical maximum possible [34], the percentage of enzyme particle suspension was calculated (see 4.10. in Materials and Methods).

As shown in Figure 7.1., when low stirrer speeds were applied (100 rpm) particles were partially suspended (35.09%) and the majority were still at the bottom of the reactor. A substantial difference was observed when stirred was set at 160 rpm and 260 rpm, since enzyme particle concentration (EPC) increase nearly to 75% and 85%, respectively; demonstrating the larger bulk recirculation done by the impeller. However, a low amount of biocatalyst was still observed to be settled at the bottom of the vessel. In contrast, when stirred was set at 360 rpm, enzyme particle suspension reached the 100% and no particles were detected at the bottom.

In order to avoid unnecessary higher speed, a new value of 300 rpm was tested. Since the obtained EPC was over 100%, this value was chosen as final agitation speed.
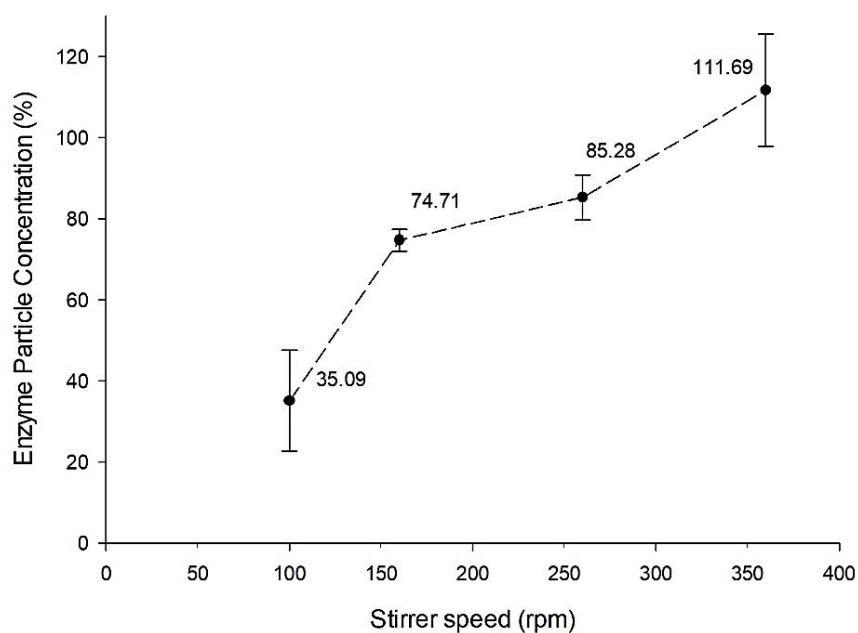
**Figure 7.1.** Enzyme Particle Concentration values (%) using 40 g of alperujo oil and the corresponding amount of biocatalyst at different stirrer rates (rpm).

### 7.2.2. Scaling-up of transesterification reactions: from vial to mini-reactor.

Results presented in Chapter 6 showed how biodiesel synthesis reactions should be performed, considering some crucial aspects such as temperature, initial water activity and finally, an addition strategy for alcohols – methanol or ethanol – to achieve the proposed objectives. As said, methanol and ethanol are the most used acyl acceptors for biodiesel production, whether chemical or enzymatic catalysis since these compounds are available and cheap [2]. However, when lipases are used as biocatalysts, methanol and ethanol can become a severe problem due to their capacity to hinder lipase activity by inhibition and/or inactivation [24].

This main drawback, which is one of the most concerning in the biocatalysis community, was effectively resolved working with 10 mL-vials obtaining satisfying results (Table 6.3) when alcohol was added in five or ten pulses.

Thus, in the present chapter, same strategies were equally implemented using a lab scale 50 mL-stirred tank trying to achieve identical results in order to demonstrate that these addition profiles could be applied in reactors with larger volumes. Nevertheless, the morphologies of previous and current vessels differed in some points. In fact, reactions using 10 mL-vials were performed inside an orbital shaker incubator, while current reactions were carried out using mechanical stirring with an impeller and temperature was maintained through a heating plate.

Three strategies were tested for scaling-up using the 50 mL-reactor: 5-pulse and 10-pulse ethanolysis and, keeping methanol as an alternative substrate, 10-pulse methanolysis reaction. It was clear that one-step reactions did not fulfilled with the expectations since stability of enzyme was severely aggrieved in both cases.

### 7.2.2.1. 5-pulse ethanolysis

Replication of 5-pulse ethanolysis reaction shown in previous chapter (see Figure 6.6.) was carried out within the same time using 40 g of alperujo oil and a total volume of ethanol of 5.3 mL (2:1 ethanol to oil molar ratio). Time evolution of FAEE yield and oleic acid amount are shown in Fig. 7.2.. A final yield of 55.85% was achieved, nearly a 4% less than using 10 mL-vials where 58.16% was reached. It was clear that, such minor differences were not strong enough to determine that using the current mini-reactor was hindering the biocatalyst activity.



**Figure 7.2**. Time evolution of FAEEs yield (●) and oleic (o) acid of 5-pulse transesterification reaction using ethanol in mini-reactor. Reaction conditions: 40g of alperujo oil, 2:1 alcohol to oil molar, 30°C and 300 rpm.

In addition, a similar behaviour on free fatty acids was observed comparing both, vial and reactor 5-pulse transesterification reactions. Since amounts of oleic acid were different due to different used volumes, molar concentrations were calculated. In vial reaction, initial concentration of

oleic acid was 0.617 M while at the end it was 0.400 M, which represented a decreasing of a 35.2%. Approximately the same behaviour was calculated for the 5-pulse ethanolysis in the reactor, since initial concentration was found to be 0.544 M and final one it was 0.331 M, with a decreasing value of 39.2%. Similar final yields and similar free fatty acid performance in the system demonstrated that the scaling up of the reaction was successful.

Therefore, stability tests were also performed to see the differences especially about how mechanical stirring would affect in contrast to orbital agitation. Figure 7.3. shows a comparison between biocatalyst activity loss along the time in vial and mini-reactor.



**Figure 7.3.** Relative yield – considering first reaction yield as 100% – of 5-pulse ethanolysis reactions in 10 mL-vial and in 50 mL-reactor. Each cycle corresponds to 200 minutes.

Lipase retained an 84.7% of its initial activity in 20 hours of reaction – 6 cycles – in the reactor, while it could maintain nearly a 90% when vials were used as vessels. Although there were not high variances between these values, the principal hypothesis was the effect of mechanical stirring – shear stress – against the biocatalyst [39], [40]. Thus, photographs using binocular loupe were taken in order to visualise the integrity of the support (Fig. 7.4). Images showed that carrier was not broken after 20 hours of reaction at 300 rpm. Although it did not explain whether

surface enzyme was leaked from the support, small differences in stability shown between vial and reactor pointed that this effect should be low.
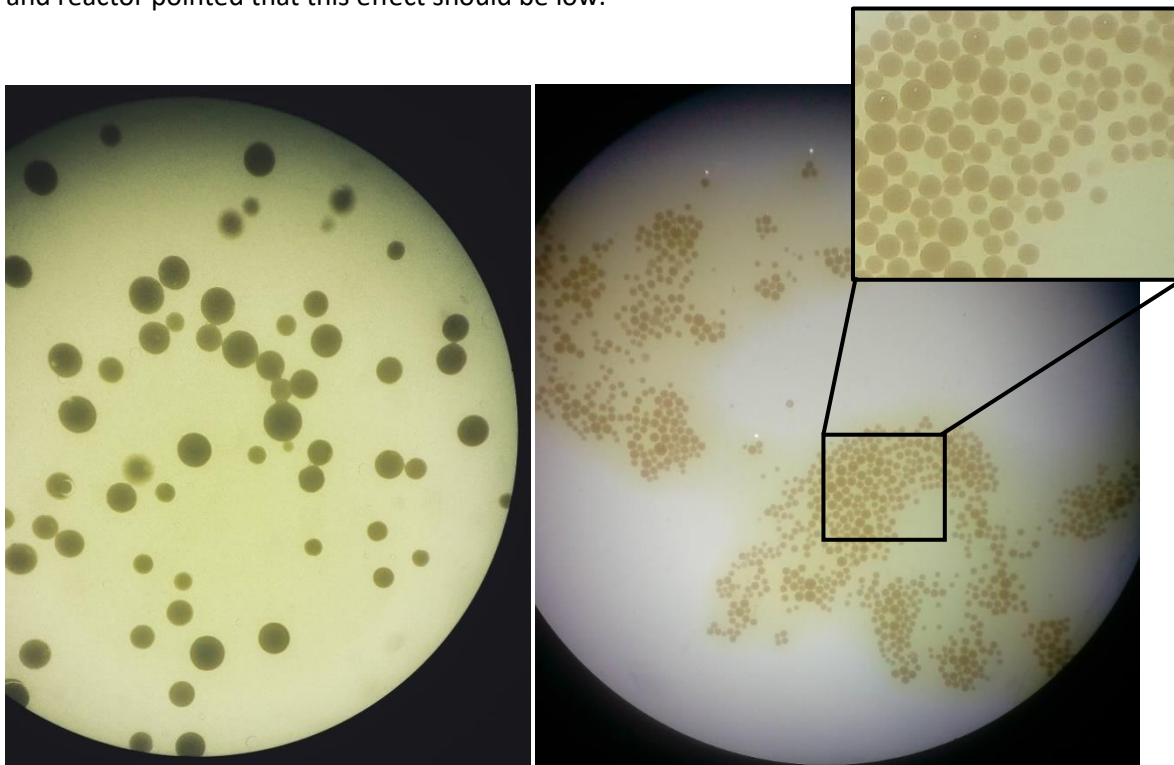


**Figure 7.4.** Binocular loupe picture of medium suspension at the beginning (left) and at the end of the 6th cycle (right) of 5-pulse ethanolyisis in the reactor, showing no breaking of the carrier.

### 7.2.2.2. 10-pulse ethanolysis

As seen before scaling up of five pulse ethanolysis reactions gave positive results. However, considering the idea that as long as more divided were the additions, better was the final yield and the enzyme stability, next experiments were performed using ethanol as acyl-acceptor but reproducing the ten pulse reactions. Figure 7.5. shows the time evolution of FAEE along the 265 minutes of reaction. Final yield achieved was 59.48%, which corresponds nearly a 90% of the total yield reaction, considering the capacity of ROL to attack only positions *sn*-1 and *sn*-3 of the triglyceride moiety. This value was pretty similar to the obtained result in 10 mL-vial, a 60.25%, demonstrating as well as in the case of 5-pulse ethanolysis the reproducible scale up for this reaction.
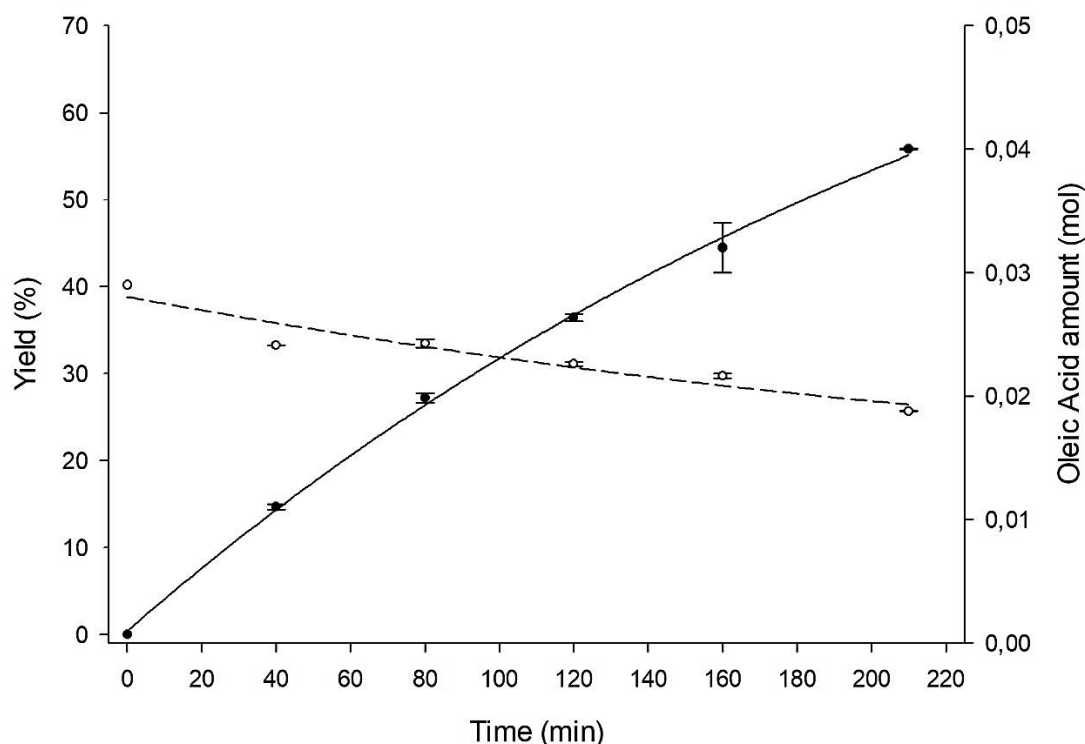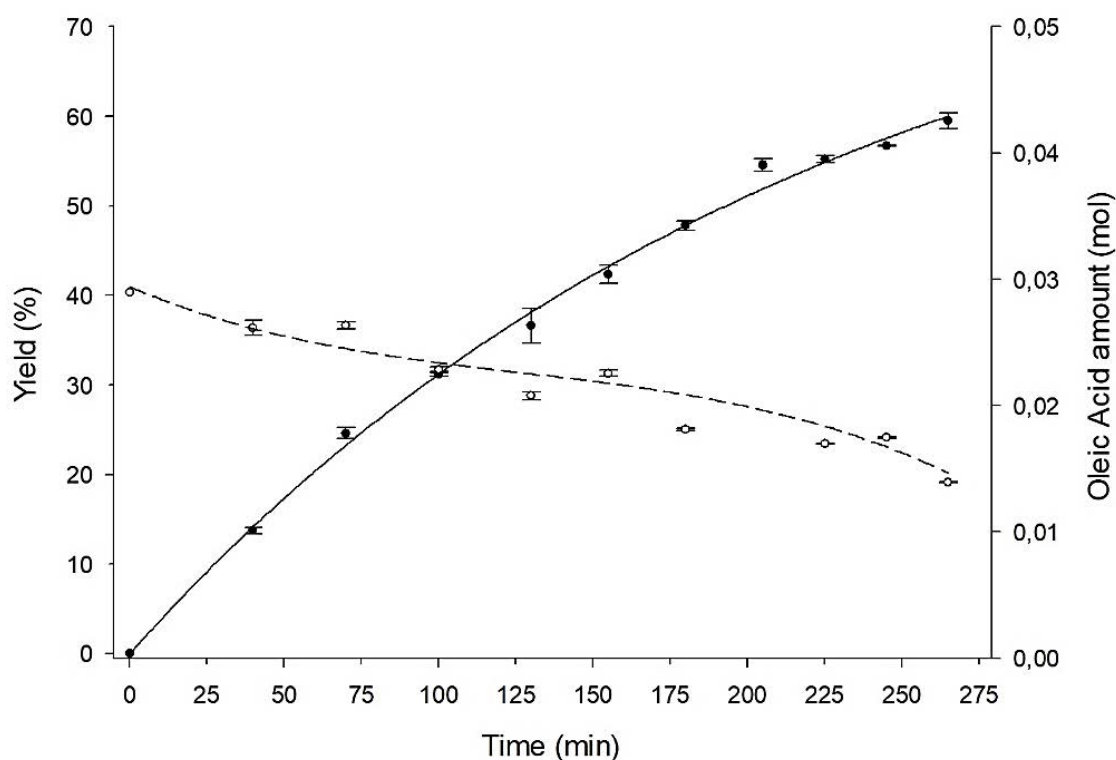
**Figure 7.5.** Time evolution of FAEEs yield (●) and oleic (o) acid of 10-pulse transesterification reaction using ethanol in mini-reactor. Reaction conditions: 40g of alperujo oil, 2:1 alcohol to oil molar, 30°C and 300 rpm.

However, comparing free fatty acids performance between vial and reactor, a slightly difference was observed. Decreasing of oleic acid concentration in vial reactions was about 34% while nearly a 50% was obtained in the reactor. Since final yield was similar for both cases, that would mean that formation of FAEE from FFA increased, reducing transesterification from triglycerides when the reactor was used in contrast to vial reactions, where FAEE seemed to be synthesised more from transesterification than esterification, as said in Chapter 6.

It is important to note that there was a positive difference between stability of lipase in 10-pulse ethanolysis reaction in the vial and in the reactor. As shown in Figure 7.6., the decrease of the global enzymatic activity is somewhat lower than when 10 mL-vials were used as vessels. The remaining activity of the rROL-HFAGlut biocatalyst was still a 90% of the initial activity for the first 31 hours of reaction (7 cycles), in contrast to the 88% when vials were used. Although this insignificant difference, it could be also stated that 10-pulse ethanolysis reaction with a 5-fold scaling up factor applied in a 50 mL-reactor with mechanical stirrer has been successfully replicated, since obtained results were consistent and robust.
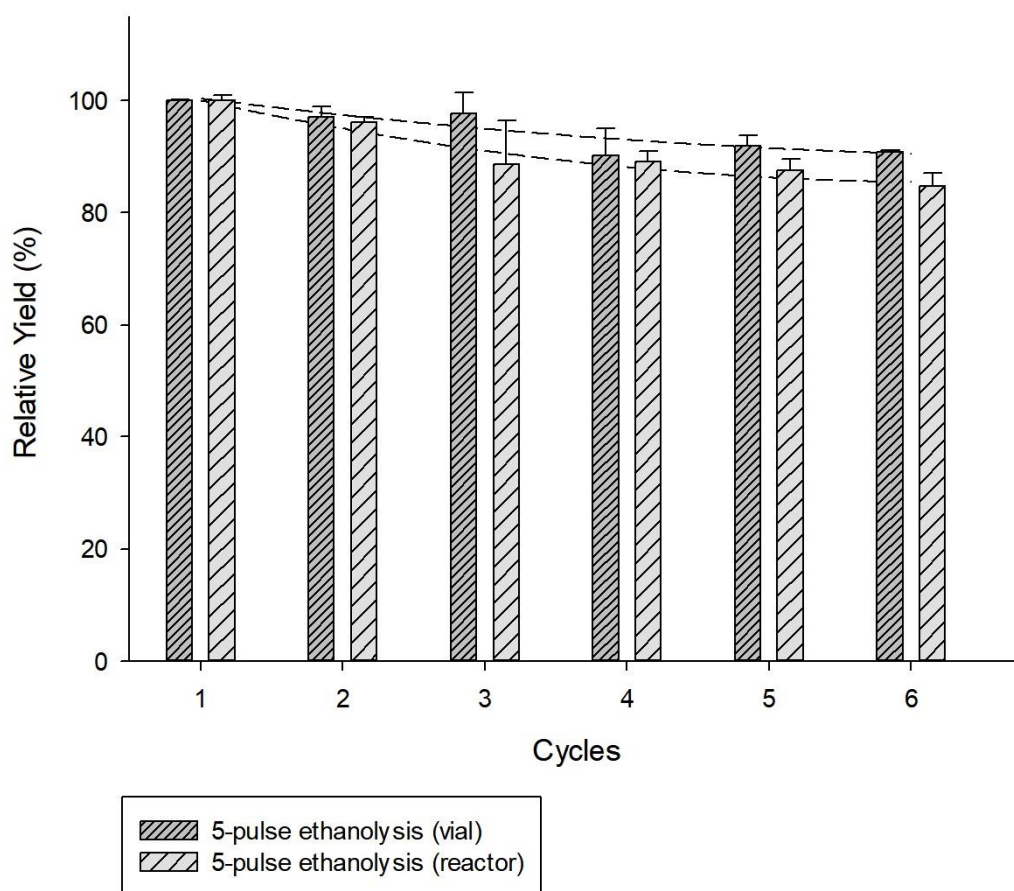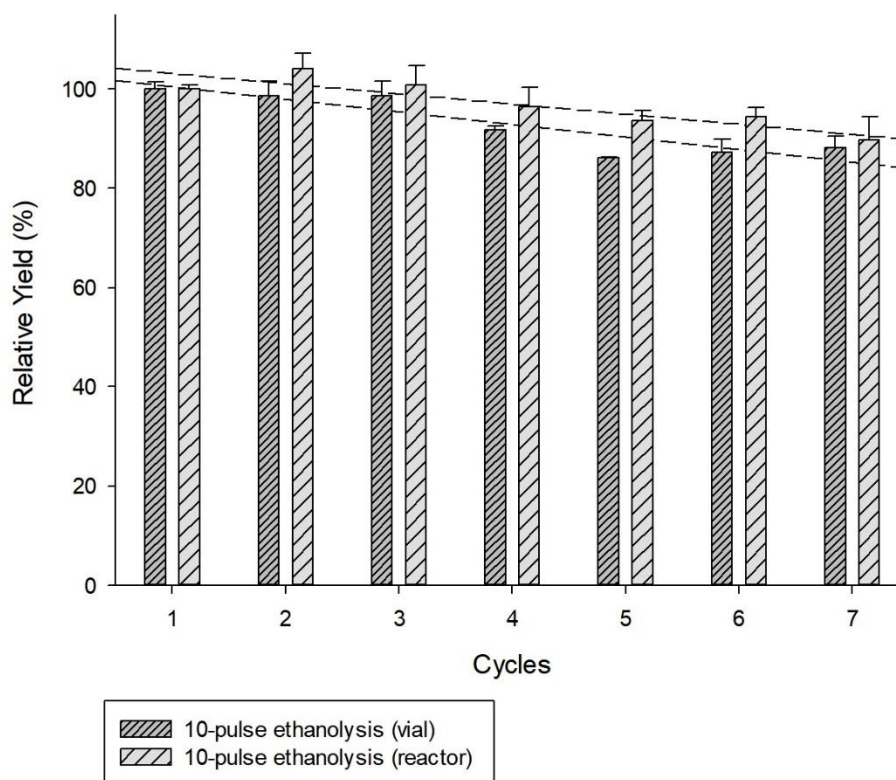
**Figure 7.6.** Relative yield – considering first reaction yield as 100% – of 10-pulse ethanolysis reactions in 10 mL-vial and in 50 mL-reactor. Each cycle corresponds to 265 minutes.

**10-pulse methanolysis**

Next experiments were performed using methanol. While adding it in one and five pulses did not show positive results in vials, it became clear that as more divided was the total added volume, better results were obtained. Similar productivities between 10-pulse methanolysis and both ethanolysis reactions shown in Table 6.3 demonstrate that it was an option to be considered.

Replication of 10-pulse methanolysis reaction was again performed with the 50 mL-reactor Same methanol addition strategy as seen in Table 6.2. was used, but with a 5-fold scaling up factor applied.

As expected, FAME yield was similar to 10-pulse reaction in vial (57.15%), with a final value of 59.87% in 375 minutes, representing a slight increase of 4.76%. These results demonstrate again the well application of the scaling-up since they are quite robust. Although some studies have stated that mechanical stirring is known to create larger fluid turbulences inside the reactor in comparison with orbital agitation [34], and methanol could be better solubilised into the organic

medium, reducing the inhibitory effect on the lipase [41], present results showed minor differences.

This consequence could be further analysed when stability tests were carried out as previous ones. As seen in Figure 7.7., stabilities using both types of vessels are very similar along the 6 cycles, corresponding to nearly 38 hours of reaction. In the case of methanolysis in the reactor, an 86.44% of the initial activity was retained, in front a 90.80% in the case of methanolysis in the vial, which signify just a decrease of 4.80%. Although these slight differences, the behaviours of both global activity losses are identical. This led to think that positive effect of dividing the total volume of methanol prevailed over the hindering consequence of the methanol itself allowing reusing the biocatalyst several times.
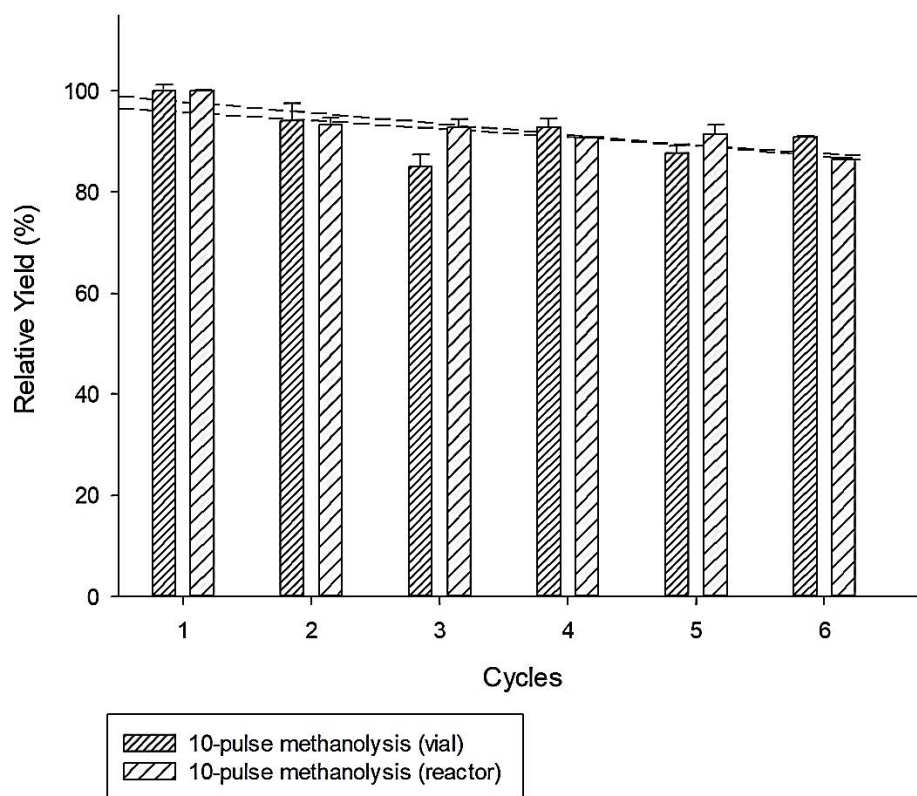


**Figure 7.7.** Relative yield – considering first reaction yield as 100% – of 10-pulse methanolysis reactions in 10 mL-vial and in 50 mL-reactor. Each cycle corresponds to 375 minutes.

Table 7.1 shows the productivity of the three reactions performed in the reactor calculated during the first 20 hours approximately. In order to a better comprehension, values obtained in vials were also included in the table. As seen before, stability of biocatalyst during the 5-pulse reactions in the reactor was slightly lower than in vial (Fig 7.3). The consequence of this stability reduction can be observed as productivity was also reduced. In contrast, productivity during 10-

pulse transesterification in the reactor resulted in a minor increasing of productivity since stability seemed to be higher than in vial, as said before (Fig.7.6). In the case of 10-pulse methanolysis productivity values were nearly the same for both cases. That matches with Figure 7.7., where stabilities showed in vial and reactor were also similar.

**Table 7.1.** Productivity values of biodiesel synthesis reactions by stepwise addition along the stability tests in reactor and vial for 20 hours.

| Reaction | Productivity ($\mu$mol biodiesel min$^{-1}$ mL$^{-1}$) | |
| --- | --- | --- |
| | Reactor | Vial |
| 5-pulse ethanolysis | 1.00 | 1.16 |
| 10-pulse ethanolysis | 1.20 | 1.13 |
| 10-pulse methanolysis | 1.07 | 1.03 |

Although minor differences between productivities were observed, further stability comparisons were performed. Several studies have stated that the most important key for a cost-effective process using biocatalysts is the price of themselves [34], [42]. In an enzyme-catalysed process, it must be ensured that lifetime of the biocatalyst is the longest possible, since it would mean more reuses and less enzyme spent.

In order to ensure this great stability, biocatalysts were reused over numerous cycles for both 10-pulse reactions seen before: ethanolysis and methanolysis. In the case of ethanolysis, 20 reuses of the biocatalyst were performed with a total extended time of 88 hours of reaction. At the end of 20th reaction, a total of 82.99% of initial yield was still retained. The difference of acyl-acceptor here was notable, since only a 64.88% was maintained when methanol was added in 10 pulses. It should be noted that, as well as in the case of ethanol, 20 cycles were carried out but final time was 125 hours of reaction instead.

Figure 7.8. was added in order to better understand and compare these results considering the time expended by the biocatalyst in contact with ethanol and methanol in the medium. As shown, both biocatalysts had a high stability along the reuses. As expected, when ethanol was used as acyl-acceptor, slightly higher global robustness than in the case of methanolysis reaction was observed. Thus, it was clear that ethanol seemed to be the best candidate as acyl-acceptor since reactions were faster and stability shown was rather higher than when methanol was used.
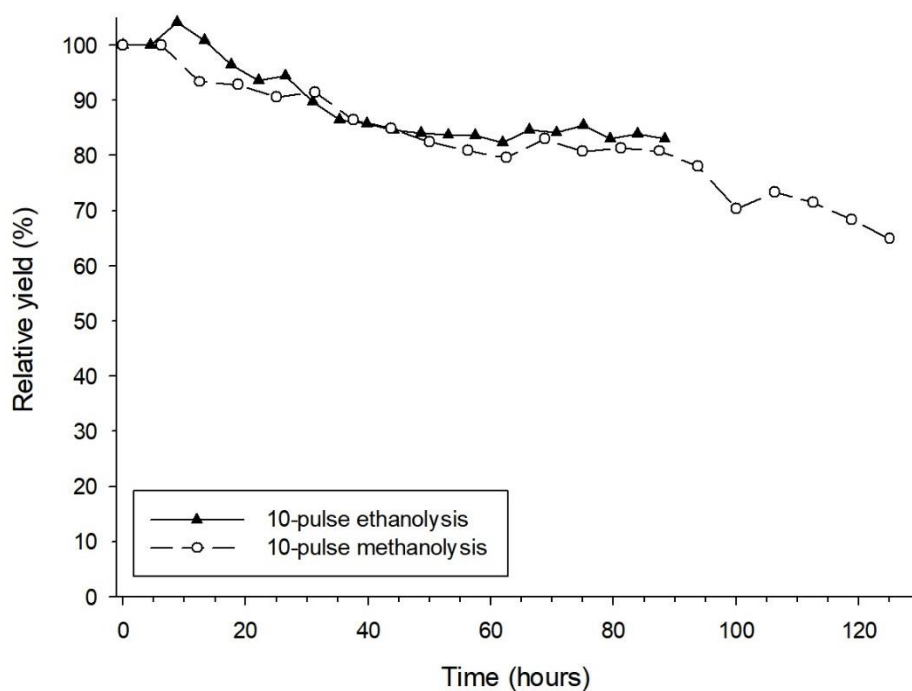
**Figure 7.8.** Evolution of relative yield obtained – considering the first one as 100% – of 10-pulse reactions using methanol and ethanol.

If an extrapolation was applied, half-life times could be determined. Table 7.2. shows these values considering different equations as well as their coefficient of determination. Two types of functions were used: linear and exponential decay, since it is known that several enzymes are inactivated using these patterns [23], [43], [44]. Higher life-time value was obtained when exponential decay was applied in 10-pulse ethanolysis resulting in nearly 266 hours. This time corresponded to 60 reaction cycles. Moreover, when linear function was applied, half-life time was reduced to 208 hours or 47 cycles, about a 22% less. Figure 7.8. shows minor differences between stability of biocatalyst using methanol and ethanol up to 88 hours, but when extrapolation was applied, these differences were higher. Again, two types of functions were used. When linear function was applied, half-life time resulted in only 170 hours that corresponded to 27 cycles. However, if exponential function was used in the extrapolation, higher life-time value was obtained, concretely 213 hours that corresponded to 34 cycles.

Therefore, if both reactions were compared using the same function it was clear that ethanol resulted in a better acyl-acceptor in terms of stability since higher half-times were achieved. Thus, biocatalyst could be reused more time, reducing final process costs.

**Table 7.2.** Half-time values of 10-pulse reactions using ethanol and methanol calculated from different extrapolations. RY and T of equations correspond to *Relative Yield* (%) and *Time* (h), respectively. Cycles were calculated based on reaction times: 265 minutes for ethanolysis and 375 minutes for methanolysis.

| Reaction | Type | Equation | $R^2$ | Half-life time (h) | Cycles |
|---|---|---|---|---|---|
| **10-pulse ethanolysis** | Linear | RY = -0.2401T + 100 | 0.7717 | 208 | 47 |
| | Exponential | $RY = 100e^{-0.00261T}$ | 0.7806 | 265 | 60 |
| **10-pulse methanolysis** | Linear | RY = -0.2935T + 100 | 0.6608 | 170 | 27 |
| | Exponential | $RY = 100e^{-0.003246T}$ | 0.7109 | 213 | 34 |

Some studies have tested the stability of several types of lipases in front the feeding strategy and the acyl-acceptor used. For instance, *sn*-1,3-regioespecific *T. lanuginose* lipase could maintain its initial activity for 3 batch cycles of 10 hours in solvent-free media and in organic solvent [45]. The same lipase used in the present thesis was immobilised in some other supports and it was used as biocatalyst of biodiesel production from a waste vegetable oil (*Jatropha carcass* oil) and methanol. Approximately, from 85% to 40% of the initial activity was kept along 40 hours using different carriers [23]. However, most of the studies present in literature that related stability of lipases in lab-scale processes were using PBRs and organic solvents. For instance, continuous biodiesel production through *Candida antarctica* (Novozyme 435) lipase in a 3-step PBR with *tert*-butanol as co-solvent was kept at 80% conversion for more than 120 hours [26]. Moreover, IIT-SARKZYME (pancreatic lipase), could maintain a 75% of its initial activity during 50 cycles, corresponding to approximately 80 hours of reaction [33]. In that way, comparing this data with exposed results, rROL-HFAGlut has demonstrated to be a promising biocatalyst in lab-scale.

Finally, as well as in the case of 5-pulse ethanolysis, a photography to visualise the integrity of the support was taken at the end of the last cycle. Figure 7.9. shows that, in contrast with the Fig.7.4., some insignificantly breaking of the carrier was observed after 88 hours (20 cycles) of using the rROL-HFAGlut biocatalyst in 10-pulse ethanolysis reaction, but still most of the particles were entire.
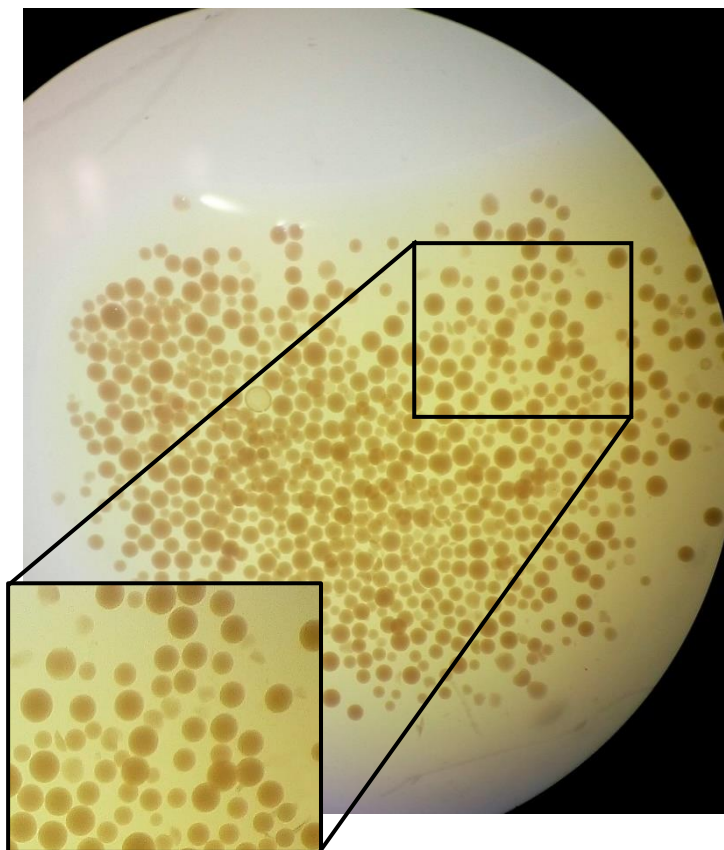
**Figure 7.9.** Binocular loupe picture of medium suspension at the end of the 20th cycle of 10-pulse ethanolyisis in the reactor, showing some minor breaking of the carrier.

### 7.2.3. Semi-continuous addition approach using automatised micro-burette

As said before, it was clear that as more divided was the total stoichiometric volume of alcohol, better was the stability achieved likewise in the reactions performed in vials. In fact, differences seen between stability of 5-pulse and 10-pulse methanolysis in vials were significant. In the case of ethanolysis, these differences in vials were not as clear as in the previous case since ethanol causes lesser hindering effect on the enzyme than methanol. However, experiments in reactor showed quite better results as more divided was the addition (Table 7.1).

Thus, once divergences between both acyl-acceptors and studies about scaling-up to larger volumes had been done, an approach to add ethanol in a semi-continuous strategy was presented. While there were many works in the literature performing continuous synthesis of biodiesel using, for instance, packed-bed reactors, studies about continuous feeding of alcohol in a fed-batch system were minor. In addition, most of works performed this strategy by adding the acyl-acceptor using a constant feeding rate. For instance, methanol droplets of 0.05 mL were

added in every 5 minutes  obtaining a 100% yield in 16 hours [46]. Furthermore, ethanol was added to esterify FFAs using one first pulse of 1/3 of the total volume, and then the remaining 2/3 was constantly fed by a pump [47]. Other work attempting to scale up a biodiesel synthesis process, methanol was continuously fed to the reactors and it was kept below 20 % in methanol mass in the heavy phase [48].

In the present thesis, a feeding strategy of ethanol using an exponential profile is presented. Since addition of 10 pulses of ethanol were not performed at a constant rate and it was demonstrated that high yield and stability were achieved by using this strategy, a new approach was proposed using an exponential equation (Eq. 1) as initial basis for the addition of the alcohol through an automatised micro-burette:

$$V_t = \int Q_0 \cdot e^{(-b \cdot t)} \, dt \qquad (1)$$

Where $V_t$ represents the total volume of ethanol added, $Q_0$ stands for the initial flow rate, $b$ is the exponential factor and $t$ represents the time of the reaction.

Time and total volume – determined by alcohol to oil molar ratio – were maintained as previous experiments in 265 minutes and 2:1, respectively, in all the following experiments in order to keep reactions comparable.

For the following equation (Eq. 2) it was assumed that the apparition of FAEE species in the medium was totally caused by ethanol consuming – evaporation was negligible –, thus:

$$\frac{dFFAE}{dt} = -\frac{dEth}{dt} \qquad (2)$$

Initial rate could be used as the value corresponding to the FAEEs apparition in order to calculate the volume of ethanol initially consumed per minute. Considering stoichiometric ratio between FAEE and ethanol, it was 12.85 µmol ethanol $min^{-1}$ $mL^{-1}$ or 34.20 µL ethanol $min^{-1}$ in terms of initial flowrate taking into account the total working volume of 45 mL. The exponential factor ($b$) was then determined as -0.2769 $h^{-1}$ by solving Eq. 1.

As shown in Figure 7.10., although initial rates seemed to be similar, Exponential 1 reaction yield obtained from these previously calculated parameters did not reach the same value as in the case of 10-pulse ethanolysis, since final yield was 54.22% instead of 59.48%. Another reaction was proposed (Exponential 2) by increasing the initial flow rate in approximately 50% in order to add higher amounts of ethanol initially, resulting in a $Q_0$ of 51.3 µL · $min^{-1}$ and a calculated

exponential factor of -0.5328 h$^{-1}$. While it is true that no significant changes were observed at the end (56.55%), differences in terms of yield were present during the reaction, where increasing $Q_0$ resulted in similar behaviour to 10-pulse reaction up to 150 minutes (Fig. 7.10.).



Exp1: $Q = 34.2 \cdot e^{-0.2769 \cdot t}$

Exp2: $Q = 51.3 \cdot e^{-0.5328 \cdot t}$

○   10-pulse ethanolysis
▲   Exponential 1
●   Exponential 2

**Figure 7.10.** Time evolution of yield obtained in 10-pulse ethanolysis, Exponential 1 and Exponential 2 using automatised ethanol addition. 40 g of alperujo were used at 350 rpm and 30°C.

Obtaining lower values of final yield compared with 10-pulse ethanolysis was not fulfilling the expectations regarding the final yield. It was probably because initial rate of FAEE appearance was calculated from the theoretically consumption of ethanol in the whole reaction medium. In fact, initial rate is proportional to the concentration of substrates. Therefore, initial concentration of ethanol in Exp. 2 was extremely lower than in the case of 10-pulse reaction: 0.11% v/v and 2% v/v, respectively.

Thus, larger amounts of ethanol should be added initially, i.e., increasing initial flow rate, $Q_0$. However, since time was fixed and exponential factor was derived from $Q_0$, the exponential addition itself would result in different addition profiles where, as higher as the initial flow rate was, large volumes were added initially, so low volumes would be added in the last minutes of the reaction.

Thus, a restriction in the initial flow rate ($Q_0$) was stablished in order to maintain the ethanol addition during the entire reaction and avoiding spending time to add lower volumes at the end of the process. Hence it was decided that more than 1% of the total volume of ethanol, which corresponds to 52.5 µL, should be added in the last 15% of the reaction time, corresponding to last 40 minutes. Using these constrictions, 91.05 µL min$^{-1}$ as initial flow rate and *b* of 1.0433 h$^{-1}$ were obtained to test Exponential 3 reaction.

Figure 7.11. shows the comparison of the time evolution of yield in 10-pulse ethanolysis and Exponential 3. Again, as happened in previous reaction (Exponential 2), higher yield was achieved in the initial times of the process due to the increase of ethanol volume added, but final yield, nearly 56.7%, was still lower than 10-pulse ethanolysis.

In addition, shown in Fig 7.11., one pulse of ethanol with the same volume as the first pulse of 10-pulse ethanolysis reactions was added and, after that, the remaining was continuously fed following the exponential profile shown in Exponential 3 [47]. As seen, Exponential 4, no substantial differences were observed in comparison with previous reaction neither higher initial rate was achieved due to the first pulse added. In addition, final yield was quite similar to Exponential 3, 56.9%, representing a very slight increase.
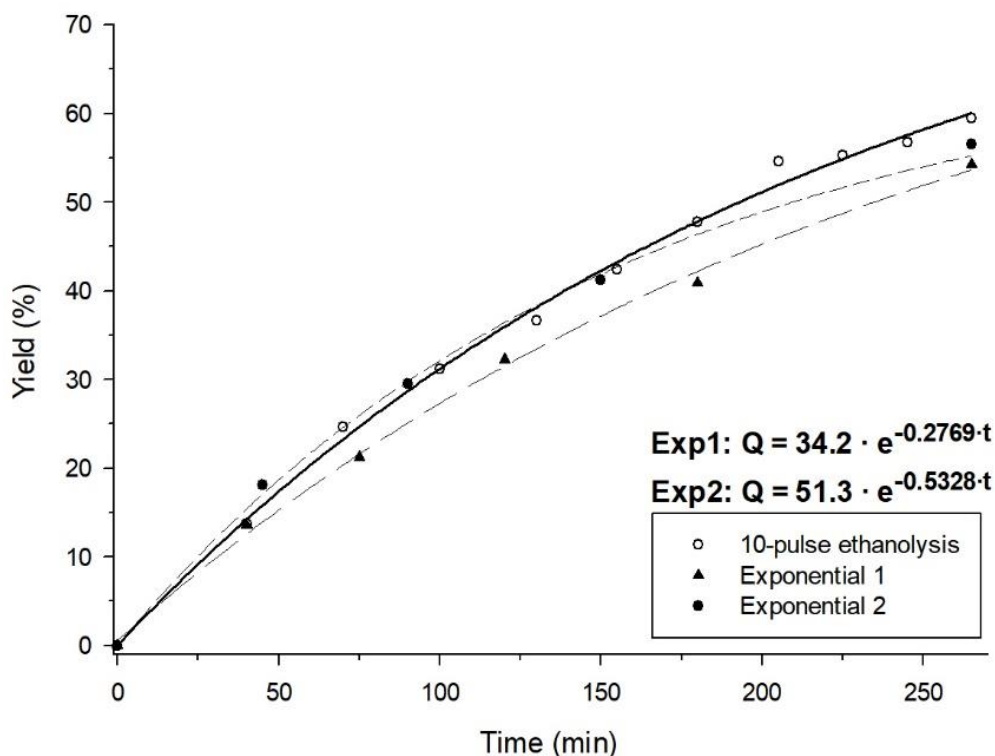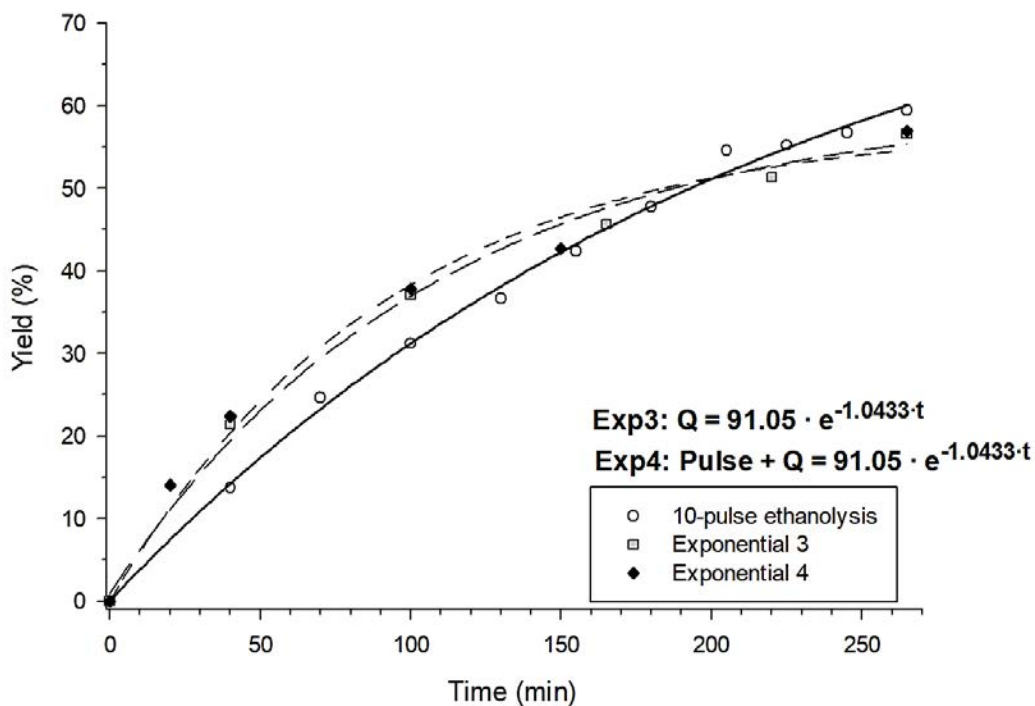


**Figure 7.11.** Time evolution of yield obtained in 10-pulse ethanolysis and Exponential 3 and Exponential 4 using automatised ethanol addition. 40 g of alperujo were used at 350 rpm and 30°C.

The previously commented results from reactions using automatised addition of ethanol demonstrated that, even modifying the most used constant feeding to the exponential one, which seemed to be more actually applicable, lower yields were achieved. In fact, trying to improve such little yield values in the final reaction, where the equilibrium was severely displaced to products, represented an important challenge without a more detailed study of the actual reaction kinetics [49].

## 7.3. Conclusions

Results showed how scaling up of biodiesel synthesis reaction in vials were successfully achieved. Three experiments were replicated using a stirred-tank reactor of 50 mL. 5-pulse ethanolysis stability in reactor showed lower values compared with same reactions in vials along 20 hours. In that way, 10-pulse ethanolysis resulted in slightly better results when it was performed in reactor.

In addition, 10-pulse methanolysis were also considered and stability obtained in reactor was identical as the same achieved in vials. Certainly, these differences are not the relevant point themselves, but the fact that these demonstrate the successfully application of the scale up.

In order to better visualise these results, productivities for each reaction along 20 hours were calculated, showing that 10-pulse ethanolysis productivity in reactor increased in a 6% compared to vial. Further stability analyses were performed, reusing rROL-HFAGlut up to 20 cycles for 10-pulse ethanolysis (88.3h) and 10-pulse methanolysis (125h), observing better results for the first case, where half-time life of 265 hours was determined. Since, as more divided was the total volume of alcohol, better was the stability, continuous feeding strategy of ethanol using a micro-burette was attempted. However, results were not as similar as it was expected which may come from the fact that alcohol concentrations at the beginning of the reaction were lower compared with stepwise addition.

## 7.4.    References

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# R E S U L T S IV

Economic evaluation of a potential industrial
plant for enzymatic biodiesel production

8

# 8. RESULTS IV. CONTENT

## 8.1.    Introduction

Currently, the high cost of pure vegetable oils, which are the main type of feedstocks for biodiesel synthesis, is hampering the implementation and economic viability of many production plants. Since catalysts are not expensive in alkali-catalysed transesterification, it has been reported that feedstocks may represent up to 70-90% of the total biodiesel costs [1]. Although the choice of feedstock is based on such variables as local availability, cost and government support; current biodiesel factories should consider the use of other low-cost feedstocks. Thus, there is a need of flexibility in biodiesel processes in order to accommodate variations in feedstock quality and availability. Designing multi-feedstocks plants where refined vegetable oils from low-value acidic fats could be used as substrates would ensure the profitability and economic viability of the production plant for a long period of time since it would be not as dependent as currently is on the type of feedstocks [2].

Several studies have focused on the optimisation of biodiesel synthesis – alkaline or enzymatic – using many different feedstocks, such as soybean oil, castor oil, sea mango oil, etc. [3]–[8]. However, there are not many studies about modelling or predict the optimal conditions for biodiesel synthesis on a production plant comparing the use of different feedstocks [9].

Currently, most of the production plants are running using low-FFA vegetable oils as feedstocks and sodium or potassium hydroxide as catalyst (alkaline). However, as said before, the key factor for a good profitability of the plants is their capability for synthesising biodiesel independently from the feedstock. For instance, Alfa Laval Ageratec systems (Sweden) are intended for industrial-scale production of biodiesel with a throughput corresponding to 330 days of full-rate operation per year. This company is able to esterify via alkaline or acid, and can incorporate low-value feedstock with a FFA content up to 10% [10].

Designing an industrial process for production of biodiesel may be complex and difficult, but despite that, some basic parts must be included. The overall process is divided into four main sections: feedstock pre-treatment, reaction, separation and purification (Fig. 8.1.). In general terms, upstream unit operations are mainly dependent on the feedstock is used, whereas the downstream operations depend on the catalyst chosen for transesterification.

**Figure 8.15.** Schematic process for industrial biodiesel production.

Feedstock pre-treatment is applied in order to make the substrate suitable for the process. This may include operations such as filtration, melting, degumming, water removal or controlling initial water activity, alkaline treatment or pre-esterification [11]–[13].

Secondly, the reaction of transesterification takes place. Stirred tanks are usually used in chemical-catalysed processes. However, as explained in Chapter 7, if enzymatic catalysis is used, transesterification is mainly carried out using two types of reactors: continuous packed bed or batch/fed-batch stirred tank reactors, considering each one's advantages. The reaction part is maybe the most important one in enzyme-catalysed processes since it requires several studies to optimise the reaction itself. Moreover, enzyme cost can represent more than 70% of the total process, thus it is necessary to extend as much as possible their catalytic life, i.e. try to maintain their activity over long periods of time.

Finally, into the downstream process two main parts are present: separation and purification. First step requires mechanical units like centrifuges or decanter systems in order to remove the main by-product of the biodiesel production – unless regioespecific lipases were used – which is glycerol [14]. Moreover, removal of alcohol excesses is needed due to several reasons to fit final biodiesel into quality standards but also in order to recycle it and reduce production costs. Normally, flash evaporators or distillation are the most common used units [11]. Another important step in alkali-based catalysis is water washing, where remaining catalysts, soaps, salts and residual glycerol are removed. By contrast, some studies have stated that this procedure is not usually required when immobilised enzymes are used [15]. Water washing is a critical step in terms of sustainability, since large amounts of water are frequently used. That is why avoiding unnecessary steps can be really important.

Purification of biodiesel includes removal of remaining mono-, di- and triglycerides, sulphur and other components above the stablished limits of quality standards. Vacuum distillations, thin-film evaporators and polishing filtration are the most common used unit operations to purify final biodiesel [11].

## 8.2.    Objective

In the present chapter a simulation and economic evaluation of an industrial plant to produce approximately 35000 tonnes of biodiesel are presented using a design software, SuperPro Designer®. In addition, since recombinant *Rhizopus oryzae* lipase was the enzyme chosen to catalyse the transesterification reaction, 22000 tonnes 2-monoglyceride was also produced as an alternative product to biodiesel, instead of glycerol. Previous results of studies performed on reaction conditions and lipase stability have been used as basis for the implementation and development of a simulated process. Evaluation of profitability and economic viability in front different scenarios have been carried out. Several factors have been initially considered such as feedstock price, reutilisation of biocatalyst in order to build up a cost-effective process.

## 8.3.    Process simulation

### 8.3.1.    Case of study and brief process description

As said before the main objective is to simulate an industrial process for biodiesel enzymatic production using alperujo oil as feedstock and rROL-HFAGlut as immobilised biocatalyst. Thus, the main goal was to produce approximately 35000 tonnes of biodiesel, which would represent nearly a 3.24% of Spain's biodiesel total forecast consumption for 2017 [16].

 Figure 8.2. shows a schematic representation of the overall process which was divided in two operational parts: firstly, upstream and reaction performed in batch mode and finally a continuous downstream. Batch mode has been chosen to make more feasible the initial water pre-equilibration of all reagents. Continuous mode has been selected to handle better the large volumes present in purification procedures.

A first step of raw feedstock pre-treatment, consisting in a simple centrifugation in order to remove impurities like gums and waxes, was applied. Then, ethanol and alperujo oil were pre-equilibrated to get a determined initial water activity. Reaction of transesterification took place in a stirred tank reactor fed by ethanol using the 10-pulse strategy, explained in Chapter 7. Biocatalyst was retained inside the reactor along the reuses and an average reaction yield within the selected reuses was considered in order to simplify the following steps.
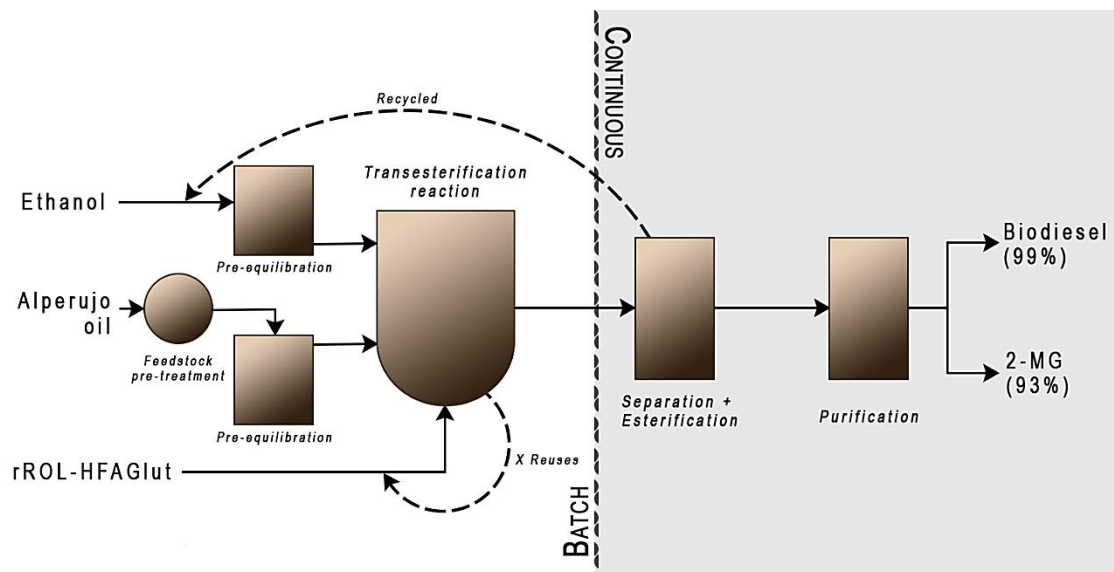
**Figure 8.2.** Schematic overview of the proposed process. From alperujo oil to biodiesel and 2-monoglyceride using rROL-HFAGlut

A storage tank (not shown in the schematic overview) was placed next to reactor to start continuous mode. Subsequent separation steps included an ethanol recovery system, followed by alkaline washing to eliminate possible remaining FFA after reaction. Soaps formed were removed by centrifugation. Last purification steps included a water washing through differential extraction to eliminate soap impurities followed by a drying of some possible water remains followed by a thin-film evaporation to extent the purification of final biodiesel and 2-monoglyceride.

### 8.3.2.  Components definition

Chemical components used during the simulation are summarised in Table 8.1. Water, ethyl alcohol, sodium hydroxide and FFA, considered as oleic acid, were available in SuperPro Designer® databases. By contrast, *FAEE*, *triolein*, *monoolein*, *biocatalyst* and other components were defined using available literature.

Biodiesel was introduced as *FAEE* using ethyl oleate external data since it is the major alkyl ester present in final product. Boiling and melting point were set to 216°C and -32°C, respectively [17]. Triglycerides were defined in form of *triolein* using external data for the same reason as *FAEE*. Boiling and melting point were set to 554°C and -5°C [18]. 2-Monoglycerides were introduced as *monoolein* with a boiling point of 239°C and a melting point of 25°C [19]. Diglycerides were considered in a minor proportion and they were not taken into account in the simulation in order to simplify it.

Raw alperujo oil was introduced as *pre-dewaxed alperujo* and it was defined as 72 %w/w of *triolein,* 18 %w/w of *FFA* and 10 %w/w of *debris* – representing inorganic matter, waxes and impurities present in the initial substrate –.

Biocatalyst, which in fact was not a reactant, was also described as *debris* in order to facilitate comprehension.

**Table 8.1.** Definition of components used for the simulation in SuperPro Designer®.

| Component | Definition |
|---|---|
| Ethyl alcohol | Available in SuperPro® components database |
| Water | Available in SuperPro® components database |
| Sodium hydroxide | Available in SuperPro® components database |
| Biodiesel | Defined as *FAEE* (ethyl oleate). Bp: 216°C Mp: -32°C |
| Triglycerides | Defined as *triolein*. Bp: 554°C Mp: -5°C |
| Free fatty acids | Defined as *FFA* (oleic acid) available in database |
| 2-monoglycerides | Defined as *monoolein*. Bp: 239°C Mp: 25°C |
| Alperujo oil | Defined as *pre-dewaxed alperujo*. 72 %w/w of *triolein*, 18 %w/w of *FFA* and 10 %w/w *debris* (waxes). |
| Biocatalyst | Defined as 100 %w/w of *debris* |

### 8.3.3. Process definition: batch operational mode

Process batch was divided into two main parts: initial water pre-equilibration of the components and the enzymatic reaction.

Considering 12 hours for the pre-equilibration, including transfer in and out of components; and taken into account that enzymatic reaction lasted approximately 6 hours, including transfer in, settling of the biocatalyst and transfer out, two storage tanks for each component's pre-equilibration were set in order to optimise total batch process time. As shown in Figure 8.3., the process works in a repeating way, starting with pre-equilibrated reactants. So, after a reaction batch (React. 1.1.) of 6 hours, another pre-equilibration (Pre-equil. 1.1.), which was started during the preceding batch, is ready to be used for a new batch reaction (React. 1.2.). This new batch also starts at the same time with another reactants' pre-equilibration (Pre-equil. 2.1.) that will last 12h to allow another batch start. As pre-equilibration 1.1. finishes, a free tank is released where pre-equilibration 2.1. will take place.
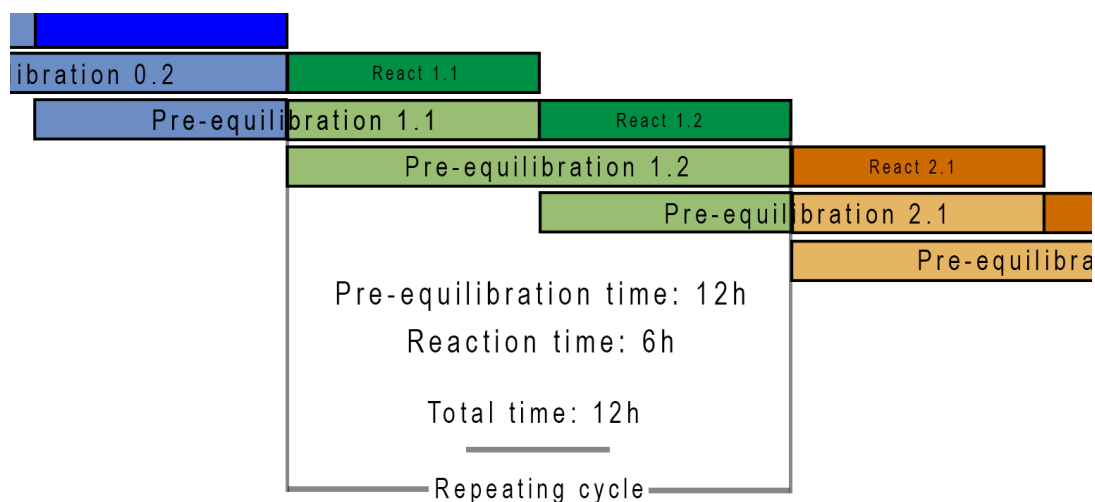
**Figure 8.3.** Schedule for the batch operational mode comprising 2 pre-equilibration processes and 2 transesterification reactions with a total time of 12 hours (repeating cycle).

As said before, annual production of 35000 tonnes of biodiesel are proposed within the 7920 hours, stablished by the software. This time corresponds to 330 days, assuming three working turns per day (24h). Then, biodiesel production rate should be around 4.42 tonnes $h^{-1}$. Considering a repeating cycle of 12 hours, at least 53.03 tonnes of FAEE must be produced during this time taking into account further purification steps.

Thus, around a 90% of purification yield at the end of the final stream was achieved assuming steps optimisation. Considering this, a value between 58-59 tonnes of FAEES should be obtained after each repeating cycle, which in fact included two enzymatic batch reactions.

Figure 8.4. shows the process flow diagram including all the units present in the batch mode comprising pre-treatment of the feedstock, pre-equilibration of ethanol and substrate at a determined initial water activity using saturated potassium hydroxide salt solution.

Ethanol was split into two vessels of 7 $m^3$ (V-101 and V-102). Alperujo oil, previously dewaxed by centrifugation (DC-101) to remove impurities and waxes, was also split into two 52-$m^3$ storage vessels (V-103 and V-104). These four vessels were as well contained by four larger sealed vessels – not showed in Fig. 8.4. – in order to achieve water activity equilibrium using the proper salts, as shown in Figure 8.5 A. The enzymatic reactor of 60 $m^3$ (R-101) was also designed to be contained by a larger vessel to pre-equilibrate initial water of fresh biocatalyst (Figure 8.5B).

**Figure 8.4.** Flow diagram of process batch mode part composed by three main parts: pre-treatment of the feedstock, pre-equilibration of all components and reaction. The circles represent the automatised pumps.
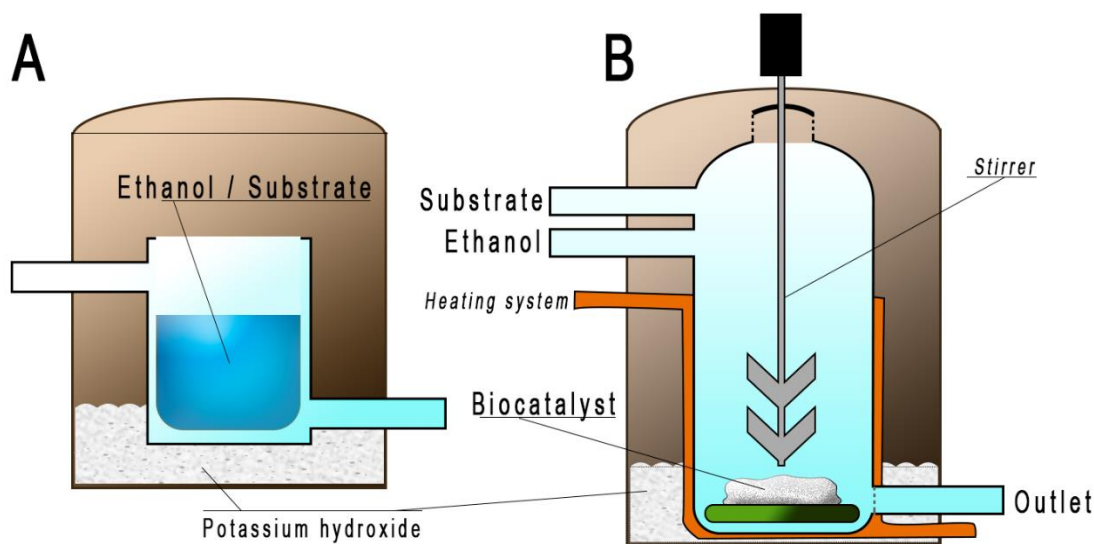
**Figure 8.5.** Schematic representation of pre-equilibration method used for ethanol/substrate (A) and for biocatalyst inside the enzymatic reactor (B) using two larger sealed vessels.

Once all components were pre-equilibrated, transesterification reaction took place inside the stirred tank using the ethanol, the alperujo oil and the rROL-HFAGlut (Table 8.2.).

Biocatalyst was used during 20 reutilisations and average FAEE yield of 88% was taken into account, as seen in Figure 7.8. However, if one considered that rROL-HFAGlut was regioespecific lipase, the actual average yield – over the 67% – was about 59%. Molar composition of 2-monoglyceride (2-MG) was calculated as the half of FAEE concentration due to this regioespecificity. Remaining FFA concentration was also calculated using the average values during the first 20 cycles. Finally, assuming that no diglycerides were present in the reaction in order to facilitate the stoichiometry, the rest was considered as unreacted triolein and ethanol (Table 8.2). Considering the extent and subsequent purification steps, 105 tonnes of raw alperujo oil and 12.3 m$^3$ of ethanol were needed per repeating cycle.

**Table 8.2.** Component definition in terms of final mass in the output stream of the reactor per cycle.

| Component | Final cycle mass (ton) |
|---|---|
| FAEE | 58.19 |
| 2-MG | 34.33 |
| Triolein | 0.462 |
| FFA | 9.039 |
| Ethanol | 0.056 |
| **TOTAL** | **102.095** |

### 8.3.4. Process definition: continuous operational mode

Following lines summarises the units that are used during the continuous mode, which corresponded basically to the downstream and purifying of the process (Figure 8.6.). In general terms, it consisted in the neutralisation of possible remaining free fatty acids into soaps. Then, a hot water washing treatment was applied to reduce the content of these by-products. Finally, a thin-film evaporator was used to separate both interest products: biodiesel and 2-monglyceride.

Operational units were dimensioned by the software SuperPro Designer®, considering the initial input flowrate of 8.506 tonnes $h^{-1}$. This value was obtained from the final cycle mass in the reactor output in the batch operational mode, 102.095 tonnes, divided by the total time spent during every repeating cycle, 12 hours.

- Storage tank (V-105): a vessel of 9.6 $m^3$ was set at the beginning of the process in order to receive and storage the flow from batch part with a residence time of 1h.

- Flash (V-106): this distillation unit consisted of a flash drum of 0.8 $m^3$. It was added to remove up to 99% of the ethanol present in the stream, which was condensed again in order to reuse it at a flowrate of 4.7 kg $h^{-1}$.

- Saponification reaction (R-102): Saponification process is applied if some amount of free oleic was still detected at the end of the reaction since most common quality biodiesel standards stated that acidic value should not exceed 0.5 mg KOH $g^{-1}$ in the final biodiesel [20], [21]. Thus, an excess of sodium hydroxide solution (1.6 tonnes $h^{-1}$) was mixed with the main stream in a 23-$m^3$ reactor with a residence time of 2 hours at 80°C. However, if no free fatty acids were detected at the end of the reaction or high-grade feedstocks were used, which usually did not contain these species, this step was no longer needed.

- Centrifugation (DC-102): using a decanter centrifuge nearly a 93% of the organic content was separated from soaps which conformed, besides water, the solid output. However, the main stream still contained an 0.24% of these components.

- Differential extraction (DX-101): this liquid extraction system with a working area of 0.7 $m^2$ using counter-current washing with heated water was placed after the centrifugation.

Water at a flowrate of 2300 kg h$^{-1}$ – relation 3:1 biodiesel to water – was heated up to 80°C. Soaps were continuously transferred to the aqueous phase and were completely removed.

- Flash (V-107): a flash distillation was set in order to remove remaining water. A flash drum of 0.7 m$^3$ was used. Vapour was released at 2425 kg h$^{-1}$ through the secondary output, which ideally would be reuse it in the heating systems. Moreover, stream with nearly the final purified products was transferred to the next unit.

At this point, concentration of triglycerides and monoglycerides was still high – 0.523% and 36.51%, respectively – to fit into the quality standards, since ASTM D6751 and EN 14214 stablished a maximum of 0.2% for the triglyceride and 0.7% for the monoglyceride content.

In addition, the main purpose of the present chapter, besides the simulation of an industrial process for biodiesel synthesis using rROL-HFAGlut as biocatalyst, was to economically evaluate the process itself. In that way, it was clear that converting ideally a 67% of the initial feedstock to the main revenue (biodiesel) and wasting the remaining 33%, the process could not be cost-effective. Therefore, it was decided to try to separate both main products in a sufficient grade to be profitably sold. Several companies stated that there were two processes for the purification of biodiesel: the cold filtration and the vacuum distillation/evaporation. The most widely used process for the purification of biodiesel is vacuum evaporation, which allows to increase FAEE content, eliminate colour and scent on it, improve the filterability test and reduce the content of mono-, di- and triglycerides [22].

- Thin-Film Evaporation (TFE-101): this vacuum evaporation system principle is to lower the boiling temperature and residence time, being an excellent method for gentle thermal treatment of heat sensitive, high boiling products [23]–[25]. In normal atmospheric conditions these components would be decomposed. Pressure inside the drum was stablished at 0.005 mbar and temperature was set to no more than 60°C, determined by the vapor pressure of ethyl esters at such low-pressure values [26]–[28].
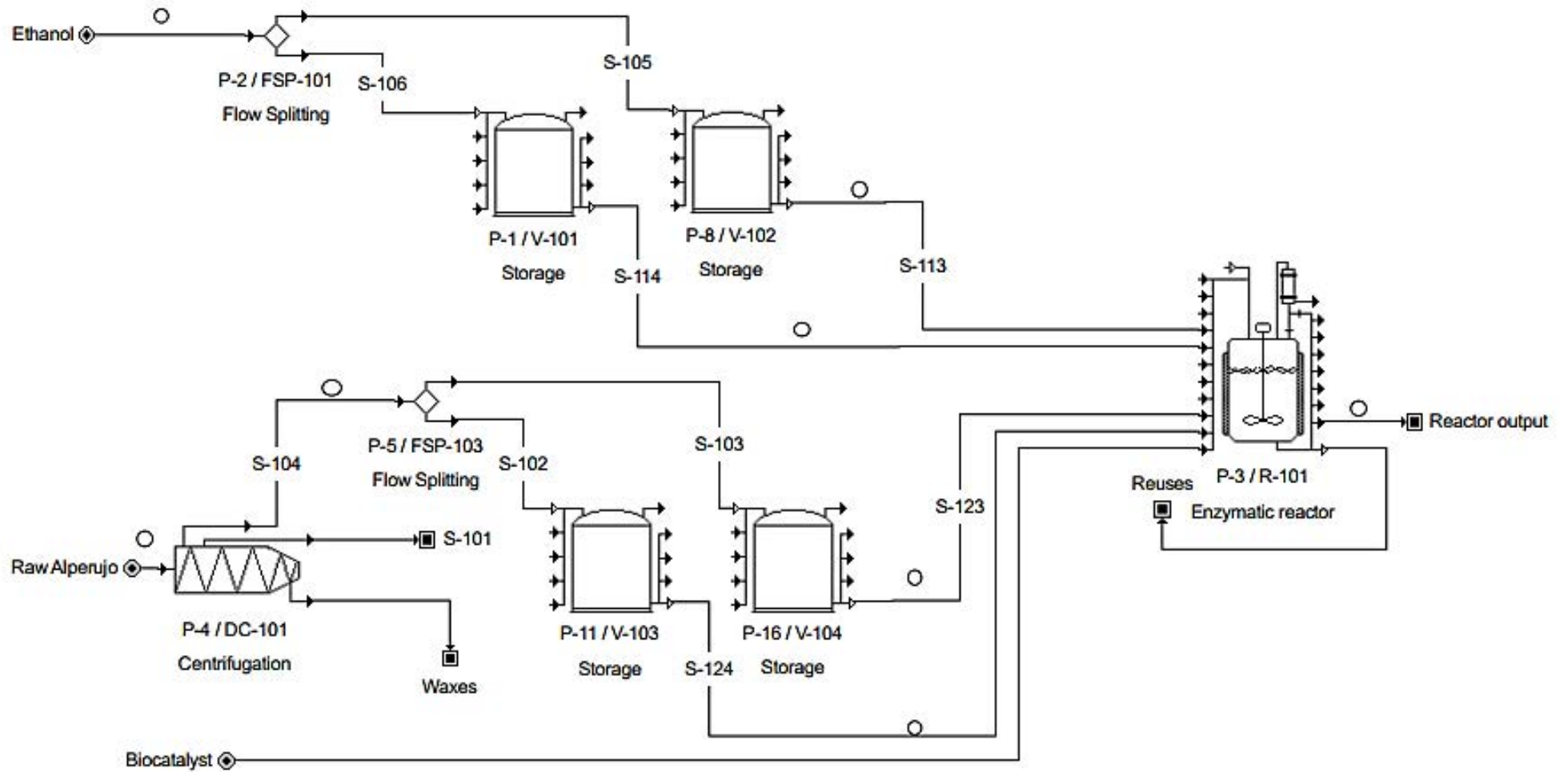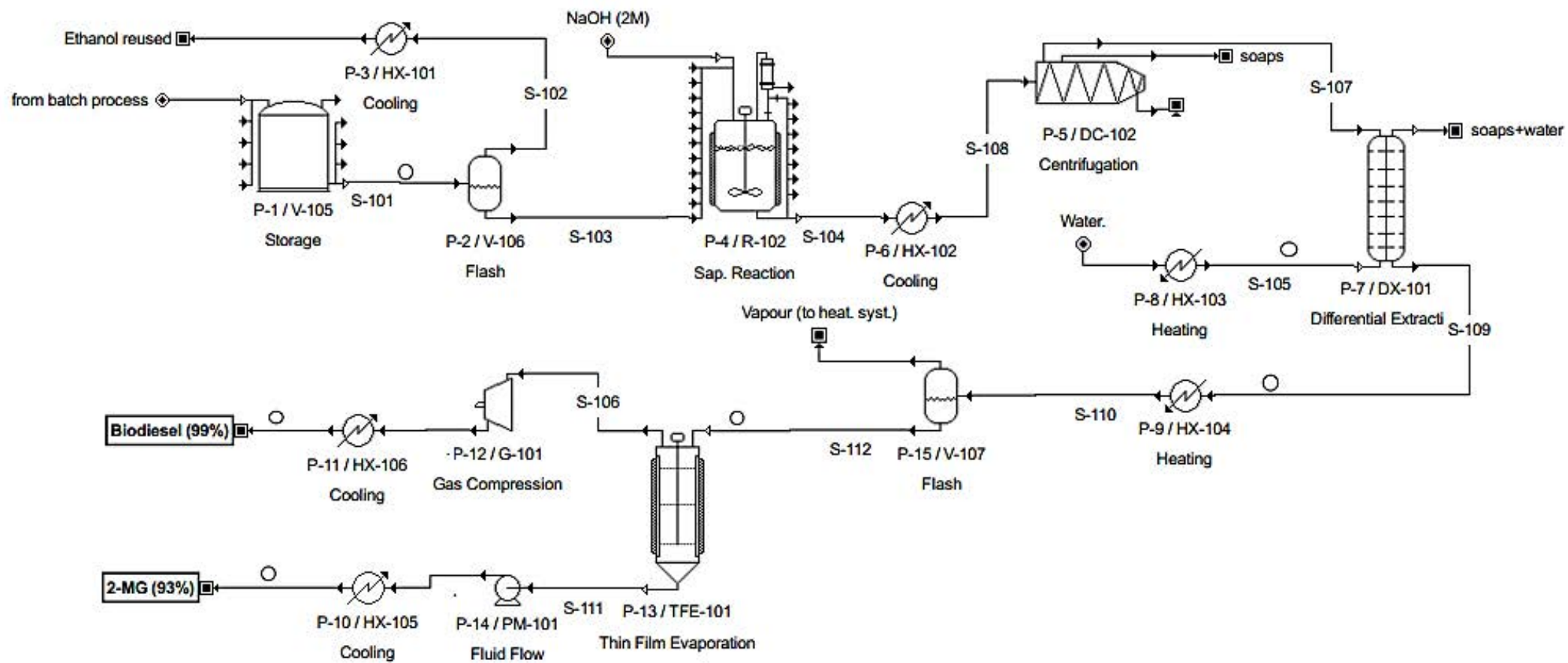
**Figure 8.6.** Flow diagram of process batch mode part composed by three main parts: pre-treatment of the feedstock, pre-equilibration of all components and reaction. The circles represent the automatised pumps.

After that, a compressor (G-101) for the gas output and a positive displacement pump (PM-101) for the liquid output were placed in order to increase pressure and keep flowrate, before cooling and condensing again the components.

On one hand, concentrate output recovered a stream of 2765 kg h$^{-1}$ which was mainly composed of 93% of monoglyceride, 5% of FAEE and 1% of triglyceride. This stream represented the secondary selling product: *2-Monoglyceride (93%)*. On the other hand, volatile output recovered a final stream of 4398 kg h$^{-1}$ which was composed by 99.3% of FAEE, 0.59% of monoglyceride and 0.01% of triglyceride. These values fitted perfectly into both biodiesel standards said previously. Thus, this stream was determined to be sold as main revenue: *Biodiesel (99%)*.

Table 8.3 summarises all the of operation units in both modes: batch and continuous.

**Table 8.3.** Description of all units present in the entire process (batch and continuous), defined by SuperPro®.

| | Unit | Name | Volume (m³) | Area (m²) | Description |
|---|---|---|---|---|---|
| **Batch** | Centrifuge | DC-101 | 230 gpm | | Feedstock pre-treatment |
| | Double Tank | V-101 | 7 | - | Ethanol pre-equilibration |
| | | V-102 | | | |
| | | V-103 | 52 | - | Feedstock pre-equilibration |
| | | V-104 | | | |
| | Reactor | R-101 | 60 | | Enzymatic reaction |
| **Continuous** | Tank | V-105 | 9.6 | - | Store mix from batch |
| | Flash | V-106 | 0.8 | - | Ethanol recovering |
| | Cooling | HX-101 | | 0.4 | Ethanol condensation |
| | Reactor | R-102 | 23 | - | FFA saponification |
| | Centrifuge | DC-102 | 45 gpm | | Mix/soaps separation |
| | Heating | HX-103 | - | 1 | Heat water to 80°C |
| | Differential Extr. | DX-101 | - | 0.7 | Wash mix |
| | Heating | HX-104 | - | 8 | Heat mix to 120°C |
| | Flash | V-107 | - | 0.7 | Evaporate water |
| | Thin Film Evap. | TFE-101 | - | 1.1 | Separate FAEE/2-MG |
| | Compress. | G-101 | 165 kW | | Vacuum discharge |
| | Cooling | HX-102 | - | 2 | Mix cooling after saponification |
| | | HX-106 | | 0.4 | Biodiesel cooling |
| | | HX-105 | | 0.4 | 2-MG cooling |

**8.4.    Process economic analysis**

The main goal of this chapter is to economically evaluate the implementation of the lab-scale process to an industrial one. The following lines describes the estimation methods used to determine: fixed investment costs – equipment, installation, etc. – and working capital, which conforms almost the Total Investment Cost (TIC), variable production costs – raw material and services – and fixed production costs – maintenance, manpower, management, etc. – which results in the Total Production Cost (PTC). Incomes from selling have been also calculated to determine the process profitability.

First simulation has been done considering data and prices as more realistic as possible. After that, a serial of study cases is presented where some conditions, prices and factors were modified. It should be noted that, all prices have been updated to 2017 following annual indexes such as Consumer Price Index (CPI) and Chemical Engineering Plant Cost Indexes (CEPCI)

**8.4.1.    Total Investment Cost (TIC)**

This parameter includes the sum of the equipment needed to implement the process, resulting from the stages of a project known as fixed investment, and of the sum of the resources necessary for initiating the production activities and maintaining them, referred to as working capital and preliminary expenses as process set up and legal compliance to start the business [35]. It is assumed that these initial overheads are minor and they can be estimated as about $0.5 million.

**8.4.1.1. Fixed Investment Cost**

The economic evaluation is carried out considering that this proposed project will be built in an already owned land. Thus, the major budget part will be equipment prices, so it is important to estimate them as realistic as possible. The following Table 8.4. shows prices estimated for all the units that were used during the whole biodiesel synthesis process. Instead of using the automatic estimation taken from the SuperPro Designer® software itself, which could be sometimes not highly precise, a combination of two main approaches have been used. Reactors, pumps and heating/cooling systems costs have been estimated via Sinnot-Towler method [31]. The rest of the prices have been calculated using Couper method [32] or direct references to actual prices.

Unit sizes and parameters (shown in Table 8.3.), construction material as well as installation costs have been taken into account in order to estimate the final unit price. For instance, all vessels have been designed to be made of stainless steel SS313. In addition, pre-equilibration tanks and the enzymatic reactor prices have been calculated also considering the cover vessel (Fig. 8.5). Pumps prices have been estimated individually depending of the flowrate, however total cost is shown in table. Finally, last available CEPCI (569.6, July 2017, [33]) was used to update prices.

Final installed equipment cost was estimated to be $4.23 million, where both enzymatic and saponification reactor and thin film evaporator represented a 43%. In addition, other relevant fact is that the four reactors used for pre-equilibration signified a 23%.

**Table 8.4.** Cost of all the units present in the process for the 2017.

| Unit | Name | Total (K$) | Reference |
|---|---|---|---|
| **2x Pre-equilibration Tanks** | Feedstock | 613.10 | |
| | Ethanol | 338.46 | |
| **Centrifuge** | DC-101 | 283.46 | [32] |
| | DC-102 | 121.54 | |
| **Compressor** | G-101 | 163.80 | |
| **Tank Mix** | V-105 | 92.32 | |
| **Differential Extraction** | DX-101 | 134.84 | |
| **Flash** | V-106 | 89.20 | [34] |
| | V-107 | | |
| **Enzymatic Reactor** | R-101 | 1238.16 | |
| **Thin Film Evaporation** | TFE-101 | 315.62 | |
| **Saponification Reactor** | R-102 | 529.00 | |
| **Cooler/Heater** | HX-101 | 3.91 | [31] |
| | HX-103 | 9.38 | |
| | HX-104 | 46.34 | |
| | HX-102 | 13.96 | |
| | HX-106 | 5.80 | |
| | HX-105 | 5.90 | |
| **Pumps** | - | 137.37 | |
| | | **4231.36** | |

Once equipment was defined and prices were estimated, the rest of fixed investment cost such as pipelining, structures, insulation, foundation, etc., should be determined. As many cases, there is not a unique way to do that. Thus, two specific methods were presented:

The Happel Method [36]: is probably the most used since it is the most accurate method among the others. It is divided into two main parts: materials, where equipment, pipelining, structures, foundation, etc. are considered, and associated labour.

Table 8.5. shows the estimated fixed investment costs of the proposed plant. Calculation percentages were stablished based on the plant characteristics themselves. As seen, the final cost raised up to \$19.88 million, including a 41% from materials, a 23% from labour and a relevant 36% from overhead costs, fees and contingencies.

The Vian Method [37]: this method is simpler than previous one. The principal difference is that all the derived costs, such as installation, instrumentation, insulation, electricity, etc. come from the equipment. After that, a serial of additional costs is applied including project, contractor and unexpected overheads.

As seen in Table 8.6., final fixed investment costs were approximately \$20.15 million, which was a cost very similar to the obtained when applying Happel method. In this case, a 63% came from equipment costs, a 15.7% came from the project cost and finally, a 21.3% came from contractor and unexpected costs. Compared with Happel method, labour costs were included in equipment percentages in this method, since the percentage is nearly the same – 63% and 64% –.

Finally, in order to simplify following calculations, the average value obtained from both methods was used as a final fixed investment cost, \$20.01 million.

**Table 8.5.** Fixed cost calculated using Happel method.

| ITEM | Material (Mat) (K$) | | Labour (Lab) (K$) | |
|---|---|---|---|---|
| Containers (A) | | 2811.042 | 10% A | 281.104 |
| Built towers (B) | | 628.869 | 30% B | 188.661 |
| Pre-built towers (C) | | - | 15% C | - |
| Exchangers (D) | | 85.286 | 10% D | 8.529 |
| Pumps and Centrifuges (E) | | 706.159 | 10% E | 70.616 |
| Instruments (F) | 15% Equipment | 634.703 | 15% F | 95.206 |
| (G) | Σ(A-F) | **4866.059** | | |
| Insulation (H) | 5% G | 243.303 | 150% H | 364.954 |
| Pipeline (I) | 45% G | 2189.727 | 100% I | 2189.727 |
| Foundation (J) | 4% G | 194.642 | 150% J | 291.964 |
| Building (K) | 4% G | 194.642 | 70% K | 136.250 |
| Structure (L) | 4% G | 194.642 | 20% L | 38.928 |
| Fire-fighting equip, (M) | 1% G | 48.991 | 700% M | 340.624 |
| Electricity (N) | 4,5% G | 218.973 | 150% N | 328.459 |
| Painting and cleaning (O) | 0,75% G | 36.495 | 600% O | 218.973 |
| Materials & labour (P) | G +Σ(H-O) +Lab | 12741.139 | | |
| Special equip cost install (Q) | | - | | |
| (R) | P+Q | **12741.139** | | |
| Overhead costs (S) | 30% R | 3822.342 | | |
| Engineering labour fees (T) | 13% R | 1656.348 | | |
| Contingencies (U) | 13% R | 1656.348 | | |
| TOTAL FIXED COST | Σ(R-U) | **19876.176** | | |

**Table 8.6.** Fixed costs estimated using Vian method.

| ITEM | COST (K$) | |
|---|---|---|
| Equipment (X) | | 4231.356 |
| Installation (X2) | 40% X | 1904.110 |
| Valves & Pipelines (X3) | 60% X | 2538.814 |
| Instrumental (X4) | 10% X | 423.136 |
| Insulation (X5) | 20% X | 275.038 |
| Electricity (X6) | 15% X | 634.703 |
| Buildings (X7) | 13.5% X | 571.233 |
| Auxiliar Installations (X8) | 50% X | 2115.678 |
| Y | Σ(X-X8) | **12694.068** |
| Project (X9) | 25% Y | 3173.517 |
| Z | Y + X9 | **15867.585** |
| Contractor (X10) | 7% Z | 1110.731 |
| Unexpected overheads (X11) | 20% Z | 3173.517 |
| TOTAL | Z + X10 + X11 | **20151.833** |

### 8.4.1.2. Working Capital

The ratio of working capital to total capital investment varies with different companies and depending on types of business. If a company manufactures and sells a product at a uniform yearly rate, then 15–25% of the total investment cost is an adequate amount of working capital [32]. Therefore, considering a 15% of the TIC, working capital was fixed as $3.62 million. Total investment cost was finally calculated as a total quantity of $24.13 million, as seen in Table 8.7.

**Table 8.7.** Total investment cost divided into fixed cost and working capital (in M$).

| Fixed | Preliminary expenses | Working capital | TOTAL TIC |
|---|---|---|---|
| 20.01 | 0.5 | 3.62 | **24.13** |

### 8.4.2.   Production Total Cost (PTC)

PTC is, in fact, the estimation of the operational expenses for a year. PTC can be divided into some different parts including: raw material costs, utilities or services which are the steam, energy, electricity requirements for the process; auxiliary materials and packaging, maintenance, direct labour, laboratory costs, supervision, plant managing, capital charges, insurances, local taxes and royalties. Finally, a percentage of this sum is applied in order to obtain R+D+I cost.

Raw Materials: considering 12 hours per repeating cycle, a total 660 cycles could be performed along the 7,920 hours per year. Table 8.8. shows the annual total amount and cost of all raw materials used – alperujo oil, ethanol and biocatalyst –, which in fact they are the main materials for the biodiesel production. In addition, secondary materials such as water, sodium hydroxide and potassium hydroxide have also been considered.

**Table 8.8.** List of unitary prices for all the raw materials used, total amount needed and total cost per year.

| Component | Price ($ m$^{-3}$) | Price ($ ton$^{-1}$) | Amount per year | Total (M$) |
|---|---|---|---|---|
| **Ethanol** | 700 | - | 8078 m$^3$ | 5.65 |
| **Alperujo Oil** | - | 951 | 68806 ton | 65.4 |
| **Water** | 2.02 | - | 32757 m$^3$ | 0.067 |
| **NaOH 99%** | - | 300 | 949 ton | 0.285 |
| **KOH** | - | 600 | 546 ton | 0.327 |
| **Biocatalyst** | - | 690000 | 91.51 ton | 63.1 |
| | | | **TOTAL per year** | **134.9** |

Some considerations about raw materials listed above have been taken into account in order to calculate costs:

- Price of alperujo (951 $ ton$^{-1}$) has been calculated using data from *Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente* [29] updated to 2017 and applying the currency change to dollar.

- Ethanol used per year (8.078 m$^3$) resulted from the total needed for the enzymatic reaction and considering a 0.6% recovery of total ethanol needed in the unit V-106.

- Price for industrial water was taken from *Agència Catalana de l'Aigua* [30] and applying the currency change to dollar. Treatment of water was not considered neither the reutilisation after the possible recovering.

- Potassium hydroxide needed for the initial water activity pre-equilibration was calculated using the actual sizes of the tanks assuming that total mass of this salt occupied a 13,33% of the total tank height assuming a density of 0.8 g cm$^{-3}$. In addition, it was assumed that fresh salt must be added every 20 repeating cycles.

- Price for biocatalyst was calculated following some considerations. Final cost was estimated to be $690 kg$^{-1}$ where support represented a total of $240 kg$^{-1}$ – data from Resindion S.r.l. – and rROL powder represented the remaining $450 kg$^{-1}$, taking into account that cost per kilogram was $300 – data from Biocon, S.A. – and the total enzyme used per kilogram of carrier was approximately 1.5 kg (experimental data).

  Thus, total amount of biocatalyst needed per year was calculated considering that it represented the 1.33% of total alperujo mass – taken from experimental data – and it

could be reused 20 reaction cycles along the 660 cycles per year, which means every 10 process repeating cycles since two batch enzymatic reactions were performed per cycle.

Taken together, the final cost per year of raw materials came to nearly $135 million, where 49% represented the cost of the feedstock and biocatalyst cost, the 47%. Actually, if one considers that the biocatalyst is usually the main part of the cost in enzymatic processes, this gives an idea about how expensive could also be the current substrate.

Table 8.9. shows total cost of production, divided into variable and fixed cost. Explanations for some calculations are laid down in the following lines.

**Table 8.9.** Variable and fixed costs of the process and Production Total Cost.

|  | **Calculation** | **Cost (M$)** |
|---|---|---|
| Raw materials (RM) | Estimated | 134.9 |
| Auxiliary | 5% Maintenance | 0.090 |
| Services | From software | 0.315 |
| Packaging | Negligible | - |
| Maintenance | 7,5% TIC | 1.810 |
| Direct labour (DL) | Estimated | 0.421 |
| Lab costs | 10% DL | 0.042 |
| Supervision | 20% DL | 0.042 |
| Plant management | 50% DL | 0.211 |
| Capital charges | - | - |
| Insurances | 1% TIC | 0.241 |
| Local taxes | 2% TIC | 0.482 |
| Royalties | 1% TIC | 0.241 |
| **Production Cost (C)** | A + B | 138.80 |
| **R+D+I & administration** | 5% C | 6.940 |
| **PTC** | A + B + C | **145.74** |

Utilities/Services: in order to simplify calculations, this value was taken from the simulating software SuperPro Designer®. It was set to $315000.

Direct labour: During the batch process, two employees for three working shifts were estimated to be optimal. In the case of continuous process, also two employees for three working shifts were stablished, resulting in a total of twelve employees. Basic salary was set to $26400 and the social insurance percentage (33%) was applied with a resulting value of $35112 per employee. Thus, a total of $0.421 million was dedicated to direct labour.

### 8.4.3. Economic viability of the process

In a free enterprise system business, companies purpose is to make a benefit. If profits aren't maintained, a company's growth is stifled [32]. Thus, total income from revenues should be high enough to compensate total production cost to get profit, but also to give back the total investment cost in a reasonable plant lifespan to start being considered a profitable project.

### 8.4.3.1. Sales

The revenue base for the present biodiesel synthesis plant consisted in two products. On one hand, the biodiesel (99%) itself produced at a flowrate of 4398 kg h$^{-1}$, resulting in a total annual production of 34828 tonnes. On the other hand, 2-monoglycerides (93%) was produced at 2762 kg h$^{-1}$, resulting in a total annual production of 21872 tonnes per year.

If selling prices for biodiesel and 2-monoglycerides were stablished to be 1.2 and 3.2 $ kg$^{-1}$, respectively, total annual revenue would be $111.8 million, where $41.79 million would come from biodiesel and $69.99 million, from 2-monoglycerides.

However, annual production total cost of $145.75 million exceeded the revenues value in nearly $34 million. This fact resulted in a negative net cash flow for the whole plant lifespan and therefore, a non-viable process.

Trying to overcome this situation by increasing revenues, was not firstly considered since market prices remained rather stable and were already stablished. In contrast, some critical points in plant total cost were determined to make this process profitable.

On one hand, as said before, the 49% of raw material costs which resulted in more than 44% of PTC came from feedstock price (*alperujo*). The main problem that appears here is that, in fact, it is not considered as a waste oil at all, since if some following steps are performed, it can become a totally vegetable edible oil [29]. However, not all this feedstock is currently converted into comestible oil, thus, companies should handle it as a by-product or waste. In this way, the present work has tried to use this oil to stablish a process to synthesise biodiesel capable to use, besides the surplus of alperujo, another high FFA feedstock with similar properties. However, the high price of this feedstock – originated as a by-product of the olive oil extraction, which is currently a valuable asset – made that this designed process became non-profitable.

On the other hand, the most common critical point that appears in biocatalytic processes are the biocatalysts themselves since they can represent a very significant part – up to 60-70% – of the final cost of the process [38]. From far, researchers have struggled to obtain optimised and robust biocatalysts in order to reuse them for long periods of time, reducing enough production cost to make the process cost-effective.

### 8.4.4. Case studies

The economic evaluation of two different scenarios are proposed and analysed. Before that, some preliminary assumptions should be contemplated. Considering that price of capital and interests are currently very low, an Internal Rate of Return (IRR) about 2% would be acceptable, but taking into account the implicit risk in the business, an IRR higher than 5% is pursued to consider the plant as a profitable project.

In this way, two scenarios are proposed taken as basis this consideration:

- The first one is to stablish the maximum price for the feedstock to get a 5% of IRR.
- The second one is related with biocatalyst. Here, the main goal is to find out how many reuses should be needed to get the previously commented IRR.

In order to determine these values, further economic analysis was performed. Net Cash Flow (NCF) analysis allows estimating the plant's potential to generate additional benefits by analysing different in and out capital flows based on the annual periods from the year that initial investment is performed. As before, some considerations must be taken into account.

- Plant lifespan: 15 years.
- Initial investment: is the total cost that must be provided at the beginning (year 0). It consists of the fixed investment cost, the working capital and preliminary expenses (Table 8.7.).
- Sales: profits obtained by selling. (See 8.4.3.1).
- Costs: production expenses (Table 8.9.) but decreasing feedstock price or increasing biocatalyst reuses to reduce costs for Case 1 and 2, respectively.
- Payback: lineal. Calculated by dividing the plant cost – fixed investment cost minus 5% as residual value – by the lifespan.
- Taxes: a 40% of taxes are applied to the taxable base of the year before.

### 8.4.4.1. Case 1: Feedstock price.

It is clear that alperujo oil costs are too high to make this process profitable, since its price represents a 48.5% of raw material costs, which exceeds about $23 million the total revenues income. In order to get the IRR of 5% at the end of the whole process – 15 years – the cost of the reactants should be lower.

Then, by trial/error-based calculation, oil price has been decreased to achieve the previously explained constrain to result in a cost-effective process. Reducing a 25.15% the annual production total cost, a final value of $109.08 million would allow an IRR of 5.28%.

To do that, feedstock price should be diminished to 0.443 $ kg$^{-1}$, representing nearly a 47% of the alperujo oil price (0.951 $ kg$^{-1}$). Although a bountiful harvest of olive may lead to plenty supplies of alperujo oil, such a drastic price drop is not foreseeable. Then, the possibility more feasible would be working with new feedstocks whose prices should be lower than the previous determined value. In this sense, waste or non-edible oils like waste-cooking oil could be used.

Table 8.10. shows the actual NCF for the designed process. The year before the starting, fixed investment must be done and working capital should be provided, which amounted a total of $24.13 million. After that, first year of the plant activity ended with a positive NCF of $2.70 million. Following years, due to applied taxes, NFC resulted in $2.14 million.

Net present value (NPV) is the current value of future cash flows, and depending on the interest, these values can be positive or negative. The interest, which makes NPV equals to zero, determines the IRR. Results are shown in Figure 8.7.
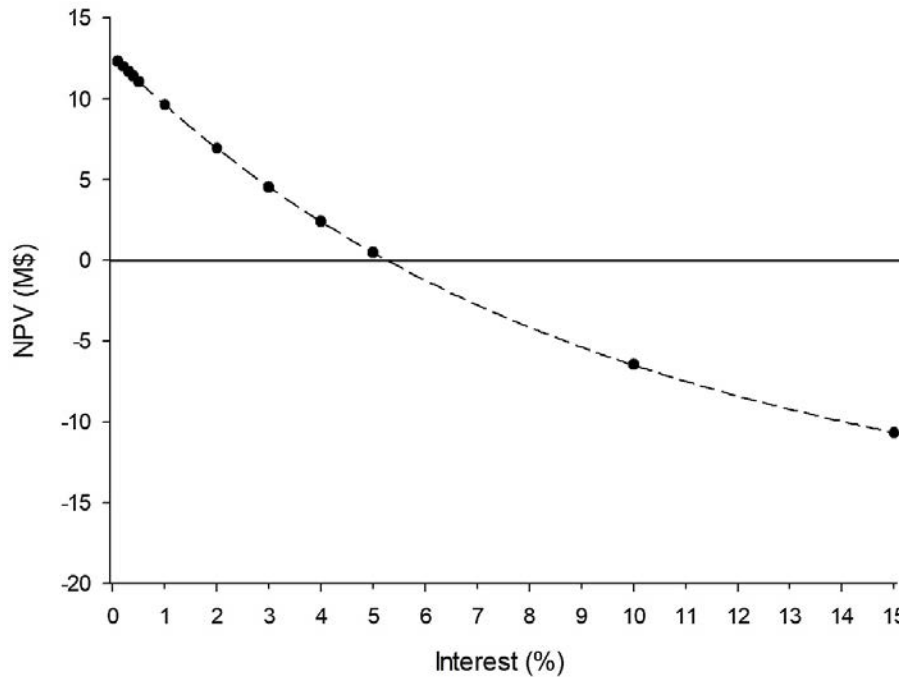
**Figure 8.7.** Sensitivity analysis using the Net Present Value (NPV) based on interest. IRR = 5.28% for case study 1 – Feedstock price.

### 8.4.4.2. Case 2: Biocatalyst reuses.

In this case biocatalyst stability during the reactions is considered keeping alperujo oil as feedstock. As long as biocatalyst is reused, the total cost of production is reduced since less amount of fresh biocatalyst should be provided. In this case, same goal has pursued, which is to get an IRR of 5%.

It was determined by trial/error that biocatalyst should be reused up to 45 times to achieve the objective IRR. This number of reutilisations represents 25 reutilisations more than in the original process. This could be quite feasible, since trend shown in Figure 7.8, led to believe that biocatalyst stability could induce to reuse it several times again without losing the retained activity. In addition, further analysis determined that expected half-life time could last up to 266 hours, which represented 60 cycles. Thus, it is not unreasonable to think that reusing biocatalyst 25 times again, it could be as active as during 20 cycles.

Then, annual plant total cost would be shortened up to 25.26% with a final value of $108.9 million by increasing 25 biocatalyst reutilisations.

Table 8.11. show the actual NCF for the designed process. Again, the year before the starting, fixed investment must be done and working capital should be provided. After that, first year of

the plant activity ended with a positive NCF of $2.87 million. Following years, due to applied taxes, NFC resulted in $2.24 million.

As in the previous case, sensitivity study was performed. Figure 8.8. shows that the final IRR was 5.88%.
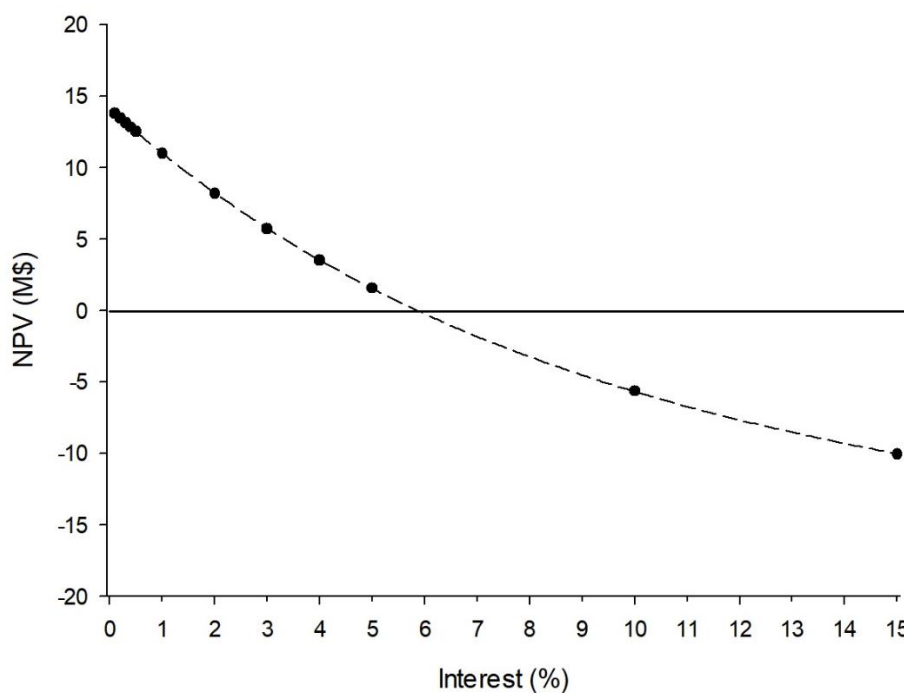


**Figure 8.8.** Sensitivity analysis for the case 2 using NPV and interest. IRR was found to be 5.88%

However, one can perform several modifications at the same time. In that way, if two previous case studies were combined. It is clear that this process would also be feasible and cost-effective by reducing, for instance, feedstock cost from 0.951 $ kg$^{-1}$ to 0.627 $ kg$^{-1}$, and reusing biocatalyst 25 times instead of 20, NCF would be $2.12 million for the 15 years of process with an Interest Rate of Return of 5.15%.

These values underline that, whether from feedstock cost or biocatalyst reuses, which in fact are the key points in the process, there is a wide range of possibilities that makes this proposed process to be cost-effective.

**Table 8.10.** Net Cash Flow for case study 1 – Feedstock price (Values in M$).

| Year | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|
| Fixed | -20.51 | | | | | | |
| Working capital | -3.62 | | | | | | |
| Payback | | -1.29 | -1.29 | -1.29 | -1.29 | -1.29 | -1.29 |
| Sellings | | 111.78 | 111.78 | 111.78 | 111.78 | 111.78 | 111.78 |
| Costs | | -109.08 | -109.08 | -109.08 | -109.08 | -109.08 | -109.08 |
| Tax base | | 1.40 | 1.40 | 1.40 | 1.40 | 1.40 | 1.40 |
| Taxes (40%) | | | -0.56 | -0.56 | -0.56 | -0.56 | -0.56 |
| | | | | | | | |
| NCF | -24.13 | 2.70 | 2.14 | 2.14 | 2.14 | 2.14 | 2.14 |

**Table 8.10. [Continuation]** Net Cash Flow for case study 1 – Feedstock price (Values in M$).

| 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | 1.02 |
| | | | | | | | | | 3.62 |
| -1.29 | -1.29 | -1.29 | -1.29 | -1.29 | -1.29 | -1.29 | -1.29 | -1.29 | |
| 111.78 | 111.78 | 111.78 | 111.78 | 111.78 | 111.78 | 111.78 | 111.78 | 111.78 | |
| -109.08 | -109.08 | -109.08 | -109.08 | -109.08 | -109.08 | -109.08 | -109.08 | -109.08 | |
| 1.40 | 1.40 | 1.40 | 1.40 | 1.40 | 1.40 | 1.40 | 1.40 | 1.40 | |
| -0.56 | -0.56 | -0.56 | -0.56 | -0.56 | -0.56 | -0.56 | -0.56 | -0.56 | -0.56 |
| | | | | | | | | | |
| 2.14 | 2.14 | 2.14 | 2.14 | 2.14 | 2.14 | 2.14 | 2.14 | 2.14 | 4.08 |

**Table 8.11.** Net Cash Flow for case study 2 – Biocatalyst reuses (Values in M$).

| Year | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|
| Fixed | -20.51 | | | | | | |
| Working capital | -3.62 | | | | | | |
| Payback | | -1.29 | -1.29 | -1.29 | -1.29 | -1.29 | -1.29 |
| Sellings | | 111.78 | 111.78 | 111.78 | 111.78 | 111.78 | 111.78 |
| Costs | | -108.91 | -108.91 | -108.91 | -108.91 | -108.91 | -108.91 |
| Tax base | | 1.57 | 1.57 | 1.57 | 1.57 | 1.57 | 1.57 |
| Taxes (40%) | | | -0.63 | -0.63 | -0.63 | -0.63 | -0.63 |
| | | | | | | | |
| NCF | -24.13 | 2.87 | 2.24 | 2.24 | 2.24 | 2.24 | 2.24 |

**Table 8.11. [Continuation]** Net Cash Flow for case study 2 – Biocatalyst reuses (Values in M$).

| 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | 1.02 |
| | | | | | | | | | 3.62 |
| -1.29 | -1.29 | -1.29 | -1.29 | -1.29 | -1.29 | -1.29 | -1.29 | -1.29 | |
| 111.78 | 111.78 | 111.78 | 111.78 | 111.78 | 111.78 | 111.78 | 111.78 | 111.78 | |
| -108.91 | -108.91 | -108.91 | -108.91 | -108.91 | -108.91 | -108.91 | -108.91 | -108.91 | |
| 1.57 | 1.57 | 1.57 | 1.57 | 1.57 | 1.57 | 1.57 | 1.57 | 1.57 | |
| -0.63 | -0.63 | -0.63 | -0.63 | -0.63 | -0.63 | -0.63 | -0.63 | -0.63 | -0.63 |
| | | | | | | | | | |
| 2.24 | 2.24 | 2.24 | 2.24 | 2.24 | 2.24 | 2.24 | 2.24 | 2.24 | 4.01 |

## 8.5.    Conclusions

It is clear that, trying to design a profitable enzymatic biodiesel synthesis plant is still a challenging purpose.

First of all, planning and scheduling of the operational units within the process have been successfully set up, considering experimental or literature available data to get closer to the reality as much as it has been possible.

In terms of profitability, results showed that some improvements should be performed before the total implantation since process was found to be non-profitable due to high cost of production in contrast to revenues amount. Two case studies were carried out, in one case feedstock price was reduced and reutilisations of biocatalyst were increased in the other.

First one, showed that reducing significantly the substrate cost, the process became viable. It should be mentioned that, apart from *alperujo*, there are lots of oil capable to be used as biodiesel substrate. In this way, the present proposed plant would be able to handle the major of all these vegetable oils, which can contain even higher amounts of FFA. However, this should not be a problem since, besides using rROL-HFAGlut, it is equipped with a saponification system able to neutralise whatever the concentration of free fatty acids at the end of reaction.

Second case study showed that biocatalyst is, as said before, a key point for the viability of the process. Results demonstrated that, the process could also become feasible increasing the total reutilisations by 25.

Finally, a combined purpose with a reduced feedstock price and reused biocatalyst assures a wide range of possibilities to get a profitable enzymatic biodiesel plant.

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# GENERAL CONCLUSIONS

**9**

Nowadays, biodiesel has arisen as one of the most important alternatives to petroleum-based energies. Although synthesis of fatty acid alkyl esters is currently well stablished, the present thesis has tried to give light to another interesting approach which is the production of biodiesel via the enzymatic way. Instead of using the most common vegetable edible oils, this thesis is focused on the utilisation of a feedstock with high content of FFA. Most of the non-edible oils have this property and biodiesel synthesis has become a new form to revalue them. Although alperujo oil is an edible oil, it is an abundant and reproducible source of a high FFA content oil to be used as a model.

Since it is clear that biocatalyst is one of the key parameters in enzymatic reactions, a functional and feasible immobilisation has been tested in order to ensure stability of the recombinant ROL in transesterification reactions. Activation of the support using ethylenediamine and treating with glutharaldehyde (HFAGlut) has resulted in an enhancement on initial synthesis rate that was not observed using the commercial ones, thus rROL-HFAGlut was chosen as a proper biocatalyst for next experiments.

Role of FFA present in alperujo has been evaluated, showing that these components provided higher initial reaction rate as well as enzyme stability, allowing more biocatalyst reuses. These results demonstrated that alperujo oil could be perfectly used as biodiesel feedstock through enzymatic synthesis using recombinant ROL in a lab-scale 10 ml-vials using orbital stirring.

To ensure a satisfying biocatalyst performance and stability, transesterification reaction conditions should be set up. It is known that lipases are influenced by water activity ($a_w$) and temperature. Thus, a serial of initial $a_w$ pre-equilibrations was performed and three temperatures were tested. Best results showed that this lipase has its peak of activity at low $a_w$ values and its major stability at 30°C.

Moreover, synthesis of biodiesel is usually carried out using two different alcohols as acyl-acceptors: methanol and ethanol, since they are highly available and not expensive. However, as many studies stated, the use of these short-chain alcohols can produce an important damage on the enzyme performance. Both acyl-acceptors showed different inactivation behaviour depending on how they were added. Three strategies for their stepwise addition were proposed where ethanol seemed to be less damaging along the three approaches. Moreover, one-pulse methanolysis resulted fatidic for ROL activity in contrast when ten pulses were added, demonstrating that stepwise addition is an excellent strategy to overcome alcohol inactivation. Best results in terms of initial rate and stability were achieved the more fractioned the alcohols were. At this point, differences between ethanol and methanol were minor.

Another important factor to consider is the scaling-up of the process to larger volumes. Since biocatalyst is really sensitive to major changes, first scale up to a 50 mL-stirred reactor was performed. The most challenging modification here was moving from orbital to mechanical stirring. Previous experiments performed in vials were replicated. While results showed slightly differences between both sizes, the major conclusion that one can draw from here is that scaling-up was successfully performed.

In addition, further analyses of the biocatalyst stability were carried out in order to elucidate how robust it could be depending on the alcohol chosen. Since ethanol caused less damage to the biocatalyst during reactions, stability was better maintained along the reutilisations compared with methanol. Thus, ethanol was finally selected as the acyl-acceptor using a 10-pulse feeding strategy.

Since results showed that the more fractioned the alcohols were, the more stability achieved, a new addition approach was attempted. Semi-continuous ethanol feeding using a micro-burette was tested but preliminary obtained results led to think that ethanol was added too slow which resulted in a low concentration in the medium.

Finally, one of the most important factors to be considering at is the transfer of the process to an industrial size in a profitable and reliable project. It is known that designing an enzymatic biodiesel synthesis plant is a challenging purpose generally due to enzyme's cost. However, the major problem that this study has been facing at is the high price of the alperujo oil, which resulted in a non-profitable process along 15 years of plant lifespan. Thus, although they are not incompatible, two modifications were suggested independently in order to find out a practical and beneficial operation. It is clear that lowering the substrate cost will be enough to make it cost-effective. As said before, alperujo oil was only chosen as a model, so other cheaper feedstocks with similar properties could be integrated into the process. In addition, results showed that biocatalyst is a relevant actor here, since reutilisations should be doubled to make the process cost-effective, even with alperujo oil as a feedstock.

SCIENTIFIC

CONTRIBUTIONS

**10**

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# NOTES