Study of the lipase-catalyzed hydrolysis of waste oleochemical streams

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One, remember to look up at the stars and not down at your feet. Two, never give up work. Work gives you meaning and purpose and life is empty without it. Three, if you are lucky enough to find love, remember it is rare and don't throw it away.

Stephen Hawking

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First of all, to my family, especially to my mother because she was my very first teacher, she did not only teach me how to write, how to read, and mathematics, but she also taught me the value of the knowledge the importance of the education and the necessity of curiosity in the world, without her kindliness and support nothing that today has been done by me would be possible. She has been the person who has believed in me since the beginning and in each step of my professional development.

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Abstract

Study of the lipase-catalyzed hydrolysis of waste oleochemical streams

Residual vegetable oils and fats have attracted attention around the world because their common mismanagement generates a complex cascade of environmental and health problems. Nonetheless, since they are mostly comprised of triglycerides, they can be used as second generation raw materials in the oleochemical industry. In particular, fatty acids that are intermediates for the manufacture of surfactants, plasticizers, biofuels, among others, can be obtained through the hydrolysis of such waste triglycerides. However, the current industrial process (i.e. Emery-Colgate) for fatty acids production has important disadvantages mainly the energy intensity and waste generation. For this reason, enzymatic processes have been explored as viable alternatives to conventional ones, since they operate under milder temperature and pressure conditions. In the same way, the interest in developing effective enzymes at the industrial level has driven new advances such as immobilization in suitable and tunable solid supports that increase their stability and facilitate their reusability. Likewise, process intensification has also been employed to improve reaction yields and to reduce waste generation. In the present study, the enzymatic hydrolysis of used cooking oils was explored, using Candida Antarctica lipase B immobilized on activated carbons. It was verified a greater enzymatic activity and immobilization efficiency was obtained by amino-functionalization with subsequent cross-linking using glutaraldehyde. Once immobilized, reaction conditions were explored by changing pH, temperature, substrate ratio and immobilized enzyme loading. Finally, a comparison with currently used commercial enzymes and reusability tests were also performed to assess the feasibility of the process.

Keywords: Enzymatic immobilization, enzymatic hydrolysis, used cooking oil, Candida antartica lipase B, activated carbons.

Resumen

Estudio de la hidrólisis catalizada por lipasas de corrientes oleo químicas residuales

Los aceites vegetales y grasas residuales han captado mundial ya que típicamente se disponen de forma incorrecta generando una compleja cascada de problemas ambientales y de salud. Sin embargo, debido a que estos residuos están compuestos principalmente de triglicéridos, estos se pueden usar como materia prima de segunda generación para la industria oleoquímica. En particular, los ácidos grasos que son un intermediario para la manufactura de surfactantes, plastificantes, biocombustibles entre otros, pueden ser obtenidos mediante la hidrólisis de triglicéridos residuales. No obstante, el proceso industrial actual (i.e. Emery-Colgate) para la producción de ácidos grasos presenta importantes desventajas como su intensidad energética y la generación de residuos. Por esta razón se han explorado procesos enzimáticos como una alternativa viable a los convencionales, ya que estos operan en condiciones de temperatura y presión más benévolas. De la misma forma, el interés por desarrollar enzimas efectivas a nivel industrial ha impulsado nuevos avances tal como la inmovilización de enzimas en soportes sólidos adecuados y modificables para aumentar su estabilidad y facilitar su reusabilidad. Igualmente, se ha empleado la intensificación de procesos para mejorar el rendimiento de la reacción y reducir la generación de residuos. En este estudio se exploró la hidrolisis enzimática de aceites usados de cocina, usando la Cándida antártica lipasa B inmovilizada en carbones activados. Se verificó que la mayor actividad y eficiencia de inmovilización se logró por medio de amino-funcionalización seguida de entrecruzamiento usando glutaraldehído. Una vez inmovilizado, se evaluaron diferentes condiciones de reacción enzimática, variando pH, temperatura, relación de sustratos y carga de enzima inmovilizada. Finalmente, se realizó una comparación de la eficiencia del proceso usando enzimas comerciales y una evaluación de factibilidad a través de ensayos de reusabilidad.

Keywords: Inmovilización enzimática, hidrólisis enzimática, aceite usado de cocina, Candida antartica lipasa B , carbonos activados.

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Abbreviations

Abbreviature	Term
AC	Activated carbons
CALB	Candida Antarctica Lipase B
CALBAC	Candida Antarctica Lipase B onto Activated Carbons
CRL	Candida Rugosa Lipase
DGs	Diacylglycerols
FFAs	Free fatty acids
FOGs	Fats oils and greases
MAC	Mesoporous activated carbons
MGs	Monoacylglycerols
PUFAs	Poly unsaturated fatty acids
TGs	Triacylglycerols
UCOs	Used cooking oils

Introduction

Vegetable oils and animal fats are the main feedstocks of the oleochemical industry, and they are widely used in the manufacture of a large variety of derivatives [1]. Oils and fats are mainly composed of triacylglycerides of fatty acids having different chain lengths and saturation character, and their concentration and nature are mainly dictated by the corresponding biobased source. While triacylglycerides (TAGs) can be directly used as feedstock in many processes, derived basic oleochemicals such as fatty acids and their methyl esters are preferred as raw materials because they can be more easily handled, fractionated, and split according to their carbon chain length and their saturated-unsaturated nature [2]. Amongst basic oleochemicals, free fatty acids (FFAs) are of major importance as they constitute a great portion of the renewable materials currently used in the chemical industry [3]. FFAs are mainly produced by sequential-parallel hydrolysis of TAGs, and they are used to obtain a large variety of molecules that are difficult to produce via petrochemical pathways [4]. These include soaps, surfactants, lubricants, plasticizers, and many other commodity, fine and specialty chemicals [5]. Amongst the different FFAs, polyunsaturated fatty acids (PUFAs) are of special interest because they have enhanced nutritional value for food/feed applications, and they are also suitable feedstocks for biopolymers production, which are currently under high and increasing demand [6,7].

Even tough biobased and renewable resources have gained significant use in the chemical industry, awareness on the associated life cycle impacts of agricultural-based raw materials is necessary. This has resulted in a better understanding of the water-energy-food security nexus that have redirected the attention to reclaiming and exploitation of biobased residues [8]. In the case of the oleochemical industry, there is an increasing interest for exploiting waste fats oils and greases (FOGs). These mainly correspond to food post consumption residues mainly from large urban centers and to industrial and agricultural wastes. On one hand, urban waste lipids correspond to inevitable and inedible fats and oils from animal rendering, grease traps, food residues, and used cooking oils (UCOs), among others [9].

On the other hand, industrial and agricultural waste lipids include rancid oils, spent filtering earths, soapstocks, mill effluents, deodorization distillates, etc. [10]. Currently, millions tons of such waste lipids are generated and mishandled worldwide, causing a cascade of environmental, health, and economic impacts, and also losing their value as oleochemical resources [11].

In spite of the potential benefits of using FOGs as industrial feedstock, there are still many logistics, supply chain and technical challenges to overcome, in particular for the reclaiming of UCOs [12]. These residues are characterized by a higher heterogeneity and a high content of impurities and acidity [13], thereby a variety of pre-treatment processes are required for their transformation into suitable oleochemical feedstocks [14]. Currently, a major goal of pretreatment is the reduction of moisture and acidity to fulfill the required specifications for biofuels production, as this is the most common valorization route implemented at the industrial scale [15]. Similar to edible oils and fats refining, FFAs removal from waste lipids is generally accomplished by physical and/or chemical processes [16,17]. However, as the acidity content can be large, pretreatment can be cumbersome, costly and residues-intensive. Alternatively, recognizing the extensive use of fatty acids, and instead of reclaiming and refining only the TAGs fraction of UCOs, these could be subjected to hydrolysis to enable the complete recovery of FFAs for further oleochemical use.

Currently, industrial FFAs are commonly manufactured by hydrolysis of edible oils and fats under extreme conditions via highly energy-intensive processes (e.g. Colgate-Emery, Eisenlohr) [18]. These processes involve the acid-catalyzed or uncatalyzed reaction with water under high temperatures and pressures (i.e., around 250 °C and 50 bar), and such conditions are required to enhance miscibility between oil and water, to reduce oil viscosity, to overcome equilibrium limitations and to achieve fast kinetics. In addition to requiring costly equipment and high operating expenses, these extreme conditions also trigger undesired side reactions such as the polymerization of valuable PUFAs [3,19,20]. In this regard, catalytic hydrolysis under milder conditions is a greener alternative to the current industrial processes. While reducing the energy of activation of reaction, the use of heterogeneous catalysts could also mitigate equipment corrosion, facilitate separation, and reduce production of undesired byproducts [3,19,21]. Additionally, operating under milder

conditions would enable to exploit low-quality and waste oleochemical feedstocks, limiting a further decomposition of such materials [22].

Amongst the variety of materials that can be used to promote the hydrolysis of low-quality feedstocks, bioderived enzymatic catalysts have attracted attention recently due to their high activity under milder conditions, high regioselectivity, and substrate specificity [23]. Among the variety of enzymes used to promote hydrolysis of waste lipids, lipases belong to a special group that exhibit high activity even in hydrophobic media. This feature gives lipases the ability to selectively catalyze different reactions besides hydrolysis including esterification, interesterification, alcoholysis, acidolysis, and aminolysis [24]. Nonetheless, despite their high activity and selectivity, the extended use of lipases in the industry is still limited mainly due to their high costs compared with typical homogeneous and heterogeneous catalysts. As a result, researchers have focused on developing new and more specific lipases, with higher activity, more resilience to the reactive media, with higher stability to operating conditions, and suitable for recycling and reuse. An effort in this direction is the use of immobilized lipases, which provide such advantages over free enzymes [25].

Despite gaining stability and reusability, when enzymes are immobilized, additional factors start to play a critical role in the hydrolysis of waste FOGs. These include the interaction of reactants with the support, the interfacial area between the immiscible reactants, the accessibility of reactants to the active sites, the stability and activity of the anchored enzymes, and the potential effects of impurities, etc. [26]. These issues have been actively studied and different approaches have been explored to overcome the resulting limitations. Some studies have been focused on developing mesoporous supports with large surface area or with tailored surface characteristics to improve reaction performance. Also, different immobilization methods have been developed to improve enzymes stability and activity [23]. Other studies have focused on the use of intensified technologies to increase compatibility and interaction between reactants, for instance by enhancing mixing and promoting the formation of stable emulsions. In this latter cases, techniques such as high-shear mixing, microwaves, and ultrasound have been proven to be effective in promoting contact between reactants and generating high hydrolysis rates of lipids, even in solvent-free systems [27].

Even the recent advances in the hydrolysis of waste lipids, there is still need for a better understanding of the enzymatic processes, immobilization techniques, supports characteristics, processing conditions, intensification methods, and reaction configurations. This in turn would enable to develop suitable technologies for industrial-scale production of FFAs from second generation feedstocks [28]. In this direction, this work focuses on the study of the production of FFAs via enzymatic hydrolysis of UCOs. In particular the process is evaluated using *Candida Antarctica* Lipase B as catalysts of reaction.

In the first part, immobilization of the *Candida Antarctica* Lipase B (CALB) onto mesoporous activated carbon (Starbon A800) was conducted. This was done based upon experimental evaluation taking into consideration reported conditions from literature that were obtained from a comprehensive review presented in Chapter one. Afterwards, performance of the immobilized biocatalyst was assessed in the hydrolysis of UCOs under different operating conditions. As presented in chapter two, reaction performance using the immobilized catalyst was evaluated varying substrate molar ratio, catalyst concentration and with/without the influence of ultrasound. Finally, in chapter three, a feasibility analysis is carried out by evaluating the reusability of the synthetized biocatalyst and its performance compared with the current benchmark commercial biocatalyst (e.g. Novozyme 435®).

Some specific outcomes and novel results from this work are:

- Assessment of the performance of Candida Antarctica Lipase B (CALB) supported on mesoporous activated carbons as catalysts for the hydrolysis of used cooking oils (UCOs).
- Evaluation of the efficiency of immobilization of CALB on an activated carbon (Starbon A800) and its potential for reusability.
- Assessment of the effect of various operating variables on the hydrolysis of refined vegetable oils and UCOs, such as water-to-oil molar ratio, catalyst concentration, time of exposure to ultrasound, and mechanical stirring.
- Comparison of hydrolysis performance using the immobilized enzyme with respect to a commercial benchmark (Novozyme 435®).

Chapter 1.

Immobilization of Candida Antarctica lipase onto mesoporous carbon (Starbon A800) via combined physical adsorption and covalent attachment.

This section includes fragments of the paper: A. Baena, A. Orjuela, S.K. Rakshit, J.H. Clark, Enzymatic hydrolysis of waste fats, oils and greases (FOGs): Status, prospective, and process intensification alternatives, Chem. Eng. Process. - Process Intensif. 175 (2022) 108930

The paper contents were reformatted, and figures and tables were enlarged to fulfill edition requirements for the dissertation document.

1. Immobilization of Candida Antarctica lipase onto mesoporous carbon (Starbon A800) via combined physical adsorption and covalent attachment.

1.1 Abstract.

Enzymes, and in particular lipases, are promising alternatives to intensify most of the current highly energy-, materials- and cost-intensive chemical processes used for the hydrolysis of vegetable oils and fats. Advantageously, lipases are highly active and selective catalysts near to ambient conditions in the hydrolysis of triacyl glycerides into fatty acids and intermediate glycerides. However, key issues when using such biobased molecules are yet to be overcome such as stability to the reactive medium, diffusional hindrance and catalyst recovery and reusability. In this regard, lipase immobilization seems to be a suitable method to solve such issues, but this requires a thorough assessment of lipases sources, supports, and suitable immobilization techniques. This study deals with the lipase immobilization of *Candida antarctica* lipase B (CALB) onto mesoporous activated carbons (Starbon A800) with three different immobilization protocols. The immobilization efficiency and enzyme loading were evaluated using amino functionalization with polyethylenimine, and covalent attachment with glutaraldehyde to enhance lipase physical adsorption. Lipase activity was measured to compare the performance of the assessed biocatalysts with respect to benchmark commercial counterpart (Lipozyme-TLIM and Novozyme 435®) and to assess the effect of pH and temperature during hydrolysis. According to results, the most promissory catalyst for hydrolysis of lipids was *Candida antarctica* lipase B immobilized on activated carbon (CALBAC3) which exhibited a lipase activity, enzyme loading and immobilization efficiency of 136.9 LU/g, 11.30% and 56.27%, respectively.

Keywords: Lipase, Activated carbons, Interfacial activation, immobilization.

1.2   Introduction

The use of enzymes as bio-derived catalysts in the oleochemical industry has been widely studied because of their well-known key role in different metabolic processes and reactions. Some of the most important industrial enzymes are lipases which are primarily used for the catalytic hydrolysis of triacyl glycerides (TAGs) into free fatty acids (FFAs) and partial glycerides. Lipases are produced by living organisms to aid the digestion, transporting and processing of dietary lipids and TAGs [29]. The industrial interest over this type of enzymes relies on their high catalytic activity at mild operating conditions (i.e. near ambient temperatures and pressures), high regioselectivity, and substrate specificity [18,30]. As a result, and in contrast with current industrial processes, lipases enable to carry out the hydrolysis of lipids under a very low energy intensity and avoiding undesired side products [5,20]. Also they allow to work with low-quality feedstocks such as waste fats, oils and greases (FOGs) owing to the fact that water and FFAs content do not represent a major limitation for the enzymatic process [31].

Despite of the above mentioned benefits, there are still some drawbacks of using lipases that limit their industrial implementation as catalysts such as their higher costs, the difficult recovery, and that they are sensitive to reaction conditions such as temperature, pH, ionic strength, and impurities [5,19]. In particular, one of the most challenging issues in the enzymatic hydrolysis of waste FOGs (e.g. used cooking oils, UCOs), is their high content of polar impurities. Those correspond to oxidized organic compounds generated during oil cooking and frying (e.g. ketones, aldehyde, alcohols), and to phosphatides, sulfur- and nitrogen-containing compounds extracted during food processing [16,17,32]. These molecules may act as enzymes inhibitors since they could compete with TAGs and water for the active sites during hydrolysis, or they can promote desorption and leaching of anchored enzymes [33]. Besides, polarity of such impurities can generate a change in the morphology of enzymes, thus affecting their activity and selectivity. For instance, it has been verified that phosphatides present in waste and nonedible oils inhibit enzymatic reactions [34].

In order to exploit enzymes advantages in the hydrolysis of waste FOGs, and to overcome the above-mentioned drawbacks, different techniques have been developed for the

synthesis, isolation, purification, and immobilization of the biobased catalysts. In particular, immobilization, which is the confinement of an enzyme to a phase (matrix/support) different from the reactive media [23], is done to overcome inherent limitations related to enzymatic reactions, providing more resilience to reaction conditions, and enabling a simpler recovery and reuse [5,35]. Some typical methods for enzymes immobilization are described in Figure 1.1, which include adsorption, crosslinking, covalent bonding, entrapment, and encapsulation. Such techniques have enabled enhancement of the enzymatic performance and to conduct continuous processes in a variety of industries, including food, dairy, detergents, and pharmaceutical [36,37]. Amongst these, the most widely used at the industrial scale is the physical adsorption because it is a simple, economic, and fast technique. Adsorption exploits the physical interactions generated between the carrier and enzyme, which occurs through Van der Waals forces, ionic interactions, and hydrogen bonding [37]. As the physical Interactions between enzyme and carrier are weak, adsorption is a suitable technique to prevent enzymes distortion and to avoid a loss of activity [23]. Nevertheless, this weak interaction also means that absorbed enzymes are also prone to desorption and leaching.
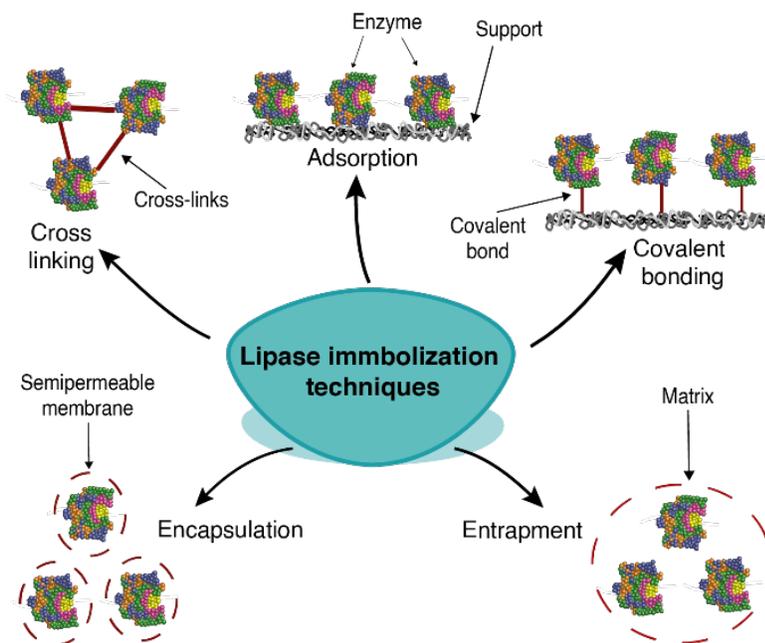
Figure 1.1. Common immobilization techniques for enzymes.

By using adsorption methods, a variety of enzymes have been immobilized on different supports such as ion exchange resins, inorganic aluminosilicates, activated carbons, etc. A particular example is Novozyme 435®, a commercial immobilized enzyme (i.e. lipase B from *Candida antarctica* on a resin Lewatit VP OC 1600; Novozymes) that currently is the most widely employed biocatalyst for research and industrial synthesis [38]. However, as Novozyme 435® support is moderately polar (i.e. polymethayacryl-divinylbenzene copolymer matrix), it would be expected that some issues would appear during TAGs hydrolysis due to excessive water and glycerol adsorption around the active sites. In contrast, it would be expected that the use of highly hydrophobic mesoporous material as enzymes supports may help improving yield of hydrolysis reactions. This is expected because large pores facilitate the access of large TAGs molecules to the active sites, and the hydrophobic nature would reduce accumulation of glycerol and water in the surface [29,35,39]. For instance, activated carbons (AC) have shown effective performances in the hydrolysis of waste TAGs [40]. These materials have exhibited good structural properties, high substrate stability, and low costs [41]. Additionally, it has been verified that one of the most promising enzymes for waste FOGs hydrolysis, *Candida Antarctica*, has less textural and conformational changes on mesoporous carbon materials [42]. Likewise, a similar study on immobilized *Pseudomonas gessardii* identified that mesoporous ACs are very effective supports for hydrolytic systems owing to their high substrate affinity, mechanical stability, and the enhanced accessibility for large TAGs [43].

In general, the interactions of lipases with AC are weak at high temperatures, which leads to a shrink in the adsorption capacity [44]. Nonetheless, lipase adsorption onto AC results optimal at temperatures in between 30 to 40 °C, enhancing stability at conditions of waste FOGs hydrolysis, and enabling to operate in wider temperature range in comparison with free lipases [45]. However, during hydrolysis of TAGs there is production of FFAs, diglycerides (DGs), and monoglycerides (MGs), within a water-rich media. Under these conditions, hydrolysis products exhibit a detergent action that can favor enzyme leaching [46]. This again indicates that the simple physical adsorption is expected to be not very effective in the immobilization of lipases that are intended for the hydrolysis of waste FOGs. In this particular case, mixed immobilization techniques can be used to overcome the leaching issues and to enhance biocatalysts stability [39,47].

Frequently, enhanced immobilization is done by combining adsorption on activated surfaces and by using glutaraldehyde; the last is a bifunctional agent that had proved to enable a strong crosslinking with minor effects on the enzymes. This strategy takes advantage of the reactivity of the carbonyl group in the glutaraldehyde with the amino groups in the enzyme molecule (Figure 1.2). On one hand, physical adsorption allows the linkage of the enzymes to the carrier, and on the other hand, crosslinking enhances the stability by producing enzyme aggregates inside the pores of the carrier, and increasing the contact area between the enzymes and bonds to the carrier [47,48].
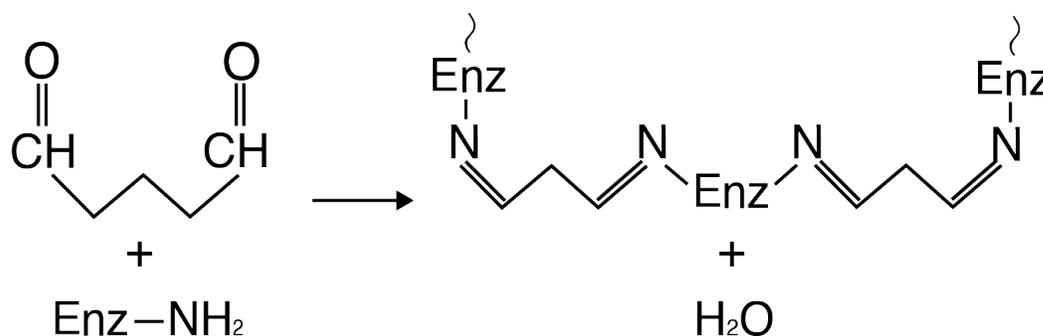
Figure 1.2. Reaction scheme of glutaraldehyde with amine groups of protein molecules in acidic or neutral media.

As previously mentioned, immobilization onto activated carbons can be carried out by direct absorption on the untreated support or after a chemical treatment of the surface. This can be done via amino-functionalization of the surface, and then a subsequent crosslinking with glutaraldehyde can be done before or after lipase absorption. The schematic representation of the immobilization of the lipase onto the activated carbon following the different methods is illustrated in Figure 1.3. Some active groups on the surface of the support are used for the functionalization with polyethylenimine, which helps providing more anchoring points either for the enzyme or the glutaraldehyde chains. It is well known that amine and carboxyl groups in the amino acids residues show an affinity for the carbonyl and amine groups respectively [40,49], but it is still unknown why some immobilization protocols use the activation with glutaraldehyde before or after the lipase immobilization. It has been suggested that while the nature of those bonds is still unknow, the final results may be related to configurational and structural changes in the enzyme as well as in the accessibility to the active site for the substrates [47].
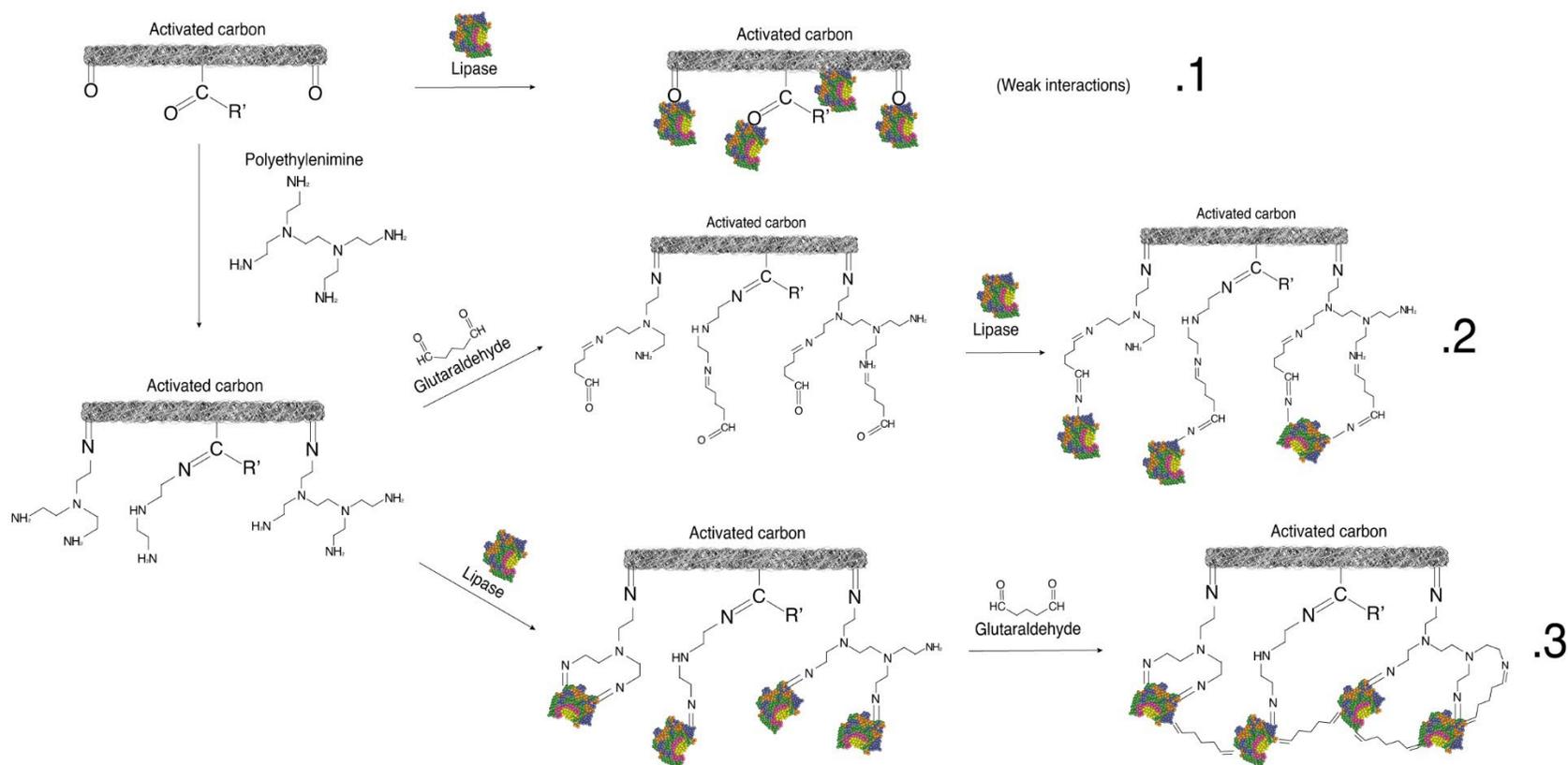
Figure 1.3. Schematic illustration of lipase immobilization. 1) Physical adsorption without amino-functionalization and activation with glutaraldehyde. 2) amino-functionalization and subsequent aldehyde-activation for lipase attachment. 3) amino-functionalization, subsequent lipase attachment and finally cross-linking with glutaraldehyde.

In the case of amino-functionalized activated carbon, it has been observed that the amino groups in the surface are condensed with glutaraldehyde, having monomers and dimers of glutaraldehyde per primary group [50]. This observation suggests that the attachment of monomers and dimers of glutaraldehyde might confer a greater stability owing to the covalent interactions of longer cross-links. At the same time, depending on the conditions of the media, glutaraldehyde behaves in different ways. For instance, in basic media glutaraldehyde tend to polymerize and the interaction between glutaraldehyde and the enzyme becomes weaker. These negative effects are fostered by large loadings of the aldehyde so it has to be optimized to reduce materials-intensity, to avoid enzyme inhibition, and to reduce costs [47].

Once immobilized, enzymes performance depends on several factors such as the employed immobilization technique, the physical and chemical properties of enzyme and carrier, the enzyme conformation and loading, the nature of the immobilization and reactive media, the diffusion of the substrate, and the water activity [23, 36]. In the special case of lipases an additional factor plays a role, namely the interfacial activation. In some lipases, there is a peptide chain called lid that in contact with non-polar solvents or hydrophobic surfaces, can unblock and expose a hydrophobic pocket, which is the active site for hydrolysis. This behavior, schematically described in Figure 1.4, has been exploited and typically used for the immobilization of lipases on hydrophobic carriers [47]. For this reason, it is expected that after the immobilization, the lipase activity as well as pH and temperature stability will be enhanced due to the interactions between the carrier and the lid. For instance, a recent study on immobilized lipases onto activated carbons identified that mesoporous activated carbons are very effective supports for hydrolytic systems owing to their high substrate affinity, mechanical stability, and the enhanced accessibility for large TAGs [43].
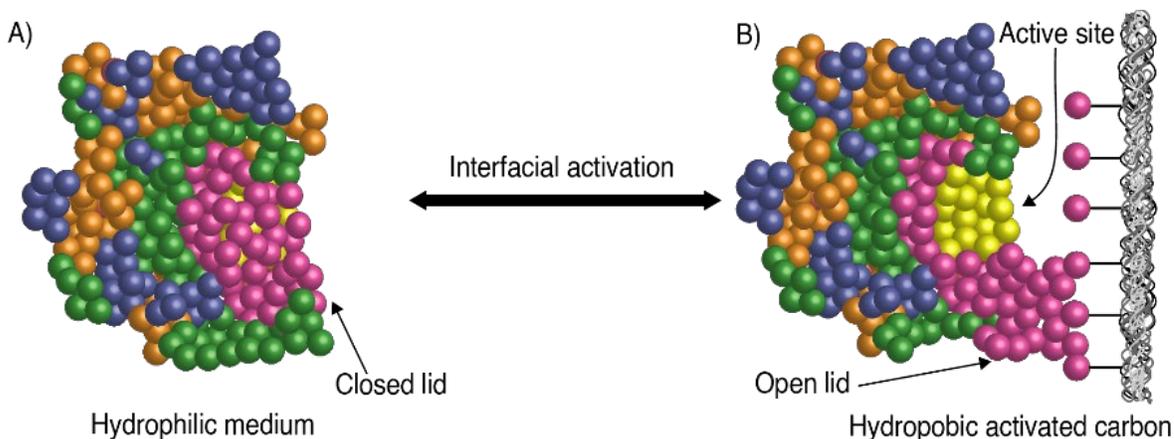
Figure 1.4. Interfacial activation of lipases on hydrophobic supports. A) Closed-form of lipase. B) Open form of lipase

In view of this context, this work aims to study the immobilization effectiveness of CALB onto mesoporous activated carbons (Starbon A800). The surface functional groups of the Starbon were modified using polyethylenimine and glutaraldehyde to form covalent interaction with the lipase and thereby improving operational stability. Ultrasound mixing was used to enhance homogenization and mobility of the enzymes within the pores of the support during the immobilization process. The effect of functionalization with glutarlaheyde and polyethylenimine along with physical adsorption on the lipase activity, enzyme loading, and immobilization yield were evaluated. The activity of the biobased catalysts was assessed in the hydrolysis of palm olein and compared with commercial benchmark products. The best performing immobilized enzymes are intended to be employed for the hydrolysis of waste FOGs, in particular of used cooking oil (UCOs).

1.3   Methods and materials

1.3.1   Materials and apparatus

Glutaraldehyde (50 % wt.), polyethylenimine, branched (99 % wt.), sodium hydroxide (98 % wt), monobasic sodium phosphate (99 % wt), phenolphthalein, bovine serum albumin (96 % wt), ethanol (99.5 % v/v.), triolein, lipase solution of Candida Antarctica lipase B (CALB) (1-5 % wt.), immobilized lipase *Thermomyces lanuginosus* (Lipozyme TLIM), and immobilized lipase Novozyme 435® were purchased from Sigma-Aldrich Co. The Starbon A800 used as supporting material for the immobilization was kindly provided by the Green

Chemistry Centre of Excellence - University of York, and its porosimetry is summarized in the table 1.1.

Table 1.1 Porosimetry data for Starbon A800.

Property	Value
Surface area (m^2/g)	374.37
Total pore volume (cm^3/g)	0.23
Mesopore (%)	41.00
Pore width (nm)	7.99 – 10.78

The immobilization process was conducted under sonication using an ultrasonic bath P30H (Cole Parmer, USA) with temperature and frequency control. Enzyme content was monitored by measuring protein content in samples, and it was monitored with pre-calibrated absorbance measurements using a ThermoFisher Genesys™ 140 UV-Vis spectrophotometer.

1.3.2   Functionalization of activated carbon and lipase immobilization

As previously described, immobilization was carried out by using amino-activation and crosslinking with glutaraldehyde. The amine solution (2.5% v/v) was prepared by mixing 1.25 mL of polyethylenimine with 48.75 mL of acetone. The glutaraldehyde solution was prepared by mixing 2.5 mL of glutaraldehyde (50% w/v) with 47.5 mL of acetone. In all the experiments the support was pretreated with ethanol to increase water activity in the micro-environment of the surface. Three different immobilizations methods were assessed:

- CALBAC1 - Physical absorption only,
- CALBAC2 - Amine functionalization followed by glutaraldehyde crosslinking, and physical absorption,
- CALBAC3 - Amine functionalization, physical absorption and final glutaraldehyde crosslinking.

First for CALBAC1, a physical adsorption of the enzyme onto the activated carbon was carried out without amino-functionalization according to previous reports [40,43]. In this case, the lipase solution (Lipozyme® CALB) was dropwise added into 50 mM phosphate buffer (pH 7.0) during 2 h at 45 °C, then to the buffer-lipase solution. Then, the non-functionalized activated carbon particles were added, using an activated carbon loading of

1g/15 mL with respect to the buffered lipase solution. For the second immobilization method in CALBAC2, functionalization of the activated carbon was previously carried out by adding 3.5 mL of amine solution per gram of Starbon A800, maintaining the agitation for 60 min. This ratio was carefully selected according to previous studies to avoid any inhibition issues [51]. To facilitate a stronger immobilization of the enzyme, the amino-functionalized activated carbon was treated with glutaraldehyde by the addition of 3.5 mL of the aldehyde solution per gram of dried amino-functionalized activated carbon, and gently stirred during 60 mins. Then the material was washed with distilled water and dried to remove unbound compounds. Finally, the physical adsorption was performed following the same procedure as in the first method. For the third adsorption method in CALBAC3 the protocols were similar, but the physical adsorption of the enzyme was carried out on the amino-activated Starbon followed by activation with glutaraldehyde as the last step.

1.3.3   Protein determination

Protein content was used as surrogate of enzyme concentration; then, by tracking the dissolved protein content in solution it was possible to estimate the remaining free enzyme after the immobilization process and the corresponding enzyme loading in the support. The measurements of protein were done via absorbance spectrophotometry at 280 nm which is typically effective to account for aromatic amino acids (e.g. tyrosine, tryptophan and cysteine) present in some lipases residues and in concentrations between 20 to 3000 µg/mL [49, 60]. Nonetheless, many other cellular and biochemical components (e.g. nucleic acids, other proteins) can absorb UV-light, for this reason the technique is limited to purified enzyme extracted or known enzyme solutions and needs to be calibrated [54]. In this case, calibration was done by characterization of standard samples prepared from a solution of Bovine Serum Albumin (BSA) in distilled water in a concentration range of 0.1 - 3.0 mg/mL. The different solutions were stirred for 180 min prior to absorbance measurements to emulate the process used in the immobilization, and the sample was characterized immediately after a final stirring at 1000 rpm. The corresponding protein content in samples from immobilization were measured in the supernatant remaining after contacting the enzyme solution with the support. The supernatant was initially diluted in a volumetric flask to complete 100 mL, and then subjected to absorbance measurement. This protocol was repeated for the determine the effectiveness of the three immobilization techniques, and also to determine protein concentration in the commercial solution of Lipozyme® CALB.

1.3.4 Lipase activity assay and optimum catalyst conditions

The lipase activity of the immobilized biocatalyst was determined by using palm olein as a substrate during a hydrolysis test. Initially, a substrate emulsion was prepared by mixing 40 mL of palm olein with 60 mL of Arabic gum solution (5% w/v), and by subjecting the mixture to ultrasound homogenization at 37 kHz and 100 W. Then, 5mL of the substrate emulsion were mixed with 5 mL of 50 mM phosphate buffer (pH 7.0), and with 0.5 g of lipase (in solution or immobilized). After 30 minutes the reaction was stopped by adding a solution of acetone and ethanol (ratio of 1:1). Finally, the produced fatty acids were quantified by titration with a 100mM NaOH solution using phenolphthalein as indicator. The specific activity of the enzyme was reported in lipase units (LU) per gram, and it corresponded to the mols (µmol) of fatty acids produced per minute and per gram of immobilized biocatalyst under assay conditions. The specific lipase activity of the immobilized lipase was obtained with Equation 1.1.

$$Specific\ activity = LU/g\ = \frac{(V_1 - V_o)*C}{t*m} * 1000 \qquad (1.1)$$

Here, LU (µmol/min) is the lipase activity, V_1 (mL) is the volume of NaOH solution consumed during samples titration, V_o (mL) is the volume of NaOH solution used in the titration of the control sample, C (µmol/mL) is the concentration of NaOH titration solution, t (min) is the reaction time, and m (g) is the total weight of enzyme used in the assay. The corresponding activity of the commercial benchmark products, namely *Candida Antractica* Lipase B of Novozyme 435® and *Thermomyces lanuginosus* lipase of Lipozyme (TLIM) was also assessed.

In order to determine the best operating pH and temperature conditions during the hydrolysis of palm olein using the immobilized enzyme, the lipase activity was evaluated at different temperatures (30, 40, 45, 50, 55, 60 °C) and pH values (6, 6.5, 7, 7.5, 8). The assays were evaluated following the above-mention protocol, and all determinations were carried out in duplicate. To enable a comparison between the initial and final amount of FFAs during reactions, a control sample was used (V_o). In this case, a sample of the reaction emulsion at the beginning and prior addition of the biocatalyst was diluted in toluene-ethanol 1:40, and it was titrated with phenolphthalein as indicator.

1.3.5   Immobilization efficiency and enzyme loading determination

Immobilization efficiency was defined as the reduction of the lipase concentration in the supernatant solution and it was calculated in a percentage basis with Equation 1.2 [31]. The corresponding enzyme loading (% wt.) was calculated with Equation 1.3 [55].

$$Efficiency = \frac{C_{CALB0} - C_{CALBf}}{C_{CALB0}} \times 100 \tag{1.2}$$

$$Enzyme\ loading = \frac{C_{CALB0} - C_{CALBf}}{C_S} \times 100 \tag{1.3}$$

Here, C_{CALB0} (mg/mL) is the initial concentration of free lipase in solution, C_{CALBf} (mg/mL) is the final concentration of free lipase in the supernatant solution after immobilization, and C_S (mg/mL) is the total loading of the Starbon support in the immobilization solution.

1.4   Result and discussion

1.4.1   Protein determination

After assessment of the spectrophotometric method for protein quantification, and as observed in Figure 1.5, a linear relationship was verified between the absorbance and the concentration of the standard solutions of bovine serum albumin (i.e. between 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 2.0 and 3.0 mg/mL). This linearity confirmed that the lipase aggregation that could reduce absorbance [49, 61] was not present, and that mixing was effective to avoid such phenomenon in the studied range of concentrations. A slightly linear deviation was observed at concentrations above 2.5 mg/mL, and this was the upper limit of quantification of the employed technique. Using the obtained correlation, it was possible to estimate the protein content in the commercial solution of free form of *Candida Antractica* Lipase B (CALB) as 111.58 mg/mL. These results were considered to define the equivalent loadings during comparative assessment of hydrolytic activity of the different enzymes.
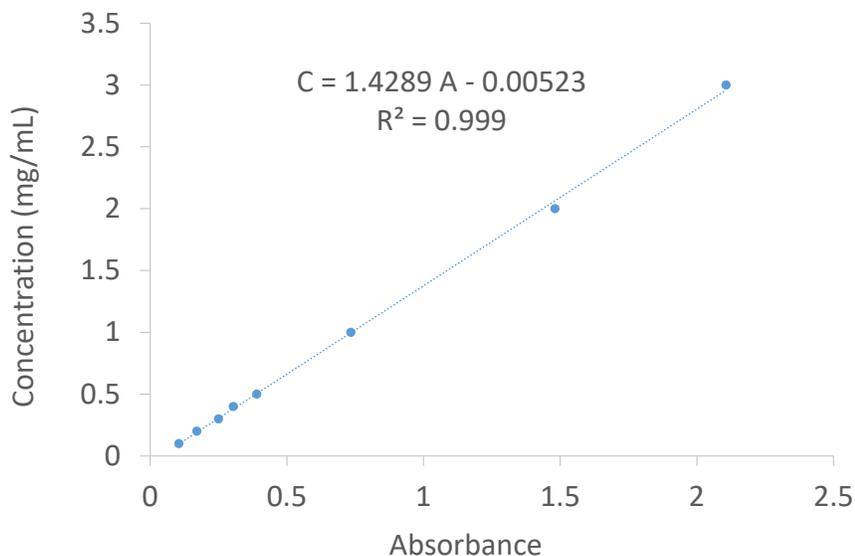
Figure 1.5. Spectrophotometric absorbance at $\lambda = 280$ nm of bovine serum albumin aqueous solutions

1.4.2   Enzyme loading and immobilization efficiency

Results of Candida Antarctica lipase B (CALB) loading on the Starbon support and the immobilization efficiency are presented in Figure 1.6 and the measured absorbance for every supernatant in each immobilization assay is reported in the table 1.2. As observed, enzyme loading and immobilization efficiency are highly improved by amino-functionalization of the surface and crosslinking. The first step helped to increase the anchoring sites for the lipase while the second strengthen enzyme binding. Compared with the physical absorption only (CALBAC1), the other two methods enabled to increase loading and efficiency in more than two-fold. In particular, the adsorption of the enzyme on an amino-functionalized Starbon followed by crosslinking with aldehyde in CALBAC3 enabled to obtain the highest immobilization efficiency. For this method the maximum loading was 11.30 % wt. and the efficiency was 56.27 %; yet it was below the recommended loadings for commercial immobilized lipases (up to 20%) [42]. This was expected because it has been reported that activated carbons do not to have as good affinity for the lipase as commercial polymeric supports [56] due to weaker bonding and reduced accessibility to the anchoring sites (i.e. smaller pores). In the case of the assessed Starbon, it has almost half or the pore volume and size compared with moderately polar and macroporous Lewatit VP OC 1600 resin commonly used as supports in commercial enzymes [57].

Table 2.2 Results of the protein determination by spectrophotometry.

Sample	Absorbance (±0.005)	Concentration (±0.01 mg/mL)
CALB solution	1.989	2.79
CALBAC 1 Supernatant	1.432	1.99
CALBAC 2 Supernatant	1.125	1.56
CALBAC 3 Supernatant	0.889	1.22
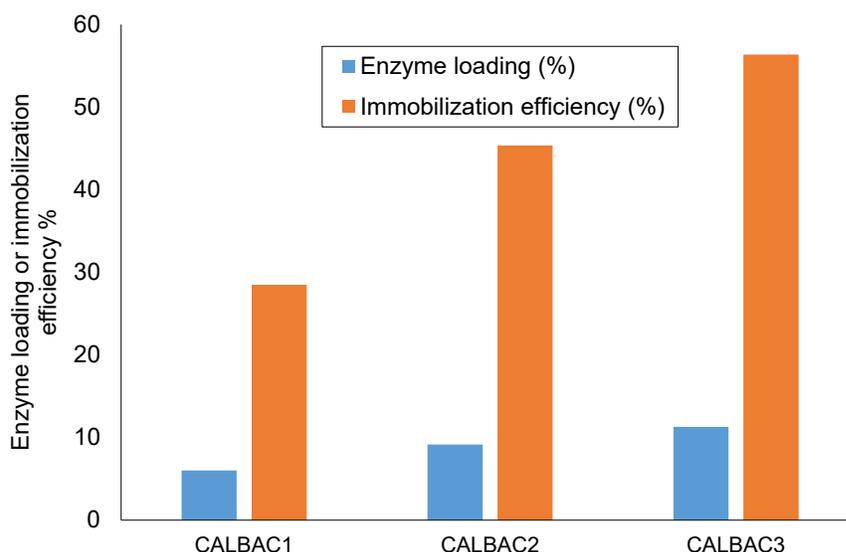
Figure 1.6. Performance of the immobilization methods for Candida Antarctica lipase B onto Starbon A800. Immobilization via physical absorption (CALBAC1), amino-functionalization followed by crosslinking and physical absorption (CALBAC2), or Amino-functionalization followed by physical absorption and crosslinking (CALBAC3).

1.4.3   Lipase activity

The specific hydrolytic activity of commercial lipases including the free forms of *Candida Antractica* Lipase B (CALB) and *Thermomyces lanuginosus* lipase (Lipozyme TLIM), the immobilized Lipozyme CALBAC and Novozyme 435®, and the corresponding of the immobilized lipases prepared in this work are presented in the Figure 1.7. As observed, Lipozyme TLIM has the highest specific activity of all assessed enzymes. This result is interesting considering that *Thermomyces lanuginosus* is generally considered to have lower activity and a higher regio-selectivity for the 1 and 3 ester bond in the triglycerides molecule [35, 54]. Despite the regio-selectivity of TLIM, this feature can be easily overcome

by acyl migration in some unsaturated fatty acids chains but with an increase in the reaction time [59]. In this case, it is expected that TLIM presented a better activity due to the nature of the triglyceride source in the assay (i.e. palm olein). On the other hand, a high concentration of MGs and DGs is representative in this kind of lipases when the equilibrium is reached [60]. In this case it exhibited higher activity than free *Candida Antractica* Lipase B (Lipozyme CALB), that is a typical benchmark lipase. Despite the better results, and as Lipozyme TLIM is marketed for its regio-specificity, it would not be recommended for the hydrolysis of waste FOGs as it would not be able to hydrolysate long saturated chains in the two ester groups producing MGs and DGs instead FFAs. Additionally, the support used in this preparation might favor esterification and transesterification reactions instead hydrolysis [58,61].
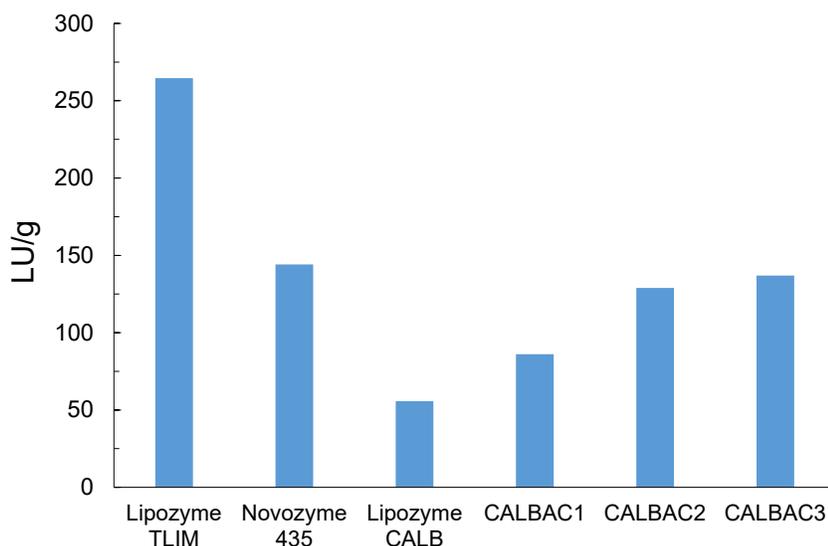
Figure 1.7. Specific lipase activity for the commercial Lipases (i.e. Lipozyme TLIM, Novozyme 435 and Lipozyme CALB) and the 3 immobilized lipases prepared with the protocols of the Figure 1.3.

Regarding the immobilized enzymes, Novozyme 435® exhibited the highest specific activity in the hydrolysis of palm olein (144.12 ± 6.22 LU/g). The partially hydrophilicity of the Lewatit® VP OC 1600 used as support in this enzyme, as well as its higher pore volume and pore size, enabled a better performance than when using Starbon supports. Comparatively, only the CALBAC3 exhibited a similar activity (136.93 ± 5.41 LU/g) to that of Novozyme 435®, while CALBAC1 and CALBAC2 had lower specific activity. In any case, it was verified that similarly to enzyme loading and immobilization, amino-functionalization

and crosslinking conferred higher enzymatic activity when compared with the free enzyme. This can be explained by two reasons, first the positive structural changes exerted in the lipase making the active site more accessible to the substrates, and second the amino-functionalization created more anchoring points producing more immobilized enzyme per area unit [62]. This also explains why immobilization of the enzyme over an amino-functionalized surface produced a more effective catalysts (CALBAC 3) that immobilization into a surface previously covered with mostly carbonyl anchoring groups (CALBAC2). Then, subsequent hydrolysis tests were performed with CALBAC 3 as catalyst.

Even though CALBAC3 specific activity (136.93 ± 5.41 LU/g) is similar to that of Novozyme 435® (144.12 ± 6.22 LU/g LU/g), in the last the enzyme is immobilized by physical adsorption approach, making it more easier to produce. Nonetheless, CALBAC preparations surpassed activity of the *Candida Antarctica* Lipase B (CALB) in its free form (i.e. Lipozyme CALB), but still lower than that of Lipozyme TLIM. This might imply that Starbon A800 used in the immobilization step lacks enough hydrophobic and carbonyl groups in the surface to effectively anchor the enzyme. Additionally the low macroporosity and pore diameter affected lipase access and attachment to the surface. It has been reported that enzyme activity is highly dependent on the pore size of the carrier, and it is required pore diameters of at least five times the kinetic diameter of a molecule to avoid diffusional issues. At the same time, larger pores than 100 nm might result in poor enzyme stability [63].

As expected, and evidencing absence of enzyme denaturing, the enzyme loading as well as immobilization efficiency were highly correlated to the enzymatic activity as shown in the figure 1.6 and 1.7. This is important because after immobilization it would be possible that a fraction of the loaded enzyme could lose its activity. This generally occurs because enzyme anchoring in a denatured form owing to the loss of conformational stability. While this did not occur in this case, this phenomena is common in covalent attachments as the one presented in this study with glutaraldehyde [36]. Furthermore, in this case it can be confirmed that activated carbons exert a positive effect in the conformation of the enzyme.

1.4.4   Effect of pH on lipase activity.

As the physical binding of enzymes and their conformation is affected by surface electric charges, pH is a key variable affecting the stability of immobilized enzymes as well as their catalytic activity in hydrolysis reaction [32]. The effect of pH in the activity of CALBAC3 is presented in the Fig 1.6.
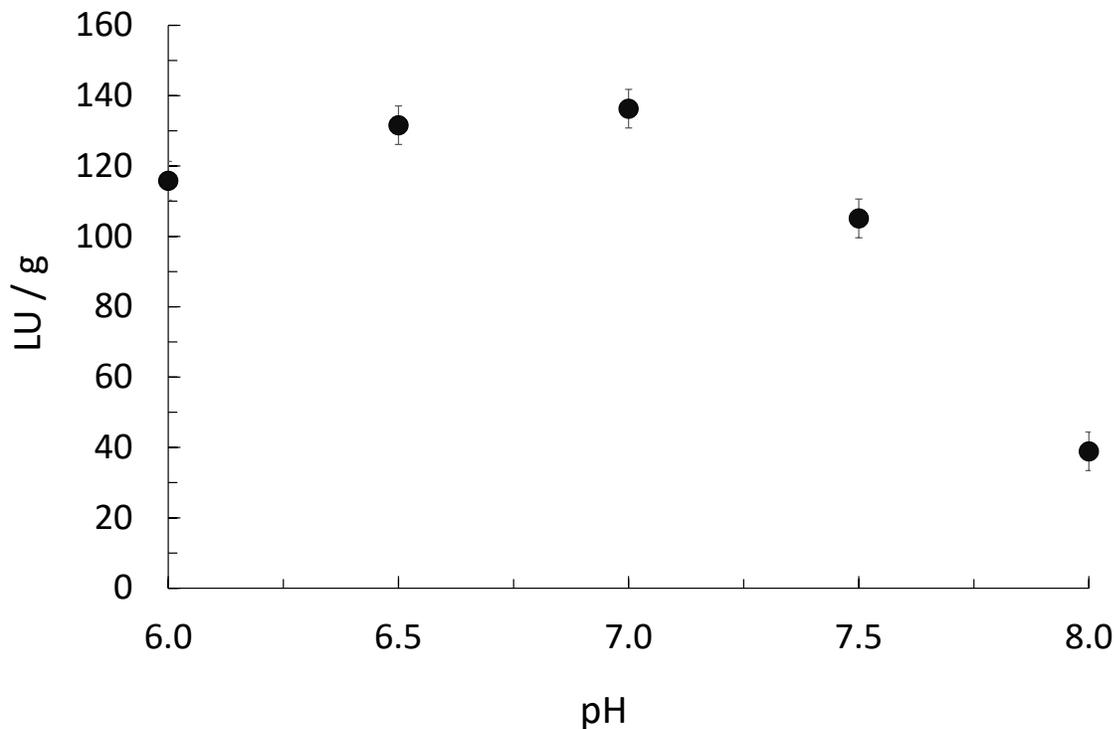
Figure 1.8. pH effect on the activity of Candida Antarctica lipase B immobilized onto Starbon A800 (CALBAC3) in the hydrolysis of palm olein. Immobilized lipase loading 5.0% wt. (based on the total amount of substrates), Temperature 45 °C.

As observed, the specific hydrolytic activity of CALBAC3 was nearly stable in between 120-140 LU/g at pH ranging from 6 to 7.3. However, similar to other studies [20], it exhibited a rapid drop above 7 with a 71.5% activity reduction at pH 8.0. Despite the rapid change, the stability of the hydrolytic activity in the observed pH range was significant considering that there was an order of magnitude change in the acidity of the reactive medium. The change of activity above pH 8 can be related with the increasing dissociation of fatty acids from palm olein near to their pKa (8.28-10.15 [64]) thus increasing the ionic strength in the environment around the enzymes. In the other hand, below pH 6, the reactive medium increases hydronium concentration and the enzyme losses some of the positive charges

that make it more stable and active [44]. This behavior has also been associated to an affinity reduction between the enzyme and the support affecting adsorption effectiveness [5, 31]. The obtained optimal activity at intermediate pH has also been reported for the hydrolysis of waste hydrogenated fat using *Rhizopus japonicus* lipase (pH 7) [21], and in the hydrolysis of beef tallow and poultry fat using free or immobilized *Rhizomucor miehei* lipase (pH 6-8) [19]. A similar behavior was reported in the hydrolysis of used cooking oils using immobilized CLR on polyvinyl alcohol-alginate-sulfate beads operating in a pH range of 7 to 8.

Taking into account that operation near neutral pH is paramount to ensure high yield during hydrolysis, there is need for using buffer solutions during reaction. While this can enhance reaction performance, this also increases operating costs and generates difficulties during downstream recovery. In particular, major issues appear during glycerol recovery from the aqueous effluent because buffer salts are soluble and nonvolatile making difficult glycerol purification [65]. Nonetheless, considering that the solubility of fatty acids in aqueous solutions at hydrolysis conditions is around 0.01 %wt. [66], and the corresponding pKa of the acids (i.e. 8.28 for palmitic acid [64]), it is expected that the pH of the solution will not drop below 6 during reaction. This enables to operate within the optimal pH range of CALBAC3 under unbuffered conditions thus facilitating catalysts recovery and glycerol purification. A recent study revealed that immobilized TLL can be used in hydrolysis of TAGs using unbuffered medium and achieving nearly 100% conversion after 24 h [65]. These results open new opportunities to work in salt-free systems (i.e. unbuffered solutions) and making the process even more sustainable.

1.4.5   Effect of temperature on lipase activity.

Temperature plays a significant role in hydrolysis reactions since it is tightly related to the solubility of TAGs and water, the fluidity of the reactive media, and to the viscosity of the substrates; all affecting compatibility and mobility of reactants [67]. In the other hand, energy of activation of physical bonding is lower than that of chemical ones, so the strength and rigidity of anchoring bonds of the enzymes are also affected by temperature, which can cause enzyme desorption. Finally, the internal bonds of enzymes are sensitive to high temperatures causing denaturation; this phenomena has been verified using CLR supported in polystyrene-divinylbenzene for the hydrolysis of UCOs [31]. All these elements

indicate that the immobilization process and hydrolytic reaction must be carried out under specific and optimal temperatures for a suitable trade-off between anchoring effectiveness and enzymes activity and stability.
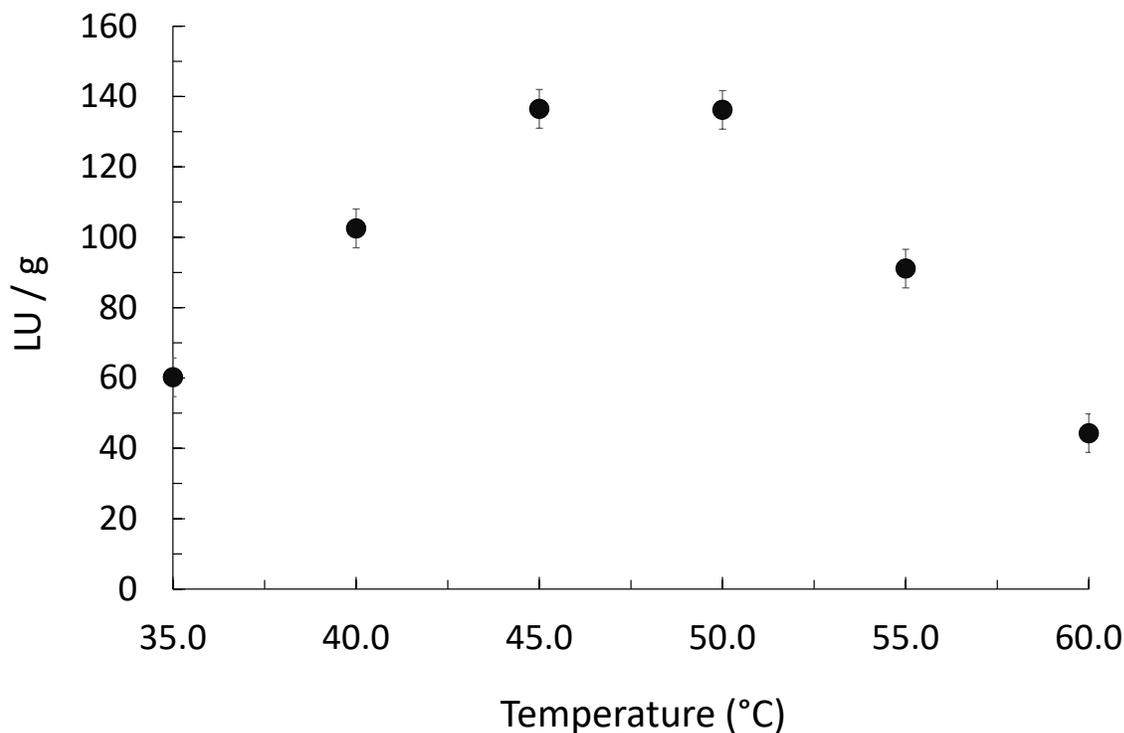
Figure 1.9. Temperature effect on the lipase activity of the immobilized preparation CALBAC3, conditions: Amount of lipase 5.0% wt. (based on the total amount of substrates), Temperature 35.0 – 60.0 °C, medium pH 7.0.

As presented in Figure 1.9, immobilized lipase (i.e. CALBAC3) exhibited the higher activity in between 136.23 ± 4.23 – 136.45 ± 5.23 LU/g and stability in the range of 45 to 50 °C. This is characteristic of immobilized enzymes as they become more rigid, stable and less prone to conformational changes than the free enzymes [45], so they can operate at higher temperatures. This characteristic is paramount when operating with different waste FOGs because they contain saturated chains with high melting points. In this case, immobilization not only enhances enzyme activity but also enables to operate at temperatures that improve fluidity and compatibility of reactants. This behavior has been observed in the enzymatic hydrolysis of different waste FOGs using immobilized lipases of *Candida rugosa* [5] and *Rhizopus japonicus* [21], and the hydrolysis of soybean deodorization distillates with

Candida rugosa lipase [68]. Reduction of activity above 50 °C has been also observed using immobilized enzymes in activated carbons [44], and it is related to weaker interactions with the support and a shrink in the adsorption capacity.

1.5   Conclusions

The aim of this chapter was to assess the immobilization of Candida Antarctica lipase B (CALB) onto activated carbon (Starbon A800), and its further evaluation as catalyst in the hydrolysis of triacylglycerides. The Starbon support had characteristics of a mesoporous material with a surface area of 374.37 m^2/g, mesoporosity of 41% and average pore diameter of 7.99 to 10.78 nm. The immobilization was done following three different methods including physisorption (CALBAC1), physisorption onto previously amino-functionalized and glutaraldehyde crosslinked surface (CALBAC2), and finally physisorption onto amino-functionalized surface and then subsequent crosslinking with glutaraldehyde (CALBAC3). From the three immobilization methods, it was verified that physisorption onto amino-functionalized surface (CALBAC3) was more effective (11.30 %wt.) and enabled a higher enzyme loading (56.36%) than physisorption on the untreated Starbon support (CALBAC1) or than in a carbonyl-functionalized Starbon (CALBAC2). Results also indicated that immobilization provided higher activity compared with the free enzyme in the hydrolysis of triacylglycerides but below to that observed for a commercial free enzyme (Lipozyme TLIM). Nonetheless, CALB has lower cost and under the specific immobilization method applied to obtain CALBAC3, it was possible to reach an activity of 136.9 LU/g. This value is similar to that observed with a commercial benchmark immobilized lipase (i.e. Novozyme 435®; 144.1 LU/g) under the same active agent loadings and operating conditions. Finally, a further exploration of the activity of CALBAC3 in the hydrolysis of palm olein was assessed at different pH and temperatures. It was found the hydrolytic activity was nearly stable in between 120-140 LU/g at pH between 6 and 7.3, with a large drop above pH 8. Regarding the operating temperature, the largest activity was around 136.50 LU/g and it was obtained in-between 45 to 50 °C. The observed activity and stability of CALBAC3 indicate that it can be regarded as a potential catalyst for the hydrolysis of triacyl glycerides present in waste fats, oils and greases, in particular for the hydrolysis of used cooking oils.

Chapter 2.

Enzymatic hydrolysis of used cooking oil by immobilized lipase onto activated carbons under ultrasound.

This section includes fragments of the paper: Baena, A., Orjuela, A., Rakshit, S. K., Clark, J. H. 2020. Enzymatic hydrolysis of waste fats, oils and greases (FOGs): Status, prospective, and process intensification alternatives. Chem. Eng. Process. - Process Intensif. Currently In revision.

The paper is reformatted and figures and tables are enlarged to fulfill edition requirements for the dissertation document.

2. Enzymatic hydrolysis of used cooking oil by immobilized lipase onto activated carbons under ultrasound.

2.1 Abstract.

The increasing global generation of used cooking oils (UCOs) is resulting in a cascade of economic, environmental, and public health issues because their mismanagement is a common practice worldwide. While exploitation via transformation into biofuels and in other biobased derivatives could help mitigating such problems, acidity of UCOs limits a feasible and profitable exploitation for different applications. Alternatively, UCOs can be subjected to complete hydrolysis for the production and recovery of free fatty acids (FFAs) that can be further used as oleochemical feedstocks. In this regard, the aim of this work is to evaluate enzymatic hydrolysis of UCO using Candida Antarctica lipase B (CALB) immobilized onto an amino-functionalized mesoporous activated carbon. Additionally, and as intensification method, the performance of the process was assessed under ultrasound-assisted mixing. Reactions were carried out at 45 ºC and pH 7, and the effect of three operating variables on the degree of hydrolysis was assessed, namely: substrate molar ratio, enzyme loading, and ultrasound-mechanical stirring effect. Based upon the experimental results, a kinetic model was proposed and the corresponding parameters were determined. It was found that the optimum operation conditions were at an immobilized enzyme loading of 7.0 % wt., and 1:30 oil: water molar ratio, reaching a conversion of 91.04 ± 3.92 %. Results also confirm the positive effect of ultrasound and mechanical stirring in the reaction performance. Based upon the kinetic experiments it was found that the assessed enzyme has a low affinity for the substrate. Nonetheless, no inhibitory effect was observed in the UCO hydrolysis, evidencing that the pretreatment process (i.e. filtering and bleaching) of UCO has a positive effect in the hydrolysis reaction.

Keywords: Enzymatic hydrolysis, used cooking oil, ultrasound, lipase, process intensification.

2.2 Introduction

As a result of their wide availability, biodegradability, and renewable character, edible oils and fats have been traditionally used as primary feedstocks of the oleochemical biorefineries. Current production of vegetable oils is around 210 Mt/yr., and about 20% is

used for biofuels and oleochemicals manufacture [11]. The main edible vegetable oils currently used for oleochemical applications are obtained from African palm, soybean, rapeseed, sunflower, palm kernel, cottonseed and coconut. These resources have been transformed in numerous derivatives for a variety of applications, and through different chemical pathways [2,69]. Nonetheless, the use of edible oils for biofuels and chemicals production involves many sustainability and ethical issues considering the water-energy-food security nexus of agricultural commodities [70], and the large life cycle impacts of agriculture, mainly in terms of land change use, fossil resources intensity, and biodiversity loss, among other indicators [28,71].

Alternatively, and following circular economy principles, waste fats, oils and greases (FOGs) can be used as second-generation feedstocks for the oleochemical industry. Despite the large content of impurities and humidity, waste FOGs are mainly composed of triacyl glycerides (TAGs) and free fatty acids (FFAs) that are suitable for further exploitation via hydrolysis, esterification, transesterification, etc. Recently waste FOGs availability has substantially increased as a result of a higher consumption of vegetable oils [11], and their trading has increased globally [72]. For instance, a well-known inevitable waste FOG is used cooking oil (UCO) which is discarded after food preparation. Recent studies indicate that global UCO generation is around 20-30% of the total production of vegetable oils (i.e. 42-63 Mt/yr.) [11]. Those corresponds to post-consumption food waste FOGs, which are mainly produced in urban centers, and they are generally disposed through the sewage systems or within the solid residues [73]. These practices trigger a cascade of environmental problems including sewage blockage, overflooding, vectors proliferation, and excessive organic loading to waste waters and solid residues [9,13]. All this results in the damage of public and private infrastructure, and in reduction of the effectiveness of waste water treatment plants and landfills, thus causing extended pollution and ecosystems damages. Additionally, in some regions UCOs are illegally collected, bleached and redistributed as new oils with the consequent impacts on public health [74]. As a result of all these problems, public agencies in different countries have deployed regulations to enforce the correct management of residual FOGs [75]. In this regard, there is need to develop alternative harnessing routes to incorporate such residues within commodity or value-added chemicals [9]. Shall this be accomplished; this would help the oleochemical industry to incorporate circular economy models and to improve sustainability performance.

A major challenge when employing UCOs as feedstock is related to their characteristic heterogeneity and the high content of impurities (e.g. humidity, hydrocarbons, polymers, polar compounds, food residues, etc.). These residues exhibit higher acidity and moisture content than commercial edible oils; however, they still have a large fraction of TAGs that contain value-added unsaturated fatty acid chains. For this reason, different alternatives other than biofuels have been reported for their industrial harnessing, including the production of soaps, fodder additives, bio-lubricants, asphalt binders, surfactants, biopolymers, biogas, etc. [18,72,76–80]. Nonetheless, in many of these applications, there is need for pretreatment to remove acidity and other impurities in order to purify the TAG fraction of UCOs. This represents a challenge mainly for highly acidified oils because the lower TAG yield and the excessive waste generation during the pretreatment process.

An alternative way to deal with the acidity of UCOs is to carry out a complete hydrolysis of the TAGs and other glycerides into FFAs by reactions with water. This corresponds to a series-parallel set of reactions, having diglycerides (DGs) and monoglycerides (MGs) as intermediate products (Figure 2.1). This is the most common chemical route to obtain FFAs from oils and fats, and it is well-known since the early days of the industrial revolution [2,81].
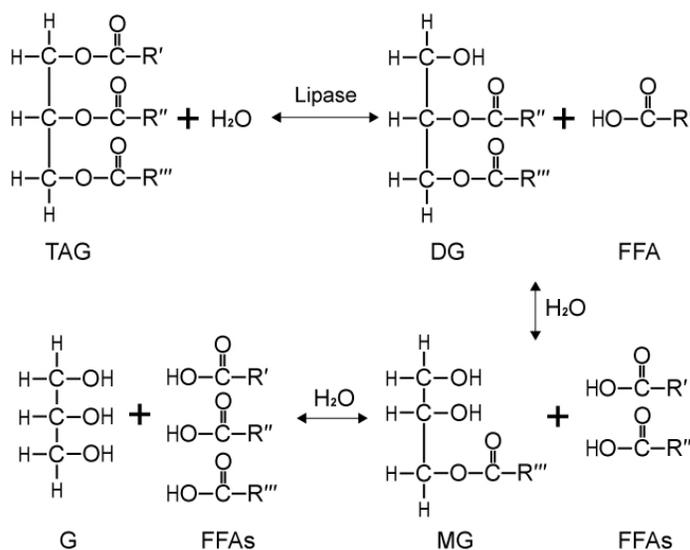
Figure 2.1. Reaction scheme for the hydrolysis of triacylglycerides (TAG) into free fatty acids (FFAs). Monoacylglyceride (MG). Diacylglyceride (DG). Glycerol (G).

In general, highly energy-intensive processes such as the Colgate-Emery has been employed in the hydrolysis fats, oils and also of waste FOGS (Figure 2.2). This process operates at high temperatures (200-300°C) and pressures (50-60 bar) to maintain reactants in the liquid phase and to achieve conversions up to 98% [82]. An important limitation is this process is that, under such severe conditions, the valuable polyunsaturated fatty acids (PUFAs) tend to polymerize, and they are also degraded into anhydrates, ketones and hydrocarbons [27]. Another drawback is the need for several separation steps including flash thanks, a bleach reactor, a distillation train to purify fatty acids, a glycerin concentrator, and a water de-aerator, among others. Also, a large amount of steam is required to obtain a high-quality product, thus increasing the overall production costs [3].
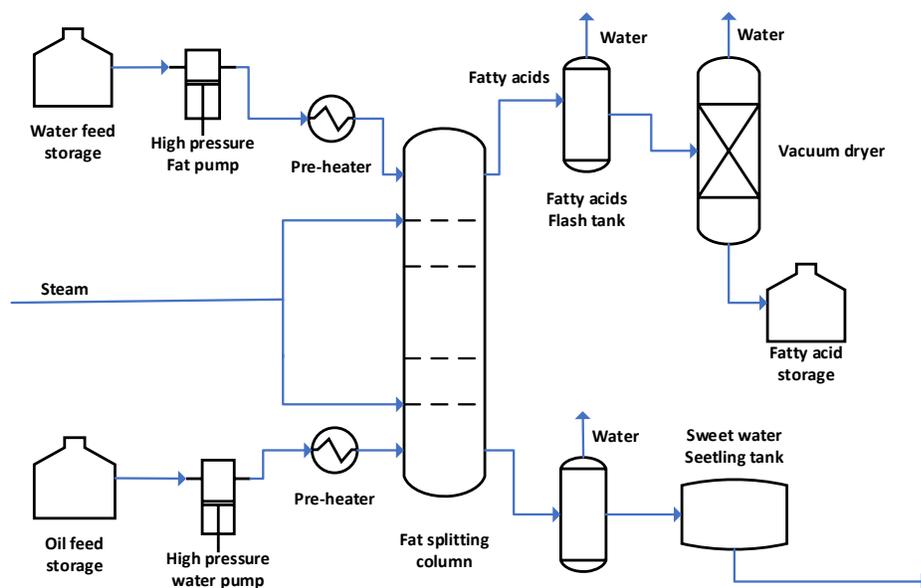
Figure 2.2. Schematic process flow diagram of the Colgate-Emery process

In general, the catalytic hydrolysis is preferred over the non-catalytic routes because the process can be run under milder operating conditions, with less energy consumption, and with a reduced product degradation. Additionally, selectivity can be tuned for the controlled synthesis of the intermediate monoglycerides (MGs) and diglycerides (DGs) which can be valuable for some industrial applications [20]. In general, heterogeneous catalysts are favored over the homogenous ones to gain control over the selectivity and to enable an easier recovery [3]. In this regard, the use of immobilized enzymes as heterogeneous

catalysts is under continuous development considering the need for overcoming the reduction of activity of the anchored enzyme, and the need to mitigate enzyme leaching. Despite these issues, immobilized enzymes seem to be a suitable heterogeneous catalysts to overcome the limitations related to impurities of waste FOGs during the hydrolysis process, and they are superior over homogenous catalysts because they avoid equipment corrosion and enable an easier separation from the products [83].

Regardless of the nature and type of catalysts, hydrolysis is highly affected by the lack of compatibility between reactants. As oil and water are immiscible, mass transfer limitations are present in the process and those are aggravated when heterogeneous catalysts are used. Historically, this has been overcome by operating under very high temperatures to increase solubility or by using solvents or emulsifiers. Alternatively, mass transfer limitations in a solvent-free media can be improved by increasing the total free interfacial area by intense mechanical stirring [27]. However, in the case of enzymatic hydrolysis, high stirring rates can derive in enzymes denaturing due to high shear stresses [24,67]. Instead, turbulence can be induced by mean of intensified techniques such as cavitation, ultrasound and microwaves. These can help increasing interfacial area and generating local hot spots for short times, boosting rates of reaction but without involving shearing stresses. These particular characteristics make such intensified technologies attractive for industrial implementation of enzymatic processes [28]. In particular, it has been stablished that ultrasound irradiated to a fluid (i.e. sonication) at frequencies above 20 kHz using a transducer (e.g. horn, probe, plate) can deliver the high energies [84]. In the case of hydrolysis, sonication promotes a greater homogeneity, increasing interface area between oil and water, and enabling high conversions of TAGs even at low water loadings at relative short reaction times [20,24].

A summary of some of the recent studies on the enzymatic hydrolysis of waste FOGs under ultrasound action is presented in Table 2.1. For comparison purposes, a specific work reporting ultrasound-assisted hydrolysis of waste FOGs with traditional inorganic homogeneous catalysts also permitted to verify high yields at reduced temperatures [85].

Table 2.1. Recent studies on the ultrasound-assisted hydrolysis of waste FOGs

Catalyst	Frequency [kHz]	Power [W]	Temperature [°C]	oil:water molar ratio	Yield (%FFA)	Catalyst concentration (%wt.) [a]	Time [h]	Ref.
Lipozyme TL IM	40.0	132	40.0	1:20	60.68 [g]	10.0	2	[20]
Novozyme 435	22.0	100	50.0	3:1	75.19	1.25	2	[27]
Aspergillus Nigger	40.0	66	45.0	1:3[f]	62.67 [e]	15.0 [b]	12	[86]
Aspergillus Nigger	40	66	45	1:3[f]	753.07[e]	15.0 [b]	12	[24]
Pseudomonas gessardii on activated carbons	20.0	--	35.0	1:100>	~100 [c]	1.66	4	[40]
KOH/Sulfuric acid	42.0	160	60.0	1:2.4 [h]	97.3	0.3 [d]	<1	[85]

[a] by weigh of substrates
[b] % v/v
[c] % hydrolysis = (Av2-Av1)/(Sv – Av1)   [Av: Acid value   Sv: saponification value]
[d] Catalyst to oil ratio
[e] FFA concentration (µmol/mL)
[f] Volume ratio
[g] FFA %p/p in the reaction sample.
[h] Oil:ethanol mass ratio

Based upon the abovementioned context, this work deals with the enzymatic hydrolysis of UCOs under the influence of ultrasound irradiation. Based upon preliminary experiments, the selected catalyst was candida antarctica lipase A, immobilized onto activated carbon via (CALBAC3). Specifically, the immobilization involved physisorption onto amino-functionalized surface of the activated carbon followed by crosslinking with glutaraldehyde. Different process parameters such as substrates ratio, catalyst loading and the influence of ultrasound and mechanical stirring were assessed to obtain the maximum performance of reaction. This also enabled to obtain a kinetic model suitable to evaluate the performance of the synthetized bioderived catalyst in the hydrolysis of UCOs and its further scale up.

2.3   Methods and materials

2.3.1   Materials

Used cooking oil (UCO) was collected from chicken restaurants in Bogotá, Colombia, and its properties are summarized in Table 2.2. The collected UCO was pretreated according to the procedures previously reported [87]. In brief, the UCO was subjected to heating, decantation to remove excess water, and filtration with a 5 µm filtering paper to remove suspended solids. Subsequently, the UCO was bleached with H_2O_2 at 90 °C to degrade polar compounds and to improve color. Finally, the pretreated UCO was stored in a closed

and opaque container under ambient conditions prior to use. For comparison purposes, experiments were also carried out with palm olein and its properties are also summarized in Table 2.2. The assessed enzyme was Candida Antarctica Lipase A immobilized onto activated carbon (CALBAC3), and it was synthetized following the protocols described in the previous chapter. Other chemicals such as monosodium phosphate (96 % wt.), Toluene (99.5 % wt.) and acetone (99.5 % wt.) were purchased from PanReac AppliChem. Potassium hydroxide (90 % wt.) used in acid value determinations, disodium phosphate (99 % wt.) used as buffers, and ethanol 99.5 % wt. were purchased from Merk.

Table 2.2 Properties and specifications of collected used cooking oil

Specification	Method	UCO Collected	UCO Pretreated	Palm olein
Acid Value (mg KOH/g)	AOCS Cd 3d-6 [88]	39.05	37.43	0.44
Moisture (% wt.)	Karl Fischer [89]	1.94	0.95	0.19
Iodine value (g I_2/100g)	Wijs method (British standard BS 684: Section 2.13) [90]	80.83	80.12	71.9
Saponification value (mg KOH/g)	AOCS Cd 3-25 [91]	199.55	198.82	194.75
Average Molecular weight (g/mol)*	[92]	896.6	905.8	864.2

* Theoretically calculated from saponification value assuming triglycerides only and discounting acidity

2.3.2. Used cooking oil enzymatic hydrolysis

The enzymatic hydrolysis of pretreated UCOs was carried out under ultrasound-assisted conditions using an ultrasonic bath (P30H Cole Parmer) with temperature control (± 0.5 °C) operating at 37 kHz. Reactions were carried out in 100 mL spinner flasks immersed in the bath, and they were mechanically agitated with an overhead motor stirrer (Cole-Parmer EW-50006-03). Experiments were carried out a at different oil:water mol ratios (1:5, 1:15. 1:30, 1:60, 1:120 and 1:180), and immobilized enzyme concentrations (1, 5, 10 %wt. based on the total amount of substrates). Reactions were carried out for 6 h at 45 °C, at pH 7.0 (controlled with a 50mM solution phosphate buffer prepared with 7.74 g/L of Sodium Phosphate Dibasic Heptahydrate and 2.91g/L of Sodium Phosphate Monobasic Monohydrate), at a stirring rate of 300 rpm using a pitched paddle propeller, and with a sonication power of 100 W. The specific pH and temperature conditions were stablished according to preliminary experiments of the previous chapter, and the remaining variables

were defined according to literature reports on the ultrasound-assisted hydrolysis of UCOs or refined oils [20, 27, 40, 86]. The ultrasound influence was evaluated by three procedures, namely: (i) without sonication but with mechanical stirring during the whole reaction, (ii) with sonication but without mechanical stirring the whole reaction, and (iii) with ultrasound only at the beginning followed by mechanical stirring at the end. The last method tried to assess the mixing effectiveness of an emulsified mixture owing to the fact that a stable emulsion was formed due to the surfactant activity of intermediate glycerides (i.e. DGs and MGs).

2.3.3. Reaction monitoring and degree of hydrolysis (conversion)

Acidity of the reactive media was used to monitor the progress of the enzymatic hydrolysis of UCOs, and the acid value determination was done according to AOCS Official Method Cd 3d-63 [88]. Samples of ~1 mL were withdrawn from the reactors at specific time intervals during reaction avoiding to remove the suspended catalyst. Afterwards, each sample was dissolved in 20 mL of a toluene:ethanol solution (1:20 v/v), and titrated with a standardized 0.1M NaOH solution using phenolphthalein as indicator. The acid value (AV, mgKOH/g) of the sample was determinate using the Eq 2.1

$$AV = \frac{56100 * C * V}{w} \qquad (2.1)$$

Here, C (mol/L) was the molarity of NaOH solution, V (L) was volume of NaOH solution required for neutralization, and w (g) was the weight of the sample taken from the reaction medium. Based upon acid value measurement and the corresponding saponification value of the UCO, the degree of hydrolysis (%) or conversion was determined using the Eq 2.2.

$$Degree\ of\ hydrolysis\ (\%) = \frac{AV_t - AV_o}{SV - AV_o} * 100 \qquad (2.2)$$

Here, AV_o and AV_t are the acid value of the sample at the beginning and at a specific reaction time, and SV is the saponification value of the initial oil.

2.3.4 Determination of kinetic parameters

The acid value profiles during the enzymatic hydrolysis of olein palm and UCO at the different operating conditions were used to obtain the kinetics parameters of reaction. In this case, a Michaelis-Menten-type kinetic model described in Equation 2.3 was evaluated

$$r = \frac{r_{max}[S]}{K_m + [S]} \qquad (2.3)$$

Here, r (mmol/L·min) is the specific hydrolytic rate, r_{max} (mmol/L·min) is the maximum reaction rate, K_m (mmol/L) is the Michaelis-Menten-type constant for the purified lipase, and $[S]$ (mmol/L) is the concentration of ester groups, which are defined as follows.

$$[S] = (SV - AV) * \frac{Oil\ density}{56100} * \frac{Initial\ Oil\ Volume}{Reaction\ Volume} * 1000 \qquad (2.4)$$

Then, after using the linerweaver-Burk representation of Equation 2.3, it is possible to linearize the model and determine the type of enzyme inhibition occurring during reaction. The linearized model is presented in Equation 2.5.

$$\frac{1}{r} = \frac{K_m}{r_{max}} \frac{1}{[S]} + \frac{1}{r_{max}} \qquad (2.5)$$

Then, by plotting $1/r$ vs. $1/[S]$ for the different experiments, it is possible to determine the corresponding kinetic constants of the model. A comparison among different vegetable oils would enable to identify if differences in inhibition occurs, mainly due to their content of impurities. This last could be the case when processing UCOs.

2.3.5 Water inhibition kinetic model

Despite water is a reactant during hydrolysis, high water concentrations or excess water can also act as a substrate inhibitor for the enzyme. As water is normally used in large molar excess during enzymatic hydrolysis, water molecules will compete with the triglyceride molecule for attaching the active site of the enzyme, and this might lead to lower conversions of triglycerides in the feedstock. While the Michael-Menten-type kinetic model can be applied for low water concentrations (10 – 15% w/t), it is not suitable to describe the

lipase-catalyzed reactions for water loading above 20 %w/v. In this case, a mechanism of anti-competitive inhibition by substrate for high water concentrations can be used to describe reaction progression [93]. The reaction mechanism is described as follows:

$$E \; + \; S \quad \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \quad ES \; \overset{k_2}{\rightarrow} \; E \; + \; P$$
$$+$$
$$I$$
$$k_{-4} \updownarrow \; k_4$$
$$ESI$$

Here, E is the immobilized enzyme, S the substrate (triglyceride), ESI is the enzyme-substrate-inhibitor complex, P the product and I is the substrate inhibitor (i.e. water). By using this approach, and recognizing is limiting behavior on the reaction rate, the corresponding kinetic model describing triglyceride consumption per unit volume of reacting fluid (r) can be represented as follows [93,94]:

$$r = \frac{r_{max}[S]}{K_m + [S] + \dfrac{[S][I]}{K_{SI}}} \qquad (2.6)$$

Here, concentrations are those described for Equation 2.3, [I] is the molar concentration of water, and K_{SI} is the dissociation or enzyme inhibition constant.

2.4   Result and discussion

2.4.1   Water to oil ratio effect

In the case of hydrolysis using lipases, water plays a key role for the accessibility of the substrate because the lipase binds to the TG molecule in the water-oil interface. Besides, it is important to consider that hydrolysis is limited by the chemical equilibrium. The chemical equilibrium constant of TAGs hydrolysis is around 0.45 at 225 °C, which limits the maximum reaction conversion below 90% [95]. Then, using a molar excess of water (> 3:1) can help overcoming chemical equilibrium limitations [72,96]. A molar excess is normally achieved because there is a very large molecular weight difference between reactants (TAG/H_2O ≈ 44-50). Nonetheless, excessively high water loadings might not conciliate well with the features of the enzyme, leading to enzyme leaching from the solid support with the consequent decrease of activity [58]. Moreover, large water loadings can also increase the

energy consumption, mainly in the downstream separation. Also, additional mass transfer limitations may appear due to the low miscibility of reactants [20,97].

For the current study, the hydrolysis degree (i.e. conversion) of UCO or palm olein at the different oil:water molar ratios are presented in Figure 2.3 and Figure 2.4, respectively. As expected, conversion is highly dependent on the water loading in the reaction media. For small values of oil to water ratio (i.e. 1:120 and 1:180), the highest conversion (25.55 ± 0.87 %) was reached after just 2 h of reaction, but it was much lower compared to that obtained for lower water loadings. In contrast, using a 1:60 ratio, a conversion of 33.63 ± 1.19 % was obtained after 4 hours of reaction, whereas for the lower water loadings of 1:30 and 1:15, the maximum attained conversions after 6 h were 61.07 ± 2.79 % and 56.49 ± 2.31 %, respectively. Moreover, at the lowest ratio water loading (i.e. 1:5), the kinetic profile did not reach a maximum in the conversion profile during the assessed time (i.e. 6 h), and the higher hydrolysis degree was 43.10 ± 2.43 %.
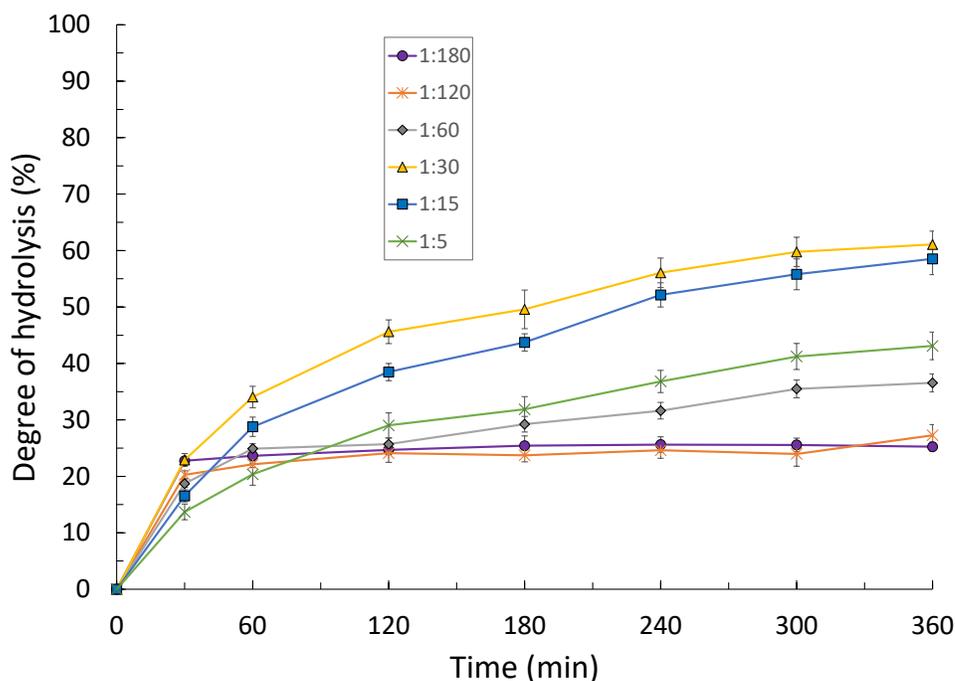

Figure 2.3. Conversion progress during enzymatic hydrolysis of used cooking oil (UCO) at different initial UCO:water molar ratios. Reactions at 45 °C, pH 7, 300 rpm, immobilized enzyme (CALBAC3) loading 5% wt., sonication power and frequency of 100 W and 37 kHz.
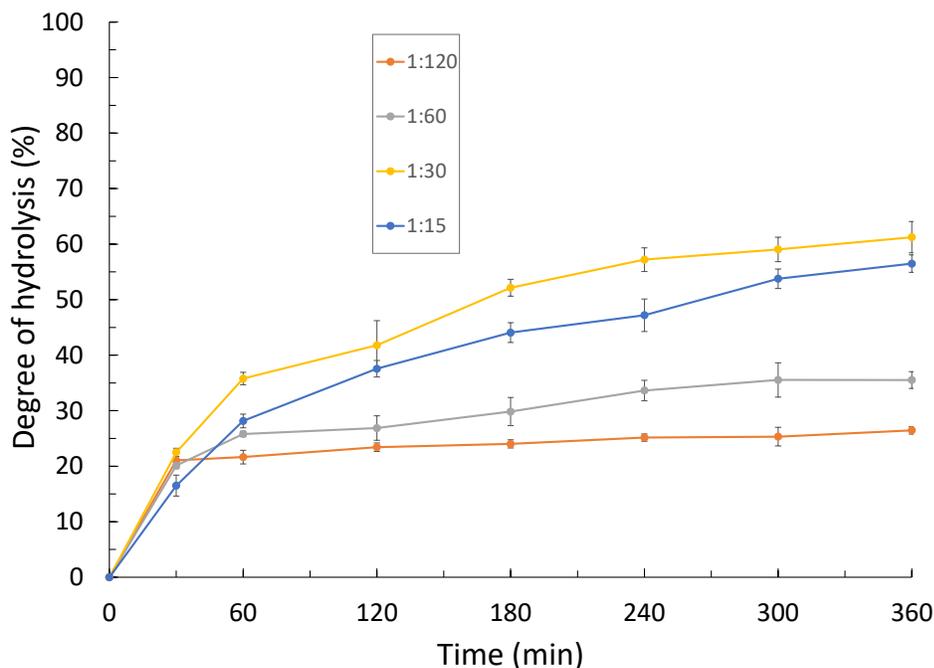
Figure 2.4. Conversion progress during enzymatic hydrolysis of palm olein at different initial olein:water molar ratios. Reactions at 45 °C, pH 7, 300 rpm, enzyme (CALBAC3) loading 5% wt., sonication power and frequency of 100 W and 37 kHz.

These results confirm that there is a trade-off in the water loading with respect to the hydrolytic activity of the enzyme for both, UCO and palm olein. At very low loadings (i.e. 1:5 and 1:15) the rate of reaction and the maximum conversion is lower than the observed at intermediate loadings (i.e. 1:30 and 1:60). Similarly, the rate of reaction and conversion drop when using large water excess (i.e. 1:120 and 1:180). As previously described, large water excess tends to saturate the microenvironment around the biocatalyst enabling to reach the maximum conversion in a short time but with poor final results. As previously mentioned, formation of a water layer via hydrogen bonding can derive in multilayer adsorption with the formation of a thick aqueous film. This might result in a poor solubility of immiscible substrates (i.e. TAGs), in a reduction of accessibility for non-polar and large molecules to the active sites, and also making the enzymes more susceptible to denaturation [20, 66]. Also, the use of a large water loading involves a larger amount of buffer salts and ions in the reactive medium [65] affecting the active sites of the immobilized enzymes. On the contrary, under low loadings the reactive media is mostly oily, and the hydrophobic support of the enzyme (i.e. activated carbon) is mainly saturated with the oil

phase. Besides blocking water binding to the active sites, the reactive media cannot be effectively emulsified to provide enough interfacial surface area for reaction between the immiscible reactants. As previously presented in Table 2.1, this behavior has also been observed in different studies of enzymatic hydrolysis. It is reported that the optimal oil:water molar loadings are in between 1:30 and 1:60 which correspond to 1:0.5 and 1:1 oil:water volume ratio, respectively.

Another important factor explaining the observed sigmoidal behavior of the oil:water ratio is the change in the emulsification effectiveness due to the changing nature of the reactive media and the surfactant action of intermediate glycerides. In the one hand, DGs have an intermediate hydrophilic-lipophilic balance (HLB) around 9-10 [98], acting as suitable oil in water emulsifier mainly for low oil:water ratios. In the other hand, MGs have a low HLB value around 3-4 [99], facilitating water in oil emulsions and being suitable for large oil:water ratios. Moreover, once glycerol is formed, there is a negative effect on the stability of the emulsion because it increases the viscosity of the aqueous phase and the size of the droplets in the dispersed phase. This results in a reduction of the contact area that affects mass transfer and hydrolysis rates [100]. This last effect is more noticeable under high oil:water loading.

While specificity of an enzyme to a substrate is currently exploited in the food and pharmaceutical industry [21,23], non-specific lipases are rather preferred for the hydrolysis of waste TAGs in order to handle impurities and the heterogeneity of the fatty acid chains in the feedstock [18]. Lipases can be regio-, stereo-, and chemo-selective, which means that not only the position of the ester bond influences the hydrolysis rate but also the saturation extent of the fatty acids in the raw material [101]. In this regard, since CALB is a non-selective lipase, it was expected a similar catalytic performance in the hydrolysis of palm olein and UCO as long as there were no inhibitors. As observed in Figures 2.3 and 2.4, this behavior was verified as the kinetic profiles during hydrolysis of UCO and palm olein were alike and both reached similar maximum conversions for all the experiments. In both cases the maximum conversions (61.07 ± 2.79 % for UCO and 61.24 ± 2.81% for palm olein) were obtained using a 1:30 oil:water molar ratio. This suggests that potential inhibitors were successfully removed in the pretreatment of UCO, nevertheless further kinetic analysis has to be made to confirm this hypothesis.

2.4.2   Catalyst loading effect

For comparison purposes, the effect of the enzyme loading on the hydrolysis was assessed using UCO and palm olein. Based upon above-mentioned results, reaction experiments were carried out using a 1:30 oil:water mole ratio because this was near the optimal for both substrates, and the corresponding results are presented in Figure 2.5 and Figure 2.6. As noticed, the maximum conversion for UCO was 91.04 ± 3.92 % and for palm olein 90.66 ± 3.56 %, both obtained at an immobilized enzyme loading (including support weight) of 7% wt. that corresponded to 9.58 LU/mL with respect to the reaction volume. Interestingly, both kinetic profiles for UCO and Olein palm were alike, exhibiting similar reaction rates and reaching nearly the same conversion. At the beginning it was expected that the impurities of UCO might have an inhibitory effect on the reaction performance. For instance, solid particles, polymers, gums, phospholipids, sterols and tocopherols typically present in waste FOGs could block the porous matrix of heterogeneous supports affecting mass transfer and rates of reaction. Additionally, enzyme activity could be affected by polar compounds and impurities in waste FOGs. Surprisingly, this was not the case as profiles were similar to those of palm olein. The similar behavior between refined oil and UCO reinforces the hypothesis of a low presence of inhibitors in UCO due to an effective pretreatment.
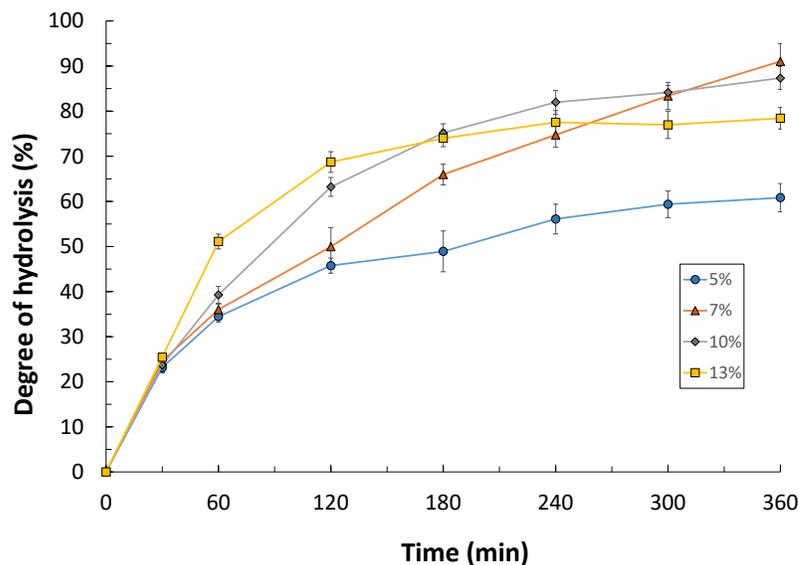

Figure 2.5. Conversion progress during enzymatic hydrolysis of UCO at different enzyme (CALBAC3) loadings. Reactions at 45 °C, pH 7, UCO to water molar ratio 1:30, 300 rpm, sonication power and frequency of 100 W and 37 kHz.
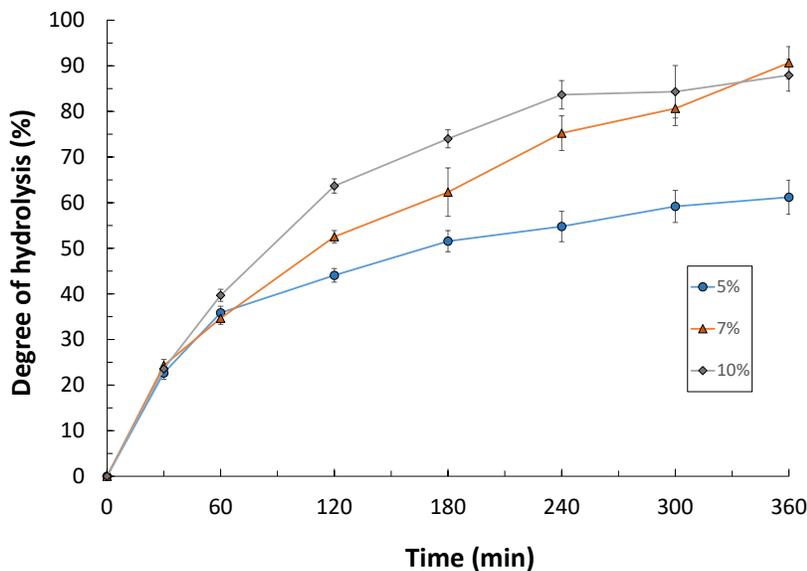
Figure 2.6. Conversion progress during enzymatic hydrolysis of palm olein at different enzyme (CALBAC3) loadings (% wt.). Reactions at 45 °C, pH 7, palm olein to water molar ratio 1:30, 300 rpm, sonication power and frequency of 100 W and 37 kHz.

Based upon the above kinetic profiles, the initial reaction rate was obtained and it was compared at the different loading of immobilized enzyme; the corresponding results are presented in Figure 2.7. As expected, faster reaction rates were obtained with palm olein compared to UCOs, but the difference is only minor. This can be caused by the presence of remaining impurities and polar compounds in UCOs that hinder or block active sites of the enzymes to the ester groups of TGAs. In Figure 2.7 is also observed that immobilized enzyme loadings > 5% wt. induce a slightly and minor enhancing effect in the rate of reaction despite the substantial change in the final achieved conversion (see Figures 2.5 and 2.6). Interestingly, there was no significant change in the observed reaction rates above a 7% wt. loading as the final conversion was similar to that of a 10% wt. Since enzyme loading has no significant effect in the initial rate, these results suggest an enzyme saturation effect in the final reaction stages due to the presence of a more polar environment. Similarly, as glycerol concentration increases, this could clog the catalyst surface hindering the entrance of the non-polar substrates (i.e. TAGs) to the active site of the enzyme [29,39]. Despite the catalyst loading has no or minor effect in the initial rate of hydrolysis, high concentrations of immobilized enzymes might result in a enzymatic saturation in the interfacial area where the reaction take place; in the case of reaction emulsions this can create aggregates or enzyme clusters which are inactive [104].
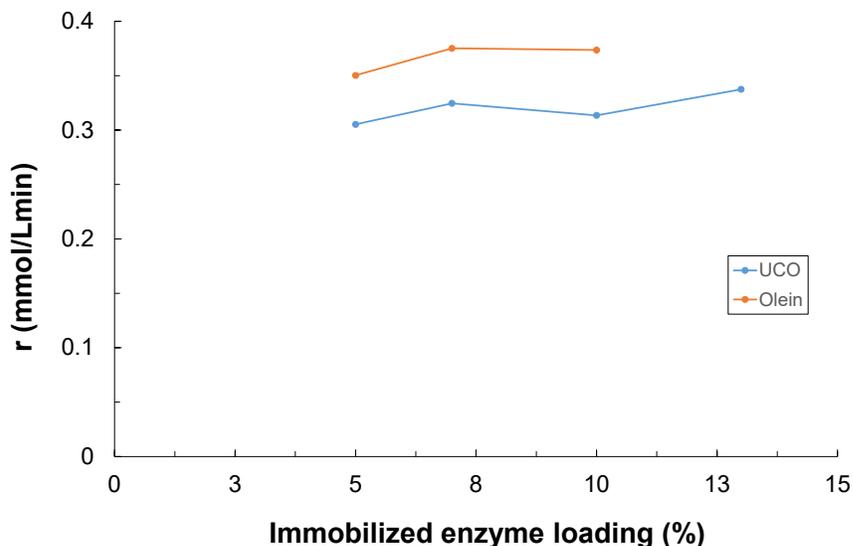
Figure 2.7. Initial reaction rates at different immobilized catalyst loading (including support).

2.4.3   Ultrasound and mechanical stirring effect

Ultrasound can generate cavitation inducing minute gaseous void cavities that suddenly collapse at several locations in the reactive medium. This also releases a vast amount of energy in high densities, highly increasing the temperatures in small volumes, and boosting mass transfer rates and reaction kinetics [28, 38]. This has been verified in the hydrolysis of fats and oils where the generation of localized hotspots have improved the process performance, even without the use of catalysts [84]. At the same time, mechanical stirring is the simplest way to overcome mass transfer limitations in biphasic reactions and can help emulsifying the mixture. However, large shear forces might induce a decrease in the overall lipase activity due to leaching and denaturation [27]. Then, considering the surfactant nature of the intermediate products, it is expected that a suitable combination of ultrasound and a certain level of mechanical stirring would enable to enhance waste FOG hydrolysis by reducing droplet sizes, increasing interfacial area, improving mass transfer coefficients and increasing kinetic constant [20]. For this reason, the effect of ultrasound-assisted mechanical stirring was studied in this section and the results are displayed in Figures 2.8 and 2.9.
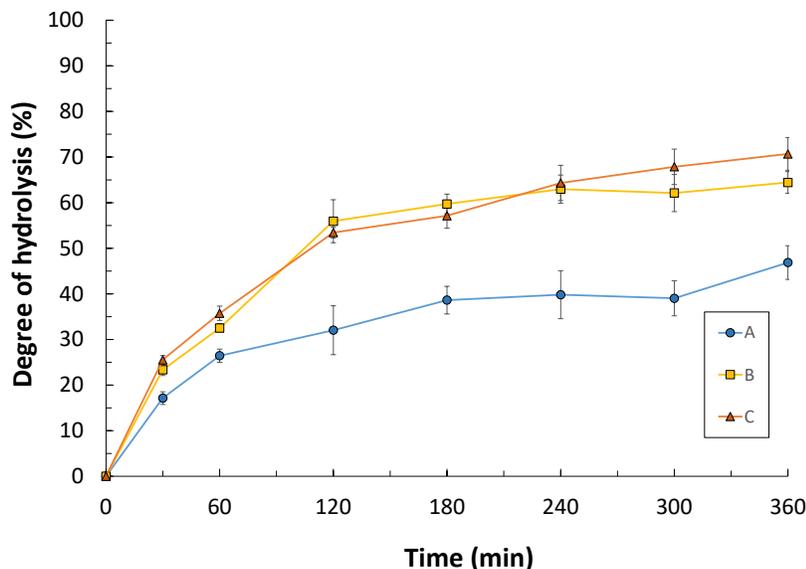
Figure 2.8. Conversion progress during enzymatic hydrolysis of UCO under ultrasound and/or mechanical stirring. Reactions at 45 °C, pH 7, UCO to water molar ratio 1:30, 300 rpm, enzyme loading 7% wt., sonication power and frequency of 100 W and 37 kHz. (A) Using mechanical stirring only. (B) Using ultrasound only. (C) Using ultrasound during the first 120 min only and mechanical stirring at the end of reaction.

Figure 2.9. Conversion progress during enzymatic hydrolysis of palm olein under ultrasound and/or mechanical stirring. Reactions at 45 °C, pH 7, palm olein to water molar ratio 1:30, 300 rpm, enzyme loading 7% wt., sonication power and frequency of 100 W and 37 kHz. (A) Using mechanical stirring only. (B) Using ultrasound only. (C) Using ultrasound during the first 120 min only and mechanical stirring at the end of reaction.
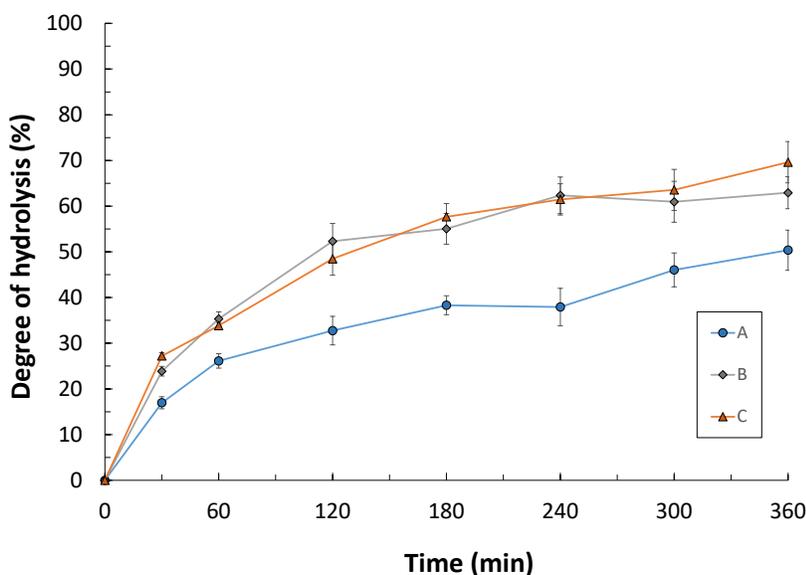
As observed in the kinetic profiles, the worst performance was obtained when using mechanical stirring only, reaching a maximum conversion below 50% even after 6h of reaction. Comparatively, and as expected, there was an evident boosting effect when using sonication. In this case, conversions were 1.5 times higher along reaction compared with mechanical stirred only, reaching maximum conversions up to 70%. Another way to confirm that the emulsion stability was induced by ultrasound is inferred by the error bars in duplicated experiments. In the case of the experiment conducted with mechanical stirring only, conversion uncertainty was in-between 3-5%, while the corresponding when using ultrasound was 1-3%. This suggests that mechanical stirring was not able to generate a completely emulsified reactive media, and the withdrawn samples had heterogeneities that generated larger deviations in repetitions during the quantification of acidity. Nonetheless, a combination of sonication in the first 120 min and subsequent mechanical stirring enabled to obtain similar performance than when using sonication only. This indicates that, once surface-active intermediate glycerides are formed at the beginning of reaction, ultrasound helps forming the emulsion and this is stabilized by mechanical stirring after sonication is removed.

Despite that in enzymatic processes continuous exposure to ultrasound could lead to severe damage to the tertiary structure of the enzyme, causing deactivation and damage due to the ultrasonic transducer [27,86], this was not evidenced in the conducted experiments. This suggests that the use of immobilized enzymes was indeed effective and it conferred structural stability to the enzyme. Regarding the obtained results, and comparing the different mixing methods, it was verified that the most effective was the use of ultrasound without mechanical assistance in the first hours followed by mechanical stirring after 3-4 hours of reaction. This combination enabled to reduce energy-intensity and to cut operating costs but maintaining emulsion stability and achieving high conversions. This confirms that intensification via sonication is an effective technology for the enzymatic hydrolysis of waste FOGs under milder conditions. Additionally, the use of ultrasound technology enables to work in solvent-free systems since ultrasound confers good mixing without using emulsifiers or solvents. This is a major benefit when considering a feasible industrial implementation of hydrolysis to exploit waste FOGs [24,27].

2.4.4   Enzyme kinetics parameters.

In the case of enzymatic processes (e.g. hydrolysis, esterification, transesterification), it has been observed that impurities in waste FOGs can inhibit the bio-catalyzed reactions [33,105]. In general, this inhibition effects have been conceptually captured by different kinetic models, in particular by using a Michaelis-Menten-type mechanism. This is based on three important assumptions:

- The product will not return to the substrate
- The total amount of enzyme (E_{total}) can be present as unbounded enzymes (E) or as an enzyme-substrate complex (ES), and finally
- Reaction proceeds in steady state, this is that the concentration of enzyme-substrate complex (ES) is constant as well as the concentration of unbounded enzyme (E).

These assumption enabled to derive Equation 2.3 [106], so the kinetic parameters K_m and r_{max} for the biocatalyst were obtained by the Lineweaver-Burk plot of Figure 2.10.
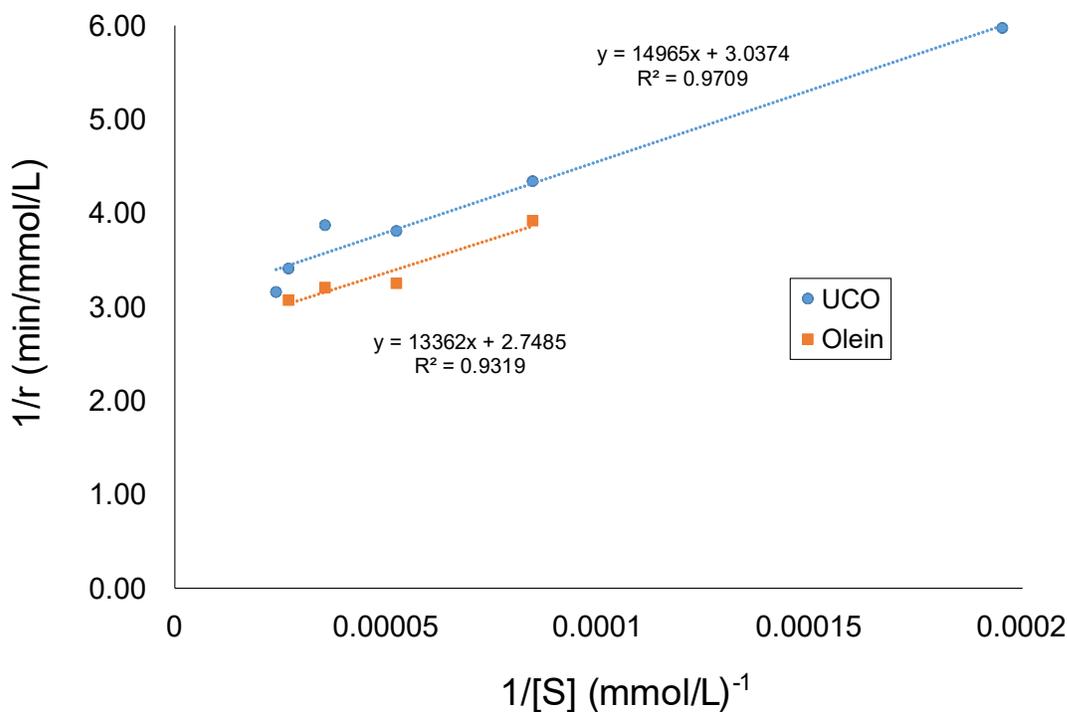

y = 14965x + 3.0374
R² = 0.9709

y = 13362x + 2.7485
R² = 0.9319

Figure 2.10. Lineweaver–Burk plot of the CALBAC immobilized lipase using UCO and palm olein as substrates.

The corresponding K_m and r_{max} values for UCO were $5.63\text{x}10^3$ mM and 0.349 mM/min, respectively, and those when using palm olein as substrate were $4.86\text{x}10^3$ mM and 0.364 mM/min, respectively. Since K_m (or the Michaelis-type constant) represents the ratio of concentrations [E][S]/[ES] in the formation of the enzyme-substrate complex [ES] ($E + S \leftrightarrow ES$), the high values of K_m for both substrates indicate a poor affinity between the substrate and the enzyme to form the enzyme-substrate complex. Thus, this step becomes a limiting factor in reaction thus explaining the relative long reaction times (6h) required to obtain high conversions. This poor affinity can be mainly due to the physical and chemical interactions of reactants with the solid matrix of the activated carbon support, and not due to a low activity of the immobilized enzyme itself. The enzyme low activity can be dismissed considering that the obtained r_{max} values between 0.1 - 0.5 mM/min were similar to those observed with other lipases from organism such as *Candida Antarctica*, *Pseudomonas gessardii* and *Rhodotorula glutinis* in their free form [107–109]. This also confirms that the immobilized enzyme used in this work is highly active for the fatty chains in the UCO and the Palm Olein [110].

Finally, the similar slopes observed in both lines of Figure 2.10 indicates that there is an uncompetitive inhibition during UCO hydrolysis, which is based on the fact that parallel lines were obtained with two different substrates [40]. In this case the slight differences of reaction rates and conversions for both substrates are due to the small difference between the K_m values (1.33%). In general, enzymes correspond to a consortium of macromolecules with catalytic action over specific bonds and reactions. Then, the changes of affinity of the enzyme with UCO is due to the chemical heterogeneity of UCOs, the presence of potential inhibitors, and the need to operate under slightly high temperatures to ensure complete melting [112]. While it has been reported that *Candida Antarctica Lipase B* (CALB) is highly active in the hydrolysis of waste FOGs and resilience to polar inhibitors (e.g. methanol, ethanol) [39, 65, 113], it is still unclear the potential effect of large molecular weight polar components present in UCOs.

2.4.5   Potential inhibition by water

High water loadings tend to clog the active sites of the enzymes causing what is known as substrate inhibition. This inhibition is generally associated to the formation of multilayers at the interface where the reaction takes place [114]. To explore this potential phenomenon,

Figure 2.11 presented the reaction rates during hydrolysis of UCO and palm olein at different concentrations of the oil substrate. It is important to recall that a large loading of oil substrate also means low loadings of the potential substrate inhibitor (i.e. water).
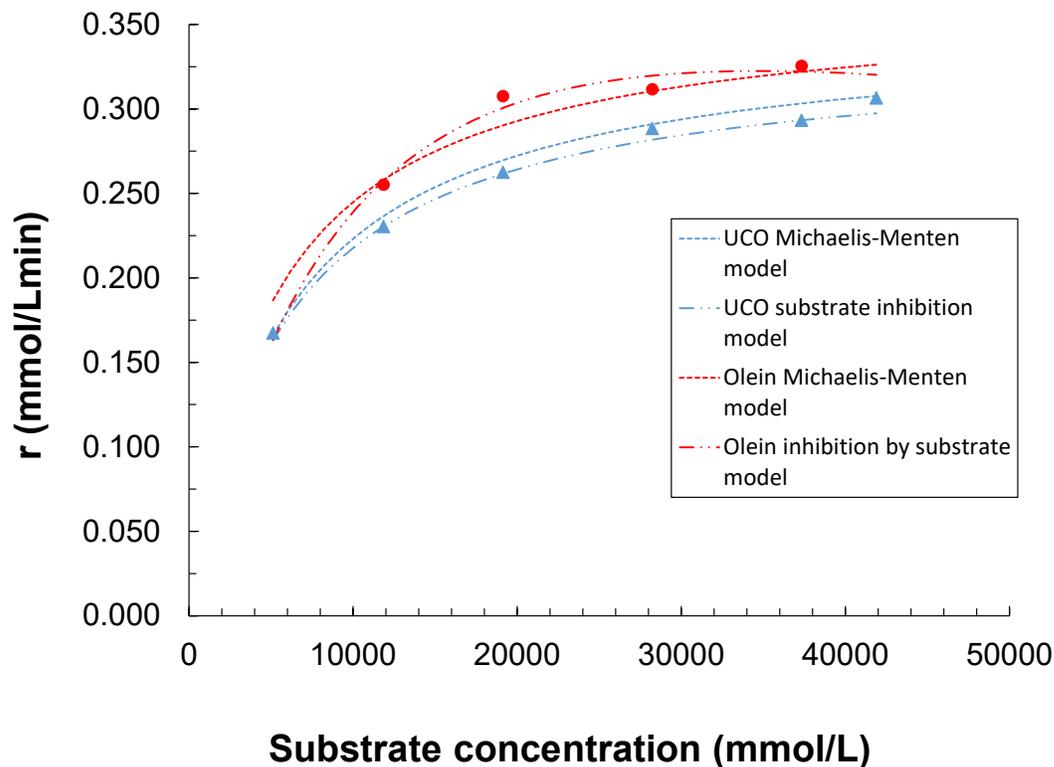
Figure 2.11. Enzymatic hydrolysis parity plot reaction rate at different concentration of oil substrate. Lines represent regressed kinetic model according with Equation 2.3 and Equation 2.6.

Table 2.3 Summary table of the results from the kinetic model representation.

Value	Michaelis-Menten model with UCO	Inhibition by substrate model with UCO	Michaelis-Menten model with Olein	Inhibition by substrate model with Olein
r_{max} [mM/min]	0.349	0.336	0.364	0.533
K_m [mM]	5.63×10^3	5.43×10^3	4.86×10^3	1.14×10^4
K_{SI} [mM]	---	3.90×10^3	---	1.06×10^4
Average model relative error [%]	1.79	1.37	2.05	1.69

As observed, reaction rate increases with the concentration of substrate (oil) and at low concentrations of inhibitor (water) confirming that excess water has a negative role in

reaction, probably associated with an anti-competitive inhibition binding the enzyme [115]. Nonetheless, at high concentrations of oil there is a plateau in the reaction rate, which is characteristic of a substrate inhibition depicted in the classic Michaelis-Menten-type model (i.e. Equation 2.3). This last phenomena may take place due three reasons, namely: high concentrations of substrates may difficult the diffusion, there is formation of an unreactive complex (e.g. ES_2), and there is inhibition by impurities presented in the substrates [94].

Using the experimental data, the corresponding parameters of kinetic model including water inhibition (i.e. Equation 2.6) were regressed. The obtained r_{max} , K_m and K_{SI} value for the hydrolysis for UCO were 0.336 mM/min, 5.43×10^3 mM and 3.90×10^3, respectively. The corresponding ones when using palm olein as substrate were 0.533 mM/min, 1.14×10^4 mM and 1.06×10^4, respectively. These results suggest that there is a slightly difference between the behavior of UCO and palm olein under the same reaction conditions which evidences the high activity of the immobilized enzyme even in the presence of impurities. Surprisingly, the higher values of K_m and K_{SI} in the olein palm suggest that reaction with UCOs tends to be less affected by substrate inhibition (water and oil), this fact can be explained due to the presence of initial acidity and some other polar compounds making the emulsion more stable even at high water concentrations [94].

The results from the kinetic regression are summarized in the table 2.3, and comparing the corresponding experimental and predicted kinetic profiles of Figure 2.11, it is observed that the Michaelis-Menten-type model tends to slightly overestimate the reaction rate at high substrate concentrations. Comparatively, the inhibition by substrate model predicted lower reaction rates as those experimentally observed, and differently from the first model, under large water loadings, it was able to predict a decrease in the reaction rates. The corresponding average relative model errors of the models are presented in the Table 2.3, and they were both lower when using the inhibition by substrate model. Nonetheless, both models are suitable to describe the hydrolysis of UCOs and palm olein using CALBAC3 as catalysts within the explored operating ranges.

2.5   Conclusions

This work focused on the evaluation of the enzymatic hydrolysis of UCO and palm olein using Candida Antarctica lipase B (CALB) immobilized onto an amino-functionalized

mesoporous activated carbon. Besides, ultrasound mixing was implemented as intensification technique to improve process performance. According to explored conditions, the best performance in terms of conversion of TAG to FFAs was obtained at 45°C, enzyme loading of 7.0% wt., ultrasonic mixing power and frequency of 100 W and 37 kHz, respectively, at 1:30 oil to water molar ratio and using assisted mechanical stirring at 300 rpm. Under these conditions a conversion of ester groups into fatty acids was 91.04 ± 3.92 %. It was found the ultrasound mixing promoted formation and enhanced the stability of emulsions in the biphasic reactions. Such emulsion was promoted by the intermediate formation of partial glycerides (i.e. mono and diglycerides) that acted as surfactants in the reacted media.

By tracking reaction evolution under variable oil to water loadings, for both UCO and palm olein, it was possible to verify a substrate inhibition effect; under low and high oil loadings there was a reduction of conversion and also of reaction rates, with a maximum around 1:30 ratio. In the one hand, excess water generates a highly polar microenvironment around the enzyme thus reducing affinity with the triglycerides. In the other hand, large excess of oil reduces accessibility of water through the non-polar support to the active sites, and affect emulsification effectiveness. It was also verified that the immobilized enzyme is not specific as it exhibited similar performance with both substrates (i.e. UCO and palm olein). Still, a slightly lower reaction rate was observed in the hydrolysis of UCO. Regarding immobilized enzyme loading, it was found that operating above 7% wt., no additional benefits were obtained. This might be related with a saturation of the enzyme (i.e. with polar glycerol and water) once high conversions are obtained. Finally, kinetic model was regressed for the hydrolysis of UCO and palm olein using a Michaelis-Menten-type mechanism that considered oil inhibition only, or both oil and water inhibition. The best model corresponded to the one involving oil and water inhibition with an average relative error of 1.37% and 1.69% in the hydrolysis of UCO and Palm Olein, respectively. The obtained model can be further use for process design in the hydrolysis of UCOs and palm olein using the immobilized CALB onto Starbon activated carbon supports.

Chapter 3.

Assessment of reusability of immobilized Candida Antarctica Lipase B onto activated carbons in the hydrolysis of used cooking oils.

This section includes fragments of the paper: Baena, A., Orjuela, A., Rakshit, S. K., Clark, J. H. 2020. Enzymatic hydrolysis of waste fats, oils and greases (FOGs): Status, prospective, and process intensification alternatives. Chem. Eng. Process. - Process Intensif. Currently In revision.

The paper is reformatted and figures and tables are enlarged to fulfill edition requirements for the dissertation document.

3.   Assessment of reusability of immobilized Candida Antarctica Lipase B onto activated carbons in the hydrolysis of used cooking oils.

3.1   Abstract.

The use of immobilized lipases as catalysts is considered an alternative for costs reduction particularly in the synthesis of different biobased chemicals, such as fatty acids from lipids. Despite immobilized enzymes can be easily recovered for reuse, a main drawback is their loss of activity due to leaching and denaturing. In this regard, this work focused on the assessment of the stability of immobilized Candida Antarctica Lipase B (CALB) onto activated carbons with functionalized surface (CALBAC3) when used as catalyst in the hydrolysis of used cooking oil (UCO). In order to determine the effect of the immobilization process on enzyme's stability, experiments were also carried out with CALB immobilized onto non-functionalized activated carbons. The immobilized enzymes were tested as catalysts in five consecutive reaction batches, and the corresponding conversions were 90.88 ± 3.19 % for the first and 76.73 ± 1.77 in the second batch. The activity was also compared with commercial immobilized enzymes Novozyme 435 and Lipozyme Tl IM, and despite the final degree of hydrolysis (conversion) was similar for all tested enzymes, reaction rates were lower with CALBAC3 and it had low reusability potential.

Keywords: Immobilized lipase, Reusability, Candida Antarctica Lipase, Hydrolysis of UCOs, Starbon

3.2   Introduction

The incorporation of immobilized enzymes as catalysts in the manufacture of some biobased products is highly desired due to their regio-, stereo- and chemo-selectivity during the transformation of different substrates. Besides, immobilization enhances stability and recoverability of the active material to last several reaction cycles, and this characteristic is paramount to ensure a suitable technical and cost-effective performance. In particular, this

is fundamental when using lipases as catalysts in the hydrolysis of vegetable oils and waste lipids because the process has to be cost-competitive with the current highly energy intensive acid- or non-catalyzed industrial processes (i.e. Emery-Colgate). For this reason, different studies have focused on evaluating the activity and stability of lipases by using them in several consecutive reaction cycles. In this regard, and as a reference point, it is important to consider the change of activity of free enzymes during several reaction cycles. Some studies attempting to recycle free lipases for the hydrolysis of FOGs have reported a ~30% loss of residual activity after the second reaction cycle. In this case, such poor reusability degree suggests that free lipases are not suitable for recycling even if they are recovered [117].

Despite the effectiveness of immobilized enzymes, their major limitation is their high cost; in general it would be required up to 100 cycles of re-use to ensure profitability at the industrial scale [34]. This have called the attention on developing suitable supports and techniques to facilitate the recovery and reuse, ensuring activity preservation. However, immobilization on such supports further increases the costs of enzymes. For instance, it has been determined that nearly 47% of the total cost of immobilized enzymes is attributed to the cost of the support in commercial Novozyme 435® [118]. Thus, selection of suitable supports must be accomplished considering technical and economic considerations.

From the technical point of view, highly hydrophobic mesoporous supports may improve the yield in hydrolysis reactions by reducing the accumulation of glycerol and water in the surface when using lipases as catalysts [29,35,38]. Also, it has been reported that hydrophobic supports improve the diffusion of the substrates and products [48]. Thus, the properties and physicochemical characteristics of a support are of upmost importance in the performance of an immobilized enzyme [23]. An ideal matrix must have specific features like inertness, physical strength, stability, regenerability, nonspecific adsorption, ability to increase enzyme specificity/activity and to reduce product inhibition, and resistance to microbial contamination [119]. As the immobilization and reaction temperature is generally low for the different lipases (i.e. around 37°C), thermal resistance is not a main factor when selecting the supporting materials [120]. From the economic standpoint, the reusability of the immobilized enzyme is paramount to reduce operating costs and also to improve sustainability indicators [121]. In this case, the immobilization method (e.g. cross-linking, adsorption, encapsulation) influences the stability of the enzyme, dictates reusability, and

affects process economics [5,23]. Several studies on commercial immobilized lipases have established that their application in different processes is not economically feasible due to inhibition, leaching and loss of immobilized catalyst [34,118]. This loss of active material prevents the required reuse in several cycles.

In the case of hydrolysis of lipids there is an additional issue because high water activity can reduce the lifetime of the catalyst [122]. Since water is a reactant, operating conditions and suitable supports are mandatory to withstand high water content and to ensure good reusability. Additionally, high shear stress caused during mixing of reactive media also cause a loss of activity and enzymes leaching. Besides, in the case of polar polymeric supports, water can swell the pores covering the actives sites. In this case, there is not only a miscibility barrier for the waste FOG, but also a reduction in the activity of lipases because the hydrophobic interactions with the lipids are broken under the high ionic strength of the surrounding aqueous medium [123].

In order to enhance reusability, rinsing and washing with suitable solvents can help to remove unreacted substrates and products that may inhibit the enzyme, thus avoiding a decrease in activity for further cycles. It has been observed that when no washing is carried out, a sudden decrease in the residual activity occurs [122]. In the selection of proper solvents, aspects such as hydrophobicity interactions, enzyme denaturing, and chemical interactions with the support have to be considered. Among the different assessed solvents (e.g. hydrocarbons, chlorinated, ketones, water, alcohols, etc.), partially polar solvents (e.g. branched alcohols) are preferred because they can solubilize MGs, DGs, TAGs, and glycerol. The branched structure also avoids the formation of a water layer around the immobilized enzyme, facilitating the recovery with no activity loss [20]. For instance, it was demonstrated that Novozyme 435® can be re-used up to 50 cycles in hydrolysis reactions, by washing with tert-butanol with no significant activity loss [124]. Nonetheless, it might be pointed out that lipases used in hydrolysis generally present a sudden decrease in the residual activity after a few cycles due to their leaching in the aqueous phase [65]. So, even when using a rinsing step with suitable solvents, it is expected that the activity of enzymes will be eventually reduced after certain degree of use. Table 3.1 summarizes results from some studies on the reusability of enzymes in the hydrolysis of waste FOGs, illustrating a clear preference for the use of branched alcohols during the sensing step.

Table 3.1. Reported reusability studies of immobilized lipases in the hydrolysis of waste FOGs

Lipase	Washing Solvent	Number of cycles	Residual activity (%)	Reference
Lipozyme TL IM	Isopropyl alcohol	10	---	[20]
CRL/ Diaion HP-20	Tert-butanol	7	>99	[31]
Lipozyme TL IM	Tert-butanol	5	42	[65]
Novozyme 435	Tert-butanol	4	62	[27]
Pseudomonas gessardii on activated carbons.	Acetate buffer	21	100	[40]
CRL on hybrid sol-gel/ calcium alginate beds	No washing	4	55	[122]

Some of the enzymes reported in Table 3.1 have reached commercial status and they have been used in a variety of processes [39]. In the case of the free and non-specific lipase used in Novozyme 435® (i.e. CALB), it has exhibited acceptable yields in the hydrolysis of FOGs [27]. Similarly, the 1,3 regioselective commercial compeer *Themomices lanuginosus* lipase (TLL) used in Lipozyme TL IM has exhibited good activity but lower reaction rates [58]. This same enzyme in its free form has shown a good performance in the hydrolysis of soapstocks and olive pomace oil, and the results suggest that the same behavior and method for monitoring the reaction could be used with other substrates [125]. However, in both cases, the commercial immobilized versions seem to be not appropriate for hydrolysis reactions because the supports are of partial hydrophilic nature. As previously mentioned, it has been established that highly hydrophobic supports are preferred for hydrolysis in order to promote interfacial activation, and to avoid inhibition by surface adsorption of glycerol or water [35,39]

Although ultrasound increases hydrolysis rates, its use must be further studied because disturbances could lead to conformational, structural changes, or desorption of enzymes from the support. However it has been found that enzymatic hydrolysis at high biocatalyst concentrations is favored by ultrasound due to the dispersion of formed enzymatic clusters [20]. In the same way, as in the previous chapter, it has been observed that the use of mechanical stirring alternated with ultrasound provides a five-fold increase of reaction rates and higher conversion when compared with simultaneous ultrasound and mechanical stirring [27]. In this context, this section deals with the use of the Candida Antarctica Lipase B immobilized onto activated carbon (CALBAC3) under ultrasound irradiation in repeated hydrolysis cycles. For comparison purposes, additional experiments were carried out with

the commercial counterpart Novozyme 435®. This assessment enabled to establish feasibility of using CALBAC3 in the valorization of UCOs via hydrolysis towards FFAs.

3.3   Methods and materials

3.3.1   Materials and apparatus.

Used cooking oil (UCO) was collected from chicken restaurants in Bogotá, Colombia. The collected UCO was pretreated according to the procedures previously reported [87]. The assessed enzyme was Candida Antarctica Lipase A, and it was immobilized onto activated carbon with (CALBAC3) and without crosslinking (CALBAC1). The corresponding synthesis was done following the protocols described in the first chapter. Monosodium phosphate (96 % wt.), Toluene (99.5 % wt.) and acetone (99.5 % wt.) were purchased from PanReac AppliChem. Potassium hydroxide (90 % wt.) used in acid value determinations and disodium phosphate (99 % wt.) used as buffers, and ethanol 99.5 % wt. were purchased from, from Merk. The immobilized lipase Novozyme 435® was purchased from Sigma-Aldrich Co. Enzymatic hydrolysis of pretreated UCOs was carried out under ultrasound-assisted conditions using an ultrasonic bath (P30H Cole Parmer) with temperature control (± 0.5 °C) operating at 37 kHz. Reactions were carried out in 100 mL spinner flasks immersed in the bath, and they were mechanically agitated with an overhead motor stirrer (Cole-Parmer EW-50006-03).

3.3.2   Used cooking oil hydrolysis

Experiments were carried out with an oil:water molar ratio of 1:30, and immobilized enzyme concentration 7 % wt. based on the total amount of substrates. Reactions were carried out for 6 h at 45 °C at pH 7.0, with a stirring rate of 300 rpm using a pitched paddle propeller, and with a sonication power of 100 W. The pH was controlled with a 50mM solution phosphate buffer prepared with 7.74 g/L of Sodium Phosphate Dibasic Heptahydrate and 2.91g/L of Sodium Phosphate Monobasic Monohydrate. Acidity of the reactive media was used to monitor the progress of the enzymatic hydrolysis of UCOs, and the acid value determination was done according to AOCS Official Method Cd 3d-63 [88]. The degree of hydrolysis was determined following the same procedure presented in the previous chapter.

3.3.3   Recovery lipase and catalyst reusability studies.

At the end of each reaction cycle, CALBAC3 and CALBAC1 particles were recovered by filtration of reaction effluent in a filter paper. The reaction product consisted of two layers, an upper oily layer of fatty acids, mono-, di- and triglycerides and a lower aqueous layer of glycerol and water. The two phases were separated by decantation using a separatory funnel. The retained immobilized enzymes on the filter were rinsed with t-butanol (~ 150 mL), and subsequently they were put into an oven at 40 °C for 1h to remove the excess solvent. No further treatment was done before reusing the catalysts in a subsequent hydrolysis batch.

3.3.4   Comparison between CALBAC3 and commercial enzymes

The hydrolysis of pretreated UCOs was carried out under ultrasound-assisted with the same conditions showed in the section 3.3.2 but using CALBAC based and commercial catalysts (Novozyme 435®, Lipozyme TL IM). While the hydrolytic specific activity of Lipozyme TL IM is higher (264.61± 5.23 LU/g), the activity of CALBAC3 (136.93 ± 5.41 LU/g) and Novozyme 435® (144.12 ± 6.22 LU/g) were similar. Nonetheless, and in spite of the activity differences, they were assessed as catalysts in different reaction cycles in a concentration of 7 % wt. based on the total amount of substrates. The reaction monitoring was carried out following the procedures described in section 3.3.2 and in previous sections.

3.4   Result and discussion

3.4.1   Reusability of catalyst.

The catalyst loading as well as the reusability thereof are main factors affecting the economic feasibility of overall enzymatic process. It is widely reported that after the repetitive use of an immobilized enzyme in different reaction cycles, a decreasing in the enzyme activity is observed, mainly caused by its inactivation, denaturation or leaching [27,40,51]. In this regard the degree of hydrolysis (i.e. conversion) of UCO with the immobilized CALB in the activated carbon was explored and compared with a commercial enzyme (Novozyme 435®). In this case, the conversion of five consecutive cycles with t-butanol intermediate rinsing are presented in Figure 3.1.
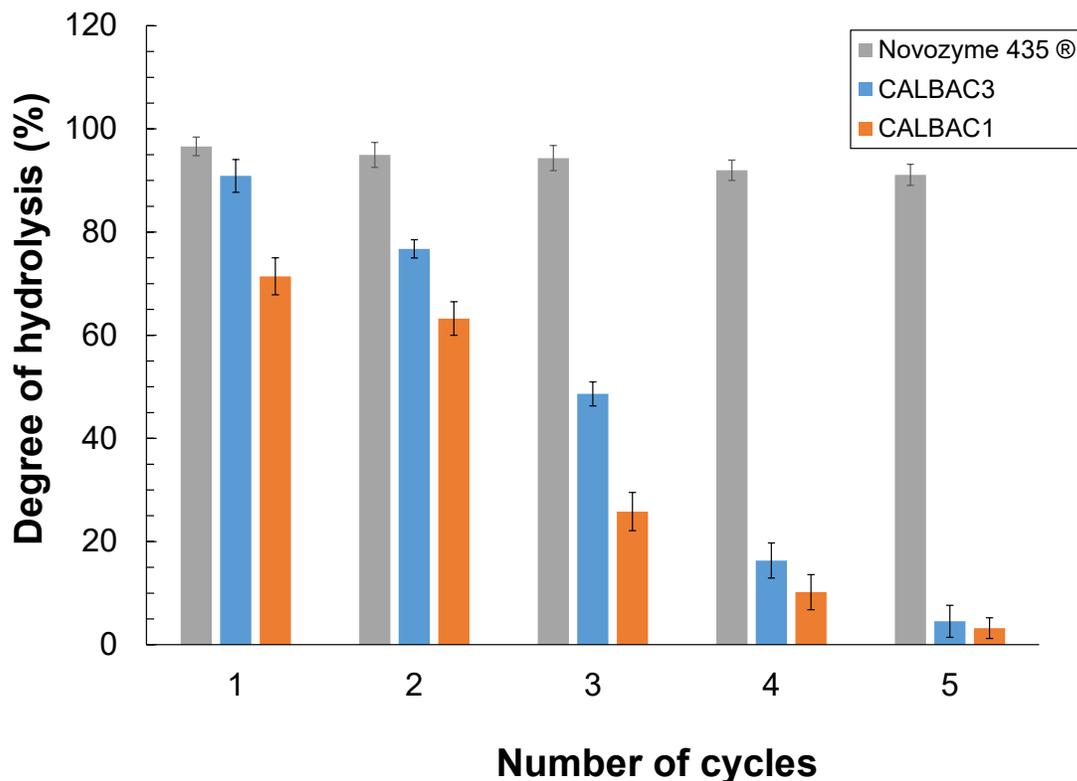
Figure 3.1. Effect of reuse of enzymes on the degree of hydrolysis (conversion) at UCO: water molar ratio 1:30, temperature 45 °C, pH 7.0, agitation speed 300 rpm, immobilized enzyme loading 7 % wt., ultrasound frequency of 37 kHz and power of 100 W.

As observed from experiments using CALBAC3 as catalysts, after the first cycle the degree of hydrolysis went from 90.88 ± 3.19 % to 76.73 ± 1.77 %, exhibiting at 15% drop in the enzyme activity. Surprisingly, this drop was even larger after several cycles and conversion at the end of the fifth cycle decreased to 4.54 ± 3.10 %. This result indicates that the assessed immobilized enzyme, using the t-butanol rinsing prior recycling, is not suitable for further deployment at the industrial scale. A heuristic of enzymatic processes indicate that ~ 100 successful reaction cycles are required to ensure the feasibility of an industrial process [34]. In the case of Novozyme 435® the reduction of activity was much lower, going from ~96 to ~90 % conversion in five consecutive cycles. This was surprising considering that it has been reported that Novozyme 435® can be employed in the hydrolysis of FOGs and it can withstand its activity even after 50 reaction cycles [34], but it generally exhibits a reduction of ~ 62% in the activity only after few successive cycles of reaction. In our case,

the observed results indicate the despite having a high initial activity, CALBAC3 exhibited poor reusability.

From Figure 3.1 it is also noticed that despite the high conversion achieved when using CALBAC3 in the first batch in comparison with CALBAC1, there is similar decrease in the activity for both catalysts with the number of reuse cycles. As the anchoring method of the enzyme is different for both catalysts, it would be expected that loss of activity would be different if the mechanism was enzyme leaching or denaturing. Besides, deactivation by saturation with polar compounds could be rule out considering that t-butanol rinsing in between cycles was precisely used to remove such compounds form the microenvironment around the enzyme. Then, the observed behavior suggests that the nature of the support (i.e. Starbon A800), its porous matrix, and the possible pore degradation during reaction is playing a main role in the stability of the enzyme and in the reaction performance. Such behavior has been previously reported when using immobilized lipases on erodible and mechanical sensitive supports [43] [126].

3.4.2   Comparison with commercial enzymes.

Novozyme 435® and Lipozyme TL IM are the most commonly used commercial immobilized lipases for hydrolysis or esterification applications at the industrial scale. While Novozyme 435® is a non-selective, lipozyme TL IM is a sn-1,3-regioselective enzyme mainly used in the dairy industry for the production of food supplements such as breastmilk [127]. However, these commercial catalysts have not been specifically designed for the hydrolysis of oils neither waste lipids. Nonetheless, considering their current applications, it was expected they would have a good activity in the hydrolysis of UCOs. Then, a comparative of their performance in the hydrolysis of UCO with respect to CALBAC3 is presented in Figure 3.2.
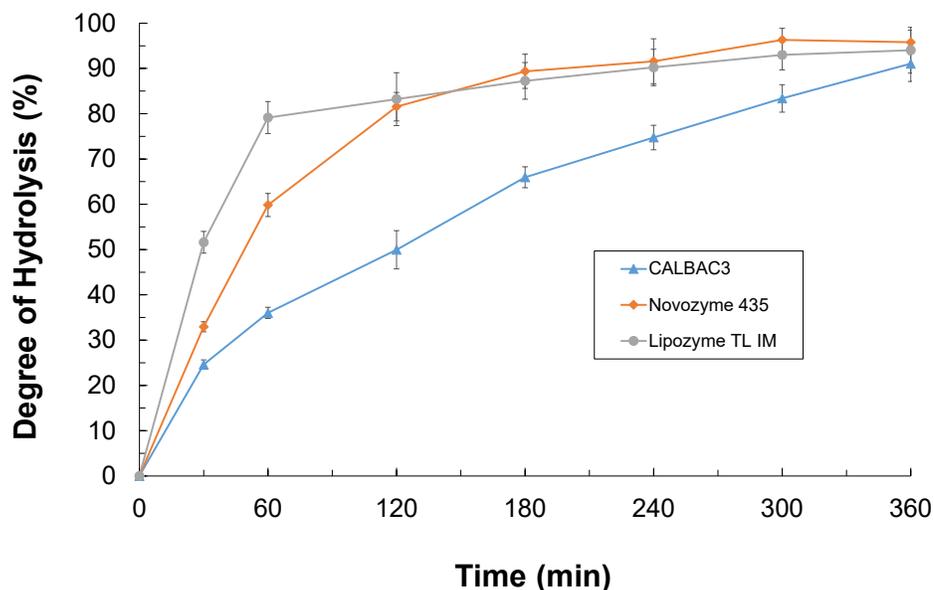
Figure 3.2. Reaction performance comparison between CALBAC3, Novozyme 435® and Lipozyme TLIM at the optimum reaction conditions

As observed, reaction rates of commercial enzymes were larger than that of CALBAC3, reaching a maximum conversion in lower time. Nonetheless, the kinetic profile during hydrolysis using Novozyme 435® and Lipozyme TL IM presented a rapid increase of conversion but a stagnation or plateau behavior after the first two hours of reaction. In comparison, CALBAC3 demonstrated a lower but constant reaction rate which enabled to achieve the same final conversion than the commercial catalysts after 6h reaction. The obtained final conversions were 91.04 ± 3.92 %, 95.77 ± 2.68 % and 94.03 ± 5.06 % for CALBAC3, Novozyme 435® and Lipozyme TL IM, respectively. Considering that enzyme loadings was done on a weight basis, it was expected that Lipozyme TL IM would exhibit a higher rate of reaction due to the higher specific activity. While this was the case at the beginning of reaction, it is clear that it suffers from some type of inhibition when conversion reaches ~80%, and this can be related to presence of a more polar environment around the enzyme or the presence of glycerides with sn-2 fatty acid chains. A similar behavior is observed with Novozyme 435®, but this occurs in longer times as the specific activity of the enzyme is lower. Regarding CALBAC3, which has a similar activity to that of Novozyme 435®, no reduction of activity was observed at large conversions. Considering the similar specific activity of Novozyme 435® and CALBAC3, this demonstrate that hydrophilic support certainly plays role reducing inhibition by hindering access of polar compounds. These

results indicate that CALBAC3 is a promising immobilized enzymatic catalyst, but further work must be conducted on improving the long-term stability for suitable recycle and reuse.

3.5   Conclusions

The reusability of immobilized enzymes is an important parameter to ensure their feasibility as catalysts in industrial process. By evaluating the reusability of immobilized Candida Antarctica Lipase B (CALB) onto activated carbons with (CALBAC3) and without (CALBAC1) functionalized surface in the hydrolysis of UCO, it was found that while the catalysts exhibited a high initial activity, its deactivation rapidly occurred after few cycles of reuse. Initially, the hydrolysis degree (i.e. conversion) of CALBAC3 was 90.88 ± 3.19 %, but it dropped a 15% after the first reuse. The same behavior was observed with CALBAC1, and this indicated that deactivation was mostly related with the nature of the support (i.e. Starbon A800), its porous matrix, and the possible pore degradation during reaction or blockage. In comparison with commercial immobilized, namely Novozyme 435® and Lipozyme TL IM, it was found that CALBAC3 exhibited lower reactions rates at the beginning of reaction, but larger when high conversions have been obtained. This enabled to achieve a similar maximum conversion after 6 hours of reaction with all assessed enzymes. This is an evidence that a hydrophobic support is less affected by the higher content of polar components along hydrolysis reaction. Despite the high activity of CALBAC3, its low reusability is still disadvantageous for a large-scale implementation. Further studies must be focused on improving reusability probably by enhancing mechanical stability of the support, enlarging pore sizes, and improving anchoring strength with the enzyme.

Chapter 4

4. Final Conclusions and recommendations

4.1 Conclusions

The aim of this study was to assess the immobilization of Candida Antarctica lipase B (CALB) onto activated carbon (Starbon A800), and its further evaluation as catalyst in the hydrolysis of used cooking oils. The immobilization process was done by combining amino-functionalization of the surface with crosslinking with glutaraldehyde in different orders. Such immobilization techniques enabled to obtain an efficiency of 11.30 %wt. and an enzyme loading of 56.36%. Results also indicate that among assessed techniques, amino-functionalization of the surface followed by adsorption of the enzyme and subsequent crosslinking with glutaraldehyde enabled to obtain a highly active immobilized enzyme (CALBAC3), having a higher specific hydrolytic activity (136.9 LU/g) than the free lipase CALB. This value is similar to that observed with a commercial benchmark immobilized lipase (i.e. Novozyme 435®; 144.1 LU/g) under the same enzyme loadings and operating conditions. Once a suitable immobilized enzyme was obtained, an experimental exploration enabled to determine the optimal pH and temperature for the hydrolysis of lipids. It was found the hydrolytic activity was nearly stable in between 120-140 LU/g at pH between 6 and 7.3, with a large drop above pH 8. Regarding the operating temperature, the largest activity was around 136.50 LU/g and it was obtained in-between 45 to 50 °C.

Regarding intensification of the process, ultrasound demonstrated a highly effective action to improve reaction performance. Under ultrasound mixing, the achieved degree of hydrolysis (i.e. conversion) was 91.04 ± 3.92 % at 45°C, with an enzyme loading of 7.0 % wt., 100W ultrasonic power, 37 kHz frequency, 1:30 oil to water molar ratio and with assisted mechanical stirring at 300 rpm. It was found the ultrasound mixing promoted formation and enhanced the stability of emulsions in the biphasic reactions. Such emulsion was promoted by the intermediate formation of partial glycerides (i.e. mono and diglycerides) that acted as surfactants in the reacted media. As a result, once an emulsion was formed using the ultrasound mixing, the mechanical stirring was capable of maintaining the stability of such emulsion and it also had a positive effect in the reaction performance.

In terms of the reaction mechanism, a comparison of enzymatic hydrolysis using used cooking oil and palm olein enable to identify a substrate inhibition effect. under low and high oil loadings there was a reduction of conversion and also of reaction rates, with a

maximum around 1:30 ratio. In the one hand, excess water generates a highly polar microenvironment around the enzyme thus reducing affinity with the TGS. In the other hand, large excess of oil reduces accessibility of water through the non-polar support to the active sites, and affects emulsification effectiveness. It was also verified that the immobilized enzyme is not specific as it exhibited similar performance with both substrates (i.e. UCO and palm olein). Still, a slightly lower reaction rate was observed in the hydrolysis of UCO. Based upon an experimental evaluation of hydrolysis of UCO and palm olein under different conditions, it was possible to obtain two kinetic models that agreed reasonably well with obtained data. Both models correspond to a Michaelis-Menten-type mechanism, one including oil inhibitions, and the second oil and water inhibition. The average relative errors of the models for hydrolysis of UCO were 1.79 % and 1.37%, respectively, and they can be used for further process design and scale up.

Finally, it was demonstrated that CALBAC3 was successfully used in 2 reaction cycles with a maximum degree of hydrolysis of 90.88 ± 3.19 % and a reduction of 15% in the second. This is a better performance than the reported in the reuse of free enzymes, but still is not suitable for further industrialization. Nonetheless, by comparison with commercial enzymes, CALBAC3 exhibited similar conversions after 6 hours of reaction without reduction in the activity along reaction. In this regard, it is considered that immobilization of Candida Antarctica Lipase onto mesoporous carbon (Starbon A800) via combined physical adsorption and covalent attachment enable to produce highly active materials for the hydrolysis of waste lipids. Nonetheless, future work must be done to improve reusability of the immobilized lipase.

4.2 Recommendations

According with the literature, among the different assessed supports, activated carbons (AC) have shown suitable performances in the hydrolysis of waste TAGs [40]. These materials have exhibited good structural properties, high substrate stability, and low costs [41]. However, taking into account the large size of the lipase (~5nm), large pore sizes supports are required for its absorption. This indicates that different mesoporous activated carbons with pore sizes >50 nm may provide a higher activities because the enzyme can

be more effectively immobilized, and the reactant might have a better accessibility to it. Also, more resistant porous matrix can help reducing support degradation after several reuse cycles, improving reusability of the enzyme. Also, it would be interesting to do the enzyme immobilization on preciously sulfonated supports; in one hand sulfonic groups have showed high affinity for the amino groups enhancing enzyme anchoring, in the other hand, sulfonic groups can act as co-catalysts during hydrolysis. Additionally, it has been verified that one of the most promising enzymes for waste FOGs hydrolysis, *Candida Antarctica*, has less textural and conformational changes on mesoporous carbon materials [42]. However, other enzymes like Candida Rugosa have shown greater activity in FOGs but lower long-term stability. Then, a lipase consortium could be co-immobilized in the same carrier to overcome the specific drawbacks of each enzyme and to exploit their synergy.

Different aspects of the hydrolysis of waste FOGs were presented mainly focusing in enzymatic catalyzed processes. An analysis of second-generation feedstocks, chemical routes, catalysts, process limitations, operating conditions for enzymatic processes, immobilization techniques, combined reactions, and current intensification approaches were covered. From the revised literature it was evidenced that the glycerol produced exerts an important effect in the whole reaction performance, on one hand glycerol at low concentrations partially prevents the leaching of the lipase because it counteracts the detergent action of fatty acids. But, on the other hand, higher glycerol concentrations could lead to enzyme inactivation, and even more important if some glycerol is removed the reaction equilibrium is shifted toward the products. For this reason, it is recommended to study the selective extraction of glycerol and to develop suitable methods to achieve this goal.

Appendix: Standard methodologies for vegetable oil characterize

A.1 Saponification value

Saponification value is based on ISO 3657 [91] standard method. Vegetable oil triglycerides reacts with an alcoholic solution of potassium hydroxide producing forming soaps or alkaline salts of fatty acids and glycerin, according to the following reaction.
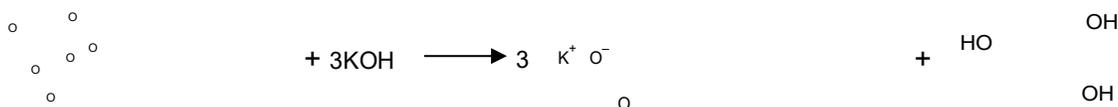
The method quantifies the amount of alkali moles utilized in the reaction by titration with HCl solution, using phenolphthalein as indicator. The saponification value is expressed in the number of milligrams of potassium hydroxide required to saponify 1 gram of vegetable oil. The saponification value is calculated using equation A.1.

$$SV = \frac{(V_1 - V_2) * C * 56.1}{m} \qquad\qquad A.1$$

Where V_1 and V_2 are blank and sample HCl volume, C is the concentration of HCl and m is the sample mass. Figure A.1 shows the procedure utilized to measure the saponification value for vegetable oils.
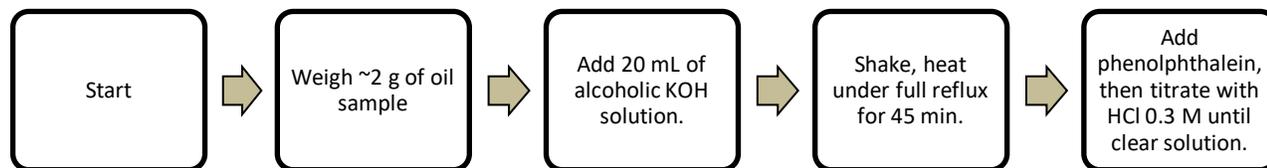
Figure A.1. Saponification value procedure

To calculate the blank volume, use the same procedure describe in figure A.1 without adding oil sample to alcoholic KOH solution.

Reagent preparation

1. Alcoholic KOH solution

   Weigh 1.683 g of KOH, then add 40 mL of anhydride ethanol absolute (99.5%), heat the solution at 40°C until KOH dissolves. If the solution cloudy it must be change due to water presence, otherwise, filtrate and storage the solution in an amber bottle. Solution must be prepared daily.

2. HCl solution (0.3 M)

   Add 12.5 ml of concentrated HCl (37%) to a 500 ml volumetric flask, complete the volume with distilled water. This process mut be performed in an extraction cabin.

3. NaOH solution (0.3 M)

   Weigh 12 g of NaOH, then add it to a 1000 mL volumetric flask, then, complete the volume with distilled water.

A.2 Acid value

Acid value is determinate following the standard method ISO 3961 [88]. Free fatty acids are neutralized with a solution of NaOH according to the following reaction.

HO   +NaOH $\longrightarrow$ Na$^+$ O$^-$ +H$_2$O

  O   O
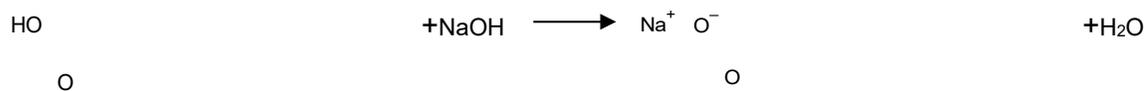
The oil is dissolved in ethanol and toluene, then the solution is titrated with NaOH. Acid value is expressed as milligrams of KOH per oil gram (equation A.2) or percentage basis of oleic acid (equation A.3).

$$AV = \frac{(V_1 - V_2) \; x \; C \; x \; 56.1}{m}$$ A.2

$$\%OA = \frac{(V_1 - V_2) \; x \; C \; x \; 28.2}{m}$$ A.3

Where V_1 and V_2 are sample and blank HCl volume, C is the concentration of HCl and m is the sample mass. Figure A.2 shows the procedure utilized to measure the acid value for vegetable oils.

| Start | | Weigh ~1.5 g of oil | | Add 20 mL of ethanol and 0.5 mL of Toluene | | Add phenolphthalein and shake the solution. | | Titrate with NaOH 0.01 M until clear solution. |

Figure A.2. Acid value procedure

To calculate the blank volume, use the same procedure describe in figure A.2 without adding oil sample to ethanol-toluene solution.
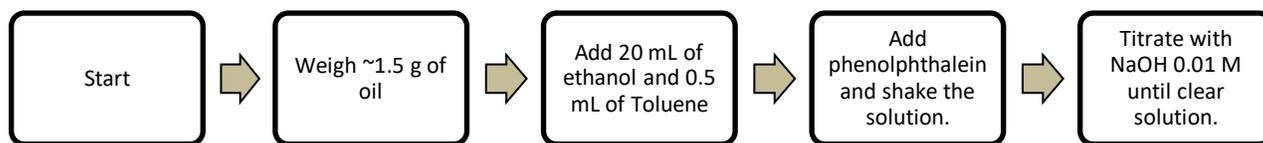
Reagent preparation

1. NaOH solution (0.01 M)

   Weigh 0.2 g of NaOH, then add it to a 500 mL volumetric flask, then, complete the volume with distilled water.

A.3 Iodine number

Determination of Iodine number in palm olein and UCO is performed by iodometric titration according to standard method ISO 3961 [90]. Oil unsaturations react with excess Wijs reagent, Wijs reagent has a concentration of 0.2 N. The excess of Wijs reagent reacts with potassium iodide to produce I_2. Molecular iodine is measured by titration with sodium thiosulfate 0.06N. Soluble starch (1% wt.) is used as indicator. The reactions involved are shown below.

$$ICl + KI \longrightarrow I_2 + KCl$$

$$I_2 + 2Na_2S_2O_4 \longrightarrow 2\ NaI + Na_2S_2O_6$$
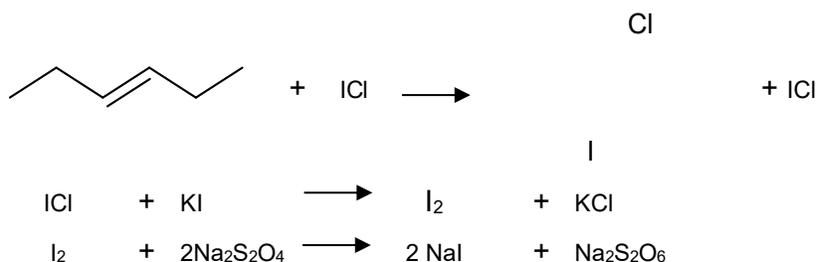
Iodine number is calculated using equation A.4, and it is reported as g I2 / 100 g oil.

$$IN = \frac{(V_1 - V_2)\ x\ C\ x\ 12.69}{m} \qquad\qquad A.4$$

Where V_1 is the blank volume of thiosulfate (ml) in blank, V_2 is the titration volume (ml) of thiosulfate, C is the concentration of thiosulfate (N), and m is the sample mass (g). Figure A.3 shows the procedure utilized to measure the iodine number in vegetable oils and epoxidized vegetable oils.

Start → Weigh ~0.06g of oil, add 5 ml of chloroform and 5 ml of Wijs → Leave in a dark place for 30 minutes, shake 3 times without incorporating air → Add 2.5 KI (15% wt.) and shake → Titrate with thiosulfate (0.06N) until light yellow → Add 1 mL of starch (the solution must turn dark purple), add dropwise thiosulfate until clear solution
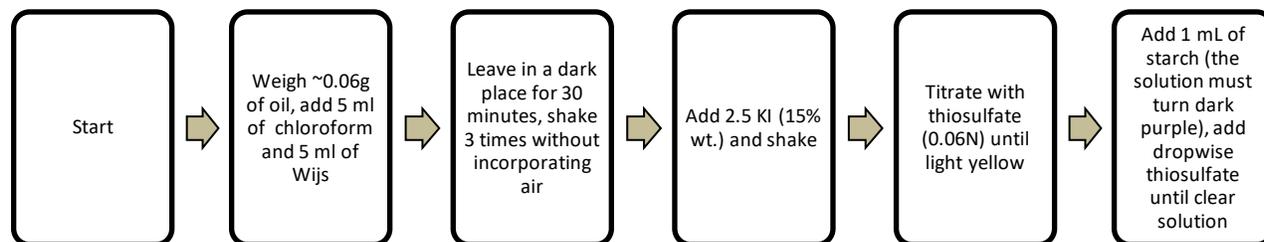
Figure A.3. Iodine number procedure

To calculate the blank volume, use the same procedure describe in figure A.3 without adding oil sample to chloroform-Wijs solution.

Reagent preparation

1. KI solution (15%)

    To prepare 100 g of solution, weigh 15 g of potassium iodide, then add 90 g of water, stir until complete dissolution.

2. Starch solution (1%)

    To prepare 50 mL of starch solution. Measure and heat to boiling point 45 mL of distillated water, meanwhile, dissolve 0.5 g of starch in 5 mL of distillated water at room temperature. Mix the starch solution with the hot water slowly with constant agitation. An emulsion should be formed, if not, the process should be repeated. This solution should be prepared daily and used when it is cold.

3. Potassium iodate solution (0.013 N)

    Weigh 0.07 g of KIO_3, add it to a 25 mL volumetric flask, complete the volume with distillated water.

4. Sodium thiosulfate (0.06 N)

    To prepare 500 mL of sodium thiosulfate. Weigh 7.5 g of $Na_2S_2O_4$ and 0.5 g of sodium carbonate. Add to a 500 mL volumetric flask and complete the volume with distillated water. This solution can be kept for a month.

A.6 Humidity

The humidity is determined following the standard method ISO 8534 [89], for this method Karl Fischer reagent is used that is a standard dissolution of iodine in water. It is Reagent is made of iodine, a base (pyridine) and SO_2 in a 1:3:10 ratio dissolved in methanol. In the reaction, 1 mole of SO_2 is oxidized for every mole of I_2 consumed. 1 mole of I_2 is consumed for each mole of H_2O, thus 2 moles of electrons are consumed for each mole of water. The whole reaction is presented below.

$$I_2 + SO^2 + CH_3OH + 3C_5H_5N + H_2O \longrightarrow 2C_5H_5NH^+I^- + C_5H_5NH^+SO_4CH_3^-$$

Humidity is expressed in mass percentage; it is calculated by equation A.7. The measurements were done Karl Fischer automatic titration equipment (Mettler Toledo Titrator Compact V10S)

$$H = \frac{V \; x \; \rho \; x \; 100}{m} \qquad\qquad\qquad A.7$$

Where V is the volume of the Karl Fischer reagent used (ml), ρ is the equivalent water in the used reagent (g/ml), m in the sample mass (g).

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