Universidade de Aveiro Departamento de Química

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, ramo Biotecnologia Industrial e Ambiental. Trabalho realizado sob a orientação científica do Professor Dr. João Manuel da Costa Araújo Pereira Coutinho, Professor Associado com agregação do Departamento de Química da Universidade de Aveiro e co-orientação da Dra. Sónia Patrícia Marques Ventura, Estagiária de Pós – Doutoramento da Universidade de Aveiro.

A todos os que me acompanharam nesta árdua caminhada, em especial aos **meus pais** pelo seu apoio incondicional…

o júri

presidente

Profª. Drª. Luísa Alexandra Seuanes Serafim Martins Leal

professora auxiliar convidada do Departamento de Química da Universidade de Aveiro

Prof. Dr. João Manuel da Costa Araújo Pereira Coutinho

professor associado com agregação do Departamento de Química da Universidade de Aveiro

Doutora Ana Paula Moura Tavares

investigadora do LSRE da Faculdade de Engenharia da Universidade do Porto

Doutora Sónia Patrícia Marques Ventura

estagiária de Pós-Doutoramento do Departamento de Química da Universidade de Aveiro

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Palavras-chave Líquidos iónicos, emulsões, estabilidade, CaLB, hidrólise de azeite comercial, superactividade

Resumo

O objectivo principal deste trabalho foi estudar a aplicação de líquidos iónicos de cadeia alquílica longa na formação de sistemas micelares, para melhorar a hidrólise enzimática de azeite comercial com o objectivo de produzir ácidos gordos de valor acrescentado. Este trabalho está dividido em duas partes. Na primeira, diferentes parâmetros, como o tipo e concentração de líquido iónicos e a razão volumétrica água/óleo, foram estudados com o objectivo de determinar as condições capazes de criar emulsões mais estáveis. A segunda parte incluiu a optimização das condições da reacção de hidrólise de forma a maximizar a produção dos ácidos gordos. Nesta fase, os parâmetros estudados foram a velocidade de agitação, a quantidade de enzima, a temperatura, o pH e a força iónica do sal usado para tamponizar a reação. Os resultados obtidos mostram que a actividade da CaLB aumentou 1.6 vezes, quando a reacção de hidrólise foi realizada a 314.2 (±0.1) K, 400 rpm, 2.08 mg proteina/mL e a formulação da emulsão era 15% azeite, 85 % (v/v) de tamão fosfato de sódio e 0.100 M de [C_{10}mim]Cl. Assim, a superactividade da lipase foi conseguida, embora o seu valor seja menor que o reportado em literatura para outros sistemas reacionais de hidrólise usando a CaLB.

Keywords Ionic liquids, emulsions, stability, CaLB, commercial olive oil hydrolysis, superactivity

Abstract The main objective of this work was to study the application of long alkyl chain ionic liquids in the formation of micellar systems to improve the enzymatic hydrolysis of a commercial olive oil to produce valuable fatty acids. This work was divided in two major parts. The first part addresses the optimization of the emulsion preparation, where different parameters were studied in order to determine the conditions capable to create the most stable emulsion, namely the ionic liquid type and concentration and different water/olive oil volumetric ratios. The second part includes the optimization of the hydrolysis reaction conditions in order to maximize the fatty acids production. Here, the stirring speed, the amount of enzyme added to the reaction systems, the temperature, the pH and the ionic strength of the buffer used to maintain the pH during all the reaction experiments, were the parameters studied. The results obtained showed that the CaLB activity increased 1.6 times, when the hydrolysis was performed at 314.2 (±0.1) K, 400 rpm, 2.08 mg protein/mL and the emulsion was composed of 15% of olive oil, 85 % (v/v) of sodium phosphate buffer and 0.100 M of [C_{10}mim]Cl. In summary, the lipase superactivity was achieved although its value was much lower than the reported in literature for other hydrolysis reactions using CaLB.

Contents

List of tables

List of Figures

List of abbreviations

AOT – bis(2-ethylhexyl)sulfosuccinate

SDS – sodium dodecyl sulphate

CTAB –cetyltrimethyl ammonium bromide

TTAB –tetradecyltrimethyl ammonium bromide

IL(s) – Ionic liquid(s)

[C_4mim][CF_3SO_3] – 1-butyl-3-methylimidazolium trifluoromethanesulfonate

[C_2mim][BF_4] – 1-ethyl-3-methylimidazolium tetrafluoroborate

CaLB–*Candida antarctica* lipase B

[$C_n$mim]$^+$ - 1-alkyl-3-methylimidazolium cations

[C_2mim]Br – 1- ethyl-3-methylimidazolium bromide

[C_{10}mim]Cl – 1-decyl-3-methylimidazolium chloride

CMC –critical micelle concentration

FFA – Free fatty acids

TFA – Total free fatty acids

List of symbols

EA –Emulsifying activity (in %)

V_w – Volume of white layer (in mL)

V_t – Volume total of emulsion (in mL)

V_s– Volume of KOH used to titrate the sample (in mL)

V_c– Volume of KOH used to titrate the control (in mL)

V_a– Volume of HCl used to titrate the sample (in mL)

V_b– Volume of HCl used to titrate the control (in mL)

M– Molarity of the KOH solution (in mol/L)

m_{oil} – Mass of olive oil (in g)

N – Molarity of the HCl used to titrate the excess of KOH (in mol/L)

I'_a – Acidity index (mmol/g)

I'_s – Saponification index (mmol/g)

U/mL – Enzymatic activity (in µmol of fatty acid per minute and mL of reaction mixture)

V_{reac} – Volume of emulsion used to perform the enzymatic assay (in mL)

t – Time of reaction (in min)

1. General Introduction

1.1. General context

The enzymes are proteins with ability to increase the reactions' rate by decreasing their activation energy, without changing the reaction equilibrium. Biocatalysis can successfully compete with chemical catalysis due to its attractive features such as high specificity promoting less side reactions and consequently reducing the amount of intermediate and contaminant products, with higher yields and, often, lower environmental impacts. Moreover, biocatalysis is considered a sustainable route[1] since the reactions are promoted at low or moderate temperatures, using preferably natural substrates and in the absence, or making use of small amounts, of toxic chemicals.[2] Until now, more than 13000 enzymes catalyzing reactions have been successfully developed in the laboratory scale[3], although the number of industrial processes is still limited.[4] The major barriers to the industrial use of enzymes are their low stability and high costs, along with the difficulty to identify the correct enzyme for a specific reaction. The major applications of enzymes are in food, pulp and paper, cosmetic and pharmaceutical industries, detergent manufacture and feed sectors as summarized in Table 1. Despite the large number of different enzymes, characterized by simple reaction mechanisms, lipases are one of the most used, not only in the laboratorial but also, at industrial scale.

Lipases (triacylglycerol hydrolases, E.C.3.1.1.3) are widely found in nature in microorganisms, animals and plants where their function is to digest lipids in order to make these available as an energy source for the cells.[5] Due to the large number of microorganisms (mainly from the *Rhizopus*, *Aspergillus*, *Mucor* and *Candida* genera) used to produce lipases by fermentation,[6] having a high production capacity and a simple purification, the number of available lipases with low costs[7] is increasing, making them good choices for a variety of studies and industrial applications. This class of enzymes is commonly known by their capacity to catalyze the hydrolysis of triglycerides to free fatty acids and glycerol. However, a number of other low- and high-molecular weight hydrophobic compounds, namely esters, thiol esters, amides, polyol/polyacid esters are also accepted as substrates by this unique class of enzymes. This class is also capable of efficiently process esterifications,[6,8] and transesterifications[9], where the alcoholysis, acidolysis and interesterification reactions are included.

Table 1 – Enzymes most used industrially[(a)].

Industry	Enzyme	Application
Food	Lipase	Enhanced cheese ripening and foam stabilization in baking
	Proteases	Reduce the protein composition in flour to baking industry and improve malt quality to brewing industry
	Glucose isomerases	Produce high fructose corn syrup by isomerization of glucose to fructose
Pulp and paper	Amylases	Cleave starch molecules to reduce the superficial viscosity
	Cellulases	Create weak spots in cellulose fibers to soften of the hydrolysis of cellulosic materials
	Lipases	Deink and control pitch in pulping processes
	Laccases	Bleach to improve brightness.
Cosmetic and pharmaceutical	Glucose oxidases	Toothpastes and mouthwashes
	Lysosymes	Disinfectant solutions for contact lens, as antibacterial
	Papain and bromelain	Moisturizer products that gives gentle peeling effects in skin care
	Disulfide isomerases	Hair waving products
Laundry detergents	Proteases	Hydrolyze protein-based stains in fabrics into soluble amino acids
	Lipases	Decompose fat of tough stains, such as oils, butter and sauces
	Cellulases	Modify the structure of cellulose fiber to increase the color brightness and soften the cotton
	Amylases	Removing resistant starch residues
Animal feeds	Xylanases	Degrade fiber in viscous diets
	α-amylases	Digeste starch
	Phytases	Degrade phytic acid to release phosphorus, calcium and magnesium
	Proteases	Degrade protein into its constituents to overcome anti-nutritional factors

(a) Adapted from Li et al.[10]

Due to their high versatility, lipases have a large range of potential applications such as in the leather[11,12] and the pulp and paper[13] processing, in the organic synthesis and production of biopolymers and flavors.[14–16] Furthermore, this class of enzymes has other applications, namely in detergent formulations (due to their lipid degradation potential),[13] in the production of single-isomer chiral drugs, food supplements and some fine chemicals (as a result of their regio-, stereo- and enantio- selectivity).[17] Moreover, lipases can be applied in the industrial wastewater treatment, mainly for the removal of fats, since they are capable of continuously hydrolyze the thin fat layers from the surface of aerated tanks allowing the oxygen transport.[18] More recently, lipases were applied in the transesterification of vegetable oils to produce biodiesel.[19–21] Finally, as described in the literature, lipases have a high affinity and performance in oil-water (substrate-media) interfaces when surface active agents, such as anionic[22,23], cationic[23,24] or zwitterionic surfactants[25–27] are applied, due to the formation of micellar systems. Those are responsible for a significant enhancement of the lipase activity, making these enzymes perfect biocatalysts for industrial applications, namely the hydrolysis of fats and oils.[28,29] This phenomenon of increasing enzyme activity is explained by structural rearrangements of the lipase active-site region, which show that, in presence of oil-water interfaces, the "lid" (structure around active site) is opened making the catalytic residues accessible to the substrate diffusion and exposing the large hydrophobic enzyme surface (stabilized by hydrophobic and electrostatic interactions).[30,31]

The higher activity and stability of some enzymes (including lipases) in different organic solvents at low water concentrations[32,33] was proved and thus, new biocatalytic processes can be performed. Furthermore, in the organic media, some undesirable side reactions caused by water can be reduced or even avoided.[32,34] However, the number of enzymes capable of carrying out reactions in an organic media is limited because these solvents sometimes impose the destabilization of the enzyme by disrupting their non-covalent interactions.[34,35] Moreover, as described in literature,[36] the application of organic solvents from the environmental point of view can be negative, since they are a source of pollution and VOC emissions[37] prompting the search for solvents with lower environmental impact, such as the supercritical fluids[38–40] or, more recently, ionic liquids.

Ionic liquids (ILs) are organic salts that are liquid at or near room temperature. By definition those salts have melting points lower than 100 ºC, and they are liquid at low temperature since they are composed by bulky asymmetric organic cations and small inorganic or organic anions[41]. These ionic compounds are considered as "*green solvents"*, since they have a large number of interesting and unique properties, which make them attractive alternatives to organic solvents. These properties include low vapor pressures, low flammability and high thermal and chemical stability.[42] These compounds are also known by their large liquidus range,[43] high ionic and thermal conductivities, and excellent solvent power for diverse substrates.[44,45] Additionally, ILs are often called *"designer solvents"*,[46] due to the large number of possible anion/cation/alkyl chain combinations allowing the manipulation of their physico-chemical, thermodynamics and also biological properties, by changing their structural characteristics.[47,48] This is an important characteristic, because it makes possible to design an IL for a specific reaction in order to improve its yield and selectivity, by increasing the substrate solubility or enzyme selectivity.[49] There are many different families of ILs that usually are based on their cation, as shown in Figure 1. Regarding the anion, it is more difficult to form categories however, they are normally divided in hydrophilic (chloride Cl, bromide Br, hydrogenosulphate [HSO_4], acetate [CH_3COO], among others) or hydrophobic (tetrafluoroborate [BF_4], hexafluorophosphate [PF_6], bis(trifluoromethylsulfonyl)imide [$(CF_3SO_2)_2N$]). Due to their interesting properties they have found application in numerous areas, namely the organic catalysis,[50–52] the polymerization,[53,54] in wastewater treatments,[55] in separation processes,[56] and biocatalysis.[41,49,57]

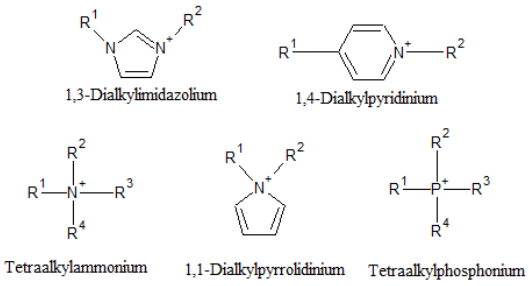

Figure 1 – Most common cations.

Recently there has been an increased interest in the use of ILs as media for diverse enzymatic reactions, because the stability and enzymatic activity parameters in these solvents are comparable or even higher than those observed in conventional organic solvents. Included in the enzyme classes already tested are the lipases from *Candida antarctica*[58,59] and *Candida rugosa*,[60] thermolysin,[61] lysozyme[62] and α-chymotripsin.[63] Many studies about the effect of ILs have been reported, most using hydrophobic compounds while considering a wide variety of enzymatic reactions. Schöfer and co-workers[64] have studied the transesterification of rac-1-phenylethanol with vinyl acetate by the application of nine distinct lipases and ten different ILs. *Candida antarctica* and *Pseudomonas sp.* lipases showed better conversion efficiencies in ILs, principally when the 1-butyl-3-methylimidazolium triflate [C_4mim][CF_3SO_3] was applied. Moreover, Dang et al.[65] observed that the increasing of 1-ethyl-3-methylimidazolium tetrafluoroborate [C_2mim][BF_4] concentration can decrease or increase the activity and stability of *Mucorjavanias* lipase, depending of the salt added to the reaction mixture or the lipase' pretreatment applied. Considering that the number of hydrophilic ILs is much larger than the hydrophobic, we have recently studied their effect on the hydrolysis of p-nitrophenyl-laurate catalyzed by *Candida antarctica* lipase B (CaLB).[66] Although the effect of the hydrophilic ILs studied in the reaction performance varies widely, the main conclusions are that the enzyme activity is decreased after the contact with diverse hydrophilic ILs.[66] Moreover, the increase in the ILs' concentration showed a negative effect on the lipase activity, although for most ILs these negative effects are smaller than the deleterious effects promoted by the presence of common organic solvents. These, and a number of other works in literature,[66–68] suggest that the increase in the alkyl chain (from C_2 to C_8) of the cation leads to a decrease in the enzyme activity and stability parameters.

Nevertheless, the inherent amphiphilic nature of some cations responsible for the interfacial and aggregation phenomena in aqueous solution is an important issue that ultimately can drive micelle formation with a specific structure, shape and properties. According to literature,[69–72] depending on the amount of water in the medium, not only the surfactants but also the ILs can form "direct" or "reverse" micelles. "Direct" micelles, corresponding to oil-in-water emulsions, can be defined as structures with the polar heads of the surfactant or IL in contact with the bulk water molecules and the nonpolar tails oriented towards the inner part of the micelle structure. When the bulk

medium has an organic nature, the hydrophilic heads are associated themselves with the water droplets and the hydrophobic tails are oriented towards the organic medium, being formed "reversed" micelles, generating water-in-oil emulsions. There are some studies that describe the application of ILs to enhance the enzymatic activity in micellar systems, typically by using "reverse" micelles.[73–75] Nevertheless, despite the significant number of papers describing the possibility of forming micelles using ILs with long alkyl chains, our group was the first to investigate the applicability of "direct" micelles formed by ILs. Thus, we reported that certain ILs can be used as surfactants, forming aggregates in presence of water and that the presence of these aggregates ("direct" micelles) is the responsible for the increase in the enzyme activity, a phenomenon known as "superactivity".[76] In that work, the application of the 1-decyl-3-methylimidazolium chloride, [C_{10}mim]Cl, promoted a 6-fold increase in CaLB activity. The increment on activity was then explained by the formation of microemulsions due to the IL surface activity. The microemulsion provided an enhanced contact between the substrate molecules and the enzyme active site, due to the large increase of the interfacial area.[76]

The aim of this work is to develop a process for the hydrolysis of vegetable oils to produce fatty acids, with high yields, using the IL induced "superactivity". This process was chosen because of the large number of potential applications of the fatty acids, especially in the production of detergents/surfactants,[77] in chemical,[78,79] cosmetic,[80] pharmaceutical and food industry.[81] Fatty acids are usually obtained by a physicochemical process (fat-splitting) involving aggressive conditions (temperatures around 250ºC and pressures between 30-60 atm) resulting in a more unstable and less pure product. Consequently, various purification steps are needed, as well as much energy and reactors with strong materials, which have high costs.[82] Alternatively, the enzymatic process promises to conserve energy and minimize thermal degradation, generating fatty acids from unstable oils containing conjugated or highly unsaturated fatty acid residues.[83] Their production by lipases is considered an advantageous process, because it is conducted under relatively mild conditions (temperatures between 35-60ºC and ambient pressures), justifying lower costs and generating products with higher purities than those obtained conventionally, due to the absence of side reactions.[84]

In this context, an enzymatic process was adopted to produce the fatty acids, but long alkyl chain ILs will be used as surfactants, to create stable emulsions which will be

used to promote the increase in the CaLB activity (used as the biocatalyst of the hydrolysis reaction). CaLB (Figure 2)[85] was here adopted because it is a good example of a largely used lipase, due to their high stability, even when compared with other lipases. Furthermore this enzyme is a valuable scientific and industrial tool because it doesn't require a hydration shell to be active, and has a quite relaxed substrate specificity and good operational stability including in ILs.[45,64,86–88]

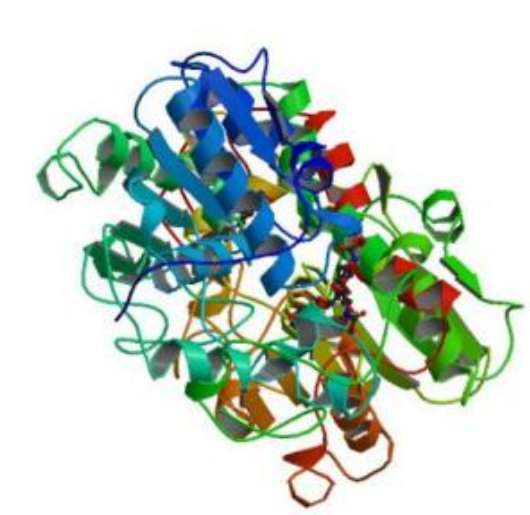

Figure 2 – Structure of lipase B from *Candida antarctica*.

An olive oil with low acidity was chosen as substrate in this case study, because it is composed by some important fatty acids and also due to its high availability in the Portuguese market. The fatty acids found in abundance in the olive oil are oleic acid (55-83%), linoleic acid (3.5-21%) and palmitic acid (7.5-20%).[89] Oleic acid is largely used in different areas, namely as an emollient in cosmetic industry,[90] an excipient[*] in pharmaceuticals,[91,92] and also as emulsifying agent of soap (as a sodium salt). The palmitic acid is mainly used to produce soaps, cosmetics and release agents. More recently, it has been found that it can be used as an anti-inflammatory agent,[93] being its use in the pharmaceutical industry nowadays a possibility. Finally, the linoleic acid is an essential fatty acid (humans cannot synthesize) mainly used in food supplements.[94]

This work will be investigating the enzymatic hydrolysis of olive oil on a heterogeneous system containing water and the oil as sketched in Figure 3. The main

[*]Excipient – pharmacologically inactive substance used as a carrier for the active substance, when the latter is not easily absorbed by the human body.

idea is to use surface active agents, in this specific case ILs, to form "direct" or "reverse" micelles in order to enhance lipase activity and increase the fatty acids' production. The formation of "reverse" or "direct" micelles will be one of the factors in study, through the manipulation of the oil/water ratio in order to optimize the emulsion systems. Together with the oil/water ratio, other parameters will be controlled, namely the type and concentration of surfactant (IL), the emulsion stability. After the optimization of the emulsion system, a kinetic study of lipase action in the media will be carried, in order to maximize the production of fatty acids.

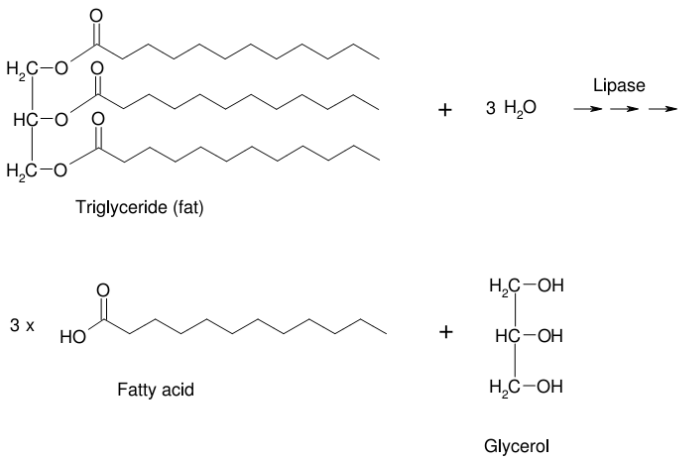

Figure 3 – Fatty acid production by enzymatic hydrolysis of triglycerides.[95]

1.2. Scope and objectives

Pioneering studies have already demonstrated the potential of long chain ionic liquids to the formation of micellar systems with the capacity to significantly improve the enzymatic activity of a lipase, a phenomenon known as lipase superactivity. This phenomenon was shown to be crucial in the hydrolysis of p-nitrophenyl-laurate, a reaction catalyzed by *Candida antarctica* lipase B (here abbreviated by CaLB). In fact, in a previous work the enzyme activity was increased 6 times in the presence of 1-decyl-3-methylimidazolium chloride, $[C_{10}mim]Cl$, due to the self-aggregation of the ionic liquid long alkyl chains.

In this context, the main objective of this work is to study the application of long chain ionic liquids in the formation of micellar systems to promote the enzyme superactivity, aiming at applying this concept to some reactions of industrial interest. In this work, the phenomenon of superactivity was applied to the hydrolysis of vegetable oils to produce valuable fatty acids. An olive oil with low acidity was chosen as substrate because it is composed by some interesting fatty acids, as describe previously. Thus, this work will be divided in three major parts described below.

The first part includes the optimization of the emulsion preparation, where different parameters will be studied in order to determine the conditions capable to create the most stable emulsion. The parameters studied will be the IL type and concentration, the water/olive oil volumetric ratio, the type and pH of the buffer solution and finally, the temperature.

The second part addresses the optimization of the hydrolysis conditions in order to maximize the reaction yield. Here, the amount of enzyme added, the stirring speed, temperature and pH of the buffers used, will be the parameters studied in terms of the reaction kinetic.

2. Experimental section

2.1. Chemicals

Trihexyl(tetradecyl)phosphonium chloride ([$P_{6,6,6,14}$]Cl) was supplied by CYTEC (Cyphos IL 101 phosphonium salt) and the 1-decyl-3-methylimidazolium chloride ([C_{10}mim]Cl) acquired at Iolitec (Ionic Liquid Technologies). The olive oil (Rosmaninho Virgem Extra) with low acid content (0.3%) produced by Cooperativa de Olivicultores de Valpaços, was purchased from a local market at Aveiro. Disodium phosphate dodecahydrate (≥99.0%) and the potassium phosphate monobasic (≥99.5%) were purchased from Sigma-Aldrich, sodium hydroxide (≥98.0%) from Eka Chemicals, pure potassium hydroxide from Pronolab, pure citric acid , ethanol and acetone (99.99%) were purchased at Fisher Scientific and, finally the hydrochloric acid (37%) was acquired at Fisher Chemical. The remaining chemicals used in this study are of analytical grade.

The enzyme used was the *Candida antarctica* lipase B (EC 3.1.1.3), here abbreviated as CaLB, which was kindly offered by the Novozymes company. This enzyme is produced in a submerged fermentation of a genetically modified microorganism, *Aspergillus niger*, and it has a specific activity of 5000 LU/g of enzymatic solution. One mL of enzymatic solution has 27.7 mg of enzyme.

2.2. Experimental procedure

2.2.1. Ionic liquid neutralization

Due to the high initial level of acids found on the ([$P_{6,6,6,14}$]Cl), a previous step of neutralization and drying before its application in the reaction system, was required. The neutralization was carried by the addition of a sodium hydroxide (NaOH) solution (0.1 M) to the IL being the mixture under constant stirring for a minimum of 24 h. Afterwards, the water and the IL phases were separated and the pH of each phase was measured. This procedure is repeated until the pH of the IL phase becomes neutral, pH 7, being thus the water phase removed. Posteriorly, the IL phase was dried under constant stirring at a moderate temperature (≈323 ±0.5 K) and by the application of high vacuum (≈10^{-1} Pa), for at least 48 h. At the end of this neutralization procedure, the purity of IL was further checked by ^{1}H, ^{13}C and ^{31}P NMR.

2.2.2. Emulsion optimization

2.2.2.1. Emulsion preparation

In order to prepare the emulsion, 10 mL of olive oil and 10 mL of an aqueous solution of each IL (0.250 M) were mixed using an ultrasonic homogenizer equipment (Sonics – Vibra Cell^{TM}, 130 watts), for 10 minutes at 20 W. During the emulsion preparation, the samples were kept in an ice bath. To optimize the preparation of the emulsions, several volumetric water/olive oil ratios [from 1 % to 50 % (v/v)], IL concentrations (from 0.020 M to 0.425 M), and buffer solutions (sodium phosphate buffer or Mcllvaine buffer) or water were investigated and adjusted.

2.2.2.2. Emulsification activity determination

After preparation of each emulsion system, 10 mL were transferred into a test tube and centrifuged at 4200 g and 298.15 and 298.15 ± 1.0 K, in a Thermo Scientific, Heraeus Megafuge 16 R. After 15 minutes, the emulsified white layer volume (V_w in ml) and total volume (V_t in ml) were determined, and the emulsification activity (EA) was calculated by the following equation:[96]

$$EA = \frac{V_w}{V_t} \times 100 \qquad (1)$$

2.2.2.3. Viscosity measurements

The viscosity of the emulsions with different water contents were carried out in the temperature range from 298.15 K to 324.15 K, at atmospheric pressure using an Anton Paar (model SVM 3000) automated rotational Stabinger viscometer-densimeter. The temperature uncertainty is ± 0.02 K in the temperature range analyzed and the relative uncertainty of the dynamic viscosity is ± 0.35%. Further details about the equipment and method can be found elsewhere.[97]

2.2.2.4. Phase inversion determination

A stainless steel electrode was connected to a conductimeter to measure the conductivity of the emulsions. Olive oil conductivity was 68 μS/cm and the conductivity of the buffer solution was 20 mS/cm. Therefore, a significant variation in

the conductivity could be observed with phase inversion. A fresh emulsion was made for each oil composition tested.

2.2.3. Hydrolysis kinetic study

2.2.3.1. Free fatty acids

750 mg of olive oil were titrated using KOH solution (0.500 M), and phenolphthalein as the acid-base indicator. The amount of free fatty acids, presented in the olive oil was then calculated by the following equation:

$$FFA\ (mmol/g) = \frac{V_s \times M}{m_{oil}} \quad (2)$$

where V_s represents the volume of KOH used to titrate the sample (in mL), M represents the molality of the KOH solution (in mol/L) and m_{oil} represents the mass of olive oil (in g) used.

2.2.3.2. Saponification of olive oil

500 mg of olive oil were mixed with 7.5 mL of an alcoholic KOH solution (0.500 M), in a boiling flask, being the mixture boiled for 30 minutes with reflux. At the end, the flask was immediately cooled and the remaining potassium hydroxide (KOH) was titrated using HCl (0.500 M) and phenolphthalein as the acid-base indicator. A control sample without oil is also prepared to serve as a blank. The total amount of fatty acids, *TFA*, presented in the olive oil was then calculated by the following equation:

$$Total\ fatty\ acids\ (mmol/g) = \frac{(V_b - V_a) \times N_{HCl}}{m_{oil}} \quad (3)$$

where, V_b and V_a represent the volume of HCl used to titrate the black and sample (in mL), respectively, N represents the molality of the HCl solution (in mol/L) and m represents the mass of olive oil (in g) added.

2.2.3.3. Time course of reaction

The hydrolysis was carried out in several 20 mL glass bottles at 310.15 ± 0.1K under constant stirring at 100 rpm, using an incubator shaker - IKA® KS 4000 ic control. Each hydrolysis reaction was carried out using emulsions (total volume of 25 mL) previously prepared for 20 minutes in the ultrasonic homogenizer at 20 W. The

reaction was initiated by adding 25 µL of lipase (CaLB) into 1 mL of the emulsion. After *x* minutes of reaction, the samples were removed from the incubator and 7 mL of an acetone-water-ethanol solution (1:1:1) is added to stop the hydrolysis reaction. Then, the amount of free fatty acids produced and released in the reaction bulk was estimated by a titration, using a KOH solution (0.5 M) and phenolphthalein as the acid-base indicator. Each experiment was performed in triplicate.

The course of reaction was expressed as percentage of hydrolysis versus time. The hydrolysis' percentage was calculated from the acidity ratio and the total amount of fatty acids.

$$\%\, Hydrolysis = \frac{I'_a \times 100}{I'_s} \quad (4)$$

The acidity index, I'_a, represents the amount of KOH (mmol) necessary to neutralize the fatty acids released from 1 g of olive oil, and its gives by the following equation:

$$I'_a = I_a(t) - I_a(t=0) \quad (5)$$

with, $$I_a = \frac{V_{KOH} \times M}{m_{oil}} \quad (6)$$

And the saponification index, I'_s, is defined as the quantity of KOH (mmol) necessary to saponify the esters and neutralize the released fatty acids per gram of olive oil.

$$I'_s = TFA - FFA \quad (7)$$

For determination of the initial rates, the slope at the origin of the fatty acids concentration against the time was estimated. For this, straight-line adjustments (by the least-squares method) were made of an increasing number of experimental points, until the value of the slope of the straight lines began to decrease or coefficient of determination, R squared, become less than 0.9.

2.2.3.4. Lipase activity determination

The hydrolysis reaction was carried out in 20 mL glass bottles at 310.15 ±0.1K and under constant stirring at 100 rpm, using an incubator shaker - IKA® KS 4000 ic control. The reaction was initiated by adding 125 µL of lipase (or water in the control

system) into the reaction mixture composed of 5 mL of the emulsion previously prepared. After *x* min (according to linear region), 350 µL of the reaction media was collected and placed in an Erlenmeyer containing 2 mL of an acetone-water-ethanol solution (1:1:1) to completely stop the reaction. Then, the amount of free fatty acids released in the reaction mixture was estimated by titration, using a KOH aqueous solution (0.500 M) and phenolphthalein as the acid-base indicator. Each experiment was performed in triplicate.[98]

One unit of lipase activity was defined as the amount of enzyme which liberated 1 µmol of fatty acid per minute at assay conditions. In this work, the CaLB activity was calculated by the following equation:

$$U/mL = \frac{(V_s/V_{reac} - V_c/V_{reac}) \times M \times 1000}{t} \quad (8)$$

where, V_s and V_c represent the volume of KOH used to titrate the sample and the control (in mL), respectively, M represents the molality of the KOH solution (in mol/L), t corresponds to the time of reaction (in minutes), V_{reac} represents the volume of the reaction solution in mL (sample or control).

In this work, when a comparison between enzymatic activity in ionic liquids and in buffer systems, the results are reported in relative activity.

$$Relative\ activity = \frac{Enzymatic\ activity\ in\ systems\ with\ IL}{Enzymatic\ activity\ in\ systems\ without\ IL} \quad (9)$$

3. Emulsion optimization

3.1. Results and Discussion

In general, the emulsions are thermodynamically unstable systems and have a tendency to break down over the time, though a variety of different mechanisms, namely creaming, coalescence and aggregation. However, to increase the reaction rates, the emulsion should be stable at least during the entire reaction time. In this context, the use of ILs as surfactants is proposed and, consequently, the study of their effects on the emulsion stability (the capacity of an emulsion to maintain its properties during the reaction time) is required. There are a number of factors that should be controlled aiming keeping or even increase its stability. Among in these factors are the water/oil ratio, the pH and ionic strength of the salt additive and the temperature.

In order to optimize the emulsion preparation, the variation/effect of several parameters was investigated, namely the IL concentration, the water/olive oil volumetric ratio, the pH and ionic strength by the addition of different buffers and finally, the temperature. Despite the large number of techniques to determine the emulsion stability[99], in this work, the emulsification activity essay described by Pearce and Kinsella, was applied since this is considered an accurate, fast and simple methodolgy.[96]

3.1.1. ILs concentration

The first parameter studied was the IL family and the IL concentration. Considering previous results obtained by our group in the study of the ILs surfactant capacity, we started this study using the $[P_{6,6,6,14}]Cl$[100] and $[C_{10}mim]Cl$.[76] These two ILs allow a comparison between two different families: the phosphonium and imidazolium, with distinct natures, being the first non-aromatic and the second aromatic and cyclic. The IL concentrations studied were comprised between 0.020 and 0.425 M (concentrations around the critical micelle concentration, CMC).

In the specific case of $[P_{6,6,6,14}]Cl$, a neutralization step was required due to the high acid content detected in the IL sample. Thus, the emulsification activity was studied having into account the imidazolium and phosphonium after and before neutralization (Figure 4). The emulsification activities of these ILs in various concentrations are also provided in Figure 4.

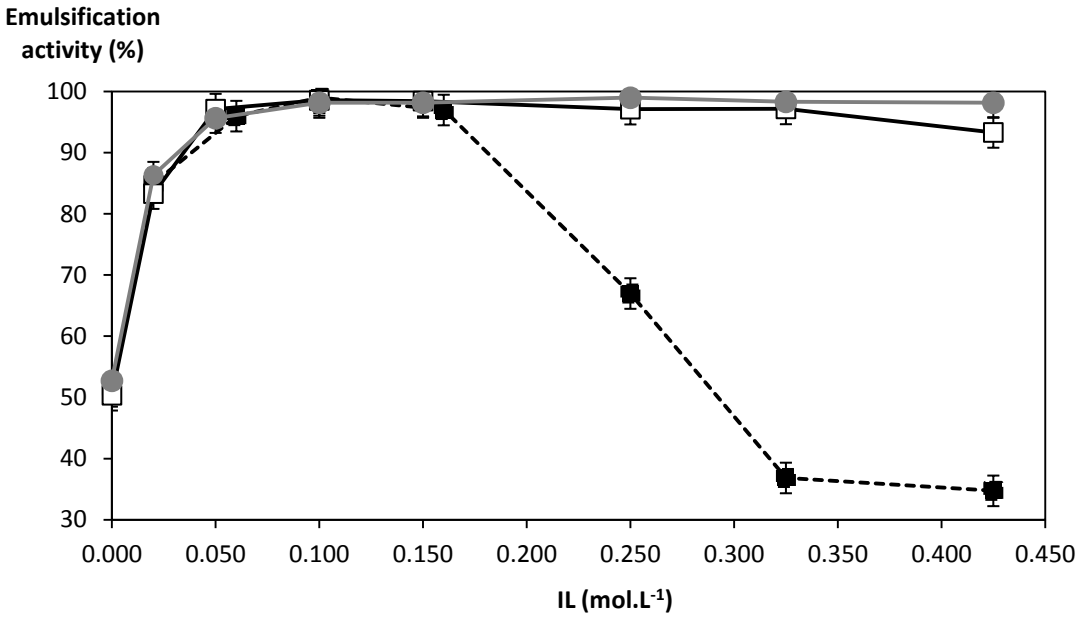

Figure 4 – Effect of the IL concentration (M) on the emulsification activity; ■ [$P_{6,6,6,14}$]Cl before neutralization, □ [$P_{6,6,6,14}$]Cl after neutralization and ●[C_{10}mim]Cl. The lines represented are only a guide for the eye.

The results suggest that a higher emulsification activity is observed when the ILs concentration is around 0.100-0.150M, independently of the IL. The decrease in the emulsification activity observed for concentrations higher than 0.160 M for the [$P_{6,6,6,14}$]Cl before neutralization is explained by the presence of some impurities, mainly acids, probably original from the synthesis of the IL. However, considering the imidazolium and the phosphonium in its neutral form, it is possible to see the absence of negative effects, which means that the emulsification activity is maintained high and constant for all the ILs concentration studied. Considering these results, the IL concentration applied in the following studies is 0.100 M for both ILs.

3.1.2. Water/olive oil volumetric ratio

Considering the optimum IL concentration, the effect of different water/olive oil volumetric ratios, from 1 to 50 % of water (v/v) was tested in the emulsification activity being the results depicted in Figure 5. One of the goals of this work was to work with low water contents (less than 50%), because we intend to work with reverse micellar systems and also because the main substrate is the olive oil. Moreover, when water is in excess an extra step of recovery and purification of the fatty acids is required.

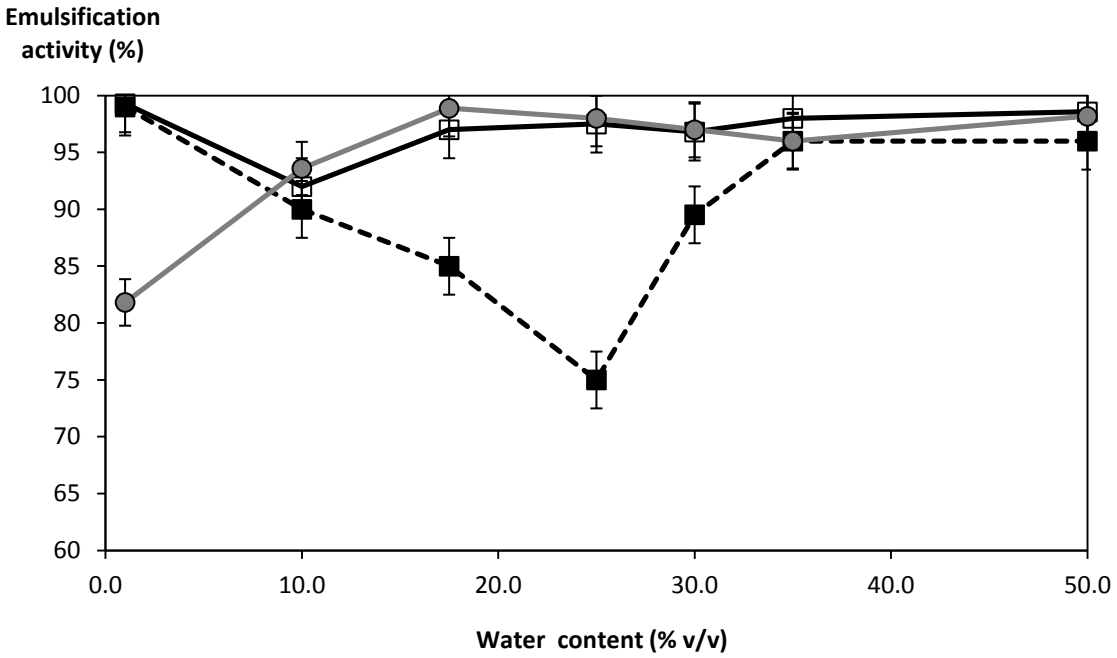

Figure 5 – Emulsification activity as a function of the water content (% v/v) presented in the emulsion formulation; ■ [$P_{6,6,6,14}$]Cl (0.100 M) before neutralization, □ [$P_{6,6,6,14}$]Cl (0.100 M) after neutralization and ● [C_{10}mim]Cl (0.100 M). The lines represented are only a guide for the eye.

Figure 5 shows the effect of the water concentration on the emulsification activity of the different IL systems. Depending on the surfactant, the effect of the water content is different. When the [$P_{6,6,6,14}$]Cl is used, the emulsification activity presents a decrease until 10 % (v/v) of water, followed by an increase until 50 % (v/v) of water. This behavior may be explained by the micelle dispersion through the system. In fact, when lower water contents are presented, free reverse micelles are dispersed in the oil bulk, but with the addition of water, the free micelles are converted in microemulsions. This change is easily detected with naked eye, because the solution with lower water contents, in this specific case is turbid and yellow, and with higher water content the solution becomes opaque and white.

Regarding the [C_{10}mim]Cl results depicted in Figure 5, the emulsification activity increases with the increase in the water content, showing a maximum at 17.5 % (v/v) of water. Above this concentration the water content has no significant effect on the emulsification activity, which is justified by the high viscosity of these emulsions, as shown in Figure 6. The viscosity increases with the water content due to the increase of the number of electrostatic interactions (e.g. hydrogen bonds), leading to a decrease in the molecular distances observed in the emulsion system, altered its stability.[101]

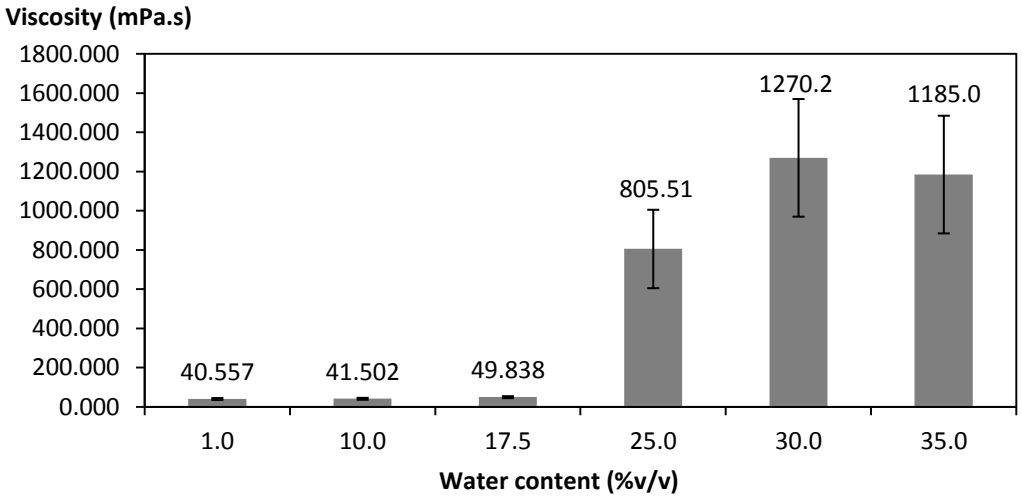

Figure 6 – Viscosity of emulsions as a function of water content (T=310.14 K, reaction temperature).

Initially it was intended to determine the droplet size of each water percentage emulsion using the ZetaSizer, to understand how the water/oil ratio can change the size of the micelles water pool, because it was been proposed that the optimum enzymatic activity occurs around a water content at which the size of micelles is similar to the size of the enzyme.[102] However it wasn't possible to determine the droplet size with DLS measurements due color and high polydispersity of the sample.

According to the emulsification activity results and taken into account that, for a complete hydrolysis 0.060 mL of water/mL of olive oil is necessary. A water content of 10.0 and 17.5 % will then be tested in the kinetic study. In the following study, where the ionic strength and the pH effects are described, 10.0 % of water will be used.

3.1.3. Ionic strength and pH

In this part of the investigation, the effect of the ionic strength and the pH, in the emulsification activity, using different salt additives, is tested. To study the ionic strength two buffer solutions [potassium phosphate (0.100 M) and Mcllvaine (0.150 M)] were used and compared with distilled water. On the other hand, the pH effect was investigated using four different pH solutions (pH 5, 6 7 and 8) based in the Mcllvaine buffer.

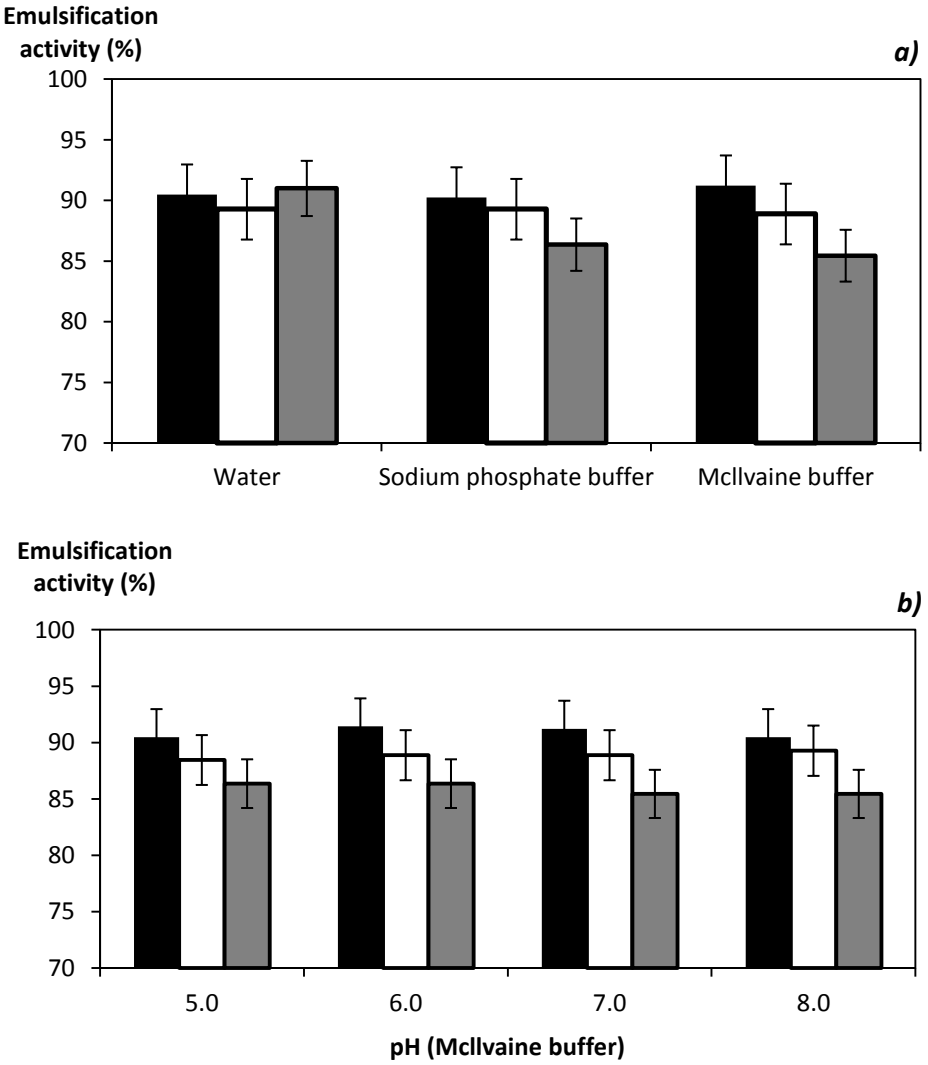

Figure 7 - Emulsification activity dependence with the *a)* ionic strength, *b)* pH using the McIlvaine buffer; ■ $[P_{6,6,6,14}]Cl$ after neutralization, □ $[P_{6,6,6,14}]Cl$ before neutralization and ■ $[C_{10}mim]Cl$.

Figure 7 suggests that the ionic strength and the pH of the aqueous phase have no significant effect (<0.025) on the emulsion stability. In the following study, where the temperature effect is described, water will be used as the aqueous phase and the optimization of pH and the type of buffer applied will be performed posteriorly in the kinetic study.

3.1.4. Temperature

Here, the temperature effect on the emulsion stability is studied in the range 298.15-313.15 (±0.50 K). Usually the temperature is responsible for the destruction of the emulsions, by its effect on the surfactant solubility in the common solvents.[103]

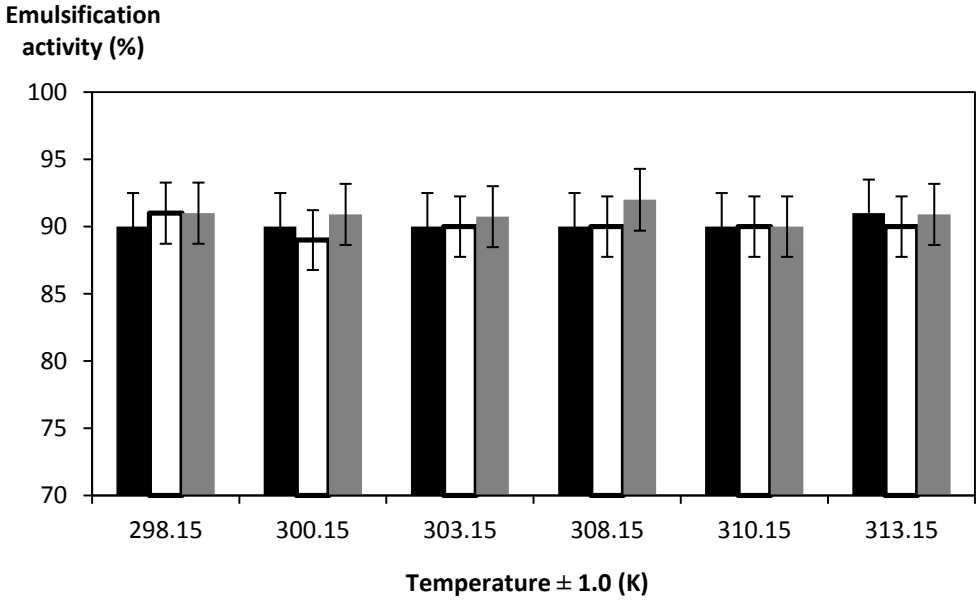

Figure 8 – Influence of temperature on the emulsification activity; ■ [$P_{6,6,6,14}$]Cl after neutralization, □ [$P_{6,6,6,14}$]Cl before neutralization and ■ [C_{10}mim]Cl.

Figure 8 shows that the effect of temperature on the emulsification activity is not significant (<0.025), as reported by Dickinson et al.[104] This result is explained by the fact that the solubility of the ILs is not affected by the temperature in the range of temperatures studied. According to these results the temperature optimization will be performed in the kinetic study.

3.1.5. Water content

The effect of the water content was studied in terms of the emulsion stability, because it was proved that low water contents are not capable to promote the superactivity phenomenon (results discussed in the next chapter – 4). It must be remarked that these results are quite different from those shown in Figure 5, due a

technical problem in the ultrasounds equipment. However, they are also relevant or even more relevant than the results aforementioned, since the enzymatic assays were performed in emulsions created when the ultrasounds processer had problems.

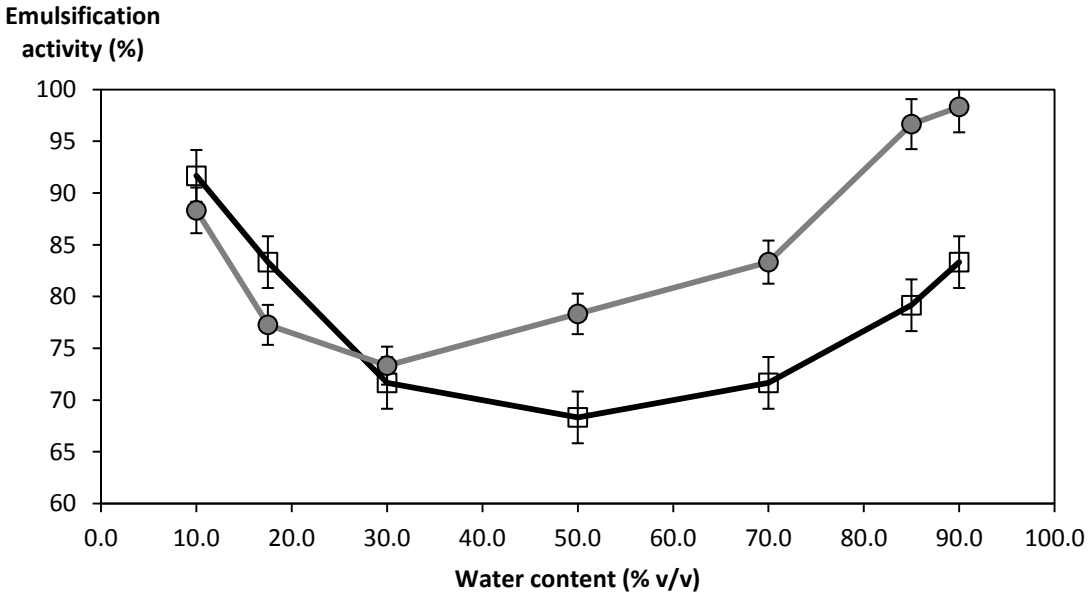

Figure 9 – Emulsification activity as a function of the water content (% v/v) presented in the emulsion formulation; □ [$P_{6,6,6,14}$]Cl (0.100 M) after neutralization and ●[C_{10}mim]Cl (0.100 M). The lines represented are only guides for the eye.

The results from Figure 9 indicate that the emulsion stability is strongly dependent of the emulsion formulation and has a bell shape behavior, *i.e.* the stability decreases with the increase of the water content until 50% (v/v), and then the stability increases with the increase in the water amount. These results suggest that emulsions with high stability are obtained when one of the two constituents is present in larger amounts. A simple justification for this behavior is the fact that in the intermediate cases occur the phase inversion, as shown in Figure 10 by conductivity measurements.

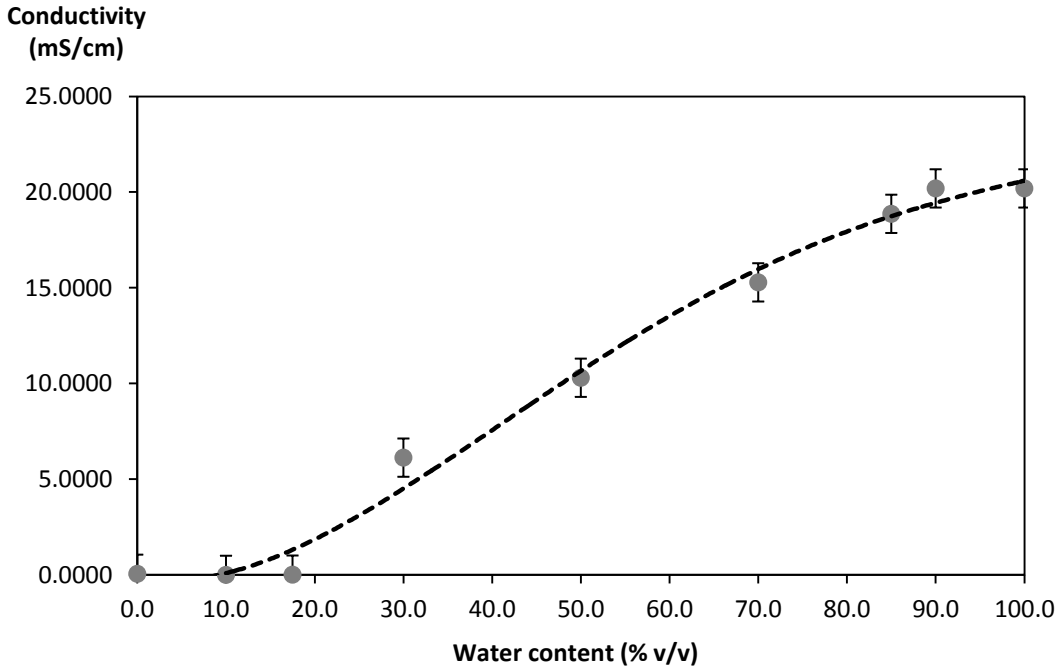

Figure 10 – Determination of the phase inversion: oil-in-water emulsion < 20.0 % (v/v) of water; water-in-oil emulsion > 80.0 % (v/v) of water. The line represented is only a guide for the eye.

3.2. Conclusions

In this work, several parameters were studied in order to obtain a high stability of the emulsions prepared, by the optimization of the parameters considered in the emulsion formulations. Thus, different conditions were studied, namely the surfactant or IL concentration (with an optimum value of 0.100 M), the water/olive oil volumetric ratio, being the optimal case found for the extreme conditions, high water amounts and low oil content and vice-versa. However, in what concerns the temperature and buffer type and pH conditions, it was not possible to identify an optimal value. In fact, it was here concluded that these variables have no visible effect in the emulsion stability, and in this context they will be only investigated in the enzymatic assays, considering the reaction performance.

4. Hydrolysis kinetics study

4.1. Results and discussion

The main goal of this study is to optimize the hydrolysis of a commercial olive oil with low acid content (0.3%) aiming to produce large amounts of fatty acids. In this context, we intent to promote the superactivity phenomenon, using an enzyme, the CaLB and micellar systems based in water-in-oil microemulsions, or in other words, based in reverse micelles. To do that, the first step is the characterization of the olive oil, the second step will be the optimization of several conditions considering the hydrolysis performance and the third step is described by the use two different IL families to promote the superactivity.

4.1.1. Preliminary experiments

In the beginning of this work, some preliminary experiments were done in order to evaluate the viability of $[P_{6,6,6,14}]Cl$ to improve the CaLB activity. However, in these initial assays, only a control system (without enzyme) was considered following the original experimental procedure described in literature[98] (condition 1 and 2 from Table 2). By an analysis of these results it was concluded that the acyclic IL $[P_{6,6,6,14}]Cl$ has a high capacity to superactivate CaLB.

Table 2 – Initial results of the enzymatic activity, following the standard protocol.[98]

Condition	Control	FFA (mmol/mL)	FFA mean ± std	Sample	FFA (mmol/mL)	Activity (U/mL)
1	Buffer	0.107	0.100 ± 0.0118	**Buffer**	0.125	4.582
		0.086			0.123	4.216
		0.106			0.129	5.034
2	Buffer	0.107	0.100 ± 0.0118	**$[P_{6,6,6,14}]Cl$**	0.305	36.846
		0.086			0.301	36.169
		0.106			0.316	38.938
3	$[P_{6,6,6,14}]Cl$	0.272	0.267 ± 0.0163	**$[P_{6,6,6,14}]Cl$**	0.305	6.717
		0.280			0.301	6.040
		0.249			0.316	8.810

Meanwhile, after a careful analysis of this experimental procedure considering our specific case, it was verified the need to perform a more detailed control for each emulsion system (absence and presence of ILs), which is described by the condition 3 from Table 2. Thus, a different control was used, where the IL is also added, to prevent

any interference of potential contaminants present in the IL bulk. In fact, after a detailed analysis of our preliminary results, it was proved the presence of some acids in the IL, which easily justifies the significant difference between the results using the control with and without $[P_{6,6,6,14}]Cl$ – Table 2.

In this context, the study of two additional parameters was performed aiming to test and understand the big difference between the results aforementioned, the acid factor as a function of time (Figure 11) and the pH of $[P_{6,6,6,14}]Cl$.

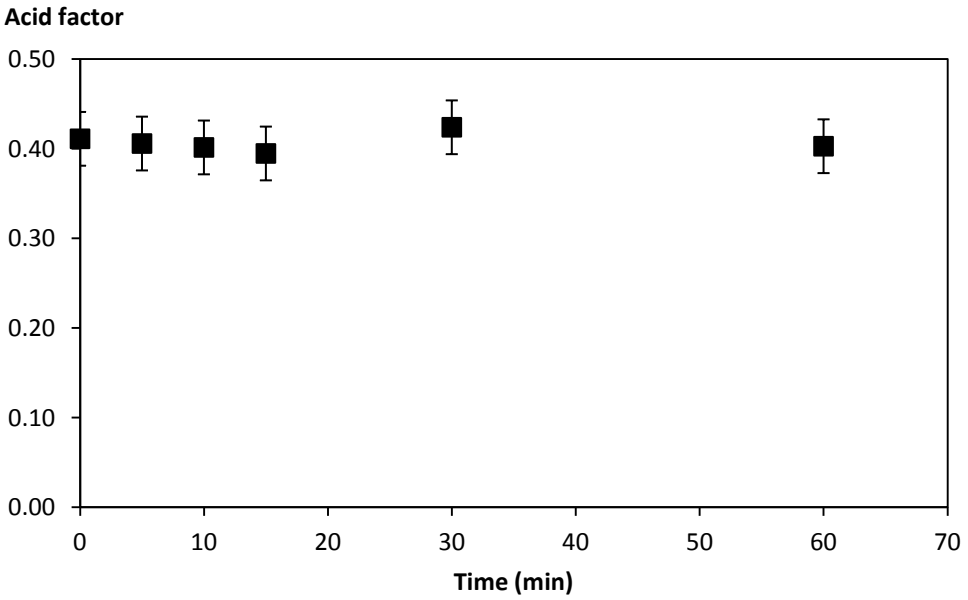

Figure 11 – Acid factor of systems with $[P_{6,6,6,14}]Cl$ as a function of time.

As it is observed in Figure 11, the amount of acids quantified in the IL systems does not present any significant variation with time, which shows that, not only the IL is not acting as a catalyst but also that the IL is not being degraded by the lipase used. However, by the pH determination and because the pH of $[P_{6,6,6,14}]Cl$ is strongly acidic (pH ≈ 1), it was concluded that the IL is contaminated with acids, that may be derived from the IL synthesis. Because the IL was impure, a careful neutralization step was carried out before all the experiments, as described in Section *2.2.1*.

4.1.2. Olive oil characterization

4.1.2.1. Free fatty acids

As previously mentioned, the substrate consists in a commercial olive oil with low free fatty acid contents (around 0.3%). However, it was decided to experimentally determine the initial concentration of free fatty acids by performing the titration of the olive oil samples with KOH.

Table 3 - Mass of olive oil and volume of KOH used in the free fatty acids experimental determination.

$m_{Olive\ oil}$ (g) ±0.0002	V_{KOH} (mL) ±0.05	[KOH] (mol/L)	mmol FFA per g oilve oil	$\rho_{olive\ oil}$ (g/mL)	[FFA] (mol/L)
0.7555	0.08	0.4621	0.0049	0.8835	0.0043
0.7511	0.09		0.0055		0.0049
0.7555	0.11		0.0067		0.0059
0.7550	0.10		0.0061		0.0054
0.7522	0.09		0.0055		0.0049

Considering five experiments, the initial concentration determined is 0.0058 ± 0.0007 mmol of free fatty acids *per* g of olive oil or 0.0051 ± 0.0006 mmol FFA *per* mL of oil.

4.1.2.2. Total fatty acids

In order to determine the total amount of free fatty acids produced when a complete hydrolysis of the olive oil occurs, the saponification of the olive oil was done using the experimental procedure described in Section *2.2.3.1.* Each experiment was performed in triplicate and a specific control was used (without olive oil addition), being the results represented in Table 4.

Table 4 – Mass of olive oil and volume of KCl used in the saponification experiments.

	$m_{Olive\ oil}$ (g) ±0.0002	V_{HCl} (mL) ±0.05	Mean ± std		$m_{Olive\ oil}$ (g) ±0.0002	V_{HCl} (mL) ±0.05	TFA±std
Black	0.0000	5.76	5.70±0.053	**Sample**	0.5000	2.56	3.43±0.033
	0.0000	5.68			0.5002	2.54	3.45±0.060
	0.0000	5.66			0.5003	2.52	3.47±0.060

The total fatty acids were calculated for each experiment, considering the molality of the HCl solution of 0.5454 M and by applying Equation 2, and are reported in the last column of Table 4. Thus, the presence of 3.45 ± 0.02 mmol of free fatty acids *per* g of olive oil or 3.04 ± 0.02 mmol per mL of olive oil (considering the olive oil density as 0.8835 g/mL) was experimentally determined.

4.1.3. Optimization of the reaction conditions

As already discussed [105], one of the main difficulties in the analysis of the enzyme performance is the fact that the common enzymatic reactions follow non-linear paths with time, which is depends on the enzyme used and the conditions adopted.[105] In this context, the study of the progress curve of the enzyme considering the hydrolysis of the olive oil was performed aiming at the determination of the linear region or the so-called steady-state.

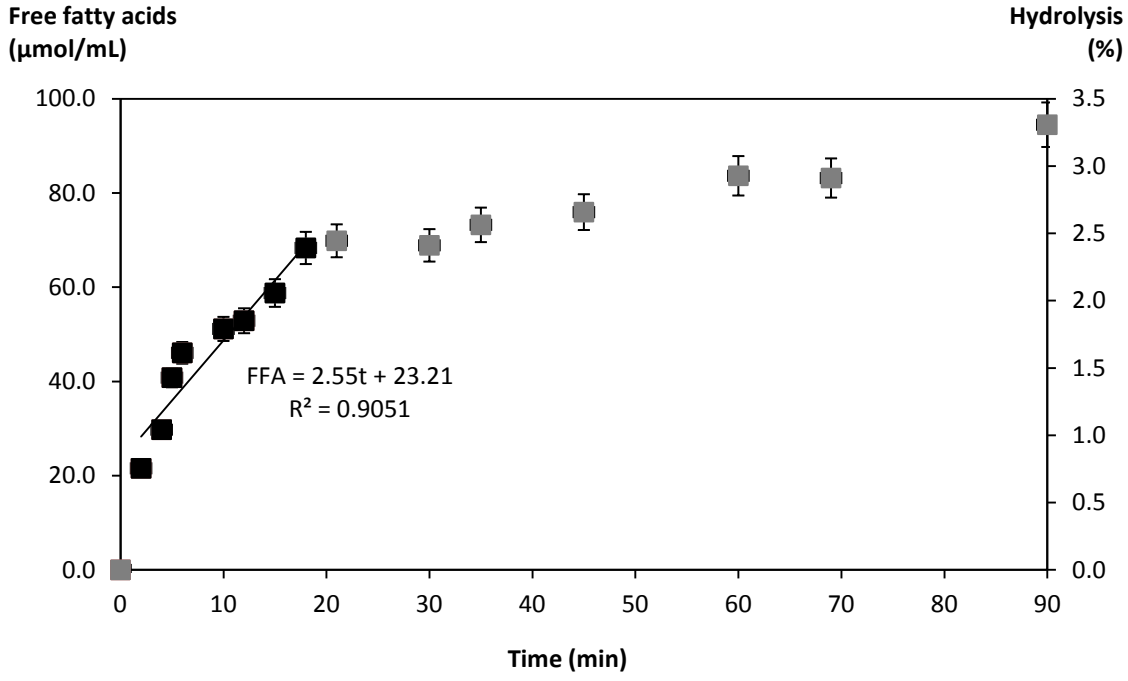

Figure 12 – Progress curve of CaLB-catalyzed hydrolysis of olive oil at 310.2 (±0.1) K and 100 rpm and 10 %(v/v) of water. The lipase concentration used is 0.69mg of protein/mL.

The curve depicted in Figure 12 can be divided into three main regions, **i)** the pre-steady-state (0-2min) where the product accumulation rate increases rapidly with time, with an extremely short duration not allowing its easily detection[105], **ii)** the steady-state,

located at 2-18min, here the product liberation increases linearly with time and consequently, the initial velocity of the reaction can be directly obtained from the slope of the linear correlation line [2.55 µmol/(mL.min)] and finally, **iii)** the concentration of the product reaches a plateau, practically not changing with time (18-90 min), which in our specific case represents the enzyme inhibition by the presence of the free fatty acids produced because they are absorbed at the oil-water interface competing with the lipase[106].

As it is shown in the Figure, the hydrolysis percentage is extremely low (less than 3.5%), which means that a detailed optimization is required. Thus, the agitation speed, enzyme concentration and temperature were considered and optimized in order to obtain higher hydrolysis percentages.

4.1.3.1. Influence of the agitation speed

The agitation speed was the first condition studied in a range from 100 to 400 rpm (at 310.2 K, 0.69 mg of CaLB/mL and buffered with sodium phosphate buffer at pH=7) being the results described at Figure 13. This condition is describing the orbital shaking rate imposed to the reaction samples, where the hydrolysis is being performed.

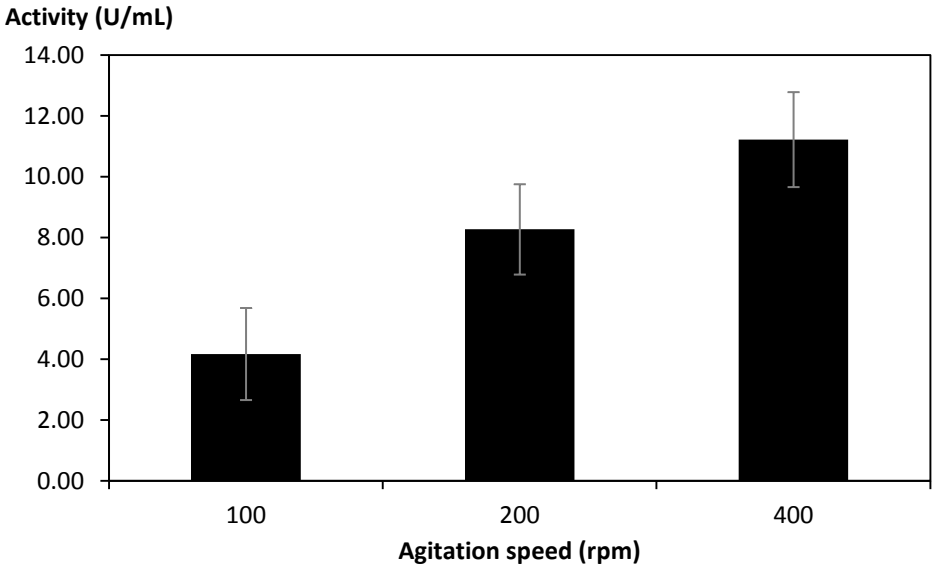

Figure 13 – Influence of the agitation speed on the enzymatic activity.

Figure 13 shows the enzymatic activity increase as a function of the agitation speed, being the best results obtained for 400 rpm. In fact, the increase in the agitation speed allows the increase of the interfacial area, trough the droplet size reduction and,

since the hydrolysis reaction takes place at the interface, a higher interfacial consequently promotes the lipase activity increase on.[107]

4.1.3.2. Influence of the enzyme concentration

Other important parameter studied in the hydrolysis optimization was the concentration of lipase (mg protein/mL). Usually, the enzyme activity increases linearly with the enzyme concentration however, there are some reactions where this profile is not verified [107].

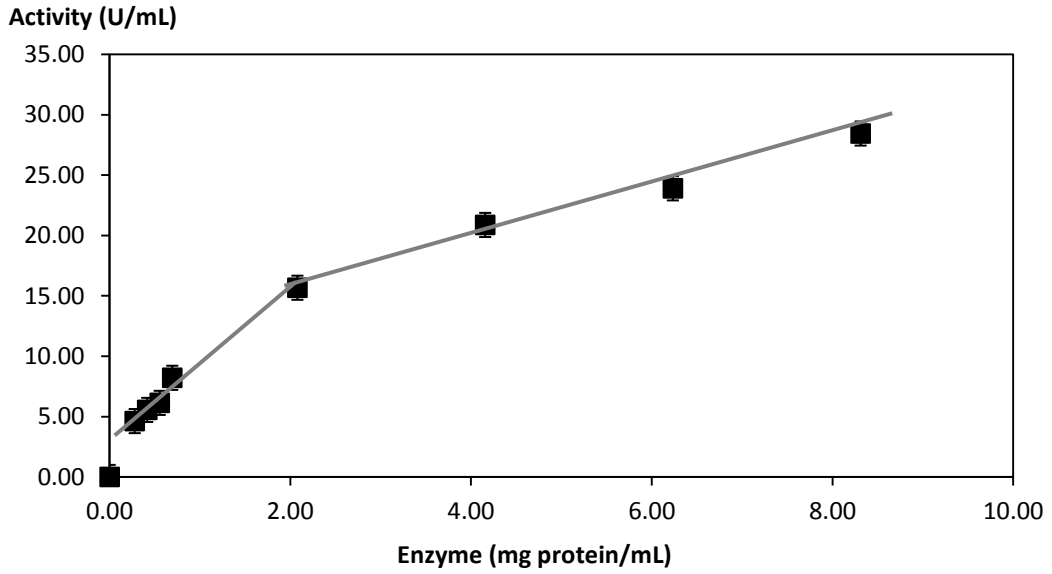

Figure 14 - Influence of the enzyme concentration on the enzymatic activity, at 310.2 (±0.1) K, pH = 7 (sodium phosphate buffer), 400 rpm and 10 %(v/v) of water.

The enzymatic activity as a function of the lipase concentration is shown in Figure 14. For the first enzyme concentration values, a rapid increase in the velocity of the reaction is verified, because the ratio between the free interfacial area and the lipase concentration is high, meaning that all the enzyme molecules are available to adsorb and catalyze the triglycerides hydrolysis at the interface. However, for enzyme concentrations higher than 2.08 mg protein/mL, the interface becomes saturated with the enzyme molecules, promoting the slowdown of this specific activity. This phenomenon was reported by other authors for the hydrolysis of various vegetable oils.[108,109] The authors comment that the slowdown in the specific activity is justified by the progressively decrease of free enzyme molecules at the interface able to promote the

formation of the enzyme-substrate complex.[108,109] In this context, the enzyme concentration adopted in the future optimization experiments was 2.08 mg protein/mL.

4.1.3.3. Influence of temperature in the reaction

The influence of temperature in the performance of the hydrolysis reaction was also checked, being the results depicted in Figure 15.

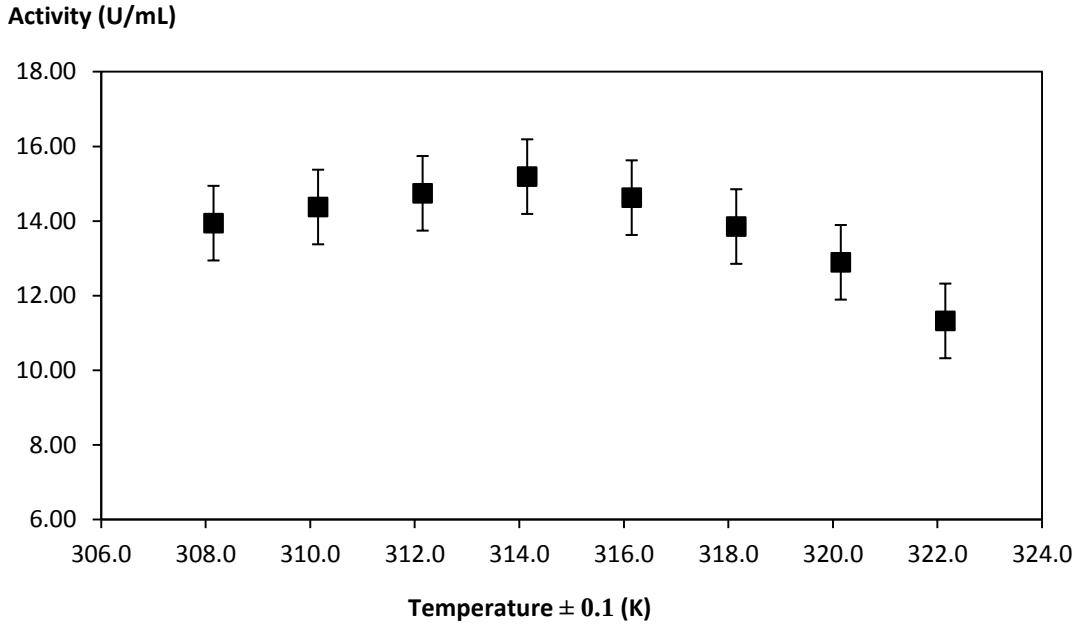

Figure 15 – Enzymatic activity as a function of temperature ± 0.1 K, with 2.08 mg protein/mL, at 400 rpm and pH=7 (sodium phosphate buffer).

Figure 15 suggests that the increase of the reaction temperature is capable of improving the production of fatty acids, since an increase in the enzymatic activity is shown, until a maximum of 314.2 (±0.1) K, because this temperature represents the maximum of enzyme activity and after this value the CaLB activity is decreasing, probably due to the enzyme denaturation (in agreement with literature [102]), this value was adopted in the remaining experiments.

4.1.3.4. Time course with the optimized conditions

As mentioned above, the profile of an enzymatic reaction curve is influenced by the reaction conditions applied. So the progress of the olive oil hydrolysis reaction, catalyzed by the lipase and applying the optimized conditions, *i.e.* 314.2 (± 0.1) K, 400 rpm and 2.08 mg protein/mL, is represented in Figure 16. Contrarily to Figure 11, this curve does not show clearly the three stages. Only two regions, the steady-state and the substrate depletion are identified. After optimization, the initial velocity of the reaction obtained directly from the slope of the steady-state phase (0-10 min) is 19.67 µmol/(mL.min). In fact, comparing the two curves, it is demonstrated the importance of the optimization experiments, since the initial velocity is 8 times higher for the optimized systems, being the hydrolysis percentage also substantially increased.

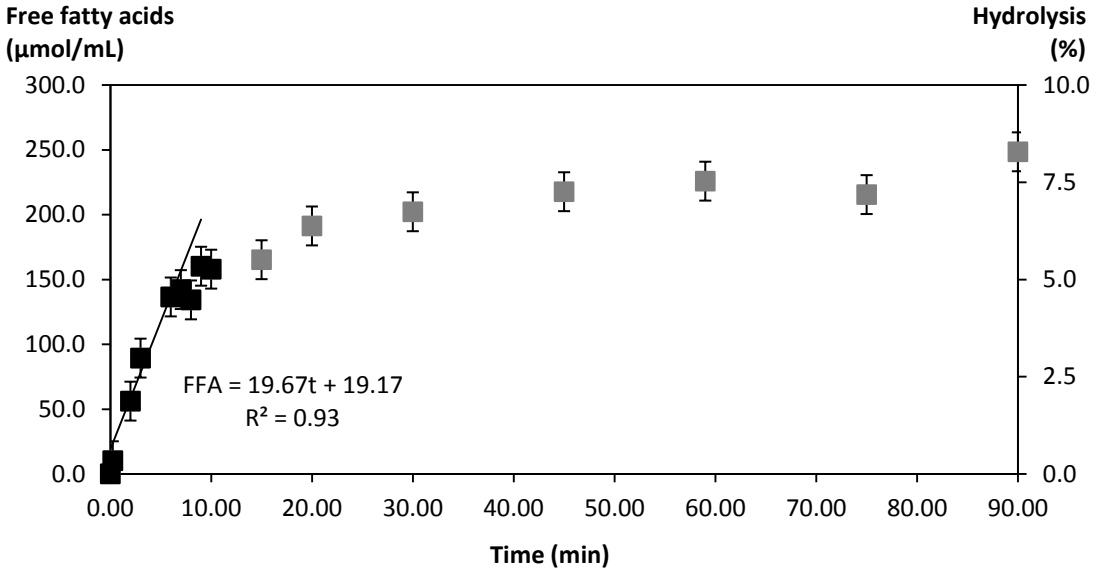

Figure 16 – Graphical representation of the progress curve of CaLB-catalyzed hydrolysis of olive oil at 314.2 (± 0.1) K, 400 rpm, 2.08 mg protein/mL (optimized conditions) and 10 %(v/v) of water.

4.1.4. Effect of ILs and salt solutions in the CaLB activity

Because the maximum of hydrolysis percentage is only around 9%, our main idea was to test the two selected ILs, to investigate their capacity to significantly improve the enzyme activity thus increasing the hydrolysis percentage. These tests were carried out during 10 min and adopting the optimized conditions [314.2 (±0.1) K, 400 rpm and 2.08 mg protein/mL].

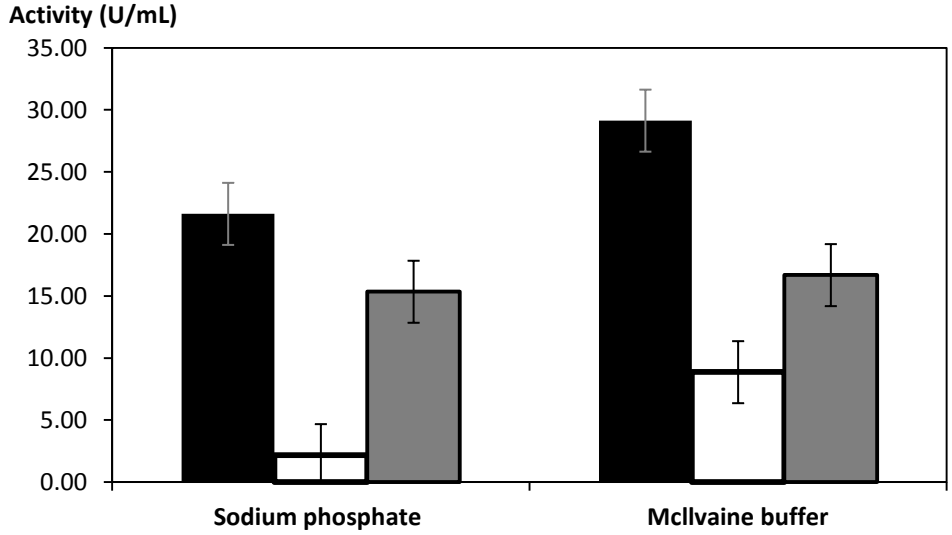

Figure 17 – Effect of two ILs in the enzymatic activity plus the buffer control; ■ Buffer, □ [$P_{6,6,6,14}$]Cl neutralized and ■ [C_{10}mim]Cl.

Figure 17 is showing how the enzymatic activity is affected by the presence of two IL families, the [$P_{6,6,6,14}$]Cl and [C_{10}mim]Cl. In an opposite way, these ILs, when applied in the hydrolysis of the olive oil show a negative effect in the enzymatic activity, when compared with their action in the hydrolysis of the *para*-nitrophenyl laurate.[76] To try to understand and to explain this behavior, the progress curve of the olive oil hydrolysis by applying the ILs was done, being the results shown in Figure 18.

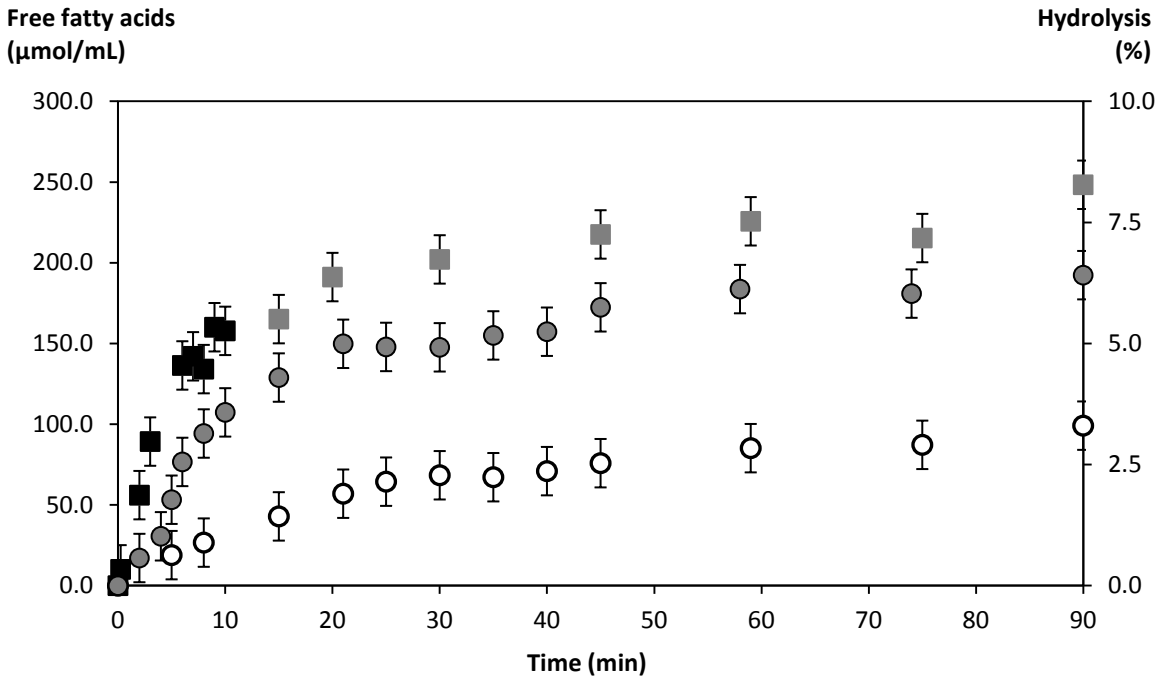

Figure 18 - Progress curve of CaLB-catalyzed hydrolysis of olive oil at 314.2 (±0.1) K, 400 rpm, 2.08 mg protein/mL and 10 %(v/v) of water using; ■ Sodium phosphate buffer, ○ [$P_{6,6,6,14}$]Cl neutralized and ● [C_{10}mim]Cl.

Figure 18 corroborates the results previously obtained, since it illustrates the negative effect of both ILs in the initial velocity of the hydrolysis, although this effect is more pronounced in the presence of [$P_{6,6,6,14}$]Cl. The fatty acids produced by the olive oil hydrolysis are mainly the palmitic and the oleic acid, *i.e.* fatty acids with long alkyl chains. Despite the low hydrolysis percentage, the amount of free fatty acids produced is high, which help us to verify the possible enzyme inhibition caused by the formation of the fatty acids with long alkyl chains, which can be agglomerated at the interface of the reverse micelles, since they are characterized by a pronounced surface activity.[106] In the presence of these ILs, the enzyme inhibition appears but with a lower content of fatty acids, because in these systems the interface is composed by the ILs, which are also competing with the lipase. The different behavior promoted by the presence of these two ILs is contradicting the theory of Kumar Das and his coworkers,[110] since they have found a better performance from the lipase when larger head-group size surfactants are applied. In fact, we can conclude that the lipase activity in this reaction is regulated by other factors that are competing with the head-group size, for example, interactions between [$P_{6,6,6,14}$]Cl and the substrate molecules, interactions that can be responsible for the change in the molecular packing of the emulsion.

Having all these experiments, principally the ILs action in the hydrolysis, into account, it is possible to conclude that the use of ILs to form water-in-oil microemulsions is not advantageous.

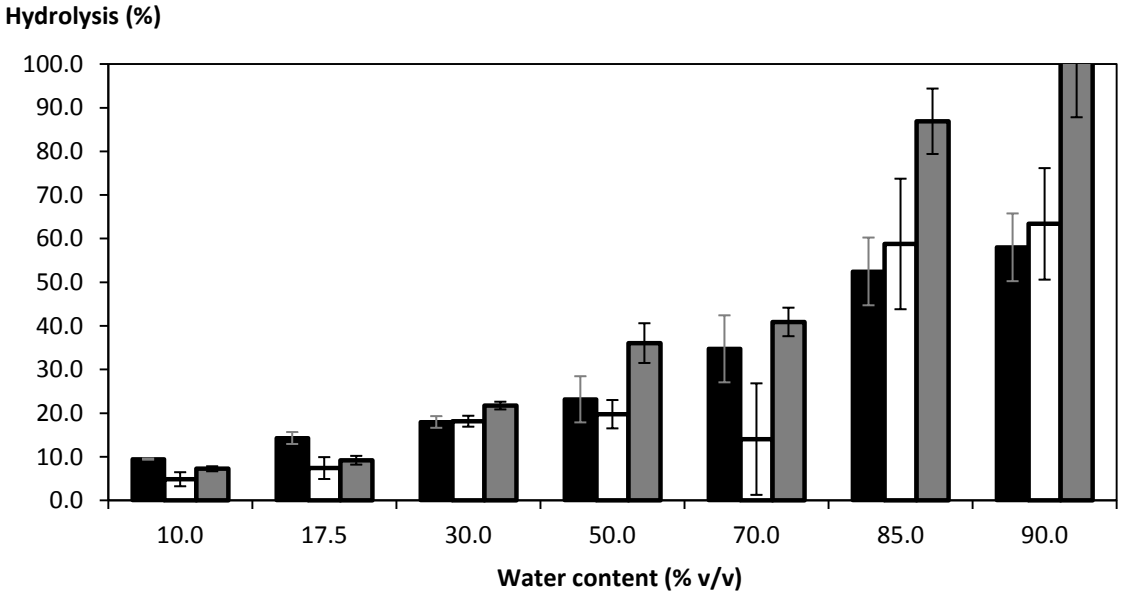

Figure 19 – Hydrolysis degree as a function of the water content (% v/v) presented in the emulsion; ■ Sodium phosphate buffer, □ [$P_{6,6,6,14}$]Cl neutralized and ■ [C_{10}mim]Cl.

Because the ILs addition was not capable of significantly increase the enzymatic activity, our conclusion was that, for this specific system and reaction the water-in-oil microemulsions were not the most adequate. In this context, our next step was to test the amount of water. In this case, different water contents were applied in the hydrolysis reaction trying to understand if the change from water-in-oil to oil-in-water microemulsions can have a significant influence in this specific reaction catalyzed by CaLB (Figure 19). Figure 19 shows an increase in the hydrolysis performance with the increase of the water content of the emulsion. This increment is verified in the absence and in the presence of these ILs however, the results suggest that this increase is more pronounced in the ILs presence, in particular when [C_{10}mim]Cl is used. In fact, a complete hydrolysis (≈100%) is achieved when [C_{10}mim]Cl is applied in the system.

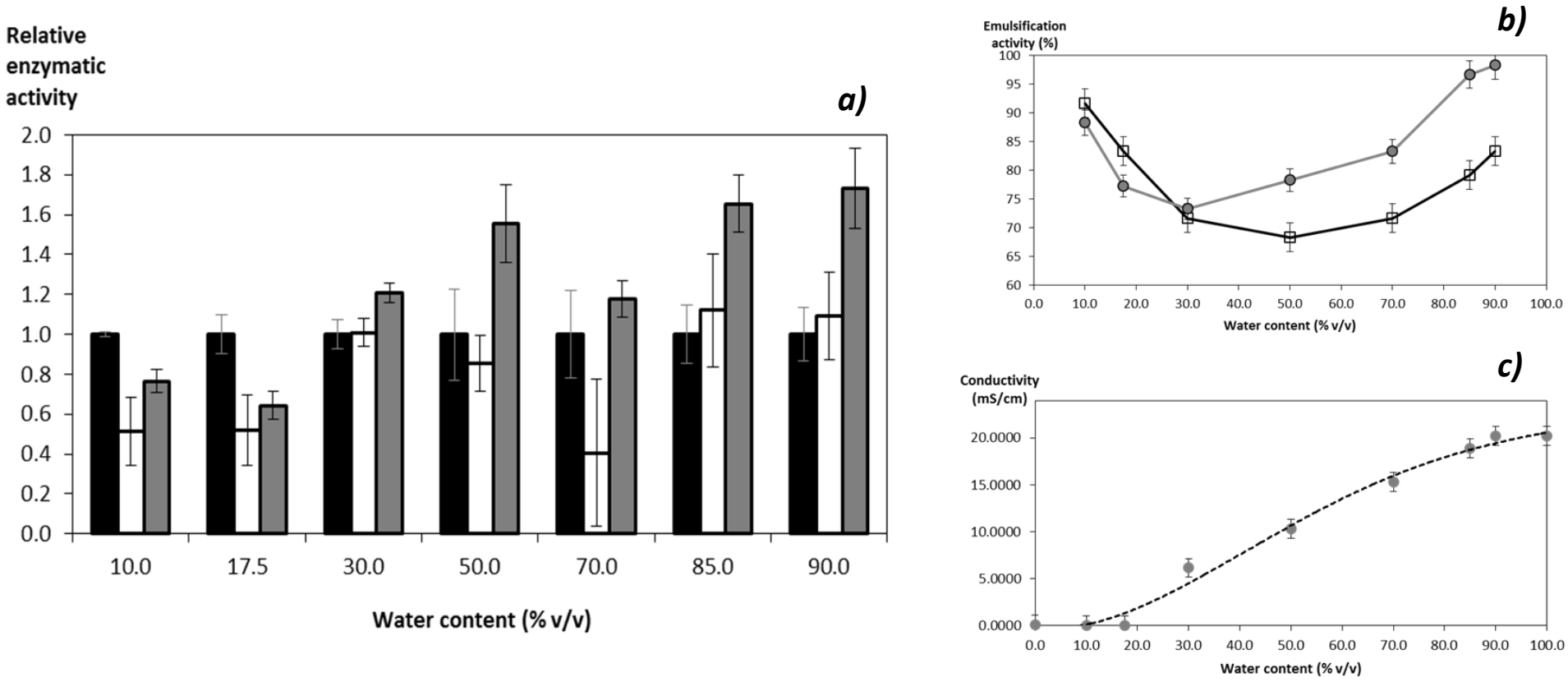

Figure 20 – a) Relative enzymatic activity of CaLB *versus* the water content (% v/v) presented in the emulsion; ■ Sodium phosphate buffer, □ [$P_{6,6,6,14}$]Cl neutralized and ■ [C_{10}mim]Cl; b) Figure 9 from section *3.1.5*; c) Figure 10 from section *3.1.5*.

The results corroborate the positive effect of the ILs presence for the high amounts of water, since the relative activity increases with the water-oil ratio, except when the water-oil ratio is 50% (v/v) and 70% (v/v). These exceptions may be justified by the phase inversion from the oil-in-water for the oil-in-water system (Figure 20 – *b* and *c*) and by the lower stability of these systems due to the characteristic phase inversion (from the oil-in-water for the oil-in-water). This significant increment in the enzymatic activity was investigated by our group in a recent work[76] and other authors [111,112] and it is known as the superactivity phenomenon. In fact, in our previous study an increase up to 6- and 14-fold was found in the relative activity of CaLB applied in the hydrolysis of p-nitrophenyl laurate, in the presence of $[C_{10}mim]Cl$[76] and $[P_{6,6,6,14}]Cl$[100], respectively. Here, the relative activity increases in the maximum up to 1.6-fold, but the complete hydrolysis is reached.

According to these results, 85% (v/v) of water content was chosen to compare the progress curve of the hydrolysis considering three different systems (the sodium phosphate buffer, the $[P_{6,6,6,14}]Cl$ and the $[C_{10}mim]Cl$) – Figure 21.

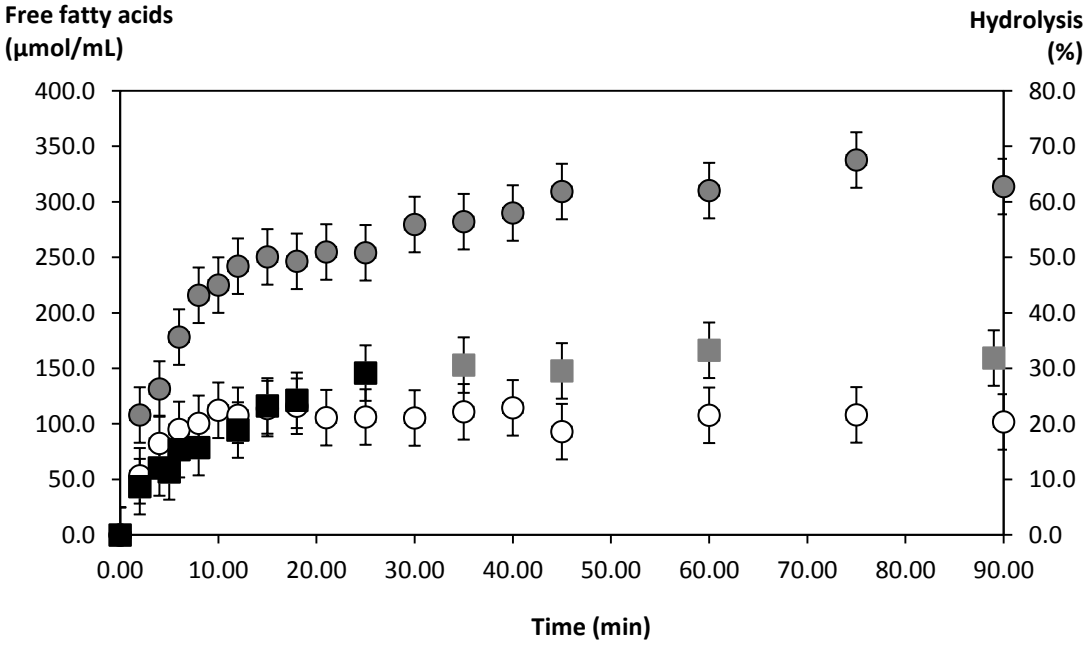

Figure 21 - Progress curve of CaLB-catalyzed catalyzed hydrolysis of olive oil at 314.2 (± 0.1) K, 400 rpm, 2.08 mg protein/mL and 85% (v/v) of water content (optimized conditions): ■ Sodium phosphate buffer, ○ $[P_{6,6,6,14}]Cl$ neutralized and ● $[C_{10}mim]Cl$.

The results suggest that both ILs increase the initial velocity of the hydrolysis reaction (6.81 and 14.12 µmol/(mL.min) for $[P_{6,6,6,14}]Cl$ and $[C_{10}mim]Cl$, respectively)

when compared with the sodium phosphate buffer system [4.40 μmol/(mL.min)]. However, when the time condition is observed, only the [C_{10}mim]Cl shows to be advantageous, since the amount of free fatty acids (≈ 325 μmol/mL) and the hydrolysis percentage (≈ 70 %) are the highest. These results can be justified by the similarity between the imidazolium cation and the histidine amino-acid, an important residue of the catalytic lipase triad responsible for facilitating the enzymatic hydrolysis. Hence, the imidazolium group when present in the interface promotes the increase of the water nucleophilicity by hydrogen-bond intercations, consequently improving the enzyme activity.[113,114]

Finally, the effect of the pH and the ionic strength of the bulk system were also verified by the application of different buffers in the hydrolysis system, being the results shown in Figure 22. Considering the pH of the Mcllvaine buffer, it is possible to conclude that the lipase is more active in an alkaline pH (≈8). However, when the sodium phosphate buffer is applied, better results are observed, despite the lower pH (≈ 7).

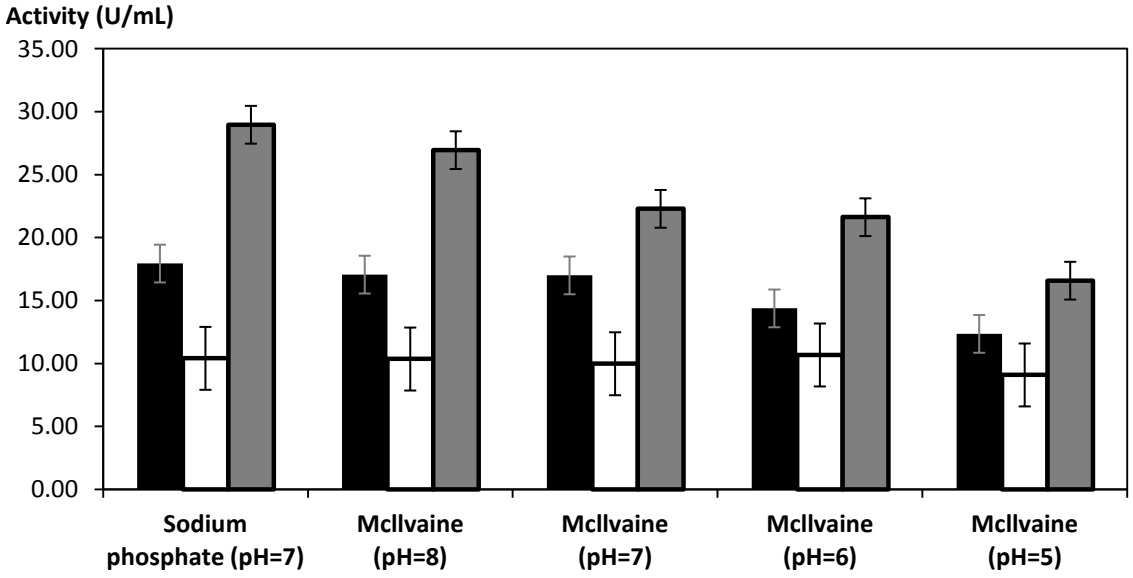

Figure 22 – Influence of type and pH buffer in the lipase activity; ■ Buffer, □ [$P_{6,6,6,14}$]Cl neutralized and ■ [C_{10}mim]Cl.

4.2. Conclusions

In this chapter, two studies were performed, the optimization of the operating conditions of the olive oil hydrolysis catalyzed by CaLB, and the study of the effect of two ILs on the lipase activity at several water/oil ratios. In fact, the optimum conditions were selected, being used 400 rpm as the optimal agitation velocity, 314.2 (± 0.1) K as the optimal temperature and 2.08 mg enzyme/mL, as the optimal enzyme concentration. Moreover, and adopting the optimal conditions aforementioned, the effect of two ILs belonging to the imidazolium and phosphonium families was investigated. It was shown that the ILs have different effects in the hydrolysis performance when distinct water contents are applied. It was proved the big influence promoted by the presence of different amounts of water, or in other words, the high influence of the type of micelles used, from the water-in-oil to the oil-in-water emusions. In fact, at low water contents the presence of these ILs does not present any advantage for the catalysis. However, at high water contents, [C_{10}mim]Cl acts as an enhancer of the lipase performance, super-activating it, promoting the complete hydrolysis of the olive oil for the highest water contents.

5. Final remarks

5.1. Conclusions

In spite of experimental problems occurring during this work, the main objective was achieved, meaning that the lipase superactivity was promoted. Thus, the lipase activity increased 1.6 times when the reactions conditions were 314.2 (±0.1) K, 400 rpm, 2.08 mg protein/mL and the emulsion was prepared using 85 % (v/v) of sodium phosphate buffer, 15 % (v/v) of olive oil and 0.100 M of [C_{10}mim]Cl. Despite the success of this work, which reports for the first time a complete study of all the procedural conditions to be taken into account, not only in the emulsion formulation but also in the reaction performance, the superactivity achieved lower values when compared with our last work, where the superactivity was promoted in the *para*-nitrophenyl laurate hydrolysis. It is our conviction that more studies should be done considering a best understand of the aggregation systems. Finally, it should be here highlighted that also for the first time the whole spectrum was considered, from the water-in-oil to the oil-in-water microemulsions

5.2. Future work

Our main goal, as already explained was to test the viability of a significant increase in the production of free fatty acids, considering the hydrolysis of a commercial olive oil, with low acid content (≈0.3%) using micellar systems. In fact this work was dealing with two main micellar systems, the water-in-oil (reverse micelles) and oil-in-water (micelles). However, and independently of the micellar approaches adopted, the appearance or absence of superactivity is not only explainable by these individual conditions. In fact, all of them should be characterized simultaneously, which means that it is extremely important to know and to understand the mechanisms behind this phenomenon. Thus, as a future work it would be of great importance to determine not only the size and shape of the microemulsions, but also the location of each component in the system, namely the water, the olive oil, the ionic liquids and the enzyme. The possible interactions should be under scrutiny considering small-angle X-ray scattering (SAXS) measurements and by the application of small-angle neutron scattering techniques (SANS).

Because this work represents just a preliminary step in the use of ILs as superactivity agents, some future works should be carried out, namely:

i) the use of other mono(-long)-alkyl chain ILs, aiming to obtain a high improvement in the enzyme activity. Thus, long alkyl chain ammonium- and cholinium-based ILs are being considered due to their high biocompatibility and benign nature;

ii) the use of di(-long)-alkyl chain ILs, where catanionic and gemini ILs can be applied. In this case, a detailed investigation should be carried out having in mind the length of the alkyl chains, the number of long alkyl chains substituted, the cation core, and in the particular case of the catanionic ILs the anion type.

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